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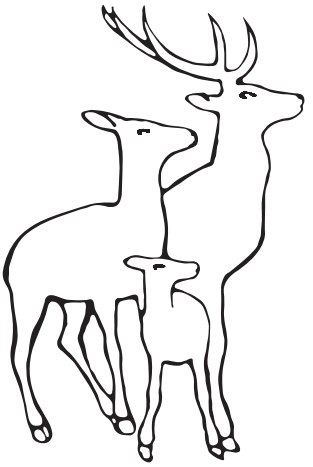

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Original Article

Evaluation of forensic toxicological characteristics of cases under the age of eighteen with substance use: a sample from Türkiye

Kerem Sehlikoğlu[1], Murat Kamalak[2], Mehmet Dağlıoğlu[2], Seval Gülçiçek[2], Fatma Köse[2], Hicran Nermin Demir[2], Tuğba Çakır[2], Duygu Ülkü[2], Melek Rüveyda Koca[2], Demet Dönmez[2], Beyza Kılınç[2], Zeynep Bilge[2]

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ABSTRACT

Background. Substance use is rapidly increasing in the pediatric age group worldwide. There is not enough toxicological data on substance use among children and adolescents in Türkiye. This study aims to reveal the forensic toxicological characteristics of cases under the age of eighteen with substance use detected.

Methods. In our study, forensic toxicological reports of biological samples taken from 587 cases brought to our institution by law enforcement officers due to allegations and/or suspicions of substance abuse between January 1, 2022, and June 30, 2024 were retrospectively examined. The cases were reviewed in terms of variables such as gender, age, age group, substance type (if any), and presence of multiple substance use.

Results. Out of the cases, 89.1% (n=523) of the cases were male and 10.9% (n=64) were female. The majority of cases (93.2%) were observed in the 15–17 age group, accounting for 547 individuals. Of the cases, 29.0% (n = 170) were identified as multiple substance users. Amphetamine-type stimulants (ATS) were present in 68.7% (n = 403) and cannabis was found in 48.2% (n = 283) of cases. It was observed that only cannabis use was significantly higher among males and only ATS use was higher among females (Cramer's V = 0.202, $p < 0.001$). The association between gender and substance type was statistically significant; however, the strength of the association was small to moderate.

Conclusion. This study assessed substance use profiles in adolescent populations through substance testing. ATS were the most frequently detected substances. The analysis revealed a significant increase in the proportion of female cases over time. While cannabis use was more prevalent among males, ATS use was more common among females. Collecting objective, valid, and definitive data will facilitate the identification of substance use issues and support the development of effective preventive policies.

Key words: amphetamine-type stimulants, adolescent, cannabis, forensic toxicology, substance use, forensic toxicological analysis.

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Substance use is a serious social issue that threatens the safety and well-being of children and adolescents, who are integral members of families, the smallest fundamental unit of society.[1] Adolescence is a period in which harmful actions and behaviors, such as suicide attempts, smoking, alcohol, and substance use, may emerge.[2] The prevalence of substance use is rapidly increasing among pediatric and adolescent age groups in Türkiye and across the world.[3,4] Epidemiological studies on community samples report that the lifetime prevalence of substance use among adolescents in Türkiye ranges from 3% to 10%. Additionally, evidence indicates that substance use is significantly more prevalent among males.[5] Substance use most commonly begins between the ages of 15 and 17; however, in some cases, it may emerge as early as age 10.[6] Factors such as low socioeconomic status, poor academic performance, thrill-seeking behavior, negative peer influence, dysfunctional family relationships, curiosity, and psychiatric disorders contribute to early substance use.[7]

Early initiation of substance abuse negatively impacts the physical and mental development of children, leading to physical, behavioral, social, and health-related problems.[8] The physical health risks associated with substance use among young individuals include accidental injuries—such as motor vehicle accidents and falls—as well as suicide attempts. Regular substance use, particularly during childhood, can severely disrupt growth and neurological development. Fundamentally, it impairs key cognitive functions, including memory, attention, and executive functioning.[1,9] Substance use in adolescents is often accompanied by psychiatric comorbidities, such as personality disorders, anxiety, and depression. The presence of these conditions is associated with a more severe clinical course and a poorer overall prognosis.[10] The necessity for individuals to engage in criminal acts and illicit behaviors to acquire substances contributes to an escalating societal threat, posing significant implications for public safety and social stability.[11] A significant concern is the heightened risk of addiction among individuals who begin using tobacco, alcohol, or illicit substances before the age of 18. Related studies indicate that a considerable proportion of adults diagnosed with substance use disorders first engaged in substance use during childhood or adolescence.[12]

A nationwide study titled "The Survey on Attitudes and Behaviors Regarding Tobacco, Alcohol, and Substance Use in the General Population in Türkiye", conducted by the Turkish Monitoring Centre for Drugs and Drug Addiction (TUBİM) in 2018 across 26 provinces, examined substance use trends in the general population. The results indicated that 3.1% of participants had used substances at least once in their lifetime. The highest proportion of lifetime substance users was in the 15–24 age group (35.4%), and the mean age of first substance use was reported as 19 years.[13] Findings from another study indicate that individuals diagnosed with substance use disorder in Türkiye accounted for 1.31% of the total population in 2005. This figure has followed a consistent upward trajectory, reaching 1.54% in 2017. Similarly, substance-related deaths have shown an increasing trend over the years.[14]

Substance use in adolescents is a critical public health priority that evolves rapidly and requires frequent monitoring, as it is a preventable contributor to both morbidity and mortality.[15,16] In Türkiye, data regarding the frequency and toxicological findings of substance use among children and adolescents remain insufficient.[4] A study by Doksat et al.[15] examined trends in substance and alcohol use, as well as gender differences, among children and adolescents receiving treatment at an addiction center. The findings revealed a substantial increase in the proportion of adolescents seeking treatment for substance use, rising from 31.4% in 2011 to 68.6% in 2014. Furthermore, the study highlighted a progressive rise in both adolescent admissions and the prevalence of polysubstance use. Notably, the use of alcohol, amphetamine-type stimulants (ATS) , and synthetic cannabinoids showed a significant upward trend.[15] Moreover,

several studies have investigated substance use trends among adolescents who present to emergency departments and other clinical settings for follow-up, treatment, or legal proceedings.[2,4] A biochemistry laboratory-based study examined the prevalence of substance use in the general population by analyzing substance data from forensic and treatment-related admissions.[17] Epidemiological surveys are regularly conducted in Türkiye to assess the prevalence and patterns of substance use among adolescents.[18,19] Although data derived from participant-reported surveys provide valuable insights, they are relatively subjective and inadequate for assessing the prevalence of substance use. Toxicological analysis of biological samples such as urine and blood offers an objective method to obtain substance-related data.[4,20] Forensic toxicological substance testing begins with the supervised collection of biological specimens by trained personnel. The process involves multiple analytical stages, including screening, detection, identification, quantification, and confirmation. It concludes with the comprehensive interpretation and documentation of all findings in an official report. Furthermore, substance analyses serve as legally significant forensic evidence.[21]

This study aims to identify substance use data among the pediatric population referred by the prosecutor's office or courts in Gaziantep, a province located in southeastern Türkiye, and thus to contribute to the development of primary preventive measures in collaboration with relevant institutions, helping children lead healthier lives in the future.

Materials and Methods

City characteristics

Gaziantep, the ninth largest city in Türkiye, holds strategic importance due to its proximity to the Middle East, its well-developed industrial base, and its accessibility to major commercial ports. The city functions as a significant transit route for various substances and is one of the two metropolitan provinces in Türkiye located along the Syrian border. The institution where the current study was conducted is recognized as the leading and most specialized center for forensic toxicological analysis in Gaziantep. Furthermore, Gaziantep was among the provinces impacted by the 2023 earthquake in Türkiye.

Sampling, setting, and procedure of the study

A total of 722 pediatric cases were referred by law enforcement for alleged or suspected substance abuse and were subjected to forensic investigation between January 1, 2022, and June 30, 2024, in Gaziantep and its surrounding regions. During the study period, a total of 36,000 toxicological analyses were performed on different antemortem or postmortem biological samples, of which 2.0% were of antemortem pediatric age group cases.

Initially, forensic toxicological reports indicating substance detection in blood and/or urine samples collected from pediatric cases were evaluated. Among the 722 cases, no substance presence was detected in the samples of 135 cases (18.7%), with their toxicological analysis results being negative; therefore, these cases were excluded from the study. The study sample consisted of 587 pediatric cases with at least one type of substance detected in their biological samples.

Characteristics of the cases

The cases were analyzed in terms of gender, age, age group, year of admission, type of substance used, and the presence of multiple substance use. The Turkish Penal Code (TPC) classifies the age of children and adolescents in terms of criminal responsibility. According to the 31st article of the TPC, children under the age of 12 cannot be held criminally responsible for their actions. For children who are at least 12 years old but less than 15 years old at the time of committing the offence, it is necessary to determine whether they have the capacity to understand the legal meaning and consequences of the offence

and whether they have the capacity to control their behaviour. The TPC recognises that the ability to understand the legal meaning and consequences of the offence and the ability to control behaviour increases in children over the age of 15. Therefore, in this study, we have grouped the age variable as <12, 12-14 and 15-17.[22] The types of substances used were categorized as cannabis, synthetic cannabinoids, opioids (heroin, morphine, codeine), psychiatric medications (benzodiazepines, antipsychotics), gabapentinoids, and ATS (amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine). The use of two or more types of substances was classified as multiple substance use.

On 6 February 2023, Türkiye and Syria were shaken by two earthquakes with magnitudes of 7.8, centered in Pazarcık, and 7.6, centered in Elbistan, in Kahramanmaraş province, causing significant losses. On 20 February 2023, a third earthquake with a magnitude of 6.4, centered in Samandağ, Hatay province, occurred. These earthquakes affected 11 provinces and caused extensive damage; more than 48,000 people lost their lives, and 14 million people were adversely affected by the disaster.[23] It should be noted that, in the aftermath of the major earthquakes, the number of applications significantly declined, with almost none recorded during the subsequent three-month period.

Analytical procedure

In this study, liquid chromatography-mass spectrometry-mass spectrometry (LC-MS/MS) was used for toxicological validation analysis of each biological sample. Each urine sample underwent an integrity test, which included assessments of urine osmolality, urinary creatinine, pH, and the presence of foreign substances in urine. Solid-phase extraction (SPE), a technique designed for rapid and selective sample preparation and purification, was employed prior to chromatographic analysis. In SPE, one or more analytes are isolated from a liquid sample by partitioning and/or adsorption to a solid stationary phase. The SPE method was used by laboratory personnel for all blood and urine samples at the center. Samples were analyzed qualitatively and quantitatively using LC–MS/MS. ATS, psychiatric drugs, gabapentinoids, cannabis (THC-COOH), synthetic cannabinoids, opiates (morphine, codeine, and 6-monoacetylmorphine [6-MAM]), and their metabolites in blood and/or urine samples were analyzed.

Ethical statement

All procedures used in this research complied with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, and its subsequent updates. The privacy rights of human subjects were respected during the implementation of the study by the authors. The study sample consisted of cases referred by the judicial authorities. Written permission for data use was granted by decision number 2024/1388 of the Forensic Medicine Institute Education and Scientific Research Commission, dated December 3, 2024. Ethical approval was obtained from the local ethics committee on December 17, 2024 (approval number 2024/10-29).

Statistical analyses

The categorical variables in the study were given as a frequency and percentage. Descriptive statistics were given as median (minimum–maximum). The categorical variables were grouped, the percentages calculated and then either Pearson's chi square test or Fisher's test as appropriate was used to compare frequencies. Post hoc analysis methods for chi-square test and Bonferroni correction were applied in the evaluation of significance between groups in multiple groups. We calculated Cramer's V for categorical comparisons as measures of effect size. Strength of association was reported using Cramer's V. Cohen[24] suggested the following guidelines for interpreting Cramer's V; if df=1; Small >0.1, Medium (Moderate) >0.3, and Large >0.5. The Kolmogorov–Smirnov test was used

for normality in continuous variables ($p > 0.05$). Kurtosis–skewness values were also evaluated.

All statistical analyses, tables and graphs were made using the SPSS 22 (IBM Corp., Armonk, NY) program. Cases with a p-value of < 0.05 were considered significant. Because of the low number of cases in some subgroups in the study, some categorical data groups were combined or were not included in the relevant analyses.

Results

A total of 89.1% (n=523) of the cases were male, while 10.9% (n=64) were female (Table I). The mean age of all cases was 16.4 ± 1.0 years. The median age was 17.0 (12–17) years for male cases and 16.0 (12–17) years for female cases. Among the cases, 93.2% (n = 547) were in the 15–17 age group, and 6.8% (n = 40) were in the 12–14 age group. Gender distribution within age groups was similar (Fisher's test, $p = 0.791$).

When examined by year of admission, the highest number of cases was recorded in 2022, with 246 cases. Gender-based analysis of annual admissions showed an increase in the proportion of female cases, from 6.9% to 15.2%, over time (Cramer's V = 0.112, $\chi^2 = 7.363$, df = 2, $p = 0.025$). Although statistically significant, the strength of the association was weak. Post-hoc comparisons using Bonferroni correction ($p = 0.02$) (adjusted significance p value = 0.025) revealed that the proportion of female admissions in 2022 was significantly lower than in other years (Table I).

Of the cases, 71.0% (n = 417) were found to have used a single type of substance, while 29.0% (n = 170) were identified as multiple substance users (Table II). The substances used were analysed, and the results revealed that ATS were present in 68.7% (n = 403) and cannabis was found in 48.2% (n = 283) of cases. Subsequent findings revealed the detection of synthetic cannabinoids in 29 cases, gabapentinoids in 23 cases, and psychiatric medications in another 23 cases (Table II). It was observed that 42.2% (n = 248) used only ATS, while 26.4% (n = 155) used only cannabis. When the chi-square test was performed, the distributions of multiple substance use by gender and age groups were similar (Cramer's V = 0.063, $\chi^2 = 2.331$, df = 1, $p = 0.127$; Cramer's V = 0.055, $\chi^2 = 1.793$, df = 1, $p = 0.181$). Among multiple substance users (n = 170), the most prevalent combination was that of cannabis and ATS, which was detected in 42.9% (n = 73) of cases. Due to the low number of cases using only gabapentinoids, synthetic cannabinoids, opioids, or psychiatric medications (n = 14 in total), these were excluded from substance use-based statistical analyses to ensure the validity of comparisons. When the post hoc Bonferroni test is applied, male cases predominantly used only cannabis ($p < 0.001$) (adjusted significance p value = 0.025), whereas female cases used only ATS ($p = 0.01$) (adjusted significance p value = 0.025) among the remaining cases (Cramer's V = 0.202, $\chi^2 = 23.407$, df = 2, $p < 0.001$; Table III). While the chi-square test indicated a statistically significant relationship between gender and substance type, the effect size pointed to a small to moderate level of association.

Analysis by age groups showed that ATS use was more prevalent in the 12–14 age group, while cannabis use was higher in the 15–17 age group (Cramer's V = 0.108, $\chi^2 = 6.642$, df

Table I. Distribution of the cases according to years and gender.

Years	Male	Female	Total
2022	229 (93.1%)	17 (6.9%)	246 (41.9%)
2023	182 (87.1%)	27 (12.9%)	209 (35.6%)
2024	112 (84.8%)	20 (15.2%)	132 (22.5%)
Total [α]	523 (89.1%)	64 (10.9%)	587 (100.0%)

Percentages were calculated as row percentages, except for the last column.

Table II. Distributions of the type of substance used

Substance	n	%*
ATS	403	68.7
Cannabis	283	48.2
Synthetic cannabinoids	29	4.9
Gabapentinoids	23	3.9
Psychiatric medications	23	3.9
Opioids	12	2.0
Cocaine	3	0.5
Multiple substance use		
Present	170	29.0
Absent	417	71.0
Total	587	100.0

* Some cases have used more than one type of substance; ATS, amphetamine-type stimulants.

= 2, p = 0.036; Table III). Although statistically significant, the strength of the association was weak.

Discussion

A study evaluating the prevalence of substance use in Türkiye based on regional illegal substance analysis results indicates the lack of nationwide studies based on laboratory analysis, and that there is a need for epidemiological studies conducted at the regional level.[17] The important advantage of this study is that it unveils the forensic toxicological data of cases involving individuals under the age of eighteen who use substances in the Gaziantep province, located in the southeastern region of Türkiye.

In studies examining children and adolescents receiving substance use disorder treatment in İstanbul, the most metropolitan city in the west of Türkiye, the proportion of male cases was observed to be 82.4% and 83.3%, respectively.[15,25] In our study, which examined pediatric forensic cases who underwent toxicological analysis due to suspected/alleged substance abuse in Gaziantep, a city in southeastern Türkiye, the male ratio was 89.1%, higher than the aforementioned studies. Becker et al.[26] stated that not only biological and hormonal factors determine substance use behavior, but also sociocultural influences affect the gender distribution in substance use. It is believed that regional sociocultural differences in Türkiye contribute to variations in gender ratios. Unlike studies conducted in Türkiye, a review evaluating adolescent substance use patterns globally, covering 70 studies, found the average male proportion to be 47%.[8] In the Turkish society, women are perceived as guardians of moral values and pillars of the family. The belief that women's substance use would degrade moral values is widespread in Turkish culture.[27] The stigmatisation of women, the paucity of social support, the challenges in accessing treatment, and the subsequent social isolation may explain the lower prevalence of substance use among women in Türkiye.

Our study highlights a notable increase in the proportion of female cases in recent years. Consistent with our results, the literature reports a growing trend in substance use among women over time.[28,29] It is believed that factors

Table III. Distribution of the type of substance used by gender and age groups.

Variables	Only ATS	Only cannabis	Multiple substance abuse	Total
Gender				
Male	210 (41.2%)	154 (30.2%)	146 (28.6%)	510 (89.1%)
Female	38 (60.3%)	1 (1.6%)	24 (38.1%)	63 (10.9%)
Age groups				
12-14 yr	24 (63.2% row, 9.7% col)	6 (15.8% row, 3.9% col)	8 (21.0% row, 4.7% col)	38 (6.6%)
15-17 yr	224 (41.9% row, 90.3% col)	149 (27.9% row, 96.1% col)	162 (30.3% row, 95.3% col)	535 (93.4%)
Total	248 (43.3%)	155 (27.1%)	170 (29.7%)	573 (100.0%)

ATS: amphetamine-type stimulants, col: column.

contributing to the increasing prevalence of substance use among girls in Türkiye include the transformation of traditional family structures, the increased accessibility of substances, the portrayal of substance use as glamorous in social media and popular culture, and the widespread engagement of young people with these platforms. Additionally, the tendency of girls to engage in risky behaviors as a means of achieving equality with and gaining acceptance from their male peers is also considered a contributing factor. Exposure to sexual abuse in childhood has been found to be associated with frequency of substance use, polysubstance use and quantity of substances consumed among girls.[30] Girls who use substances have higher rates of childhood traumatic experiences, post-traumatic stress disorder, and other psychiatric comorbidities.[31] There is a critical need for large-scale studies in Türkiye that comprehensively examine factors associated with substance use among girls, including reasons for use, age of first use, parental education and economic status, criminal history in the child or family, co-occurring psychiatric disorders, and suicide attempts.

The mean age of the cases in this study was 16.4 years, aligning with studies on adolescent substance use data from İzmir and İstanbul.[4,15] In another study investigating substance use characteristics among adolescents presenting to the emergency department in İzmir, the mean age of cases was 15.3 years, younger than in our study.[2] It has been reported that adults' substance use habits and progression to addiction often begin during adolescence. The 10–20 age range plays a decisive role in individuals adopting high-risk and health-threatening behaviors.[32,33] The similarity in age data between both genders may be attributed to the widespread accessibility of substances in society. To reduce and prevent substance use among young individuals, it is recommended to increase awareness and provide education on substance use for youth, teachers, and parents. Additionally, strengthening security measures in school environments and areas with high youth presence, enhancing psychosocial support services, and implementing cybersecurity measures to prevent the dissemination of harmful content on social media are also suggested.

The 2023 World Drug Report by the United Nations Office on Drugs and Crime stated that, in 2021, globally, one in 17 people aged 15–64 used substances at least once in the past year. The estimated number of users reached 296 million (5.8% of the global population aged 15–64), with cannabis remaining the most commonly used substance. Globally, the majority of cannabis users are male (approximately 70%), while the proportion of women is relatively higher for ATS (45% women) and the non-medical use of medications (45%–49% women).[34] In a study by Aslan et al.[4] conducted in İzmir, Türkiye, cannabis was found to be the most commonly used substance among children and adolescents, followed by multiple substance use and ATS. Cannabis was more frequently used among males. Other studies conducted in Izmir and Kayseri, Türkiye, reported ATS as the most frequently used substance.[2,17] In studies based on toxicological screening results among adolescents presenting to emergency departments in the United States and Canada, cannabis and ATS were identified as the most frequently used substances.[35,36] It has been reported that methamphetamine use has become increasingly preferred and used in adolescents due to the fact that the substance is both affordable and easily accessible.[37] In our study, the most frequently observed substance use was ATS alone, followed by multiple substance use and cannabis alone. The commonality across all studies is that ATS and cannabis are the most frequently preferred substances among children and adolescents. According to the 2024 Türkiye Drug Report[38], the sharp increase in methamphetamine seizures, which began in 2019, continued through 2020, 2021, and 2022, culminating in the highest recorded methamphetamine seizure in the country's history in 2023. In addition to the overall rise in the total amount

of methamphetamine seized, the quantity per incident also reached its peak. Similarly, the number of methamphetamine-related incidents and suspects increased. The report further highlights that while methamphetamine was detected in 7.7% of overdose deaths in 2017, this figure surged to 46.3% in 2023. Moreover, deaths caused solely by methamphetamine use rose from 0.3% in 2017 to 42.6% in 2023, underscoring the growing significance of methamphetamine use as a major public health concern.[38] In our study, the higher prevalence of cannabis use among males and ATS use among females aligns with the literature.[34] It is believed that the ease and rapid onset of oral ATS use contribute to its higher preference among females. Women tend to have more negative attitudes toward cannabis use compared to men. A survey conducted in Norway found a gender difference among participants who did not report cannabis use. While 40.9% of men perceived cannabis as risk-free or low-risk, only 16.4% of women shared this perception.[39] It was suggested that male participants in this study were more likely to view cannabis as a safe and low-risk substance.

In a study examining substance use among children and adolescents, polysubstance use was identified in 14.9% of cases, with the most common combination being ATS and cannabis (36.0%).[4] In our study, the rate of polysubstance use was higher at 29.0%, and similarly, the most frequently observed combination was cannabis and ATS (42.4%). The fact that our sample consisted exclusively of criminal cases may be related to the higher polysubstance use rate observed. The prevalence of ATS and cannabis as the most commonly used substances also leads to their frequent co-use in polysubstance combinations.

In a study on the use of cannabis among adolescents in the United States, it was reported that 12–17-year-olds used cannabis at rates of 16%, 13%, and 7% over their lifetime, the past year, and the past month, respectively.[40] Among older adolescents aged 16 and 17, 31% reported lifetime use, 25% reported use in the past year, and 14% reported use in the past month, with the highest rates of use observed in these age groups.[40] Similarly, in our study, cannabis use alone was found to be higher among the older adolescent group aged 15–17. It is thought that as a result of repeated substance use, the likelihood of individuals becoming addicted increases. Moreover, they turn to classic substance types that are easier to access.

Our study has some limitations. First, data on variables such as the age of onset of substance use and the reasons for substance use were not available. Another limitation is the lack of data on alcohol use among the cases. However, we would like to highlight the strengths and unique aspects of our study. This is the first study in Türkiye to analyze forensic toxicological data of individuals under the age of eighteen who were referred for forensic reasons. Another significant advantage is the large sample size of the study.

Conclusion

Substance use is a significant social issue. It is known that substance use among individuals under the age of eighteen is rapidly increasing worldwide and in Türkiye.

In the present study, the majority of cases were male and aged between 15 and 17 years, and ATS was the most commonly used substance. The prevalence of polysubstance use among adolescents, affecting approximately one in three individuals, highlights the ongoing importance of addressing substance use as a major issue in this population. Cannabis use was found to be more prevalent among males, while the use of ATS was more prevalent among females. The increase in substance use rates among girls over time was notable.

In conclusion, obtaining definitive data from biological samples of substance users will aid in identifying substance use-related challenges and serve as a foundation for developing effective preventive measures.

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Ethical approval

The study was approved by Non-Interventional Clinical Research Ethics Committee of Adıyaman University (date: 17.12.2024, number: 2024/10-29).

Author contribution

The authors confirm contribution to the paper as follows: Study conception and design: All authors; data collection: All authors; analysis and interpretation of results: KS, MK, MD; draft manuscript preparation: KS, Statistics: KS; Supervisor: KS, MK, MD. All authors reviewed the results and approved the final version of the article.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Original Article

Association of socio-demographic factors with measles vaccination coverage among Indonesian children aged 12-23 months: a nationwide study

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ABSTRACT

Background. This study aimed to examine the socio-demographic factors associated with measles vaccination coverage among Indonesian children aged 12–23 months, using data from a nationally representative survey.

Methods. A cross-sectional analysis was conducted using the 2018 Indonesian Basic Health Survey (Riskesdas), including 19,425 children aged 12–23 months. Multivariate logistic regression was used to identify factors associated with measles vaccination status, and subgroup analyses were performed across three regional clusters.

Results. Of the children surveyed, 73.46% had received measles vaccination, 68.14% had at least one antenatal care visit per trimester, and 53.59% had received at least one postnatal care visit. The most significant predictors of measles vaccination were frequent postnatal care (adjusted odds ratio [AOR]: 2.36, 95% confidence interval [CI]: 1.86-2.99) and higher maternal education (AOR: 2.31, 95% CI: 1.30-4.10). Other associated factors included the age and employment status of the head of the household (as defined by the Riskesdas study), travel time to healthcare facilities, household expenditure, and urban–rural residence.

Conclusion. Utilization of postnatal care and higher maternal education were key determinants of measles vaccination coverage. Improving maternal healthcare access and promoting female education may enhance vaccination uptake among Indonesian children.

Key words: vaccination, immunization, measles, children, survey, Indonesia.

Measles remains a significant concern for healthcare professionals in low- and high-income countries, including nurses, physicians, and other medical personnel.[1,2] It is a primary cause of high morbidity and mortality in children because of its high transmissibility and potential for severe complications such as pneumonia, encephalitis, acute diarrhoea, visual impairment, central nervous system infections, and mortality due to gastroenteritis.[3,4] The risk is particularly elevated for children with compromised immune systems or malnutrition. Therefore, increased measles vaccination coverage is crucial for reducing the disease burden.[5,6] However, challenges persist, with low vaccination rates often attributed to

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misinformation, vaccine hesitancy, and various socio-demographic factors.[7,8]

Globally, the measles vaccination program has significantly reduced the incidence of disease and mortality associated with measles. From 2000 to 2018, there was a 66% decrease in measles cases and a 73% reduction in fatalities attributable to routine vaccination with measles-containing vaccines (MCVs).[9] Despite these advancements, measles remains a substantial cause of morbidity and mortality in children, particularly in low-income countries.[10,11] In Indonesia, measles vaccination coverage, as observed in routine Indonesian Basic Health Research (Riskesdas) conducted since 2007, has shown fluctuating values. It was 81.6% in 2007, increased to 82.1% in 2013, and declined to 77% in 2018 among children aged 11-23 months.[12-14] Furthermore, owing to the coronavirus cisease 2019 (COVID-19) pandemic, a decrease in vaccination coverage has led to an increase in the number of measles cases in Indonesia.[15]

Previous studies in Indonesia employing national data sources such as the National Socioeconomic Survey, the Village Potential Survey, and the Indonesia Demographic and Health Survey, examined through multivariate analysis, have revealed that maternal education[16], skilled birth attendance[16-19], and socioeconomic status[16] significantly influenced measles vaccination coverage. In Indonesia, variables such as insurance ownership have been positively linked to vaccination coverage[16,20], child's age, postnatal visits, decision-making, partner's education[16], mother's age, parity, number of healthcare facilities (hospitals and health centers) per 1000 population[17], number of health centers, and residential areas (rural/urban).[21] These factors play a significant role in enhancing vaccination coverage and protecting children from vaccine-preventable diseases.

The measles vaccine plays a pivotal role in mitigating morbidity and mortality associated with measles. Children aged 12-13 months, who exhibit particular susceptibility to measles require specific attention and sufficient vaccine coverage. This study aimed to evaluate the prevalence of measles vaccination and to identify factors associated with vaccination among Indonesian children aged 12 to 23 months. The objective was to enhance existing knowledge and address the paucity of evidence regarding the determinants of vaccine coverage within this population.

Materials and Methods

Study design and data sources

A cross-sectional analysis was performed utilizing secondary data from the 2018 Riskesdas, a nationwide survey executed by the National Institute of Health Research and Development under the Ministry of Health of the Republic of Indonesia. The 2018 Riskesdas was integrated into the National Socioeconomic Survey, conducted by Statistics Indonesia (BPS) across 34 provinces in March 2018. Riskesdas represents a comprehensive national health survey conducted at the regency/municipal level. The extensive dataset encompasses various health-related factors, including health indicators, assessments, healthcare availability, health-related behaviors, environmental conditions, and hygiene. This study is based on the analysis of the 2018 Riskesdas, which has already received ethical approval (No. LB.02.01/KE.267/2017) from the Health Research Ethics Committee of the National Institute of Health Research and Development, Ministry of Health, Republic of Indonesia. Informed consent was obtained from all participants prior to data collection during the survey. Consequently, no additional ethical approval was obtained for this study. This study adhered to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.

Sampling

The sample selection in the 2018 Riskesdas employed a two-stage sampling design utilizing probability proportional to size (PPS) with linear systematic sampling. In the first stage, census

blocks (clusters) were selected using systematic PPS within each urban and rural stratum across all regencies and cities in Indonesia. In the second stage, from each selected census block, ten households were chosen through systematic sampling. This selection process incorporated implicit stratification based on the highest educational attainment of the head of the household to ensure representativeness, defining the head of the household as the person in the household who is responsible for acquisition of daily needs, and acts as the main decision-maker, or someone appointed or recognized in that role, regardless of gender. Overall, the Riskesdas encompassed approximately 300,000 households drawn from 30,000 clusters across 34 provinces.[14] For the present study, a total of 19,425 children aged 12–23 months from 514 regencies or cities were analyzed. Children who had already received booster measles vaccinations were excluded.

Variables

To enhance comprehension of the variables employed in this study, each variable was classified according to standard categorizations pertinent to maternal and child health research. The dependent and independent variables were operationalized in alignment with the structure of the 2018 Riskesdas dataset and extant literature. Table I delineates the grouping, definitions, and categories for each variable analyzed in this study.

Data analysis

This study delineated children's characteristics using frequency and percentage metrics. Cross-tabulation analysis was conducted to explore the association between independent variables and measles vaccination coverage. Simple logistic regression was employed for bivariate analysis, serving to identify candidate variables for inclusion in a multiple logistic regression model. Variables with a p-value below 0.25 were considered candidates for further analysis. Multiple logistic regression was utilized for multivariate analysis. Model selection for multivariate analysis employed a stepwise backward elimination strategy, commencing with the variable exhibiting the highest alpha value. Variables were retained and considered confounding if the odds ratio (OR) changed by more than 10%; otherwise, they were excluded from the analysis. This process was repeated until all candidate variables significantly influenced (alpha < 0.05) the dependent variable. The survey data necessitated weighting during the analysis to address sampling considerations.

Results

Demographic characteristics

Across all regions, most participants received the measles vaccine (73.46%), antenatal care (ANC) at least once every trimester (68.14%), and at least one postnatal care (PNC) visit (53.59%). The majority of mothers were aged 25–34 years (48.52%), had primary education (50.54%), and were unemployed (57.42%). Most heads of household were aged ≥45 years (35.44%), had primary education (57.77%), and were employed in the informal sector (76.48%). Most households had convenient access to primary health centers (PHCs, ≤15 minutes, 73.39%) and clinics/general practitioners (≤15 minutes, 81.13%), while 39.11% lived ≥31 minutes from hospitals. A significant proportion of households fell within the first quintile of monthly expenditure per capita (26.65%) and resided in rural areas (59.69%) (Table II).

In Sumatera, Java, and Bali, the majority of mothers were aged 25–34 years, had primary school education, and were unemployed. The heads of household generally fell within the 35–44 age group, had primary school education, and were employed in the informal sector. Many households had convenient access to PHCs and clinics/general practitioners within ≤15 minutes, while hospitals were within 15–30 minutes. Most households belonged to the second quintile of monthly expenditure per capita, and the majority resided in rural areas. Most participants in Sumatera, Java, and Bali had

Table I. Definition and categorization of variables used in the study.

Variable	Categories	Description
Dependent Variables		
Measles vaccination	Vaccinated Not vaccinated	Indicates whether the child aged 12–23 months received measles vaccination. The information about vaccination data was obtained from the family especially mother and verified using the child's vaccination card
Independent Variables		
Antenatal care frequency	At least once per trimester Checked by non-healthcare personnel No ANC	Assesses adequacy of ANC received during pregnancy
Postnatal care frequency	PNC per period At least one PNC visit No PNC	Measures access to care provided after childbirth
Mother's age	<25 years 25–34 years 35–44 years ≥45 years	Maternal age at the time of survey
Maternal education	No schooling Primary Secondary Higher	Formal educational attainment of the mother
Maternal employment	Unemployed Informal sector Formal sector	Employment status of the mother
Age of the head of the household	<25 years 25–34 years 35–44 years ≥45 years	Age of the primary household decision-maker
Education status of the head of the household	No schooling Primary Secondary Higher	Formal educational attainment of the head of the household
Employment status of the head of the household	Unemployed Informal sector Formal sector	Employment status of the head of the household
Time to nearest hospital	≤15 minutes 15–30 minutes >30 minutes	Self-reported time required to reach the nearest hospital

ANC, antenatal care; GP, general practitioner; PHC; public health center; PNC, postnatal care.

Table I. Continued.

Variable	Categories	Description
Time to nearest PHC	≤15 minutes	Time required to reach the nearest primary health center
	15–30 minutes	
	>30 minutes	
Time to nearest Clinic/GP	≤15 minutes	Time to reach the nearest clinic or general practitioner
	15–30 minutes	
	>30 minutes	
Monthly expenditure per capita	Quintile 1 (lowest) to Quintile 5 (highest)	Total monthly household expenditure per person, categorized into quintiles
Residential classification	Urban	Classification based on household location
	Rural	

ANC, antenatal care; GP, general practitioner; PHC; public health center; PNC, postnatal care.

Table II. Participants' characteristics across three regions in Indonesia.

Characteristics	Sumatera, Java, and Bali (n = 11,523)	Nusa Tenggara, Kalimantan, and Sulawesi (n = 6,317)	Maluku and Papua (n = 1,589)	Total (n = 19,429)
	n (%)	n (%)	n (%)	n (%)
Measles vaccination				
Not vaccinated	3,107 (26.96)	1,423 (22.53)	627 (39.46)	5,157 (26.54)
Vaccinated	8,416 (73.04)	4,894 (77.47)	962 (60.54)	14,272 (73.46)
Antenatal care frequency				
At least once every trimester	8,635 (74.94)	3,941 (62.39)	663 (41.72)	13,239 (68.14)
Checked by non-healthcare personnel	313 (2.72)	244 (3.86)	91 (5.73)	648 (3.34)
No ANC	2,575 (22.35)	2,132 (33.75)	835 (52.55)	5,542 (28.52)
Postnatal Care Frequency				
PNC per period	3,718 (34.61)	1,722 (29.97)	294 (21.84)	5,734 (32.15)
At least once in a period	5,886 (54.78)	3,056 (53.19)	615 (45.69)	9,557 (53.59)
No PNC	1,140 (10.61)	967 (16.83)	437 (32.47)	2,544 (14.26)
Mother's Age				
<25 years	2,613 (23.47)	1,630 (27.07)	385 (26.14)	4,628 (24.84)
25-34 years	5,626 (50.53)	2,775 (46.08)	637 (43.25)	9,038 (48.52)
35-44 years	2,771 (24.89)	1,521 (25.26)	405 (27.49)	4,697 (25.21)
≥45 years	124 (1.11)	96 (1.59)	46 (3.12)	266 (1.43)
Maternal Education				
No schooling	175 (1.57)	165 (2.74)	113 (7.67)	453 (2.43)
Primary	5,381 (48.33)	3,304 (54.87)	730 (49.56)	9,415 (50.54)
Secondary	3,902 (35.05)	1,752 (29.09)	458 (31.09)	6,112 (32.81)
Higher	1,675 (15.05)	801 (13.30)	172 (11.68)	2,648 (14.22)

ANC, antenatal care; GP, general practitioner; PHC; public health center; PNC, postnatal care.

Table II. Continued.

Characteristics	Sumatera, Java, and Bali (n = 11,523)	Nusa Tenggara, Kalimantan, and Sulawesi (n = 6,317)	Maluku and Papua (n = 1,589)	Total (n = 19,429)
	n (%)	n (%)	n (%)	n (%)
Maternal employment				
Unemployed	6,619 (59.45)	3,384 (56.19)	694 (47.11)	10,697 (57.42)
Informal sector	3,283 (29.49)	2,127 (35.32)	650 (44.13)	6,060 (32.53)
Formal sector	1,231 (11.06)	511 (8.49)	129 (8.76)	1,871 (10.04)
Age of the head of the household				
<25 years	435 (3.78)	296 (4.69)	79 (4.97)	810 (4.17)
25-34 years	3,202(27.79)	1,647 (26.07)	435 (27.38)	5,284 (27.20)
35-44 years	3,873 (33.61)	2,035 (32.21)	542 (34.11)	6,450 (33.20)
≥45 years	4,013 (34.83)	2,339 (37.03)	533 (33.54)	6,885 (35.44)
Education status of the head of the household				
No schooling	455 (3.95)	393 (6.22)	99 (6.23)	947 (4.87)
Primary	6,558 (56.91)	3,820 (60.47)	847 (53.3)	11,225 (57.77)
Secondary	3,417 (29.65)	1,519 (24.05)	461 (29.01)	5,397 (27.78)
Higher	1,093 (9.49)	585 (9.26)	182 (11.45)	1,860 (9.57)
Employment status of the head of the household				
Unemployed	855 (7.42)	427 (6.76)	110 (6.92)	1,392 (7.16)
Informal sector	8,731 (75.77)	4,929 (78.03)	1,200 (75.52)	14,860 (76.48)
Formal sector	1937 (16.81)	961 (15.21)	279 (17.56)	3,177 (16.35)
Time to nearest hospital				
≤5 minutes	3,955 (36.65)	1,497 (26.41)	322 (25.91)	5,774 (32.62)
15-30 minutes	3,250 (30.12)	1,487 (26.23)	268 (21.56)	5,005 (28.27)
>30 minutes	3,586 (33.23)	2,685 (47.36)	653 (52.53)	6,924 (39.11)
Time to nearest PHC				
≤5 minutes	8,721 (76.89)	4,337 (69.5)	986 (63.49)	14,044 (73.39)
15-30 minutes	2,156 (19.01)	1,418 (22.72)	351 (22.60)	3,925 (20.51)
>30 minutes	465 (4.10)	485 (7.77)	216 (13.91)	1,166 (6.09)
Time to nearest clinic/GP				
≤5 minutes	8,940 (86.54)	2,826 (70.09)	385 (62.60)	12,151 (81.13)
15-30 minutes	1,106 (10.71)	750 (18.60)	93 (15.12)	1,949 (13.01)
>30 minutes	284 (2.75)	456 (11.31)	137 (22.28)	877 (5.86)
Monthly expenditure per capita				
Quintile 1	2,669 (23.16)	1,987 (31.45)	521 (32.79)	5,177 (26.65)
Quintile 2	2,711 (23.53)	1,321 (20.91)	310 (19.51)	4,342 (22.35)
Quintile 3	2,294 (19.91)	1,152 (18.24)	287 (18.06)	3,733 (19.21)
Quintile 4	2,046 (17.76)	996 (15.77)	249 (15.67)	3,291 (16.94)
Quintile 5	1,803 (15.65)	861 (13.63)	222 (13.97)	2,886 (14.85)
Residential classification				
Urban	5,405 (46.91)	2,016 (31.91)	411 (25.87)	7,832 (40.31)
Rural	6,118 (53.09)	4,301 (68.09)	1,178 (74.13)	11,597 (59.69)

ANC, antenatal care; GP, general practitioner; PHC; public health center; PNC, postnatal care.

received measles vaccination (73.04%), received ANC at least once every trimester (74.94%), and PNC per period (34.61%) (Table II).

In Nusa Tenggara, Kalimantan, Sulawesi, Maluku and Papua, Mothers were typically aged 25–34 years, had primary school education, and were unemployed. The heads of household were usually aged 35–44 years, had primary education, and were employed in the informal sector. Hospitals were generally reachable within >31 minutes, PHCs within 15–30 minutes, and clinics/general practitioners within ≤15 minutes. The monthly expenditure per capita was in the first quintile, and most respondents lived in rural areas. Most respondents in Nusa Tenggara, Kalimantan and Sulawesi, received the measles vaccine (77.47%), had ANC at least once every trimester (62.39%), and PNC per period (53.19%). In Maluku and Papua, 60.54% of children received the measles vaccine, but 52.55% of mothers reported no ANC visits (Table II).

Multivariate analysis

In Sumatera, Java, and Bali, the final model incorporated several variables, including the frequency of ANC and PNC, maternal education,the age and employment status of the head of household, travel time to the nearest hospital, clinic/general practitioner, and PHC, monthly expenditure per capita, and residential classification. The most significant predictors in this model were PNC per period (OR = 2.36; 95% CI: 1.86–2.99), higher maternal education (OR = 2.31; 95% CI: 1.30–4.10), and employment of the head of the household in the formal sector (OR = 2.00; 95% CI: 1.38–2.90; Table III).

For the Nusa Tenggara, Kalimantan, and Sulawesi regions, the final model identified key service-related variables, particularly the regularity of ANC and PNC appointments, underscoring the importance of consistent maternal healthcare. The most influential predictors were PNC per period (OR = 2.51; 95% CI: 1.92–3.28), secondary maternal education (OR = 2.87; 95% CI: 1.81–4.55), and higher maternal education (OR = 2.47; 95% CI: 1.50–4.08; Table III).

In Maluku and Papua, the final model included ANC and PNC frequency, maternal education, characteristics of the head of household, travel time to the nearest hospital, clinic/general practitioner, and PHC, monthly expenditure per capita, and residential classification. The most impactful factors were PNC per period (OR = 2.40; 95% CI: 1.89–3.04) and higher maternal education (OR = 2.57; 95% CI: 1.47–4.49; Table III).

The final model for all regions combined identified multiple factors influencing measles vaccination coverage. These included ANC and PNC frequency, maternal education, the age and employment status of the head of household, travel time to health facilities (hospital, clinic/general practitioner, and PHC), and residential classification. Among all variables, maternal education emerged as the most influential predictor. Children of mothers with the highest education level had significantly greater odds of receiving the measles vaccine (OR = 3.01; 95% CI: 1.87–4.84) after adjusting for all other covariates (Table III).

Discussion

Socio-demographic factors play a significant role in shaping routine childhood vaccination coverage, including measles immunization, and ultimately contribute to achieving herd immunity, which is essential for reducing morbidity and mortality among children. Understanding these determinants is crucial for identifying and eliminating barriers to immunization uptake.

PNC and maternal education were the strongest predictors of measles vaccination. Mothers who attended PNC services were significantly more likely to complete their child's vaccination schedule, consistent with prior evidence showing that postnatal and adequate ANC enhance immunization uptake.[22-25] Additionally, mothers with secondary or higher education

Table III. Comparison of measles vaccination characteristics among children aged 12-23 months in Indonesia.

Characteristic	Sumatera, Java, and Bali (n = 11,523)			Nusa Tenggara, Kalimantan, and Sulawesi (n = 6,317)			Maluku and Papua (n = 1,589)			Total (19,429)		
	%	COR (95% CI)	AOR (95%CI)	%	COR (95% CI)	AOR (95%CI)	%	COR (95% CI)	AOR (95%CI)	%	COR (95% CI)	AOR (95%CI)
Antenatal care frequency												
At least once every trimester	78.27	1.96 (1.70-2.25)***	1.45 (1.21-1.75)***	81.39	1.84 (1.55-2.18)***	1.48 (1.21-1.8)***	79.31	3.31 (2.45-4.49)***	1.45 (1.2-1.75)***	78.86	1.98 (1.78-2.2)***	1.42 (1.22-1.65)***
Checked by non-healthcare personnel	62.16	0.89 (0.63-1.26)	0.73 (0.50-1.06)*	70.97	1.03 (0.69-1.52)	1.00 (0.67-1.48)	69.73	1.99 (1.06-3.74)**	0.74 (0.5-1.08)	65.21	0.99 (0.77-1.28)	0.81 (0.59-1.10)
No ANC	64.78	1.00 (Reference)	1.00 (Reference)	70.42	1.00 (Reference)	1.00 (Reference)	53.64	1.00 (Reference)	1.00 (Reference)	65.35	1.00 (Reference)	1.00 (Reference)
Postnatal care frequency												
PNC per period	81.38	2.83 (2.30-3.49)***	2.36 (1.86-2.99)***	84.68	3.04 (2.35-3.94) ***	2.51 (1.92-3.28)***	79.95	3.67 (2.33-5.78)***	2.40 (1.89-3.04)***	81.89	2.89 (2.47-3.39)***	2.17 (1.78-2.64)***
At least once in a period	74.62	1.9 (1.56-2.31)***	1.69 (1.36-2.11)***	79.04	2.07 (1.66-2.58) ***	1.83 (1.46-2.28) ***	70.36	2.18 (1.49-3.2)***	1.69 (1.35-2.12)***	75.42	1.96 (1.70-2.26)***	1.59 (1.33-1.90)***
No PNC	60.71	1.00 (Reference)	1.00 (Reference)	64.53	1.00 (Reference)	1.00 (Reference)	52.08	1.00 (Reference)	1.00 (Reference)	61.00	1.00 (Reference)	1.00 (Reference)
Mother's age												
<25 years	74.85	1.00 (Reference)	-	76.34	1.00 (Reference)	-	63.30	1.00 (Reference)	-	74.72	1.00 (Reference)	-
25-34 years	76.04	1.07 (0.91-1.24)	-	78.86	1.16 (0.95-1.41)	-	66.92	1.17 (0.80-1.72)	-	76.27	1.09 (0.96-1.23)	-
35-44 years	75.68	1.05 (0.88-1.25)**	-	79.25	1.18 (0.94-1.48)	-	68.89	1.28 (0.83-1.99)	-	76.16	1.08 (0.94-1.24)	-
≥45 years	61.85	0.54 (0.33-0.91)	-	76.56	1.01 (0.50-2.05)	-	69.24	1.31 (0.57-3.00)	-	66.70	0.68 (0.46-1)**	-
Maternal education												
No schooling	52.78	1.00 (Reference)	1.00 (Reference)	61.20	1.00 (Reference)	1.00 (Reference)	26.47	1.00 (Reference)	1.00 (Reference)	50.78	1.00 (Reference)	
Primary	72.43	2.35 (1.52-3.63)***	1.98 (1.16-3.38)**	74.06	1.81 (1.20-2.73)**	1.71 (1.11-2.62)**	62.32	4.59 (2.51-8.42)***	1.97 (1.17-3.32) **	72.43	2.55 (1.92-3.37)***	2.20 (1.42-3.43)***
Secondary	78.09	3.19 (2.05-4.95)***	2.16 (1.25-3.72)**	83.75	3.27 (2.11-5.07)***	2.87 (1.81-4.55)***	74.83	8.26 (4.31-15.81)***	2.25 (1.33-3.81)**	78.99	3.65 (2.73-4.87)***	2.71 (1.73-4.24)***
Higher	81.80	4.02 (2.54-6.36)***	2.31 (1.30-4.10)**	83.18	3.14 (1.94-5.08)***	2.47 (1.50-4.08)***	79.35	10.67 (5.07-22.45)***	2.57 (1.47-4.49)**	82.03	4.42 (3.26-6.01)***	3.01 (1.87-4.84)***
Maternal employment												
Unemployed	74.41	1.00 (Reference)		78.22	1.00 (Reference)	-	68.88	1.00 (Reference)	-	74.97	1.00 (Reference)	-
Informal sector	75.27	1.05 (0.91-1.20)	-	76.98	0.93 (0.78-1.11)	-	60.70	0.70 (0.51-0.96)**	-	74.84	-	-
Formal sector	82.13	1.58 (1.28-1.96)***	-	82.02	1.27 (0.89-1.81)	-	79.33	1.73 (0.89-3.39)	-	82.03	-	-
Age of the head of the household												
<25 years	79.80	1.00 (Reference)	1.00 (Reference)	67.80	1.00 (Reference)	1.00 (Reference)	63.30	1.00 (Reference)	-	76.40	1.00 (Reference)	-
25-34 years	74.25	0.73 (0.52-1.02)*	0.64 (0.42-0.97)**	78.38	1.72 (1.22-2.43)**	1.63 (1.15-2.32) **	66.92	0.88 (0.42-1.85)	-	74.50	0.90 (0.7-1.16)	-
35-44 years	74.91	0.76 (0.55-1.05)*	0.72 (0.48-1.07)	77.42	1.63 (1.16-2.29)**	1.60 (1.12-2.28) **	68.89	1.22 (0.59-2.50)	-	75.19	0.94 (0.74-1.19)	-
≥45 years	75.69	0.79 (0.57-1.09)	0.86 (0.57-1.29)	78.90	1.78 (1.27-2.49)**	1.79 (1.26-2.53) **	69.24	1.08 (0.52-2.24)	-	76.07	0.98 (0.77-1.25)	-
Education status of the head of the household												
No schooling	69.41	1.00 (Reference)	-	71.54	1.00 (Reference)	-	30.86	1.00 (Reference)	-	67.89	1.00 (Reference)	1.00 (Reference)
Primary	73.73	1.24 (0.93-1.64)	-	75.27	1.21 (0.91-1.62)	-	60.11	3.38 (1.80-6.34)***	-	73.60	1.32 (1.07-1.62)***	0.86 (0.63-1.17)
Secondary	77.04	1.48 (1.10-2.00)**	-	81.43	1.74 (1.25-2.43)**	-	76.15	7.15 (3.63-14.11)***	-	77.77	1.65 (1.33-2.06)***	0.77 (0.55-1.07)
Higher	79.90	1.75 (1.24-2.48)***	-	84.44	2.16 (1.42-3.29)***	-	73.71	6.28 (2.86-13.78)***	-	80.63	1.97 (1.52-2.55)***	0.75 (0.50-1.12)

ANC, antenatal care; AOR, adjusted odds ratio; CI, confidence interval; COR, crude odda ratio; GP, general practitioner; PHC; public health center; PNC, postnatal care; ***p <0.01; **p <0.05; *p < 0.1.

Table III. Continued.

Characteristic	Sumatera, Java, and Bali (n = 11,523)			Nusa Tenggara, Kalimantan, and Sulawesi (n = 6,317)			Maluku and Papua (n = 1,589)			Total (19,429)		
	%	COR (95% CI)	AOR (95%CI)	%	COR (95% CI)	AOR (95%CI)	%	COR (95% CI)	AOR (95%CI)	%	COR (95% CI)	AOR (95%CI)
Employment status of the head of the household												
Unemployed	70.13	1.00 (Reference)	1.00 (Reference)	77.90	1.00 (Reference)		71.14	1.00 (Reference)		71.74	1.00 (Reference)	1.00 (Reference)
Informal sector	73.61	1.19 (0.95-1.49)	1.26 (0.93-1.71)	76.75	0.94 (0.67-1.30)	-	62.40	0.67 (0.34-1.34)	-	73.83	1.11 (0.92-1.34)	1.10 (0.85-1.42)
Formal sector	82.49	2.01 (1.52-2.65)***	2.00 (1.38-2.90)***	81.28	1.23 (0.83-1.83)	-	75.88	1.28 (0.59-2.78)	-	82.03	1.8 (1.43-2.26)***	1.64 (1.19-2.24)***
Time to nearest hospital												
≤15 minutes	79.64	1.65 (1.41-1.93)***	1.54 (1.22-1.93)***	79.40	1.11 (0.89-1.39)	-	79.69	2.53 (1.62-3.95)***	1.57 (1.25-1.97)***	79.60	1.53 (1.35-1.74)***	1.47 (1.21-1.80)***
15-30 minutes	76.76	1.40 (1.18-1.65)***	1.29 (1.06-1.57)**	78.89	1.08 (0.87-1.33)	-	72.72	1.72 (1.11-2.66)**	1.29 (1.06-1.57)**	77.05	1.32 (1.15-1.51)***	1.26 (1.06-1.50)**
>30 minutes	70.30	1.00 (Reference)	1.00 (Reference)	77.64	1.00 (Reference)	-	60.80	1.00 (Reference)	1.00 (Reference)	71.80	1.00 (Reference)	1.00 (Reference)
Time to nearest PHC												
≤15 minutes	77.17	2.13 (1.61-2.83)***	1.51 (1.00-2.28)**	79.15	1.58 (1.21-2.07)**	-	71.48	3.02 (1.93-4.71)***	1.51 (0.99-2.29)*	77.37	2.07 (1.7-2.51)***	1.49 (1.07-2.08)**
15-30 minutes	70.91	1.54 (1.14-2.09)	1.24 (0.81-1.89)	76.65	1.37 (1.01-1.86)*	-	63.29	2.08 (1.26-3.42)**	1.24 (0.8-1.9)	71.89	1.55 (1.25-1.91)***	1.25 (0.89-1.76)
>30 minutes	61.31	1.00 (Reference)	1.00 (Reference)	70.59	1.00 (Reference)	-	45.38	1.00 (Reference)	1.00 (Reference)	62.34	1.00 (Reference)	1.00 (Reference)
Time to nearest clinic/GP												
≤15 minutes	76.91	2.35 (1.63-3.39)***	1.86 (1.21-2.86)***	79.56	1.18 (0.87-1.61)	-	79.75	2.12 (1.19-3.78)**	1.82(1.17-2.85)**	77.33	1.71 (1.36-2.17)***	1.27 (0.95-1.70)
15-30 minutes	71.77	1.79 (1.20-2.67)***	1.78 (1.12-2.84)**	79.63	1.19 (0.82-1.72)	-	79.76	2.12 (0.98-4.58)*	1.74(1.08-2.81)**	74.00	1.43 (1.10-1.86)***	1.27 (0.92-1.75)
>30 minutes	58.64	1.00 (Reference)	1.00 (Reference)	76.67	1.00 (Reference)	-	64.99	1.00 (Reference)	1.00 (Reference)	66.56	1.00 (Reference)	1.00 (Reference)
Monthly expenditure per capita												
Quintile 1	73.54	1.00 (Reference)	1.00 (Reference)	73.19	1.00 (Reference)	-	55.75	1.00 (Reference)	1.00 (Reference)	72.63	1.00 (Reference)	-
Quintile 2	73.84	1.02 (0.86-1.19)	0.94 (0.77-1.15)	78.16	1.31 (1.05-1.64)**	-	61.92	1.29 (0.86-1.93)	0.94 (0.77-1.15)	74.30	1.09 (0.96-1.24)	-
Quintile 3	72.01	0.93 (0.77-1.12)	0.76 (0.61-0.94)**	78.37	1.33 (1.06-1.67)**	-	67.21	1.63 (1.06-2.50)**	0.76 (0.61-0.95)**	73.09	1.02 (0.88-1.19)	-
Quintile 4	75.50	1.11 (0.91-1.35)	0.82 (0.65-1.04)	79.03	1.38 (1.08-1.77)**	-	72.73	2.12 (1.30-3.44)**	0.86 (0.68-1.09)	76.06	1.20 (1.02-1.4)**	-
Quintile 5	81.93	1.63 (1.32-2.02)***	1.02 (0.77-1.35)	83.00	1.79 (1.32-2.43) ***	-	75.59	2.46 (1.49-4.05)***	1.09 (0.83-1.43)	81.88	1.70 (1.43-2.02)***	-
Residential classification												
Urban	76.38	1.16 (1.03-1.32)**	0.70 (0.59-0.84)***	78.42	1.08 (0.90-1.30)	-	78.57	2.54 (1.76-3.66)***	0.72 (0.61-0.87)***	76.75	1.18 (1.06-1.31)**	0.71 (0.61-0.84)***
Rural	73.55	1.00 (Reference)	1.00 (Reference)	77.09	1.00 (Reference)	-	59.07	1.00 (Reference)	1.00 (Reference)	73.66	1.00 (Reference)	1.00 (Reference)

ANC, antenatal care; AOR, adjusted odds ratio; CI, confidence interval; COR, crude odda ratio; GP, general practitioner; PHC; public health center; PNC, postnatal care; ***p <0.01; **p <0.05; *p < 0.1.

were more likely to adhere to recommended vaccination schedules, likely due to better health literacy and access to information.[24,26,27] This underscores the importance of integrating maternal health services and education in immunization programs.

The characteristics of the heads of households, also influenced vaccination uptake. Our findings showed higher vaccination rates among children in households where the heads of household were older and employed in the formal sector. According to research conducted in Ghana, children are more likely to receive all recommended vaccinations when fathers are actively involved in the decision making process.[28,29] In line with this, our analysis indicates that additional factors also influence vaccination coverage among children. Higher vaccination rates were observed among children in households where the heads of household were aged more than twenty five years and employed in the formal sector across regions such as Sumatra, Java, Bali, Nusa Tenggara, Kalimantan, and Sulawesi. A previous study reported that parents within the younger age group of twenty to twenty nine years were less likely to vaccinate their children due to concerns about the safety and effectiveness of vaccines. This study further shows that fathers who are employed are generally less likely to refuse childhood vaccination.[30] Moreover, fathers who held negative perceptions toward child immunization were often employed in manual labor or physically demanding occupations.[31]

The Riskesdas 2018 study did not specifically collect information about the fathers of the children; instead it obtained data on the head of household and the mother. Consequently, both the original dataset and the present analysis derived from it carry an inherent gender bias, as they operate under the assumption that heads of household are rarely the mother. Given the observed differences in the demographic profiles of the heads of household and of the mothers, it can be reasonably inferred that mothers were generally not considered heads of household. However, the absence of paternal data limits the ability to examine theinfluence of the fathers on child health outcomes, including vaccination behaviors.

Access to healthcare services, particularly shorter travel times to hospitals, clinics, or health centers, significantly increased the likelihood of timely vaccination. This is supported by previous findings that even small increases in travel distance can reduce vaccine uptake.[24,32] Furthermore, an additional one kilometer in travel distance to the nearest health facility has been shown to reduce the likelihood of vaccination by five percent. Although no direct correlation was found between the nearest health facility and dropout rates in vaccination, long distances may contribute to delayed immunization, especially during the final stages of the vaccination schedule.[33] In Maluku and Papua, lower measles vaccination coverage may be attributed to several interrelated factors beyond individual socio-demographic characteristics. These regions face systemic challenges, including limited healthcare infrastructure, geographic isolation, and difficult terrain, which hinder access to routine immunization services. Additionally, there is a chronic shortage of health professionals and inadequate distribution of vaccines in remote areas. Sociopolitical dynamics, such as historical marginalization and underinvestment in public health, further exacerbate these disparities. Addressing these challenges requires region-specific strategies that prioritize infrastructure development, community outreach, and equitable allocation of healthcare resources.

Socioeconomic status and area of residence significantly influenced vaccination rates. Families experiencing economic hardship are often less likely to complete childhood vaccinations, resulting in lower immunization coverage.[34] In contrast, vaccination rates tend to be higher among families with better financial conditions and usually improve as the family's economic situation becomes more stable.[35] Children from wealthier families and urban areas were more likely to be vaccinated, consistent with findings from both sub-Saharan Africa

and Indonesia.[26,36] This finding underscores the persistent disparity in immunization coverage between urban and rural populations. It also highlights the increased risk faced by children in rural areas, who are more likely to experience missed or delayed vaccinations.[37]

This study presents several notable strengths that enhance the significance and impact of its findings. Firstly, the utilization of nationally representative data facilitates a high degree of generalizability, rendering the conclusions applicable not only at the local or regional level but also across the entire population. Such a comprehensive data source ensures that the findings accurately reflect the true dynamics of the population, which is particularly crucial for informing evidence-based public health planning and policymaking. By employing large-scale population data, this study offers a comprehensive depiction of vaccination coverage and the social factors influencing it in Indonesia. The insights derived are valuable not only for national stakeholders but also contribute to the global understanding of how social determinants shape vaccination outcomes. This evidence can assist policymakers and public health professionals in making strategic decisions aimed at designing inclusive and effective immunization programs.

Despite its strengths, the study has certain limitations. A primary limitation is the absence of specific behavioral and attitudinal variables, such as trust in vaccines, parental beliefs, and the impact of misinformation. These elements are increasingly recognized as critical in understanding vaccine acceptance and behavior, and their absence limits the ability to fully explain variations in vaccine uptake. Additionally, the study may be affected by unmeasured confounding factors, including cultural beliefs, community-level influences, the quality of healthcare services, or gender inequality, which could bias the results. Furthermore, the analysis did not assess potential interaction effects between variables due to constraints in the available dataset. Including such analyses in future research could provide deeper insights into the complex relationships affecting vaccination coverage.

This study provides meaningful evidence for policymakers and public health professionals, particularly in Indonesia. It contributes to the global understanding of how social determinants shape vaccination coverage and supports the integration of maternal healthcare with immunization programs. The identification of PNC and maternal education as strong determinants of vaccination uptake has clear implications for policy. These findings may guide targeted interventions in maternal and child health services. Future research should expand the current analysis by exploring behavioral and psychological drivers of vaccine uptake, such as health beliefs, perceived barriers, and trust in healthcare systems. Additionally, mixed-methods studies that incorporate qualitative data from caregivers and healthcare providers may offer deeper insights into contextual challenges and facilitate the development of culturally sensitive interventions. Strengthening maternal education and expanding access to PNC services, particularly in underserved and remote areas, should also be prioritized as part of policy efforts aimed at improving routine childhood immunization.

Conclusion

This study revealed that measles vaccination coverage among children aged 12–23 months in Indonesia is influenced by maternal healthcare utilization, maternal education, and access to health services. Specifically, frequent PNC visits and higher maternal education significantly increased the likelihood of vaccination. Household characteristics, travel time to healthcare facilities, and socioeconomic factors also played important roles. Regional disparities in coverage emphasize the need for targeted interventions, especially in underserved areas like Maluku and Papua. Strengthening maternal and child health services, improving accessibility, and promoting health education are critical steps toward achieving higher

vaccination coverage and reducing preventable childhood illnesses.

Ethical approval

The study was approved by Health Research Ethics Committee of the National Institute of Health Research and Development, Ministry of Health, Republic of Indonesia (date: July 28, 2017, number: LB.02.01/KE.267/2017).

Author contribution

The authors confirm contribution to the paper as follows: Study conception and design: AH, IRI, MW; data collection: AH; analysis and interpretation of results: AH, IRI, MW, HA; draft manuscript preparation: AH, HA, RR. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Original Article

Differences between multi-triggered and single-triggered food anaphylaxis in children: a real life study

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ABSTRACT

Background. Food-induced anaphylaxis (FIA) is a severe form of food allergy, and literature data about multi-triggered FIA is scarce. This study aimed to evaluate the differences between multi-triggered and single-triggered food anaphylaxis in children.

Methods. The study included pediatric patients (age <18 years) who were diagnosed with FIA between January 1, 2015 and February 28, 2023. Demographic data, clinical features, laboratory findings, and allergological work-up results were evaluated from the patients' records.

Results. A total of 459 patients (64.1% male) were evaluated. The median age at onset (first anaphylactic reaction) was 12 months (interquartile range [IQR]: 6-24 months). Food anaphylaxis with multiple foods was reported in 114 of the 459 children (24.8%). Multi-triggered FIA was most commonly associated with combinations of milk and egg (n=21) and tree nut and tree nut (n=21). Atopic disease, asthma, higher total IgE level, and higher eosinophil count were more frequent in patients with multi-triggered FIA. In multivariate regression analysis, total IgE >100 IU/mL was identified as a predictive factor for multi-triggered FIA (Odds ratio [95% confidence interval]: 2.46 [1.40-4.30], p=0.001).

Conclusions. FIA with multiple trigger foods was detected in approximately a quarter of the children with FIA. Multi-triggered FIA was associated with higher rates of atopic disease, asthma, eosinophilia, and increased total IgE levels. A total IgE level higher than 100 IU/mL was a risk factor for multi-triggered FIA. This suggests that high IgE levels may be a warning sign for clinicians to be vigilant for multiple food triggers in the screening and follow-up of FIA patients.

Key words: anaphylaxis, food induced anaphylaxis, multiple triggered, single triggered, total IgE.

Anaphylaxis is a severe, life-threating disease and foods are the main triggers of anaphylaxis in children.[1-5] Food-induced anaphylaxis (FIA) can sometimes occur with more than one food, which is referred to as multi-triggered FIA. However, there is limited information about this in the literature, and the true prevalence of multi-triggered FIA remains unclear.

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Identifying genuine cases of anaphylaxis to multiple foods is challenging due to the severity of the reaction, fear of oral challenge testing, and/or cross-reactivity. McIntyre et al. reported that approximately a third of anaphylaxis cases occurred with multiple substances, and approximately two-thirds (66%) of the reactions in patients with multiple allergies were related to multiple foods.[6]

A recent study indicated that multi-food allergic individuals were epigenetically distinct from single-food allergic individuals.[7] Other studies reported that patients with multiple food allergy (MFA) had more food allergy-related adverse events[8], as well as more severe food-induced allergic reactions and more frequent emergency department visits.[9] Patients with MFA were also reported to have an increased prevalence of atopic comorbidities and asthma morbidity[10-12], while highly atopic children may be prone to having allergies to multiple foods.[13] MFA can result in increased accidental ingestions and may cause fatal/near-fatal reactions.[9] Several studies reported that MFA imposes a greater physical health impact, quality of life impairment, and economic burden than single food allergy (SFA).[11,13,14] These impacts may be exacerbated in patients with multi-triggered FIA because of the rigid elimination requirements and later development of tolerance. Although data about multi-triggered FIA are scarce, we hypothesized that these patients may exhibit differences from those with single-triggered FIA, like in MFA.

It is known that having one atopic disease can lead to having another; thus, a SFA may predispose to additional food allergies.[15] However, we are aware of no study published to date that demonstrates predictive factors for multi-triggered FIA.

In the present study, we examined the differences in the characteristics of children with FIA to single and multiple foods and assessed the value of certain factors in predicting multi-triggered FIA.

Materials and Methods

In this single-center retrospective study, children (age <18 years) who presented to our pediatric allergy and immunology clinic with FIA between 2015 and 2023 were identified by screening patient records in the hospital's electronic database. If the records were incomplete, we contacted the patient's family to obtain missing data.

We recorded patient data in a standardized form that included their demographic and clinical characteristics, laboratory results (eosinophil count and percentage; tryptase levels and total IgE levels at presentation), and allergological work-up results (baseline specific IgE [sIgE] levels, skin prick test (SPT) wheal diameter, and oral food challenge (OFC) results. Results closest to the anaphylaxis reaction were recorded.

FIA was defined according to the criteria established by the European Academy of Allergy and Clinical Immunology (EAACI) task force position papers on childhood anaphylaxis management.[1,16] All cases were evaluated by pediatric allergology physicians based on clinical history and complementary allergy tests (skin prick test / sIgE / Tryptase levels / open food challenge) cases were deemed to involve anaphylaxis triggered by a specific food when these assessments confirmed the diagnosis. Tryptase levels were considered if the patient was admitted within six hours after the reaction or if the reaction occurred during an open food challenge. Open food challenge tests were conducted in appropriate cases, provided parental informed consent was obtained and the case was considered non severe.

Trigger foods were identified through reaction history and allergological testing, including SPT, serum sIgE levels (measured using the Immulite® 2000 System; Siemens Healthcare Diagnostics, Tarrytown, New York), and/or OFC tests. sIgE levels were regarded as positive when equal to or greater than 0.35

kU/L. SPT was performed at least four to six weeks after the anaphylactic reaction, with antihistamines discontinued one week before testing. SPT was conducted using commercial preparations (ALK, Abello, Madrid, Spain) or fresh foods (e.g., vegetables, fruit, and fish) via the prick-to-prick method following EAACI guidelines.[17] SPT was performed on the back in younger children and on the inner forearm in older children. Maximum horizontal and vertical wheal diameters were measured after 15 minutes, with a reaction considered positive if the mean diameter exceeded the negative control by at least 3 mm.

After obtaining informed parental consent; open OFCs were performed in our allergy unit under the supervision of an allergist according to EAACI guidelines.[17] A stepwise OFC was preferred for milk (baked, then yogurt, then milk) and eggs (baked, then boiled). In the open OFC, the culprit food was freshly prepared and administered orally in incrementally increasing doses every 15 minutes. OFCs were considered positive if objective symptoms occurred within two hours of the last challenge dose.

Trigger foods were categorized into eight groups: milk, egg, treenuts, legumes, seafood, fruits/vegetables, seeds, and other. Treenuts included hazelnuts, walnuts, cashews, almonds, and pistachios. Legumes comprised lentils, peanuts, chickpeas, beans, peas, and soy. The seeds group included sesame, poppy, sunflower, and pumpkin seeds. Fruits/vegetables included banana, kiwi, potato, peach, coconut, strawberry, tomato, apple, melon, olive, and red pepper. The "other" category encompassed honey, meats (chicken and beef), sumac, cinnamon, and wheat.

Anaphylaxis triggered by more than one food was classified as multi-triggered FIA.[18] However, due to the retrospective nature of the study, cross-reactivity could not be ruled out and food-specific components could not be measured in our hospital. Nevertheless, as all included patients met EAACI anaphylaxis criteria, demonstrated clinical reactivity and had confirmed allergens based on sIgE, SPT and/or OFC, all cases of anaphylaxis involving multiple foods were included in the statistical analysis as part of the "multi-triggered FIA" group. This classification applied even when multiple tree nuts or legumes were involved.[18] Patients with multiple food allergies who experienced anaphylaxis triggered by only one food were classified as having single triggered anaphylaxis.

All patients were assessed for other atopic diseases according to international guidelines by a pediatric allergy immunology specialist.[19-21] Patients were also evaluated for mastocytosis based on clinical history (recurrent, venom-induced or unexplained anaphylaxis), physical examination (typical skin lesions, hepatosplenomegaly) and/or baseline tryptase levels.

Ethics

This study protocol was reviewed and approved by Ankara Bilkent City Hospital No. 2 Clinical Research Ethics Committee (date: 15.03.2023, number: E2-23-3593).

Statistics

SPSS version 22.0 (IBM Corp, Armonk, NY) was used for statistical analyses. The data were presented as median and interquartile ranges (IQR) because the data were not normally distributed. For comparisons of groups, we used the chi-squared (χ^2) and Fisher's exact tests for qualitative variables and the Mann-Whitney U and Wilcoxon rank sum tests for quantitative variables. Univariate and multivariate analysis were used to identify risk factors for multi-triggered FIA. Results with P values < 0.005 were considered statistically significant.

Results

A total of 459 patients (599 reactions) met the inclusion criteria. The median age at the time of the first anaphylactic reaction was 12 months (IQR: 6-24 months) and 64.21% (n = 294) were male. The baseline characteristics of the patients are summarized in Table I and characteristics of the reactions are delineated in Table II.

Multi-triggered FIA

Food anaphylaxis involving more than one food was observed in 114 of the 459 children (24.8%), accounting for 254 reactions. Among these, ninety-two patients experienced anaphylactic reactions at different times with two different foods, 19 with three different foods, 2 patients with four different foods, and 1 patient with five different foods (Supplementary Table: Triggered foods in patients with multi-triggered FIA). The most common food combinations associated with multi-triggered FIA were milk/egg (n=21) and treenut/treenut (two different treenut co-anaphylaxis) (n=21; with walnut/hazelnut (n=15) being the most frequent pairing; combinations are shown in the Supplementary Table).

Children with multi-triggered FIA had higher rates of atopic disease and asthma, as well as elevated IgE and eosinophil levels compared to those with single-triggered FIA, (p= 0.049, p= 0.010, p= <0.001, p=0.010, respectively). The similarities and differences between these groups are detailed in Table I.

Multivariate regression analysis identified, a total IgE level >100 IU/mL as a risk factor for multi-triggered FIA (Odds ratio [95% CI]: 2.46 [1.40-4.30], p=0.001, Table III).

Severity of reactions

Among the 345 patients with single triggered FIA; and 57.1% (n=197) of reactions were moderate; 26.1% (n=90) of them were mild and 16.8 % (n= 58) were severe. In contrast, the 114 patients with multi-triggered FIA experienced 254 reactions; of which 67.7% (n=172) were moderate, 20.9 % (n:53) were mild and 11.4% (n=29) were severe.

Table I. Demographic characteristics of children and comparison between anaphylaxis cases triggered by single versus multiple food allergens.

Parameters	All patients (n: 459)	Patients with single triggered food allergy (n: 345)	Patients with multiple triggered food allergy (n: 114)	p
Age at first anaphylaxis (months), median (IQR)	12 (6-24)	12 (6-24)	24 (12-48)	0.052
Male gender, n (%)	294 (64.1)	223 (64.6)	71 (62.2)	0.649
Atopic diseases, n (%)	365 (79.5)	267 (77.3)	98 (85.9)	0.049
Asthma	206 (44.9)	143 (41)	63 (55)	0.010
Atopic dermatitis	227 (49.5)	166 (48.1)	61 (53.5)	0.318
Allergic rhinitis	85 (18.5)	57 (16.5)	28 (24.5)	0.055
History of family atopic disease, n (%)	118 (25.7)	87 (25)	31 (27)	0.642
Multiple food allergy	301 (65.5)	187 (54.2)	114 (100)	NA
Co-anapyhylaxis with different foods, n (%)	114 (24.8)	-	114 (100)	NA
Recurrent anaphylaxis with same food, n (%)	161 (35.1)	124 (35.9)	37(33.3)	0.652
Anaphylaxis was the first symptom, n (%)	290 (63.2)	221 (64.1)	-	NA
Total IgE (IU/mL), median (IQR)	680 (49.9-560)	115 (33-404)	273 (89-872)	<0.001
Eosinophil count ($\times 10^9$/L), median (IQR)	400 (200-640)	360 (200-600)	480 (280-700)	0.011

IQR, interquartile range; NA, not analyzed.

Table II. Characteristics of reactions (n: 599).

	Total reactions (n: 599)	Reactions with single triggered food (n: 345)	Reactions with multi-triggered food (n: 254)
Types of food, n (%)			
Milk	178 (29.7)	137 (39.7)	41 (16.1)
Egg	114 (19)	76 (22)	38 (15)
Tree nut	156 (26)	58 (16.8)	98 (38.6)
Hazelnut	58 (9.6)	17 (4.9)	41 (16.1)
Walnut	39 (6.6)	15 (4.3)	24 (9.4)
Pistachio	34 (5.6)	18 (5.4)	16 (6.3)
Cashew	15 (2.5)	6 (1.7)	9 (3.5)
Almond	10 (1.6)	2 (0.6)	8 (3.1)
Legume	61 (10.1)	20 (5.8)	41 (16.1)
Lentil	27 (4.5)	11 (3.2)	16 (6.3)
Peanut	14 (2.3)	5 (1.5)	9 (3.5)
Chickpea	13 (2.1)	4 (1.2)	9 (3.5)
Beans	5 (0.7)	-	5 (3.5)
Peas	1 (0.1)	-	1 (0.4)
Soy	1 (0.1)	-	1 (0.4)
Fish	16 (2.6)	14 (4.1)	2 (0.8)
Fruits/vegetables	30 (5)	14 (4.1)	16 (6.8)
Banana	7 (1.1)	4 (1.2)	3 (1.2)
Kiwi	6 (1)	2 (0.6)	4 (1.6)
Potatoes	5 (0.8)	2 (0.6)	3 (1.2)
Peach	3 (0.5)	2 (0.6)	1 (0.4)
Coconut	2 (0.3)	1 (0.3)	1 (0.4)
Strawberry	2 (0.3)	1 (0.3)	1 (0.4)
Tomato	1 (0.1)	1 (0.3)	-
Apple	1 (0.1)	-	1 (0.4)
Melon	1 (0.1)	-	1 (0.4)
Red pepper	1 (0.1)	1 (0.3)	-
Olive	1 (0.1)	-	1 (0.1)
Seeds	26 (4.3)	19 (5.5)	7 (2.8)
Sesame	20 (3.3)	15 (4.3)	5 (2)
Poppy	3 (0.5)	1 (0.3)	2 (0.8)
Sun flower seed	2 (0.3)	2 (0.6)	-
Pumpkin	1 (0.1)	1 (0.3)	-
Other	18 (30)	7 (2)	11 (4.3)
Meats (5 chicken, 5 beef)	10 (1.6)	2 (0.6)	8 (3.2)
Honey	4 (0.6)	3 (0.9)	1 (0.4)
Wheat	2 (0.3)	-	2 (0.8)
Sumac	1 (0.1)	1 (0.3)	-
Cinnamon	1 (0.1)	1 (0.3)	-
Symptoms, n (%)			
Skin	571 (95.3)	333 (96.5)	238 (93.7)
GIS	274 (45.7)	171 (49.6)	103 (40.6)
Respiratory	478 (79.7)	260 (75.4)	218 (85.8)
Neurological	51 (8.5)	36 (10.4)	15 (5.9)
Cardiovascular	13 (2.1)	8 (2.3)	5 (2)
Severity of anaphylaxis, n (%)			
Mild	143 (23.8)	90 (26.1)	53 (20.9)
Moderate	369 (61.6)	197 (57.1)	172 (67.7)
Severe	87 (14.5)	58 (16.8)	29 (11.4)
Reaction time (minute), median (IQR)	5 (5-15)	5 (5-15)	5 (5-15)

GIS, gastrointestinal system; IQR: interquartile range, sIgE: specific immunoglobulin E, SPT: skin prick test.

Table III. Predictive factors for food-induced anaphylaxis with multiple triggering foods.

Parameters	Univariant			Multivariant		
	OR	95% CI	p	OR	95% CI	p
Gender (male)	0.90	0.58-1.40	0.649			
>2 years of age	2.08	1.30-3.32	0.002	1.31	0.71-2.44	0.307
Presence of other atopic disease	1.78	0.99-3.21	0.051	1.40	0.62-3.18	0.177
Atopic dermatitis	1.24	0.81-1.89	0.318			
Asthma	1.74	1.13-2.67	0.011	1.29	0.72-2.31	0.121
Allergic rhinitis	1.64	0.98-2.74	0.057	1.11	0.56-2.22	0.327
Family atopic disease history	1.10	0.68-1.78	0.67			
Total IgE >100 IU/mL	2.56	1.52-4.33	0.000	2.46	1.40-4.30	0.001
Eosinophil ≥400×10^9/L	2.079	1.24-3.48	0.005	1.62	0.93-2.81	0.081

CI, confidence interval; IgE, immunglobulin E; OR, odds ratio.

Unusual triggers

Four patients experienced anaphylaxis after consuming honey, none of whom had pollen atopy, although one had a history of venom anaphylaxis. Five patients had anaphylaxis triggered by potatoes with all reactions occurring after consuming baked potatoes. Two patients experienced anaphylaxis triggered by red meat, both reacting to well-cooked meat despite no known exposure to ticks.

Discussion

This single-centered, real-life study, examines the differences between multi-triggered and single-triggered food anaphylaxis in children. Multi-triggered FIA was detected in 24.8% of the patients with FIA. Patients with multi-triggered FIA were more likely to have atopic disease, asthma, higher total IgE levels, and higher eosinophil counts. Additionally, a total IgE level above 100 IU/mL was associated with an increased risk of multi-triggered FIA.

Food allergens are common triggers of anaphylaxis in children.[1,2,4,5] While the exact prevalence of FIA remains uncertain, non-fatal FIA reactions have been reported at an incidence of 0.5-16 per 100000 person-years.[22] In a study on epinephrine administration for life-threatening allergic reactions in school settings, McIntyre et al. reported that over one-third (36%) of reactions occurred in individuals allergic to multiple substances, with approximately two-thirds (66%) of these reactions involving multiple foods.[6] Although there is limited data about the prevalence of multi-triggered FIA, several studies reported MFA in 30-40% of children with food allergy.[9,18,23] In the current study, 24.8% of FIA cases involved multiple trigger foods.

Individual food allergen types in FIA may vary according to culture and population.[22] The most commonly reported triggers in FIA included cow's milk, hen's egg, peanut, tree nuts, shellfish, and fish.[2] McIntyre et al. reported that among patients who reported multiple allergies, the most common allergens listed were tree nuts (54%) and peanuts (51%).[6] In our study, multi-triggered FIA was most frequently associated with milk and eggs or a combination of tree nuts.

Many studies have investigated co-allergy and co-sensitization to treenuts. The most common treenut co-allergens vary by country depending on consumption patterns, with pistachio/cashew and walnut/pecan among the most frequently reported combinations.[24,25] In contrast, we observed in this study that walnut and hazelnut were the most common tree nut combinations associated with multi-triggered FIA. This is not surprising, considering that walnut and hazelnut are the most consumed treenuts in

our country.[26] Another study from our country showed that walnut-allergic children have a higher rate of concomitant hazelnut allergy.[25] Furthermore, unlike the aforementioned studies, our study focused on the most severe cases (anaphylaxis), and reaction severity may differ according to the combination of foods. Two components of hazelnut, Cor a 9 and Cor a14, are members of a protein family known to cause severe anaphylactic reactions.[24]

In our study, milk/egg was the most common combination of anaphylaxis-inducing foods overall. This may be attributed to the age distribution of our sample, which was predominantly composed of infants. The intake ratio of a food is a determining factor in the development of allergic reactions[24], and infants typically consume milk and egg more than other types of foods. We also observed that egg in particular was the most common co-allergen food in children with multi-triggered FIA. A mouse model study illustrated that primary ovalbumin sensitization enhanced sensitization to a secondary unrelated allergen (latex).[27] Our findings may support this observation. In addition, Cetinkaya et al. reported egg white as the most common co-allergen other than tree nut/peanut in patients with tree nut/peanut allergy, but only 41.4% of the patients in their study had anaphylactic reactions.[25] Masthoff et al. reported that peanut allergy was common among hazelnut-sensitized patients but was not primarily due to IgE cross-reactivity. They indicated that it may be a result of having an atopic predisposition, and being exposed to tree nuts and peanuts most likely increased the risk for co-sensitization to both foods.[28] They also proposed that T-cell cross-reactivity could contribute to this phenomenon.[28] Further research is needed to understand why egg or tree nuts tend to cause multi-triggered FIA as observed in our study and previous investigations.

A study indicated that individuals with MFA exhibited epigenetic differences from individuals allergic to a single food, detected as variations in DNA methylation between these groups.[7] Therefore, there may also be some epigenetic differences between multi-triggered FIA and single-triggered FIA. Furthermore, certain immunological differences were reported previously between single- and multi-triggered food allergies.[8,27,29] It is well established that DOCK8 deficiency is likely associated with anaphylaxis with multiple foods.[29] However, there is limited information about the pathophysiologic, genetic/epigenetic, and clinical differences between single-triggered and multi-triggered FIA. Understanding these differences and identifying predisposing factors for multi-triggered FIA could aid in prevention and management. Previous studies have shown that MFA is linked to greater impairment in quality of life, higher rates of concomitant allergic disease, and increased reaction severity.[9,11,18] Warren et al. reported that the prevalence of physician-diagnosed atopic comorbidities increased significantly as the number of reported convincing food allergies increased.[18] Wang also stated that highly atopic children may be at greater risk of developing allergies to multiple foods[13], while Hill et al. reported that individuals with MFA were at increased risk of developing asthma and rhinitis compared to patients with a SFA.[11] In our study, concomitant atopic disease, especially asthma was more frequent in children with multi-triggered FIA. Asthma may contribute to the development of anaphylaxis by increasing the severity of reactions. Moreover, we detected that total IgE levels and eosinophil counts were higher in children with multi-triggered FIA than in those with single-triggered FIA. Similarly, Blumchen et al. reported that total IgE levels and Th2 responses were higher in double allergen sensitized mice than single allergen sensitized mice, and that the strength of the primary sensitization is an important factor in increasing Th2 responses to the secondary allergen.[27]

Data about predictors of multi-triggered FIA are also scarce. In previous tree nut studies investigating multiple nut allergies, patient age was reported to be an important factor

influencing the prevalence of MFA.[2,24,30] It was also noted that because the number of nuts consumed increased with age, older age may be associated with multiple nut allergies.[30] Çetinkaya et al. reported that a current age of 6-10 years and a family history of atopy were risk factors for multiple tree nut/peanut allergies.[25] Waren et al. determined that MFA was more prevalent among non-Hispanic Black and Asian children compared to non-Hispanic White children.[18] Ruran et al. suggested that social and environmental risk factors and other comorbidities may also predispose patients to develop MFA.[9] As mentioned above, atopic children may have a higher risk for MFA.[13] In addition, Allen et al. reported that infants with vitamin D insufficiency were more prone to developing MFA rather than SFA.[29] Unlike these previous studies, we specifically investigated predictors of multi-triggered FIA, rather than MFA, and found that total IgE levels above 100 IU/mL were associated with multi-triggered FIA. This suggests that for food-allergic children with high total IgE levels, clinicians should be more vigilant for recurrent anaphylaxis with other types of foods and may follow them more closely. However, the causality of this relationship remains unclear, as elevated IgE levels may both contribute to and result from FIA, as seen in DOCK8-deficient patients. We hope that our results may guide new studies on multi-triggered FIA in terms of its prevalence, mechanisms, characteristics, and management. However, IgE levels alone are insufficient to predict multi-triggered FIA; as they are influenced by factors such as allergen exposure frequency and duration. Therefore, a large-scale cohort study is necessary to better understand these relationships.

Component testing can be useful in distinguishing between cross-reactivity and true sensitization, especially in patients with allergies to multiple foods such as nuts. However, since these tests were not performed on the patients in our study, we could not make a clear distinction between cross-reactivity and sensitization. This is the most important limitation of our study. Another limitation is that we classified patients into single- or multi-triggered groups based on the number of anaphylaxis episodes. However, confounding factors—such as protective parental behaviors (e.g., eliminating suspicious allergens from the child's diet) or the child's age (e.g., not yet exposed to certain foods)—may have influenced the occurrence of new anaphylaxis events, potentially limiting our findings.

Additionally, baseline serum tryptase levels were not available for all patients, which restricted our ability to assess the potential presence of systemic mastocytosis. However, systemic mastocytosis is extremely rare in children, with most pediatric cases limited to cutaneous forms that typically resolve spontaneously.[31,32] None of our patients exhibited clinical features suggestive of systemic mastocytosis, such as characteristic skin lesions, hepatosplenomegaly, or unexplained recurrent anaphylaxis. In the absence of clinical signs indicating systemic involvement, we did not pursue further diagnostic evaluations, such as bone marrow biopsy, in accordance with current guidelines.[32,33]

The strongest aspect of this research is that it is a single-centered, real-life FIA study. Since there are a limited number of studies in the literature reporting the differences between single and multiple food anaphylaxis, we believe that our study makes a significant contribution to the literature.

Furthermore, our study highlights the considerable rate of multi-triggered FIA among anaphylaxis cases. This should encourage new studies on this subject; because further longitudinal, multi-layer, case-control and molecular-based studies are needed.

In conclusion, multi-triggered FIA was detected in approximately a quarter of children with FIA. Higher rates of atopic disease and asthma and higher levels of total IgE and eosinophilia were observed in patients with multi-triggered FIA when compared with children with single-

triggered FIA. Moreover, higher total IgE was found to be a significant predictor of multi-triggered FIA.

Supplementary materials

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Ethical approval

The study was approved by Ankara Bilkent City Hospital No. 2 Clinical Research Ethics Committee (date: 15.03.2023, number: E2-23-3593).

Author contribution

The authors confirm contribution to the paper as follows: Study conception and design: ŞBY, İKÇ, ZŞE, MT, EDM, EC; data collection: ŞBY, BK, TG, SAA, EDM, EC; analysis and interpretation of results: ŞBY, İKÇ, ZŞE, MT, EDM, EC; draft manuscript preparation: ŞBY, İKÇ, ZŞE, MT, EDM, EC. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Original Article

Off-label use of recombinant factor VIIa for neonatal pulmonary hemorrhage; a single-center experience

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ABSTRACT

Background. Pulmonary hemorrhage (PH) leads to acute and catastrophic deterioration in neonates, and there is no curative treatment available. Off-label use of recombinant Factor VIIa (rFVIIa) is a promising treatment to control bleeding. The aim of this study was to investigate the efficacy and safety of rFVIIa in neonatal massive PH.

Methods. We used rFVIIa for PH in our neonatology unit during October 2022. We compared demographic and prognostic data of neonates with PH, for two years prior to and following this time point. Intravenous rFVIIa (50-90 μg/kg/dose) was administered to patients with life-threatening PH that was unresponsive to conventional therapies including surfactant administration, vitamin K treatment, blood product transfusion, increasing airway pressure, high frequency ventilation, and endotracheal adrenaline. Potential side effects, such as thromboembolism, were monitored for one week.

Results. We present 16 neonates (7 females; 14 preterm) treated with rFVIIa in addition to conventional treatments and compared their clinical outcomes with the rFVIIa-untreated group (n=21). Median (interquartile range [IQR]) birth weight (960 [775-2377] vs 910 [710-1360] g, p=0.20) and gestational age (29 [27-32] vs 27 [27-29] weeks, p=0.25) did not significantly differ between the groups. Median (IQR) postnatal day of PH occurrence was 7.5 (3-15) in the rFVIIa-treated group and 3 (1.5-6) in the rFVIIa-untreated group (p=0.019). Overall, six neonates died of PH complications in the intervention group. All neonates responded to rFVIIa to varying degrees (cessation of bleeding, n=11; reduced bleeding, n=5). A second dose was required in three. No thromboembolism was observed during the treatment period. Death attributable to PH [6 (37%) vs 16 (76%), p=0.042] and overall mortality (7 [43%] vs 18 [86%], p<0.001) were lower and median hospitalization duration (37 [10-95] vs 4 [3-9] days, p=0.001) was longer in the study group than in the control group.

Conclusions. Until proven otherwise by further prospective studies, rFVIIa may be effectively and safely administered at higher doses (90 μg/kg), with repeat dose if necessary, when neonatal life-threatening PH does not respond to conventional treatment.

Key words: massive pulmonary hemorrhage, off-label, recombinant factor VIIa , neonatal intensive care unit.

Pulmonary hemorrhage (PH) is a devastating disorder with sudden deterioration of the patient's clinical condition. The incidence is 1–12 per 1,000 live births.[1] The condition features the release of hemorrhagic secretions from the respiratory system, with simultaneous respiratory decompensation. The magnitude of bleeding can vary from slight to extensive bleeding. Hypovolemic shock and death are inevitable if severe hemorrhage persists. PH occurs particularly in preterm neonates who often have patent ductus arteriosus (PDA)

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within a few days after birth.[1,2] Intrauterine growth restriction, chorioamnionitis, respiratory disorders, coagulopathy, asphyxia, mechanical ventilation, surfactant therapy, and sepsis are the other risk factors.[1,3] The mortality rate can be as high as 50% in severe cases, depending on the severity of the hemorrhage, the infant's gestational age, and the timeliness and effectiveness of medical interventions.[1] As there are no curative treatments, treatment often focuses on stabilizing the infant's respiratory status, addressing the underlying cause, and providing supportive care, such as high pressures in mechanic ventilation, surfactant, transfusion with blood products, vitamin K, and local ephinefrine. Early detection and management are key to improving outcomes.

Recombinant activated factor VII (rFVIIa), a leading candidate in current therapies, is a promising agent for survival.[1,2] rFVIIa is primarily used to manage bleeding in patients with hemophilia or factor VII deficiency.[4] rFVIIa facilitates thrombin production when its formation is impaired.[5] Thrombin formation is essential for providing a stable fibrin plug.[4,5] The successful use of rFVIIa to stop bleeding in adults without congenital hemorrhagic disorders has been reported.[6-10] A few case reports have described rFVIIa as a life-saving drug in children and neonates with acquired bleeding disorders.[5,10-16] In these reports, rFVIIa was successfully administered intravenously or locally. However, systemic use and high drug doses may increase the risk of thromboembolic events.[17,18] The only study, besides case reports, examining the efficacy of rFVIIa in neonates with PH was a retrospective study by Cosar et al.[17] In this study, rFVIIa was administered intravenously at a low dose in addition to conventional treatments to a limited number of neonates with PH, and favorable results were obtained.[17] Gkiougki et al. focused on investigating the factors affecting the response to rFVIIa in neonates who received higher and repeated doses of rFVIIa in their retrospective study.[19] The optimal dose of rFVIIa that should be used has not yet been identified. Therefore, an increase in the number of published cases may facilitate the development of more effective treatment protocols. In such studies, it is crucial to present the cases in detail. We aimed to: 1) share our experience with patients in our unit, where rFVIIa is mostly given via systemic administration at a higher dose, which may make a significant contribution to both increasing the number of cases treated with rFVIIa in the literature; and 2) to evaluate the effects and side effect profile of treatment in newborns at higher doses, and different treatment regimens.

Materials and Methods

The patient data were collected retrospectively after local ethics committee approval (KU GOKAEK 2024/12.25/343). Written informed consent was obtained from the parents before off-label use of rFVIIa. We have been using rFVIIa to control bleeding as an off-label drug in our level III neonatal intensive care unit (NICU) since October 2022. Neonates with PH, both those treated with rFVIIa and those not treated, were compared based on demographic and prognostic data to determine the efficacy and safety of rFVIIa treatment for massive PH, in addition to conventional treatment. The neonates who were treated with rFVIIa due to unresponsiveness to conventional treatment for massive PH during NICU hospitalization between October 2022 and December 2024 were the study group. The neonates who did not receive rFVIIa treatment for PH during the two years prior to October 2022 were included in the control group, as the medication was added to the treatment protocol in October 2022. The exclusion criteria were newborns who had major congenital anomalies incompatible with life, metabolic diseases, or incomplete patient data. Antenatal, natal, and postnatal risk factors, the etiology of PH, conventional treatment options for PH, dose and frequency of rFVIIa administration, response to treatments, morbidities, and mortality were recorded. Possible adverse reactions to rFVIIa, such as fever, thromboembolic events, and

hypersensitivity reactions were also recorded. Thromboembolic events were monitored for one week; fever and hypersensitivity reactions were monitored for 24 hours after the administration of the drug.

There are no standard diagnostic criteria for life-threatening PH in the literature. We used diagnostic criteria like those established by Cosar et al.[17] Life-threatening PH was diagnosed as the presence of: (1) aspiration of fresh blood via endotracheal tube; (2) acute deterioration of pulmonary functions; (3) hemodynamic instability; and/or (4) pulmonary bleeding as evidenced by the appearance of new pulmonary shadows observed on chest radiographs. Early-onset PH was defined as PH present in the first seven days postnatally. Neonates who had significant bleeding from the respiratory tract or endotracheal tube and whose clinical status simultaneously deteriorated were treated primarily with a combination of conventional treatment options, including vitamin K administration, high-frequency ventilation (HFV), increasing positive end-expiratory pressure in conventional ventilation, or endotracheal adrenaline, transfusions with blood products, and surfactant administration. Neonates in the study group who were unresponsive to the combination of these conventional treatments were given a slow intravenous bolus over five minutes of rFVIIa (NovoSeven®, NovoNordisk, Copenhagen, Denmark). When we added the treatment to our NICU's protocol, we initially preferred lower doses (50-60 μg/kg/dose), but later standardized the application to 90 μg/kg/dose, as routine. If the response to rFVIIa was insufficient after two hours of the initial dose, the patient received repeated doses. Physician's observation on the bleeding volume determined the response to rFVIIa.

Bronchopulmonary dysplasia (BPD) was defined as the need for oxygen support beyond 28 postnatal days. Necrotizing enterocolitis was diagnosed using Bell's criteria.[20] Sepsis in neonates was defined as the presence of at least two clinical and two laboratory findings listed below, with or without a positive blood culture;

a) Clinical signs: 1) Body temperature ≤36 or ≥38.5 °C; 2) Bradycardia, tachycardia, or rhythm instability; 3) Oliguria; 4) Hypotension; 5) Sclerema, petechia; 6) Apnea or tachypnea or increased oxygen demand or ventilation support requirement; 7) Poor sucking, feeding intolerance, abdominal distention; and/or 8) Hypotonia, irritability, lethargy.

b) Laboratory findings: 1) White blood cell count >20,000 $x10^9$ cells/L or <4000 $x10^9$ cells/L; 2) Immature to total neutrophil ratio >0.2; 3) Platelet count <100,000 $x10^9$ cells/L; 4) Lactate >2 mmol/L, base excess <-10 mEq/L; 5) Procalcitonin levels ≥ 2 ng/mL and/or C-reactive protein levels >15 mg/L or; 6) Blood glucose levels <45 or >180 mg/dL. Survival was defined as survival at discharge.

Statistical analysis

All statistical analyses were conducted using IBM SPSS for Windows, version 29.0 (IBM Corp., Armonk, NY, USA). The assumption of normality was assessed using the Shapiro-Wilk test. As the normality assumption was not met, continuous variables were reported as median and interquartile range (IQR). Categorical variables were summarized as counts and percentages. Comparisons between groups were performed using the Mann–Whitney U test, while associations between categorical variables were analyzed using the chi-square test. A p-value of 0.05 was considered statistically significant.

Results

Only one in 17 neonates in the study group was excluded due to inadequate data on the response to rFVIIa. Thus, we present 16 neonates (Study group; 7 females; 14 preterm) who were treated with rFVIIa in addition to conventional treatments (Table I) and compare their demographic and clinical data with those of 21 neonates who received treatment without

Table I. Characteristics of individual patients with massive pulmonary hemorrhage treated with rFVIIa.

Case	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Gestational age, weeks	28	31	26	27	26	30	26	35	33	27	30	28	33	39	29	37
Birth weight, g	945	1859	500	960	960	720	720	3260	2500	880	750	850	1750	2790	980	3340
Age at PH onset, days	15	37	2	15	2	3	8	3	15	13	4	25	7	5	27	0
Etiology																
Sepsis	+	+	-	+	-	-	+	+	+	+	+	+	+	-	-	-
PDA	-	-	+	-	+	-	-	-	-	+	-	+	-	-	+	-
Pneumonia	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
Asphyxia	-	-	+	-	+	-	-	-	-	-	-	+	-	+	-	+
Postoperative	-	NEC	-	-	-	-	-	Myelocele	-	-	-	-	-	-	-	+
NEC	-	+	-	+	--	+	-	-	-	-	-	+	-	-	-	-
Dose (μg/kg/dose)	90	90	90	60	90	50	90	90	50	90	90	90	90	90	90	90
Total doses	1	1	1	1	1	1	1	1	2	1	1	2	1	1	2	1
Response to rFVIIa	+[1]	+[1]	+[2]	+[2]	+[2]	+[1]	+[3]	+[1]	+[4]	+[4]	+[4]	+[4]	+[2]	+[1]	+[4]	+[4]
Initial F-VII, %	25.6↓	29.8	-	-	-	11.4↓	41	-	50	150	-	-	43	69	112	38,6
Initial			-	-												
PT (sec)	10.9	29.8			11	28	11	11	11	9	17	13	11	11	17	18.6
aPTT (sec)	28.6	70			58	79	31	30	33	14	25	33	29	35	74	40
INR	1.2	1.9			1.7	2.8	1	1	1	0.7	1.3	1.2	1.1	1	1.5	1.6
After rFVIIa	-		-	-	-		-	-	-	-		-	-			
PT (sec)		14				18					17			10	14	10.8
aPTT (sec)		63				33					27			28	33	32
INR		1.2				1.6					1.3			0.8	1.3	0.9
Initial Platelets, $10^3/\mu L$	180	95	-	-	136	33	162	180	235	213	169	162	322	341	50	169
rFVIIa complications	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1]Stopped and non-repeated, [2]Decreased but repeated, [3]Decreased and did not repeat, [4]Stopped but repeated; *Both of invasive, non-invasive ventilation, and supplemental oxygen; aPTT, activated partial thromboplastin time; ET, endotracheal; ES, erythrocyte suspension; FFP, fresh frozen plasma; HFV, high frequency ventilation; INR, international normalies ratio; NEC, necrotizing enterocolitis; PDA, patent ductus arteriosus; PEEP, positive end expiratory pressure; PH, pulmonary hemorrhage; PS, platelet suspension; PT, prothrombin time; rFVIIa, recombinant activated factor VII.

Table I. Continued.

Case	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Management of PH																
Vitamin K	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HFV	+	-	+	+	+	+	-	+	-	+	-	-	-	+	+	-
PEEP↑	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
ET-Surfactant	+	-	+	+	+	+	-	+	-	+	+	+	-	-	+	-
ES	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	-
PS	-	+	+	-	-	+	-	-	-	-	-	-	-	-	+	-
FFP	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+
ET-adrenaline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inotropic drugs	-	-	-	+	-	-	-	+	-	+	-	-	+	+	+	+
Invasive ventilation, days	34	17	5	15	2	21	77	4	26	33	9	32	30	19	61	6
Supplemental oxygen , days*	67	65	5	15	2	64	136	4	37	50	10	32	30	19	111	8
Hospitalization, days	100	180	4	15	2	83	145	4	42	66	10	32	78	19	122	10
Death attributable to PH	-	-	+	+	+	-	-	+		-	+	+	-	-	-	-

[1]Stopped and non-repeated, [2]Decreased but repeated, [3]Decreased and did not repeat, [4]Stopped but repeated; *Both of invasive, non-invasive ventilation, and supplemental oxygen; aPTT, activated partial thromboplastin time; ET, endotracheal; ES, erythrocyte suspension; FFP, fresh frozen plasma; HFV, high frequency ventilation; INR, international normalies ratio; NEC, necrotizing enterocolitis; PDA, patent ductus arteriosus; PEEP, positive end expiratory pressure; PH, pulmonary hemorrhage; PS, platelet suspension; PT, prothrombin time; rFVIIa, recombinant activated factor VII.

rFVIIa, referred to as the control group (Table II). Eight neonates presented with early-onset PH in the study group. Overall, six neonates died of PH complications. All neonates responded to rFVIIa to varying degrees; bleeding was stopped in 11 neonates (68%) and reduced in the other five (31%) (Table I). Nine of the rFVIIa-treated patients (56%) experienced bleeding recurrence, most of whom responded to conventional treatments, while only three (19%) required a second dose of rFVIIa. Only two of the 10 patients whose initial FVII levels were checked had low levels, and the median (IQR) FVII level before treatment was 42% (28%-80%).

Median (IQR) birth weight (960 [775-2377] vs 910 [710-1360] g, p=0.20) and gestational age (29 [27-32] vs 27 [27-29] weeks, p=0.25) did not significantly differ between the study and control groups. Demographic findings are summarized in Table II.

Median (IQR) postnatal day of PH occurrence was 7.5 (3-15) in the rFVIIa-treated group and 3 (1.5-6) in the rFVIIa-untreated group (p=0.019). The requirement for HFV for impaired oxygenation (9 [56%] vs 19 [90%], p=0.024) and requirement for inotropic support for persistent hemodynamic instability (9 [56%] vs 19 [90%], p=0.024) due to PH was lower in the rFVIIa group than in the control group. Median (IQR) hospitalization duration of survivors and nonsurvivors (37 [10-95] vs 4 [3-9] days, p=0.001), death attributable to PH (6 [37%] vs 16 [76%], p=0.042) and overall mortality (7 [43%] vs 18 [86%], p<0.001) differed significantly between the study and control groups. The incidence of retinopathy of prematurity (ROP) was significantly lower in the study group than in the control group (20 [95%] vs 5 [31%], p<0.001). The clinical outcomes of the neonates are summarized in Table II.

Discussion

We present our experience with off-label use of rFVIIa in sixteen neonates with life-threatening PH. This is the second clinical study comparing outcomes in two groups of neonates with acute PH, one receiving rFVIIa and the other not, in addition to conventional treatments. The present study used higher doses than the single study conducted previously in the literature, which administered rFVIIa at 50 μg/kg/dose in addition to conventional treatments in the study group. In the present study, all neonates with severe PH, which persisted despite conventional treatments, responded to rFVIIa within two hours, to varying degrees. The requirement for HFV for impaired oxygenation and inotrope for persistent hemodynamic instability was lower in the rFVIIa group than in the control group. Although the group treated with rFVIIa had significantly lower death rates attributed to PH and all other causes compared to the group not receiving rFVIIa, their hospitalization duration was significantly longer.

Therapeutic levels of rFVIIa can enhance thrombin generation, which is essential for the formation of a stable fibrin clot that is resistant to fibrinolysis.[5] rFVIIa triggers thrombin formation by interacting with tissue factor and activating factor X during impaired thrombin production.[5] Currently, rFVIIa is an approved treatment for bleeding in patients with hemophilia, factor VII deficiency, and Glanzmann thrombasthenia.[4,6-16] The only retrospective case-control study that investigated the off-label use of rFVIIa reported 21 premature infants born before 30 gestational weeks who were treated with a single dose of 50 μg/kg rFVIIa in addition to conventional treatment for PH.[17] The mortality attributable to PH, was 23%, and total mortality was 42% in the rFVIIa-treated group. However, these outcomes did not significantly differ between treated and untreated groups. The main benefit of rFVIIa administration was observed in the stopping of hemorrhage, reducing blood product requirement, and improving coagulation test parameters in this study. It was also reported that 66% of cases experienced a complete cessation of bleeding, with a recurrence rate of 42%. However, the authors did not mention a reduction in bleeding as an outcome.[17] In another case-control retrospective study,

Table II. Demographic and clinical findings of neonates with PH treated and not treated with rFVIIa.

	Treated with rFVIIa n=16	Not treated with rFVIIa n=21	P
Gestational age, weeks, median (IQR)	29 (27-32)	27 (27-29)	0.20
Birth weight, g, median (IQR)	960 (775-2377)	910 (710-1360)	0.25
Male, n (%)	10 (63)	13 (62)	1
Early membrane rupture > 18h + chorioamnionitis, n (%)	4 (25)	4 (19)	0.77
Small for gestational age, n (%)	9 (56)	13 (62)	0.99
Oligo/anhydroamnios, n (%)	4 (25)	6 (29)	1
Preeclampsia/eclampsia, n (%)	5 (31)	5 (24)	0.71
5th min APGAR, median (IQR)	6 (5-8)	7 (6-8)	0.78
Antenatal steroids, n (%)	10 (63)	17 (81)	0.27
Maternal diabetes mellitus, n (%)	3 (19)	2 (10)	-
Perinatal asphyxia, n (%)	4 (25)	3 (14)	0.43
Early-onset PH, n (%)	8 (32)	17 (68)	0.10
High-frequency ventilation for PH, n (%)	9 (32)	19 (68)	0.024
Persistant hemodynamic instability due to PH, n (%)	9 (32)	19 (68)	0.024
Surfactant for respiratory distress syndrome, n (%)	13 (81)	20 (95)	-
Patent ductus arteriosus, n (%)	12 (75)	12 (57)	0.43
Necrotizing enterocolitis, n (%)	5 (31)	2 (9.5)	0.20
Sepsis, n (%)	10 (63)	12 (57)	1
Intraventricular hemorrhage, n (%)	3 (19)	10 (48)	0.14
Bronchopulmonary dysplasia, n (%)	8 (53)	1 (5)	-
Retinopathy of prematurity, n (%)	5 (31)	20 (95)	<0.001
PH onset, days, median (IQR)	7.5 (3-15)	3 (1.5-6)	0.019
Erythrocyte transfusion number for PH, median (IQR)	1 (1-7)	1 (1-1)	0.96
Fresh frozen plasma transfusion number for PH, median (IQR)	1.5 (1-2)	1 (1-1)	0.15
Total transfusion number during hospitalization, median (IQR)*	5 (4-16)	5 (3-7)	0.35
Invasive ventilation, days, median (IQR)	20 (7-34)	4 (3-8)	0.001
Non-invasive ventilation, days, median (IQR)	1 (0-10)	0 (0-1)	0.018
Total oxygen supplementation (days), median (IQR)	32 (10-65)	4 (3-9)	<0.001
Death attributable to PH, n (%)	6 (38)	16 (76)	0.042
Over-all mortality, n (%)	7 (44)	18 (86)	<0.001
Hospitalization, days, median (IQR)	37 (10-95)	4 (3-9)	0.001

IQR, interquartile range; PH, pulmonary hemorrhage; * Erythrocyte, thrombocyte, and fresh frozen plasma.

Gkiougki et al. primarily aimed to determine the factors affecting the rFVIIa response. To this end, 29 neonates with PH who received rFVIIa were divided into two groups: those who survived until discharge and those who died.[19] In this study, the mean gestational week of patients treated with rFVIIa was 31 weeks and 4 days, and all of them received the drug at 100 μg/kg/dose as a bolus injection and thereafter 100 μg/kg every four hours until cessation of hemorrhage. All-cause mortality was reported as 48%. They stated that coagulation test parameters improved after rFVIIa.[19] These two studies reported no side effects. Since

October 2022, we have treated neonates with refractory PH, with rFVIIa as a final resort to stop bleeding. Initially, we were cautious and started with a low dose of 50-60 μg/kg for the initial three patients. However, due to reports showing no side effects at higher doses, we increased the dose to 90 μg/kg. We did not observe thrombosis in our patients, like the previous two studies. Sixteen neonates, 14 of whom were preterm, significantly responded in various degrees to the off-label use of this agent regardless of the dose of rFVIIa we administered. Unlike the study of Cosar et al., we accepted a significant decrease or cessation of bleeding as a response.[17] In 68% of our cases, bleeding completely stopped, and in 31%, it had significantly decreased. Bleeding recurred in 56% of these patients but only 19% required a second dose of rFVIIa. If we accept only the cessation of bleeding as a response, the rates of cessation and recurrence of bleeding are similar to those reported by Cosar et al.[17] However, we believe that the significant decrease in bleeding in the remaining 31% of patients in the present study represents a valuable outcome. The satisfactory response we achieved in all patients who received this treatment, may be due to the higher dose relative to that used by Cosar et al.[17]

In one case series, 13 patients aged between 2 days and 15 years who did not have congenital hemorrhagic disorders were treated with rFVIIa for acute, life-threatening bleeding from various sites.[4] The median PT time was 32.9 s prior to rFVIIa administration and 11.6 s after infusion. The bleeding completely ceased for at least 24 hours in 10 patients, whereas three patients experienced a reduction in bleeding approximately 45 minutes after receiving rFVIIa. In addition, the need for blood products significantly decreased following rFVIIa treatment. Greisen et al. investigated the effects of rFVIIa on PT time and reported that rFVIIa with an 80 μg/kg/dose partially normalized PT time in preterm babies with a gestational age less than 33 weeks.[21] However, unlike these cases and Cosar et al's clinical study, we could not show any significant effect of rFVIIa on blood transfusion requirement.[4,17] The mean clearance of factor VII is approximately 50% higher in children than in adults, and the terminal half-life is very short, approximately two hours. Although there was a shortened prothrombin time (PT) / international normalized ratio (INR) and activated partial thromboplastin time (aPTT) after rFVIIa application, no direct correlation was shown between rFVIIa efficacy and the PT/INR or aPTT values.[22] The control coagulation test was not routinely performed after the treatment in our unit. In the six patients we assessed coagulation tests after rFVIIa treatment, we found that coagulation parameters improved in four of them. The initial FVII serum levels before rFVIIa administration were low in only two of the 10 neonates. The physiological level for Factor VII in premature neonates is low, ranging from 0.14 to 0.57 IU/mL.[23] Furthermore, rFVIIa also works by strengthening the fibrin clot, even if there is a sufficient level of Factor VII. Therefore, it will be more valuable for the clinician to observe the decrease in bleeding volume than improving laboratory values. Brady et al. reported their experience with rFVIIa in nine infants suffering from severe hemorrhage due to various causes, including postoperative complications from cardiac surgery, vitamin K deficiency, and intracranial hemorrhage, suspected necrotizing enterocolitis and abdominal hemorrhage, as well as PH.[14] The infants' age ranged from 2 days to 4 months. The dosages administered in this series were between 90 and 100 μg/kg. All patients experienced clinical resolution of their bleeding after receiving rFVIIa, and seven out of the nine patients survived.[14]

In the present study, persistent hemodynamic instability and requirement for HFV to improve oxygenation due to massive pulmonary bleeding were significantly less common in the treatment group than in the control group. However, rFVIIa treatment is expensive and difficult to procure for off-label use. Thus, we used this drug as a last resort when we could not control the patient's clinical status with standard treatment options in our unit. Therefore, mild

findings before treatment were not expected in rFVIIa-treated patients, and the lower incidence of HFV or persistent inotrope requirement should be secondary to the response to rFVIIa. Moreover, since the control group consisted only of patients who received conventional treatment during the period when rFVIIa was not included in the treatment protocol, and included both responders and non-responders, the patients in the control group who survived might have had a somewhat milder clinical condition. In addition, our results showed that hospitalization and mechanical ventilation days were significantly prolonged in the treatment group compared to the control group. We believe that the increased rate of survival with treatment led to longer hospital stays and prolonged ventilation periods. On the other hand, Cosar et al. did not show a significant difference between the groups regarding these two parameters.[17] The higher incidence of hemodynamic instability and impaired oxygenation in the group not treated with rFVIIa, along with the increased need for high pressure and oxygen, may have led to a more frequent occurrence of ROP in this group.

The optimal dose of rFVIIa for neonates has not been determined. The dose of rFVIIa differs in the literature, ranging from 50 to 200 μg/kg/dose.[3,5,16-18,24,25] It was usually administered intravenously and rarely intrapulmonarily in these reports. Systemic use of the drug, especially at higher doses, has been associated with a risk of thrombosis.[16,18,26] However, pharmacokinetic studies have indicated that young children may require higher doses of rFVIIa because of its shorter half-life and increased clearance rate in this age group.[27] Almost all reports, including ours, found no adverse events attributable to rFVIIa.[3,5,13,16,24,26] Yilmaz et al. reported the efficacy of rFVIIa use in 13 children without hemophilia, including four premature neonates with life-threatening bleeding.[4] One of the two patients in this series who experienced a thrombotic complication after receiving rFVIIa was a premature newborn who had a central venous catheter. He developed respiratory distress syndrome, along with gastrointestinal and intracranial hemorrhage caused by disseminated intravascular coagulation. A total of four doses (100 μg/kg/dose) were administered with a 1-day interval. A few hours after the last dose, he developed thrombosis in the brachial veins.[4]

A second dose was required in the present study in three neonates because of the recurrence of massive bleeding two hours after the first dose. Cetin et al. reported that active bleeding significantly subsided after the second dose of rFVIIa (120 mg/kg per dose), and an improvement in the oxygenation index was observed eight hours after the third dose, in their case.[3] Grizelj et al. described a neonate who experienced massive postoperative hemorrhage following ileostomy, as well as three patients who had severe PH during mechanical ventilation for meconium aspiration syndrome and during postoperative resuscitation after cardiac surgery.[5] In three of the cases, the first bolus of rFVIIa completely and immediately halted the bleeding. Despite the cessation of bleeding after the first dose, the patients continued to receive rFVIIa to prevent rebleeding. All infants received rFVIIa 100 μg/kg and thereafter 100 μg/kg every four hours, until cessation of bleeding in the case-control study by Gkiougki et al.[19] After performing future randomized controlled trials, regular administration of FVIIa at 2-hour intervals, regardless of response to treatment, may be an alternative method of treatment in the acute phase of bleeding. However, the concern about increased thromboembolic complications should be addressed in cases where the drug is administered frequently.

To date, there have been reports successfully using rFVIIa in infants suffering from different etiologies, including umbilical or pulmonary hemorrhage, postoperative bleeding including cardiac surgery, gastrointestinal and intracranial hemorrhage, liver diseases, and coagulopathy.[11,15,16,24] However, further standardized studies are needed before this approach can be introduced into routine

practice in the NICU. Our results are promising in controlling pulmonary bleeding; however, the retrospective nature of the study and the low number of cases are limitations. However, due to the low incidence of PH, the small number of neonates with PH who have been treated with rFVIIa and reported, and the limited number of clinical studies, we believe that this sample size is valuable in increasing the number of cases to reflect the effectiveness and safety profile of this form of rFVIIa treatment. In addition, the optimal dose, frequency of application, and timing of rFVIIa in the treatment of neonatal PH have not yet been clarified. Furthermore, to our knowledge there is no other study with such a wide range of cases in terms of the dose and management of repeated doses that we applied, which increases the value of our results. The results of the present study may be a guide for future prospective standardized studies.

In conclusion, PH is a severe clinical condition characterized by a high mortality rate and significant pulmonary and neurological morbidities.[26] We successfully treated 16 neonates with rFVIIa who had life-threatening PH, without experiencing significant adverse events. Our results are promising for the control of life-threatining bleeding with rFVIIa in neonates, especially premature ones. However, to validate and generalize the results of this study, systematic prospective studies are needed to investigate the efficacy and safety of rFVIIa administration for PH, as well as to determine the optimal timing and dosage.

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Ethical approval

The study was approved by Kocaeli University Non-Interventional Clinical Research Ethics Committee (date: December 25, 2024, number: 2024/12.25/343).

Author contribution

The authors confirm contribution to the paper as follows: Study conception and design: OSP, AG; data collection: AA, OSP; analysis and interpretation of results: OSP, AG, SB; draft manuscript preparation: OSP, SB. All authors reviewed the results and manuscript, and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Original Article

Balancing intervention and complications: management of otitis media with effusion in children with cleft palate

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ABSTRACT

Background. Children with cleft palate (CP) are at high risk for otitis media with effusion (OME), which may impair hearing, speech, and development. Although ventilation tube (VT) insertion during palatoplasty is common, its universal use is debated due to uncertain long-term benefits and potential complications. This study aimed to identify preoperative audiological predictors of VT necessity and evaluate VT-related complications.

Methods. A retrospective review was conducted on 65 non-syndromic CP patients who underwent palatal repair without prior or concurrent VT placement. Preoperative audiological evaluations were performed, and patients were followed postoperatively for VT insertion and complications. Preoperative hearing thresholds, cleft severity (Veau classification), and VT related complications were analyzed statistically.

Results. The likelihood of VT insertion rose significantly in parallel with the severity of preoperative hearing loss, ranging from just 5.9% in patients with normal hearing to 75% in those with moderate conductive hearing loss (CHL) ($p < 0.001$). Pairwise comparisons showed significant differences between normal hearing and both mild ($p = 0.0026$) and moderate CHL ($p = 0.01$). CP severity was not associated with preoperative hearing but correlated with higher VT placement (Veau I: 10%, Veau IV: 69.2%; $p = 0.035$). Complications included otorrhea (45.2%), early extrusion (35.5%), and tympanic membrane perforation (12.9%), with no significant associations to preoperative hearing level and CP severity.

Conclusion. Preoperative hearing level at the time of palate repair is a strong predictor of VT need in CP patients. Mild to moderate CHL significantly increases the risk of persistent OME, supporting early intervention. Normal or slight loss often resolves without treatment, favoring a conservative approach. Higher cleft severity is associated with increased VT placement rates; it does not correlate with preoperative hearing levels or increased VT-related complications. These findings highlight the value of individualized, hearing-based decisions over routine tube placement.

Key words: cleft palate, otitis media with effusion, ventilation tube, audiology, conductive hearing loss.

Cleft lip with or without cleft palate (CLP) represents one of the most prevalent congenital anomalies, with an incidence ranging from 1/500 to 1/1000 births.[1] Children with cleft palate (CP) are at increased risk of developing otitis media, with rates approaching 100%.[2] A decrease in eustachian tube function, particularly an impairment in the opening function, results from malfunction of the tensor veli and levator veli palatini muscles, which are compromised in patients with CP. This causes a disruption in the middle ear airflow, which

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facilitates the development of otitis media with effusion (OME). OME is well-documented to impede speech and language development, underscoring the critical need for accurate diagnosis and prompt treatment in patients with CP.[3-5]

The standard approach to addressing OME in patients with CP involves the insertion of ventilation tubes (VT) during CP repair procedures.[6] VT insertion is mostly performed to restore hearing immediately in order to prevent or minimize developmental impairment in children with OME.[7] However, recent reports suggest that not all patients with OME require VTs during palate surgery, as the procedure itself has demonstrated the capacity to improve middle ear ventilation and Eustachian tube function.[8,9] Thus, the meticulous selection of patients who are suitable candidates for VT insertion is paramount for healthcare providers and patients alike, aiming to mitigate potential complications such as recurrent otorrhea, permanent alterations to the tympanic membrane, and iatrogenic cholesteatoma formation.[10-13] Furthermore, research indicates that prophylactic VT insertion may not offer significant advantages over vigilant monitoring of middle ear status in patients with CP and OME.[14] The current trend leans towards a 'wait-and-see' approach regarding middle ear effusion, steering away from prophylactic VT insertion during palate surgery.[11,15-17]

This study aimed to assess the significance of ontological and audiological findings in patients who had undergone palate surgery, with the objective of identifying potential candidates for VT insertion.

Materials and Methods

This article is a retrospective case review, and all interventions were performed in the Departments of Audiology, Otolaryngology-Head and Neck Surgery, and Plastic and Reconstructive Surgery at Hacettepe University Hospital, a tertiary reference center. The study was approved by the Hacettepe University Ethics Committee for Non-Interventional Clinical Investigations with the number 21/859.

Patients

A retrospective review was conducted on the medical records of patients with CP who underwent surgery at our institution between February 2016 and September 2021. Inclusion criteria required children (<18 years) with CP not associated with a genetic syndrome, to have undergone palate repair, to have completed regular otological and audiological follow-up, and to have completed at least one year of follow-up. Exclusion criteria included patients with isolated cleft lip, those who had a VT placed prior to or during palate surgery, those with sensorineural or mixed hearing loss, those with unilateral hearing loss, and those who did not meet the one-year follow-up requirement. Patients were excluded if they had syndromic CP, as this condition is frequently linked to craniofacial skeletal abnormalities that increase susceptibility to multifactorial middle ear disease and other etiologies of hearing loss. A total of 65 patients with CP who met these criteria were included in the study.

Interventions

All newborns, including those with CP, were screened with the automatic auditory brainstem response (AABR) test using the MB 11 BERAphone (CE certificate 0123, Berlin, Germany) shortly after birth as part of the nationwide screening program. Infants with CP are at increased risk of conductive hearing loss, often due to middle ear effusion. Therefore, comprehensive audiological evaluations at 3 and 6 months of age are recommended, even if the initial newborn screening is passed. For patients with CP who fail the screening test, diagnostic tests such as air and bone conduction ABR and behavioral observation audiometry have been performed at around 3 months of age. Patients continued to receive audiological evaluation every 3 months until palate surgery. The patients included in the study consisted

of patients who did not receive a VT before or during palate repair. The last audiological evaluations of the patients before palate repair were noted. The relationship between hearing levels before palate repair and the rate of VT placement during follow-up was analyzed. Early extrusion, need for re-tube placement, VT-related otorrhea, permanent perforation and cholesteatoma development were noted in the follow-up of patients with VT placement.

CP were classified according to the Veau classification.[18] According to Veau, defects of the soft palate only are classified as Group I, defects involving the hard palate and soft palate are classified as Group 2, defects involving the soft palate up to the alveolus, usually with involvement of the lip, are classified as Group 3, and complete bilateral clefts are classified as Group 4. Between months 9 and 12, patients underwent palate repair at the Department of Plastic and Reconstructive Surgery. Furlow palatoplasty was preferred in Veau I clefts, given its adequacy for narrow defects; however, in cases with wider clefts, Dorrance palatoplasty was employed due to insufficient tissue mobilization with the Furlow method alone. For Veau II clefts, Dorrance was the standard approach, but in clefts too wide for tension-free closure, a two-flap palatoplasty was used. Conversely, a minority of patients with narrow Veau II clefts underwent primary repair without flap elevation. In more complex Veau III and IV clefts, two-flap palatoplasty was routinely selected, as it allows for greater tissue mobilization and tension-free closure, which simpler techniques could not achieve. These decisions were made on a case-by-case basis following intraoperative assessment of cleft width and tissue characteristics.

Otological examination and pneumatic otoscopy findings were complemented with impedance and audiometric measurements. GSI TympStar Version 1 (CE certificate 0344; Smørum, Denmark) was used to measure middle ear impedance. Type A tympanograms, with their peak pressures within -50 daPa and compliance ranging from 0.2 to 1.4 cc, were considered normal. Pathological curves included tympanograms Ad, As, B, and C.

For children older than 5 years of age, pure tone averages (PTA) at 500, 1000, 2000, and 4000 Hz were calculated, and the level of hearing loss was classified based on Clark's evaluation[19]: 0–15 dB as normal hearing, 16–25 dB as slight hearing loss, 26–40 dB as mild hearing loss, 41–55 dB as moderate hearing loss, 56–70 dB as moderately severe hearing loss, 71–90 dB as severe hearing loss, and more than 91 dB as profound hearing loss.

Statistical analysis

Data analysis was performed using SPSS 25 (SPSS Inc., Chicago, IL, USA). The chi-square (χ^2) test was utilized to investigate the relationship between categorical variables. Post-hoc analysis with Bonferroni correction was employed to compare every subset further. Subgroup analysis utilized the phi test, and Cramer's V was used to calculate effect sizes in a 4x2 table. Effect sizes of 0.1, 0.3, and 0.5 were classified as small, medium, and large, respectively. A p-value below 0.05 indicated statistical significance.

Results

Patient demographics

Of the patients included in the study, 60% (n=39) were male and 40% (n=26) were female. The age of the patients at the time of palate surgery ranged from 8 to 35 months, with a mean age of 10.7±4 months. Of the patients, 15.4% (n=10) had Veau type 1, 33.8% (n=22) had Veau type 2, 30.8% (n=20) had Veau type 3 and 20% (n=13) had Veau type 4 CP anomalies. The most preferred surgical technique for palate repair in patients with Veau 1 CP was Furlow palatoplasty (90%, n=9), in patients with Veau 2 CP it was Dorrance palatoplasty (54.5%, n=12), and in patients with Veau 3 CP and Veau 4 CP it was two flap palatoplasty (95%, n=19 and 100%, n=13 respectively). Preoperative hearing levels were normal in 26.1% of patients (n=17),

while 15.4% of patients (n=10) had bilateral slight conductive hearing loss (CHL), 46.2% of patients (n=30) had bilateral mild CHL, and 12.3% of patients (n=8) had bilateral moderate CHL. VTs were not inserted during the follow-up period, as the hearing of 52.3% of patients (n=34) remained within normal limits or the hearing of patients with hearing loss returned to normal. However, a VT was placed in 47.7% of patients (n=31) because their hearing did not improve, or their hearing loss progressed. While 30.8% of patients (n=20) had a VT placed only once, 16.9% of patients (n=11) required VT insertion more than once because the VT extruded early, and OME persisted (Table I).

CP type and its effect on preoperative hearing levels and progression of hearing loss

The percentage of patients with normal hearing before CP repair was 50% (n=5), 27.3% (n=6), 15% (n=3) and 23.1% (n=3) in the Veau 1, 2, 3 and 4 groups, respectively. No significant relationship was found between the CP type and the preoperative hearing levels of the patients (χ^2=4.83 and p=0.848, Table II). After CP surgery, VT was placed in 10% (n=1), 45.5% (n=10), 55% (n=11) and 69.2% (n=9) of patients in the Veau 1, 2, 3 and 4 groups, respectively, because hearing did not improve or worsened. A significant relationship was found between the severity of the CP and the insertion of VT during the patients' follow-up (χ^2=8.585 and p=0.035, Table II).

Effect of hearing levels before CP surgery on VT insertion rate

All patients underwent an otological examination, tympanometry and age-appropriate audiometric testing (including bone conduction ABR) prior to palate surgery. While 26.1% of patients (n=17) had normal hearing, 73.9% of patients (n=48) had varying degrees of CHL (Table I). VTs were placed during follow-up in 5.9% (n=1) of patients with normal hearing, 50% (n=5) of those with slight hearing loss, 63.3% (n=19) of those with mild hearing loss and 75% (n=6) of those with moderate hearing loss (Table III). A significant and strong relationship was found between the level of hearing loss in patients before CP surgery and the insertion of VTs during follow-up (χ^2=17.26, p=0.0006, Cramer's V: 0.515, Table III). In other words, as the degree of hearing loss prior to CP surgery increased, the likelihood of OME resolution decreased and the need for VTs increased. In pairwise comparisons, there was a significant difference in the need for tube insertion between normal vs. mild HL (χ^2=12.39; p=0.0026, phi=0.513) and normal vs. moderate HL (χ^2=9.69, p=0.01, phi=0.622, Table IV).

Table I. Patient demographics, n (%).

Gender	Male		39 (60%)
	Female		26 (40%)
CP type	Veau 1		10 (15.4%)
	Veau 2		22 (33.8%)
	Veau 3		20 (30.8%)
	Veau 4		13 (20%)
Hearing levels before CP Repair	Bilateral normal hearing		17 (26.1%)
	Bilateral slight CHL		10 (15.4%)
	Bilateral mild CHL		30 (46.2%)
	Bilateral moderate CHL		8 (12.3%)
Ventilation tube insertion	No		34 (52.3%)
	Yes	Only once	20 (30.8%)
		More than once	11 (16.9%)

CHL: conductive hearing loss, CP: cleft palate

Table II. Hearing status before palatal repair and rates of ventilation tube insertion by cleft palate type.

Cleft palate type	Total number of patients	Hearing Status, n (%)						Ventilation Tube Insertion, n (%)				
		Normal hearing	Slight HL	Mild HL	Moderate HL	Chi- square	p	No VT	VT inserted	Chi- square	p	Cramer's V
Veau 1	10	5 (50%)	1 (10%)	3 (30%)	1 (10%)			9 (90%)	1 (10%)			
Veau 2	22	6 (27.3%)	4 (18.2%)	10 (45.4%)	2 (9.1%)			12 (54.5%)	10 (45.5%)			
Veau 3	20	3 (15%)	3 (15%)	11 (55%)	3 (15%)	4.83	0.848	9 (45%)	11 (55%)	8.585	0.035*	0.363
Veau 4	13	3 (23.1%)	2 (15.4%)	6 (46.1%)	2 (15.4%)			4 (30.8%)	9 (69.2%)			

Chi-square test, *p<0,05 accepted as statistically significant, Cramer's V represents strength of association, HL: hearing loss.

VT-related complications

The follow-up period of the 47.7% of patients (n=31) who had a VT inserted ranged from 12 to 118 months. The median follow-up was 42 months. No significant relationship was found between cleft severity and VT complications (Table V). Although early extrusion was more frequently observed in patients with greater preoperative HL, the association was not statistically significant (p = 0.118). Similarly, no significant correlations were found between hearing levels and otorrhea (p = 0.706) or persistent perforation (p = 0.443, Table VI). In 16.9% of patients (n=11), the VT extruded early before the OME resolved and these patients required more than one episode of VT insertion. These patients required an average of 2.82 VT insertions. 45.2% of patients (n=14) had at least one episode of VT otorrhea. 12.9% of patients (n=4) had a permanent perforation of the tympanic membrane after extrusion of the VT (Table V). In all reported cases, the VT had extruded spontaneously rather than being removed surgically. The diagnosis of "permanent perforation" was made at least 12 months after extrusion, during routine postoperative follow-up. All 4 patients who developed a persistent perforation were found to have had at least one episode of VT otorrhea.

Discussion

CP type and its effect on hearing status and progression

It is reasonable to expect that more severe CP types (e.g., BCLP, Veau IV) would be associated with poorer initial hearing status and less favorable OME prognosis.[6] Previous studies have indicated that a higher proportion of patients with complete clefts require repeat VT placements compared to those with incomplete clefts.[7,20,21] Conversely, Shaffer et al. found no association between multiple VT insertions and CP type or Veau classification.[22] Similarly, Nomura et al. observed no correlation between the recurrence of OME after palatoplasty and CP type.[23]

Table III. Rates of ventilation tube insertion during follow-up of patients by hearing level of patients before palatal surgery.

Hearing Level	Ventilation Tube Insertion , n (%)		Chi-square	p	Cramer's V
	No	Yes			
Bilateral normal	16 (94.1%)	1 (5.9%)	17.26	0.0006*	0.515
Bilateral slight HL	5 (50%)	5 (50%)			
Bilateral mild HL	11 (36.7%)	19 (63.3%)			
Bilateral moderate HL	2 (25%)	6 (75%)			

Chi-square test, *p<0.05 accepted as statistically significant, Cramer's V represents strength of association, HL: hearing loss.

Table IV. Pairwise comparison of ventilation tube insertion rates by hearing level of patients before palatal surgery.

Pairwise Comparisons	Chi-square	p value (raw)	p value (corrected)	Phi
Normal vs. slight HL	4.76	0.029	0.174	0.42
Normal vs. mild HL	12.39	0.0004*	0.0026*	0.513
Normal vs. moderate HL	9.69	0.0018*	0.01*	0.622
Slight vs. mild HL	0.13	0.71	1	0.058
Slight vs. moderate HL	0.35	0.55	1	0.14
Mild vs. moderate HL	0.04	0.84	1	0.03

Chi-square test, the adjusted p cut-off for raw p was accepted as 0.0083 (0.05/6). *p<0.05 accepted as statistically significant for p-values corrected by Bonferroni method, Phi represents strength of association, HL: hearing loss.

Table V. Frequency of complications in patients with ventilation tubes by cleft palate type.

Complication	Veau 1		Veau 2		Veau 3		Veau 4		Total		p (χ2)
	Yes (n)	No (n)	Yes (n)	No (n)	Yes (n)	No (n)	Yes (n)	No (n)	Yes n (%)	No n (%)	
Early extrusion	0	1	2	8	6	5	3	6	11 (35.5%)	20 (64.5%)	0.339
Otorrhea	1	0	7	3	3	8	3	6	14 (45.2%)	17 (54.8%)	0.131
Permanent perforation	0	1	1	9	1	10	2	7	4 (12.9%)	27 (87.1%)	0.787
Cholesteatoma	0	1	0	10	0	11	0	9	0 (0%)	31 (100%)	n/a

χ2: Chi-square test, *p<0.05 accepted as statistically significant.

Table VI. Frequency of complications in patients with ventilation tubes by pre-operative hearing level.

Complication	Normal hearing		Slight HL		Mild HL		Moderate HL		Total		p (χ2)
	Yes (n)	No (n)	Yes (n)	No (n)	Yes (n)	No (n)	Yes (n)	No (n)	Yes n (%)	No n (%)	
Early extrusion	0	1	0	5	7	12	4	2	11 (35.5%)	20 (64.5%)	0.118
Otorrhea	0	1	3	2	8	11	3	3	14 (45.2%)	17 (54.8%)	0.706
Permanent perforation	0	1	2	3	1	18	1	5	4 (12.9%)	27 (87.1%)	0.443
Cholesteatoma	0	1	0	5	0	19	0	6	0 (0%)	31 (100%)	n/a

χ2: Chi-square test, *p<0.05 accepted as statistically significant, HL: hearing loss.

In our study, although a statistically significant increase in VT insertion rates was observed with higher Veau classifications (from 10% in Veau I to 69.2% in Veau IV; p = 0.035), we found no direct association between cleft severity and preoperative hearing levels or complication rates. This partially aligns with the findings of Iemura-Kashiwagi et al., who reported that patients with more extensive clefts—particularly those involving the alveolus—were at greater risk of OME recurrence and thus more likely to require repeated tympanostomy tube insertion.[6] However, Schwarz et al. found no statistically significant correlation between cleft width, cleft type, and VT insertion prevalence, suggesting that additional anatomical or functional factors may influence surgical decisions.[24] Yoshitomi et al. further supported this by showing that cleft width was significantly associated with the severity and nature of middle ear effusion prior to palatoplasty, but not with the overall incidence or duration of OME.[25] Together, these findings emphasize the complexity of predicting VT needs based solely on cleft morphology and support the use of audiological criteria—such as hearing thresholds—as a more reliable indicator, as proposed in our individualized management strategy.

Prophylactic (early) vs. late grommet insertion

Several studies have demonstrated the beneficial effects of early tympanostomy tube placement on hearing, speech, and language development in children with CP, supporting a proactive approach to the management of OME.[4,21,26-28] However, other researchers have raised concerns regarding this strategy, citing potential complications such as myringosclerosis, tympanic membrane perforation, and cholesteatoma formation.[9,11,29,30]

Proponents of early tympanostomy tube placement include Frisina et al. who identified the absence of a VT at the time of palate repair as an independent prognostic risk factor for hearing loss in patients with CP.[31] Similarly, Azman et al. reported favorable otological outcomes in younger children who underwent selective VT insertion during palatal closure before the age of one.[32] Valtonen et al. demonstrated that early tympanostomy performed at six months of age yields comparable otological and audiological outcomes, as well as mastoid air cell system development, in both cleft and non-cleft patients with OME, without significant long-term otologic complications.[33] Klockars et al. suggested that early VT placement, even before palatal closure, may offer better outcomes.[34] Inoue et al. evaluated the long-term otological and audiological outcomes in children with and without CP who underwent tympanostomy for OME before the age of 2.[35] They concluded that outcomes were similar in both groups, affirming the positive effects of VT on hearing and language development in patients with OME.

Conversely, Robson et al. reported no significant advantage of early VT on developmental outcomes and even observed worse hearing in the treated group, supporting a conservative approach.[11] In their retrospective series of 213 CP patients with OME, Gani et al. reported that they placed VT in only 41 patients (19.2%), 22 at the time of palatal surgery and 19 at follow-up, and treated the remaining 22 patients (10.3%) with hearing aids.[30] In another study, a conservative approach resulted in a 29% tube insertion rate, with more frequent grommet use observed in patients with severe clefts.[21] These findings indicate that prophylactic VT placement is not universally required for all patients with CP. Systematic reviews by Ponduri et al. and Kuo et al. concluded that evidence for early VT benefits remains limited.[9,29] Maina et al., in their recent review, emphasized that while VT insertion may be associated with increased complication rates, conservative management can be a safe alternative when hearing is closely monitored.[36]

It is intuitively plausible that CP repair may improve Eustachian tube function by restoring the integrity of the palatal muscles and soft palate[9], Supporting this, D'Andrea et al. observed that early interventions, such as Sommerlad intravelar veloplasty, reduced the

need for VT insertions by decreasing persistent OME.[8] Additionally, some studies indicate that as Eustachian tube function matures with age, patients with CP require fewer VT insertions later in life.[37] However, the majority of patients with CP develop OME at an early age, making early intervention critical to ensuring their hearing, speech, and motor development progress in line with their peers.[32,38,39] At this stage, it is imperative to establish clear, evidence-based indications to determine which patients would benefit from VT placement and the optimal timing for intervention.

A recent guideline clearly stated that VT insertion may not be required for all CP patients and should be based on tympanic membrane status and hearing loss assessment prior to palatoplasty.[40] In line with this, our data suggest that audiological tests are reliable tools for differentiating risk groups and should be included in the armamentarium of every clinician treating these patients. Patients, especially those with mild and moderate hearing loss, could benefit from VT insertion during CP surgery. Because these patients are less likely to experience resolution of the effusion compared to patients with normal hearing. In patients with hearing loss less than 25 dB, watchful waiting may be a more appropriate approach to avoid complications of unnecessary VT insertion.

Complications of VT in patients with CP

VT complication rates may reach 80%, with otorrhea being the most common and burdensome.[41] Studies indicate that otorrhea rates are notably higher in patients with CP compared to those without.[29] Ungkanont indicated that the group undergoing routine VT insertion exhibited a greater prevalence of tympanic membrane abnormalities and an increased number of grommets placed.[42]

Conversely, a large-scale study involving 3,003 patients found no statistically significant differences in complication rates, including otorrhea or the need for ear nose throat (ENT) follow-ups, between CP patients with tube insertion and non-CP patients.[43] Similarly, a retrospective study of 285 patients reported a low rate (7.5%) of persistent tympanic membrane perforation following VT insertion, with only 3 cases of cholesteatoma due to tympanic membrane retraction.[31] Another study with 116 patients and a 72-month follow-up concluded that VT insertion did not influence cholesteatoma development in patients with CP.[44]

In line with the literature, we found that the most common complication of VT was otorrhea with rates of 45.2% (n=14). In our study, the median follow-up of patients who had ventilation tubes placed was 42 months, and no patients developed cholesteatoma. However, it was found that 12.9% of patients placed in VT developed a permanent perforation. The reason why this rate is higher than in the literature may be due to the use of Paparella type 2 VTs. In our clinic, because of the longer duration of effusion and the higher need for repeated VTs in patients with CP, medium to long term Paparella type 2 tympanostomy tubes are preferred. It is known that permanent perforation rates increase as the duration of VT increases.[23]

In our study, no significant relationship was found between the type of cleft palate and VT-related complications. Although the results of the study by Shaffer et al.[22] are parallel to this finding, there are articles in the literature arguing that the early extrusion rate increases with increasing cleft palate severity.[7,21] Our analysis showed that while complication rates, particularly early extrusion, appeared higher in patients with moderate hearing loss, no statistically significant relationships were established between preoperative hearing levels and VT-related complications. Although several studies have examined the timing of VT placement and its impact on hearing outcomes in patients with cleft palate, there is a lack of research specifically investigating the direct relationship between preoperative hearing levels and VT-related complications. Therefore, further studies are warranted in this area.

Limitations

The study adds valuable information to the ongoing debate about prophylactic versus selective VT insertion during CP repair, emphasizing evidence-based patient selection. The study has several limitations. The heterogeneity in surgical techniques, including Furlow palatoplasty, Dorrance palatoplasty, and two-flap palatoplasty, may have influenced outcomes differently due to varying effects on Eustachian tube function and middle ear ventilation. The limited sample size reduces the statistical power and may obscure significant differences or relationships between subgroups. Additionally, the retrospective design introduces potential bias and limits the ability to establish causality. As a single-center study, the findings may not be generalizable to other populations or healthcare settings. The use of medium-to-long-term Paparella type 2 tympanostomy tubes, which are associated with higher rates of permanent perforation, may have influenced the reported complication rates, limiting the comparability of the results with studies using short-term tubes.

Conclusion

This study underscores the importance of individualized management strategies for OME in patients with CP. Audiological assessments proved critical for stratifying risk groups and identifying candidates for VT insertion. While mild to moderate hearing loss was significantly associated with persistent OME and the need for VTs, patients with normal or slight hearing loss benefited from a conservative approach, minimizing unnecessary interventions and related complications. In addition, the literature presents some inconsistencies; however, it can be reasonably inferred that individuals with more severe cleft deformities are less likely to achieve resolution of OME. Such cases may necessitate repeated VT insertions and, therefore, demand closer and more frequent monitoring.

Tailoring VT insertion to patients' audiological profiles may optimize outcomes by balancing the benefits of early intervention against potential complications. Further prospective studies are needed to refine these guidelines and evaluate long-term impacts on hearing, speech, and language development.

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Ethical approval

The study was approved by Hacettepe University Non-Interventional Clinical Investigations Ethics Committee (date: 29.06.2021, number: 21/859).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: OK, EP, MÇK, BT, GÇ, MY, MK, FFÖ; data collection: BT,MÇK, EP; analysis and interpretation of the results: BT, EP, MÇK, GÇ, MY, MK, FFÖ; draft manuscript preparation: BT, EP, MÇK, GÇ, OK, MY, MK, FFÖ. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Original Article

Toll-like receptor 7 single nucleotide polymorphism rs3853839 in pediatric patients with immune thrombocytopenia

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ABSTRACT

Background. Immune thrombocytopenia (ITP) is a multifactorial disease involving environmental and genetic factors. This study aimed to evaluate the association of a single nucleotide polymorphism (SNP) rs3853839 in the Toll-like receptor 7 (*TLR7*) gene with susceptibility to ITP and its clinical features.

Methods. This retrospective, observational, case-control study was conducted on 172 pediatric patients with ITP and 170 healthy children. Genomic DNA was extracted from peripheral blood and genotyped via a snapshot technique.

Results. The serum *TLR7* mRNA in the case group (1.129±0.536) was significantly higher than that in the control group (0.851 ± 0.298) ($p<0.001$). Female patients with the GG genotype and male patients with the G/(-) genotype demonstrated the highest level of *TLR7* mRNA (1.478±0.522 and 1.280±0.590, respectively) ($p<0.0001$), whereas female patients with the CC genotype and male patients with the C/(-) genotype showed the lowest level of *TLR7* mRNA (0.752±0.171 and 0.732±0.218, respectively) ($p<0.0001$). The severity and chronic progression of ITP was significantly increased in female patients with the GG genotype and male patients with the G/(-) genotype ($p<0.05$). However, *TLR7* rs3853839 polymorphism was not significantly associated with corticosteroid sensitivity and disease recurrence ($p>0.05$).

Conclusions. This study suggests that *TLR7* rs3853839 may be a key genetic factor in the susceptibility and severity of ITP disease, providing new insights into disease progression and severity prediction. These findings present significant insights into the pathogenesis of ITP and may serve as a foundation for developing personalized treatment strategies tailored for pediatric patients with ITP.

Key words: immune thrombocytopenia (ITP), *TLR7*, genetic polymorphism, genotype.

Primary immune thrombocytopenia (ITP) is a highly complex autoimmune disease.[1] Its etiology is still not fully understood. Genetic and environmental factors play essential roles in the pathogenesis of ITP.[2] The clinical features of ITP can include increased antibody-mediated platelet destruction or antibody-mediated inhibition of platelet production, leading to insufficient platelet production.[3] The International Working Group (IWG) defines ITP as newly diagnosed (diagnosis to 3 months), persistent (3 to 12 months from diagnosis), or chronic (lasting >12 months).[4]

Toll-like receptors (TLRs) play essential roles in responses against microbial agents,

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inflammatory pathways, and the regulation of innate immune responses.[5] Disturbances in the innate immune response can precipitate the onset and progression of ITP.[6] TLRs induce the development and differentiation of T-cell subsets, including Th1, Th2, Th17, and Tregs. They also modulate the development and functions of Tregs through mediated signals and can impact the development of atopic disorders. Dysregulated TLR expression or genetic variations can contribute to imbalances in Th1 or Th2 immunity levels.[7-9] In patients with ITP, there is an observed imbalance in CD4+ T cell subsets, characterized by a skewed Th1/Th2 balance that favors Th1 and a skewed Th17/Treg balance that favors Th17. Consequently, there is an elevated production of Th1 and Th17 cytokines.[10,11]

Toll-like receptor 7 (*TLR7*) gene is found in X chromosome Xp22.2, which encodes the TLR7 protein. TLR7 serves as an intracellular pattern recognition receptor and is associated with multiple polymorphisms that are potentially associated with human disease.[12] In the investigation into the pathogenesis of ITP, whole-blood gene expression profiling from ITP patients was conducted, revealing the involvement of TLR7 in the pathogenesis of ITP.[13]

Although studies have shown that TLR7 may play an important role in the pathogenesis of ITP, the specific genetic variation and its functional effects still need further exploration. Previous studies on ITP gene polymorphism mainly focused on the *PTPN22* gene[14], *HDAC3* gene[15], and *FOXP3* gene[16], while the study of *TLR7* gene polymorphism and ITP has not been reported.

TLR7 expression is associated with *TLR7* rs3853839 C/G polymorphism, which has been shown to impact *TLR7* mRNA turnover in genetic studies.[17] The rs3853839 C/G SNP, which is located in the 3' untranslated region of the *TLR7* gene, has been associated with increases in *TLR7* mRNA and protein expression.[18] Numerous studies have indicated a correlation between the rs3853839 C/G SNP and various autoimmune diseases, including systemic lupus erythematosus (SLE)[12], knee osteoarthritis[19], and autoimmune thyroid disease.[20] To our knowledge, to date no studies have been undertaken to clarify the relationship between *TLR7* rs3853839 C/G polymorphism and ITP. Therefore, it is essential to investigate the rs3853839 C/G single nucleotide polymorphism to enhance our understanding of the pathogenesis and clinical characteristics associated with ITP.

The main objective of this study was to evaluate the association of the *TLR7* rs3853839 C/G SNP with susceptibility to ITP and its clinical features.

Materials and Methods

Study design

This retrospective, observational, case-control study was conducted on 172 pediatric patients with ITP, and 170 healthy children were included as a control group. The study was conducted from January 2020 to January 2023. All the patients agreed to participate in the study and signed a written informed consent form before enrollment. The diagnosis of ITP is based on the updated international consensus report.[21] In this study, the clinical information of the children was collected, and all the children were followed for at least 1 year. Patients with a follow-up duration of less than one year or incomplete records were excluded from the study.

These definitions were developed in accordance with ITP Working Group guidelines to study the clinical characteristics and follow-up treatment outcomes of ITP.[21]

Study definitions

1. Primary ITP: isolated thrombocytopenia (peripheral blood platelet count $<100\times10^9$ /L) in the absence of other conditions associated with thrombocytopenia.

2. Newly diagnosed ITP: within 3 months from diagnosis.
3. Persistent ITP: between 3 and 12 months from diagnosis.
4. Chronic ITP: lasting for >12 months.
5. Severe ITP: presenting with bleeding sufficient to start treatment or occurrence of new bleeding symptoms necessitating additional treatment mediation with an alternative agent or an increased dose.

Response to treatment

All children with ITP were administered high-dose dexamethasone through intravenous infusion. The treatment was given at 0.6 mg/kg daily, with a maximum dose allowed as 40 mg over four days. The response after steroid treatment was evaluated as follows:

Corticosteroid sensitivity: after standard glucocorticoid treatment, the platelet count significantly increased and stabilized at a safe level ($\geq 30\times10^9$/L), effectively managing bleeding symptoms.

Corticosteroid resistance: platelet counts below 30×10^9/L, less than a twofold increase of baseline platelet count, or the presence of bleeding symptoms.

Recurrence: decrease in platelet counts below 30×10^9/L or less than twice the baseline or the presence of bleeding symptoms following treatment response.

Sample collection

The sterile venipuncture was used to extract 3-5 mL of venous blood from each patient into an EDTA anticoagulant tube. A blood DNA extraction kit (Qiagen, Maryland, USA) was used to store the samples in the same vacuum tank at -20 °C until DNA extraction.

DNA amplification and isolation

Whole-blood DNA was extracted via the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). DNA concentration and purity were determined using a spectrophotometer NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA), and all DNA samples were diluted to working concentrations of 50 ng/μL.

Amplification PCR and snapshot assay

The SNaPshot assay is a mini-sequencing method widely used to detect polymorphisms in individual genes or the whole genome. The assay is sensitive and straightforward, and the results can be automatically evaluated using software.[22] Genotyping of the *TLR7* rs3853839 gene locus was performed via the Snapshot technique.

The total volume of each PCR mixture was 25 μL: 5 μL of DNA, 12.5 μL of prepared Taq Red PCR master mix (Bioline), 1 μL of each primer, and 5.5 μL of nuclease-free water. The forward primer sequence of *TLR7* rs3853839 was as follows: AACCAATTGCTTCCGTGTCA. The reverse primer sequence of *TLR7* rs3853839 was as follows: GTTGCTGTATCAAGTGTGCAGA. The PCR cycle conditions were as follows: 1 cycle of 95 °C predenaturation for 30 seconds followed by 40 cycles of 95 °C denaturation for 10 seconds and 60 °C annealing and extension for 30 seconds. The PCR products of each SNP were examined via 2.0% agarose gel electrophoresis and digested with the corresponding restriction enzymes (Thermo Scientific, USA) according to the manufacturer's protocol. The digested products were electrophoresed on a 3% agarose gel containing ethidium bromide and then visualized via UV transmission.

Statistical analysis

The Helmholtz Centre website (Germany) was used to calculate the Hardy-Weinberg equilibrium (HWE) of *TLR7* rs3853839 genotypes. SPSS 23 software (IBM Corp, NY, USA) was used for statistical analysis of the current data. Normally distributed measurement data are presented as the means ± SDs. The chi-square test was used to compare

categorical variables and assess the distribution of *TLR7* SNPs in the association between *TLR7* rs3853839 and ITP susceptibility, corticosteroid sensitivity, disease severity, disease recurrence, and disease progression. The risk associated with individual genotypes or alleles was calculated as the odds ratio (OR) with their 95% confidence intervals (95% CI). Differences between various groups were compared via a one-way analysis of variance and $p<0.05$ indicated a statistically significant difference.

Results

Essential characteristics of the research subjects

This study included 172 pediatric patients with ITP and 170 children in the control group. Cases and controls were matched in age and gender. The mean age of disease onset among cases was 6.065±3.283 years, while in the control group, it was 5.939±3.344 years ($p=0.688$) (Table I). The male-to-female ratio in the case group was 1.17:1, while in the control group, it was 1.24:1 ($p=0.725$) (Table I). The serum *TLR7* mRNA in the case group (1.129±0.536) was significantly higher than that in the control group (0.851±0.298) ($p<0.001$) (Table I and Fig. 1). Female patients with the GG genotype and male patients with the G/(-) genotype demonstrated the highest level of *TLR7* mRNA (1.478±0.522 and 1.280±0.590, respectively) ($p<0.0001$). Whereas female patients with the CC genotype and male patients with the C/(-) genotype showed the lowest level of *TLR7* mRNA (0.752±0.171 and 0.732±0.218, respectively) ($p<0.0001$) (Table I and Fig. 1). The remaining clinical and laboratory data of the case group encompassed platelet count, initial bleeding event, corticosteroid sensitivity, disease severity at the time of sampling, disease recurrence, and disease progression, which are shown in Table I.

TLR7 rs3853839 PCR amplification products and SNaPshot sequencing results

SNaPshot sequencing results indicate that blue peaks correspond to the G base, and black peaks correspond to the C base. Both blue and black peaks signify the CG base. As the *TLR7* gene is situated on the X chromosome, female children

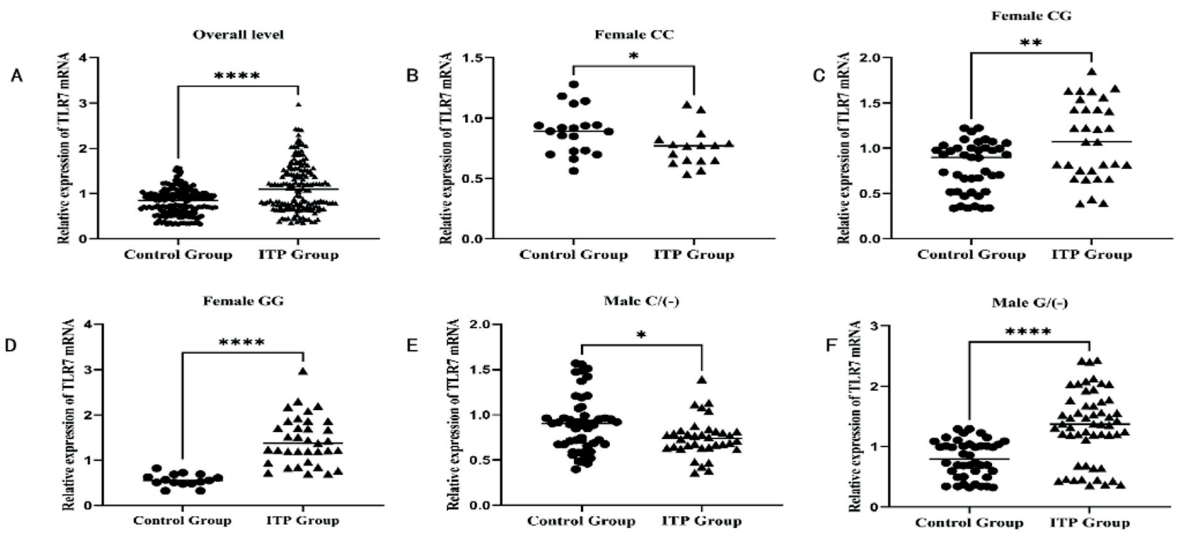

Fig. 1. Comparison of relative expression of *TLR7* mRNA between control and ITP groups with different genotypes.

A: Children in the control and ITP groups. B: Female children in the control and ITP groups with *TLR7* rs3853839 CC genotype. C: Female children in the control and ITP groups with *TLR7* rs3853839 CG genotype. D: Female children in the control and ITP groups with *TLR7* rs3853839 GG genotype. E: Male children in the control and ITP groups with *TLR7* rs3853839 C/(-) genotype. F: Male children in the control and ITP groups with *TLR7* rs3853839 G/(-) genotype.
ITP, immune thrombocytopenia; *TLR7*, toll-like receptor 7.

Table I. Clinical and demographic characteristics of participants.

Characteristics	ITP group (n=172)	Control group (n=170)	*p* value
Age of disease onset (yrs, M±SD)	6.065±3.283	5.939±3.344	0.688
Sex (male: female)	1.17:1	1.24:1	0.725
Relative expression of *TLR7* mRNA (M±SD)	1.129±0.536	0.851±0.298	<0.0001
TLR7 mRNA expression by genotype (M±SD)			
Female			
GG	1.478±0.522	0.583±0.143	<0.0001
CG	1.068±0.421	0.917±0.304	0.0604
CC	0.752±0.171	0.903±0.151	0.0146
Male			
G/(-)	1.280±0.590	0.831±0.295	<0.0001
C/(-)	0.732±0.218	0.889±0.325	0.0495
Platelet count ($\times10^9$ /L)	37.34±23.14	151.6±39.38	N/A
≤30×10^9 /L (n, %)	90 (52.33)	N/A	
>30×10^9 /L (n, %)	82 (47.67)	N/A	
Initial bleeding event (n, %)			N/A
Purpura	108 (62.79)	N/A	
Ecchymosis	38 (22.09)	N/A	
Wet purpura	26 (15.12)	N/A	
Corticosteroid sensitivity (n, %)			N/A
Complete response	75 (43.60)	N/A	
Response	32 (18.60)	N/A	
No response	65 (37.80)	N/A	
Disease severity at sampling (n, %)			N/A
Severe	70 (40.70)	N/A	
Non-severe	102 (59.30)	N/A	
Disease recurrence (n, %)			N/A
Recurrent	50 (29.07)	N/A	
Non-recurrent	122 (70.93)	N/A	
Disease progression (n, %)			N/A
Chronic ITP	55 (31.98)	N/A	
Non-chronic ITP	117 (68.02)	N/A	

ITP, immune thrombocytopenia; M±SD, mean ± standard deviation; N/A, not applicable; *TLR7*, Toll-like receptor 7.

exhibited the GG, CC, and CG genotypes (Fig. 2, A, B, and C), while male children showed the G/(-) and C/(-) genotypes (Fig. 2, D and E).

TLR7 rs3853839 C/G genotype, allele, and gene frequency distributions

The *TLR7* rs3853839 GG genotype frequency in female patients was significantly higher compared to the control group (χ^2=6.505, *p*=0.039) (Table II). The *TLR7* rs3853839 G/(-) genotype frequency in male patients was significantly higher compared to the control group (χ^2=3.968, *p*=0.046; OR=1.857, 95% CI: 1.034-3.336) (Table II). The *TLR7* rs3853839 G allele gene frequency in ITP patients was significantly higher compared to the control group (χ^2=6.146, *p*=0.013; OR=1.567, 95% CI: 1.098-2.238) (Table II). The *TLR7* rs3853839 GG

carrying gene frequency in ITP patients was significantly higher compared to the control group (χ^2=5.826, p=0.016; OR=2.337, 95% CI: 1.164-4.693) (Table II).

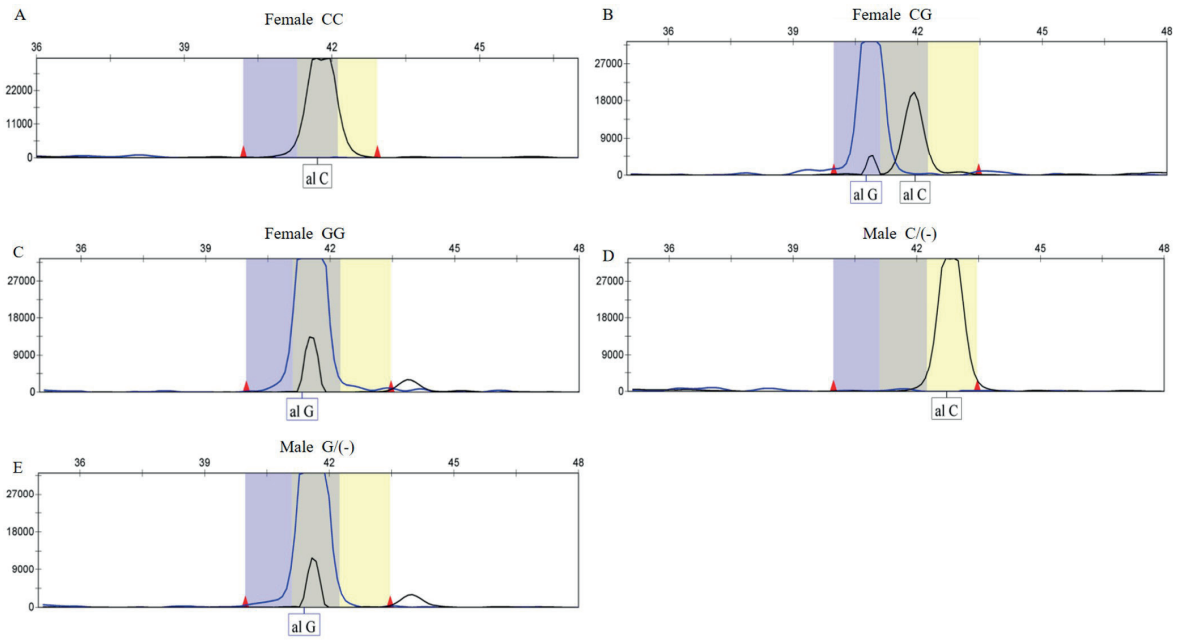

Fig. 2. Sequencing peak map of the *TLR7* rs3853839 gene locus.

A: Female children with *TLR7* rs3853839 CC genotype. B: Female children with *TLR7* rs3853839 CG genotype. C: Female children with *TLR7* rs3853839 GG genotype. D: Male children with *TLR7* rs3853839 C/(-) genotype. E: Male children with *TLR7* rs3853839 G/(-) genotype. Denoting blue peaks signify the G base, and black peaks signify the C base. Both blue and black peaks signify the CG base.

Table II. Frequency distribution of the *TLR7* rs3853839 G/C genotype, allele, and carrying gene in the ITP group and control group (n, %).

TLR7-C/G SNP Genotype	ITP group (n=172)	Control group (n=170)	χ^2	p value	OR	95% CI
Female	82	76	6.505	0.039	-	-
GG	33 (40.24)	17 (22.37)				
CG	33 (40.24)	44 (57.89)				
CC	16 (19.52)	15 (19.74)				
Male	90	94	3.968	0.046	1.824	1.007-3.303
G/(-)	59 (65.56)	48 (51.06)				
C/(-)	31 (34.44)	46 (48.94)				
Allele	254	246				
G	158 (62.20)	126 (51.22)	6.146	0.013	1.567	1.098-2.238
C	96 (37.80)	120 (48.78)				
Carrying gene	82	76	5.826	0.016	2.337	1.164-4.693
GG	33 (40.24)	17 (22.37)				
CG+CC	49 (59.76)	59 (77.63)				

CI, confidence interval; ITP, immune thrombocytopenia; OR, odds ratio; SNP, single nucleotide polymorphism; *TLR7*: Toll-like receptor 7.

The relationship between TLR7 rs3853839 C/G gene polymorphism and corticosteroid sensitivity in pediatric patients with ITP

All cases were classified into corticosteroid-sensitive and corticosteroid-resistant cohorts based on their reactivity to corticosteroid therapy (n=107 and 65, respectively). The distribution of genotypes between the two groups was compared; however, no significant correlation was observed between the *TLR7* rs3853839 genotypes and corticosteroid sensitivity in pediatric patients with ITP (p>0.05, Table III). This indicates that the *TLR7* rs3853839 polymorphism is not associated with ITP in relation to the response to corticosteroid therapy.

The relationship between TLR7 rs3853839 C/G gene polymorphism and the severity of pediatric patients with ITP

All cases were classified into the severe ITP group and non-severe ITP group (n=70 and 102, respectively). Among female ITP patients with different genotypes of *TLR7* rs3853839, the proportion of GG genotype in severe ITP patients was 19 (55.88%), which was significantly higher than that in non-severe ITP patients 14 (29.17%), and this difference was statistically significant (χ^2=6.371, p=0.041, Table III). Among male ITP patients with different genotypes of *TLR7* rs3853839, the proportion of G/(-) genotype in severe ITP patients was 28 (77.78%), which was significantly higher than that in non-severe ITP patients 31 (57.41%), and this difference was again statistically significant (χ^2=3.969, p=0.046, Table III). This indicates a potential correlation between the *TLR7* rs3853839 polymorphism and the severity of ITP disease.

The relationship between TLR7 rs3853839 C/G gene polymorphism and disease recurrence

All cases were classified into the recurrent ITP group and the non-recurrent ITP group (n=50 and 122, respectively). The distribution of genotypes between the two groups was compared; however, no significant correlation was observed between the *TLR7* rs3853839

Table III. Relationship between *TLR7 rs3853839* genotype and clinical indicators in pediatric patients with ITP, n (%).

Clinical indicators	Genotype (Female)					Genotype (Male)			
	GG (n, %)	CG (n, %)	CC (n, %)	χ^2	p value	G/(-) (n, %)	C/(-) (n, %)	χ^2	p value
Corticosteroid sensitivity	33 (40.24)	33 (40.24)	16 (19.51)	3.038	0.219	59 (65.56)	31 (34.44)	0.891	0.345
Corticosteroid-sensitive ITP group (n=107)	19 (41.31)	21 (45.65)	6 (13.04)			38 (62.30)	23 (37.70)		
Corticosteroid-resistant ITP group (n=65)	14 (38.89)	12 (33.33)	10 (27.78)			21 (72.41)	8 (27.59)		
Disease severity	33 (40.24)	33 (40.24)	16 (19.51)	6.371	0.041	59 (65.56)	31 (34.44)	3.969	0.046
Severe ITP group (n=70)	19 (55.88)	9 (26.47)	6 (17.65)			28 (77.78)	8 (22.22)		
Non-severe ITP group (n=102)	14 (29.17)	24 (50.00)	10 (20.83)			31 (57.41)	23 (42.59)		
Disease recurrence	33 (40.24)	33 (40.24)	16 (19.51)	1.183	0.553	59 (65.56)	31 (34.44)	0.300	0.584
Recurrent ITP group (n=50)	10 (37.04)	13 (48.15)	4 (14.81)			14 (60.87)	9 (39.13)		
Non-recurrent ITP group (n=122)	23 (41.82)	20 (36.36)	12 (21.82)			45 (67.16)	22 (32.84)		
Disease progression	33 (40.24)	33 (40.24)	16 (19.51)	8.968	0.011	59 (65.56)	31 (34.44)	4.333	0.037
Non-chronic ITP group (n=117)	17 (31.48)	28 (51.85)	9 (16.67)			37 (58.73)	26 (41.27)		
Chronic ITP group (n=55)	16 (57.14)	5 (17.86)	7 (25.00)			22 (81.48)	5 (18.52)		

genotypes and recurrence in pediatric patients with ITP (p>0.05, Table III). This indicates that the *TLR7* rs3853839 polymorphism is not associated with ITP in relation to the recurrence of ITP disease.

The relationship between TLR7 rs3853839 C/G gene polymorphism and disease progression

All cases were classified into the chronic ITP group and the non-chronic ITP group (n=55 and 117, respectively). Among female ITP patients with different genotypes of *TLR7* rs3853839, the proportion of GG genotype in chronic ITP patients was 16(57.14%), which was significantly higher than that in non-chronic ITP patients 17(31.48%), and the difference was statistically significant (χ^2=8.968, p=0.011, Table III). Among male ITP patients with different genotypes of *TLR7* rs3853839, the proportion of G/(-) genotype in chronic ITP patients was 22(81.48%), which was significantly higher than that in non-chronic ITP patients 37(58.73%), and the difference was statistically significant (χ^2=4.333, p=0.037, Table III). The results showed that ITP patients with *TLR7* rs3853839 GG genotype and G/(-) genotype had a significantly increased risk of developing chronic ITP.

Discussion

Recent information has shown that activation of *TLR7* leads to increased levels of Th1 and Th17 cells, and their cytokines play a pivotal role in the pathogenesis of ITP.[23,24] However, to the best of our knowledge, there is a lack of studies exploring the specific mechanisms involved. In this study, we aimed to clarify the characteristics of this mechanism associated with the *TLR7* rs3853839 single nucleotide polymorphism. Our results indicate that the *TLR7* rs3853839 GG genotype in female patients and the G/(-) genotype in male patients are linked to an increased risk of ITP, a more serious disease condition, and chronic disease progression. This finding is reported for the first time in the literature, as far as we are aware.

The pathogenesis of primary ITP involves multistep procedures, with genetic factors playing a pivotal role despite it being an autoimmune disease.[25] The principal function of the *TLR7* gene is to regulate the innate immune response.[4,26] Excessive activation or impaired function of *TLR7* may lead to innate immune dysfunction and the breakdown of immune tolerance, subsequently resulting in the onset of autoimmune diseases.[27] The immune system homeostasis in ITP patients typically relies on the dynamic equilibrium between Th1 and Th2 cells. However, any disruption to this balance can potentially induce changes in ITP.[11] Yang's research indicates that the activation of *TLR7* can lead to the polarization of Th1 immune cells, potentially influencing the development of ITP.[28] *TLR7* has also been found to recognize endogenous single-stranded RNA. When it is activated, a series of reactions occur through the classical myeloid cell differentiation factor 88 pathway, which promotes an increase in inflammatory factors such as IL-6, TNF-α, and IFN-γ, thus triggering the differentiation of Th17 cells and dendritic cells and ultimately leads to the immune inflammatory response.[29,30] Previous research has indicated a notable increase in the quantities of Th17 cells and their associated cytokines, IL-6 and TNF-α, in patients with ITP.[23,24] IL17 can induce the production of inflammatory cytokines such as IFN-γ, IL-1, and IL-6, further increasing the production of antiplatelet antibodies in mice[3,31] and patients with ITP.[32,33] Therefore, the *TLR7* gene may cause ITP through these pathways, and its specific mechanism needs further study.

Rs3853839, located in the 3′ UTR of *TLR7*, has been proven to be functional and related to an increase in *TLR7* mRNA and *TLR7* protein expression and the stimulation of IFN gene upregulation to cause the occurrence of disease.[18,34] The 3′ UTR is an essential regulatory region for expressing many genes that regulate mRNA translation, degradation, and subcellular localization by influencing RNA-binding proteins or noncoding RNAs.[35] Raafat et al.[36] reported that miRNAs could

regulate TLR signaling through direct effects on expression or by modulation of downstream regulators, adaptor molecules, and cytokines. The *TLR7* rs3853839 SNP can potentially impact gene expression by abolishing, weakening, or creating miRNA binding sites.[37] The non-risk C allele of *TLR7* rs3853839 matches a predicted binding site of microRNA-3148 (miR-3148), resulting in fast transcript breakdown and reducing *TLR7* mRNA levels.[38]

TLR7 rs3853839 SNP in Non-Hematological Disorders

It has been reported that the *TLR7* rs3853839 SNP is associated with human SLE.[39] Azab et al.[12] reported that the *TLR7* rs3853839 GG genotype and G allele were significantly associated with the pathogenesis of SLE in Egyptian patients. Yue et al.[18] reported that the *TLR7* rs3853839 C allele polymorphism is sex-specific and has a protective effect on the persistence of hepatitis C prevention in Chinese women. Xi et al.[19] revealed that the *TLR7* rs3853839 C/G polymorphism may play a role in susceptibility to knee OA. Shen's study conducted on patients with systemic lupus erythematosus revealed that individuals carrying the G mutant allele exhibited elevated *TLR7* mRNA transcripts. Heterozygous participants also had elevated levels of *TLR7* mRNA containing the G allele. These findings support the role of *TLR7* rs3853839 SNP in regulating *TLR7* mRNA expression.[34] El-Hefnawy et al.[40] indicated that the GG genotype of the *TLR7* rs3853839 SNP may be a genetic risk factor for severe COVID-19 and adverse clinical outcomes. Elevated *TLR7* mRNA expression in severe cases indicated its potential as a biomarker for COVID-19 prognosis. These findings also support the role of *TLR7* rs3853839 SNP in regulating *TLR7* mRNA expression in non-hematological disorders.

To our knowledge, this study represents the first demonstration of the association between *TLR7* rs3853839 C/G SNP and the susceptibility and clinical manifestations of ITP within the Chinese Han population. We also observed that the GG and G/(-) genotypes are associated with an increased risk of ITP. The GG and G/(-) genotypes may result in heightened activation of *TLR7*, thus enhancing the self-reactivity of the immune system and increasing the risk of ITP. The GG genotype and G/(-) genotypes were shown to be substantially more common among severe patients in the current investigation. This phenomenon may be explained by excessive activation of *TLR7*, which may lead to a stronger inflammatory and autoimmune response, aggravating platelet destruction and production inhibition. This study also demonstrated that the GG and G/(-) genotypes may be predictive indicators for the chronic progression of ITP in pediatric patients. The continuous activation of *TLR7* may lead to chronic disorders of the immune system, promoting the chronicity of ITP. This finding provides new insights into the individualized treatment of ITP.

Our study had certain limitations. Firstly, the participants included in our study were exclusively from the Chinese Han population. A more comprehensive investigation involving multi-racial populations is imperative to authenticate the correlations between *TLR7* rs3853839 gene polymorphisms and ITP. Secondly, the biological functions of *TLR7* rs3853839 remain unknown, and further mechanistic research is essential to elucidate the impact of *TLR7* rs3853839 gene polymorphisms on the transcription, splicing, and translation efficiency of *TLR7*. The third and last limitation is that the time required to draw blood is not standard. Moreover, treatment modalities other than steroids could not be examined in this study. Despite this, we believe that our results accentuate the role of immunological derangements in its pathogenesis and will lead to further studies. The course of *TLR7* mRNA individually in the same patient throughout the disease course and the cut-off levels at presentation, which predict the potential severity and progressivity of ITP in an individual patient, even at presentation, can be examined in further studies. These will enable

individualized therapy in ITP, like avoidance of steroids but treatment with *TLR7* inhibitors.

In conclusion, this study suggests *TLR7* rs3853839 gene polymorphisms may be a potential genetic marker for ITP, affecting disease susceptibility, severity, and treatment response. These findings provide new insights into the pathogenesis of ITP and may provide a basis for developing personalized treatment strategies.

Ethical approval

The study was approved by Medical Ethics Committee of Linyi People's Hospital (date: 10.26.2022, number: YX200343). Moreover, the participants' families were informed about the study, and their written consent was obtained.

Author contribution

The authors confirm contribution to the paper as follows: Study conception and design: JW, JL; data collection: JW, SW, GL, HS; analysis and interpretation of results: JW, JL; draft manuscript preparation: JW, SW, GL, HS, JL. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Original Article

Change in Gasdermin-D gene expression in familial Mediterranean fever compared to healthy children with or without acute infections

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ABSTRACT

Background. Gasdermin-D (GSDMD) is an inflammasome regulator. Pyroptosis and GSDMD-mediated interleukin (IL)-1β secretion abolish in GSDMD-deficient familial Mediterranean fever (FMF) knock-in mice. We aimed to investigate *GSDMD* gene expression (GSDMD-Δ), acute phase reactants (APRs), serum IL-1β, and IL-18 levels in FMF patients during attacks and attack-free periods.

Methods. We tested GSDMD-Δ, serum APRs, and serum IL-1β and IL-18 in 16 FMF patients (G1), during attack (G1-V1) and at attack-free visits (G1-V2). The GSDMD-Δ, serum IL-1β and IL-18 were measured in febrile controls with acute infections (G2) and healthy children (G3).

Results. Age and sex distribution of patients and controls were similar. Median GSDMD-Δ was 10 times higher in G1-V1 compared to G1-V2 ($p<0.001$). GSDMD-Δ was four times higher in G1-V1 than those observed in G2 ($p=0.026$); however, serum APRs were similar between these groups. GSDMD-Δ in G1-V2 and G3 did not differ significantly ($p>0.05$). GSDMD-Δ in G1 strongly correlated with serum C-reactive protein and amyloid-A ($r>0.60$, $p<0.01$) but did not correlate with serum IL-1β and IL-18. Median GSDMD-Δ and serum APRs were similar in patients carrying biallelic and monoallelic 'exon 10' mutations in *MEFV* gene both during attacks and attack-free visits ($p>0.05$).

Conclusion. We showed a significantly increased GSDMD-Δ for the first time in humans, thereby indicating the distinct role of GSDMD-Δ as a biomarker similar to APRs in FMF attacks. It was even higher than levels detected during acute infections, supporting the functional involvement of GSDMD-Δ in FMF attacks. GSDMD-Δ correlated with APRs but not with serum IL-1β and IL-18 levels.

Key words: Familial Mediterranean fever, gasdermins, *MEFV*, infection.

Systemic autoinflammatory diseases (AIDs) are characterized by recurrent, spontaneous, and inflammatory febrile attacks, primarily driven by dysregulation in the innate immune system.[1] There has been a shift from a gene-centric perspective to a systems-based classification to understand the intricate molecular mechanisms underlying AIDs.[2] Gasdermin-D (GSDMD) functions as a critical molecule in inflammasome response. Its activation through autoinhibition

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requires the cleavage of its N-terminal domain by caspase-1. Upon activation, GSDMD mediates the secretion of activated proinflammatory cytokines and intracellular components to the extracellular area by forming pores in the cell membrane, thus contributing to inflammasome-related pyroptosis.[3] Additionally, GSDMD plays a crucial role in neutrophil-extracellular trap generation and release, which further amplifies the inflammatory response.[4] Besides, GSDMD functions as a feedback regulator for inflammasome activation, highlighting its significance in various inflammatory, infectious, and autoimmune disease mechanisms and presenting it as a promising therapeutic target.[5]

Familial Mediterranean fever (FMF), classified under pyrin-related AIDs, is the most common AID.[6] The gain-of-function (GOF) mutations in the *Mediterranean Fever (MEFV)* gene encoding pyrin lead to a lowered activation threshold of the pyrin inflammasome, resulting in inflammatory manifestations characteristic of FMF.[7] The pyrin-caspase-1-GSDMD pathway is the central pathway of autoinflammation in FMF. The release of interleukin (IL)-1β, IL-18, and alarmin molecules is related to GSDMD through pyrin and caspase-1 activation.[8,9]

Typical inflammatory manifestations of FMF include non-periodic recurrent fever, serositis, and synovitis, which last for 6-72 hours, accompanied by increased serum acute phase reactants (APRs).[10] While there remains a lack of a clear genotype-phenotype correlation, it is noteworthy that biallelic 'exon 10' mutations in the *MEFV* gene are associated with higher disease activity, earlier disease onset, and renal amyloidosis compared to monoallelic mutations or mutations in non-'exon 10` regions.[11-15] Furthermore, pyrin inflammasome response defined by the magnitude of IL-1β secretion has been found to be higher in patients bearing biallelic `exon 10` mutations compared to those with a monoallelic `exon 10` mutations when stimulated by a bacterial toxin specifically activating the pyrin inflammasome.[7]

The primary objective of this study was to investigate the changes in GSDMD during FMF attacks compared with attack-free periods and healthy children with and without acute infections, in relation to serum APRs and serum IL-1β and IL-18. Additionally, we aimed to explore whether biallelic or monoallelic `exon 10` mutations in the *MEFV* gene exhibited a gene dosage effect on *GSDMD* gene expression (GSDMD-Δ).

Materials and Methods

Study design

Patients diagnosed with FMF according to Turkish pediatric FMF criteria were eligible for the study.[16] The *MEFV* gene mutations were examined through next-generation sequencing (NGS, QIAseq Targeted DNA FMF kit, Qiagen, Germany), and we further used an expanded periodic fever syndrome/AID NGS panel for a patient with a nonconfirmatory genotype of FMF.[17] The pathogenicity of the genetic variants in the *MEFV* gene was classified.[18] Patient recruitment and sample collection were completed between October 2022 and May 2023.

For the study, we evaluated FMF patients (Group 1 [G1], n=16) admitted during a disease attack (Group 1-Visit 1 [G1-V1]) and during a symptom-free period at least two weeks following the resolution of the disease attack (Group 1-Visit 2 [G1-V2]). We excluded FMF patients presenting with attack-like symptoms originating from other etiologies, such as infections and FMF-associated diseases, and those treated with biologics. We noted the compliance with colchicine for patients with a previous diagnosis of FMF. We grouped the FMF patients further according to the *MEFV* gene mutations (biallelic and monoallelic 'exon 10' mutations).

The febrile control group and healthy control group were recruited as age- and sex-matched to the FMF group. Healthy children without

any chronic disease were enrolled during a febrile acute infectious disease and classified as the febrile control group (Group 2 [G2], n=8) and healthy children without symptoms as the healthy control group (Group 3 [G3], n=10). The duration of fever was noted for the FMF group-attack visit (G1-V1) and febrile control group (G2) and their blood samples were collected after at least 8 hours but before 48 hours of fever. Children with a history of AIDs, both in themselves or their families, were excluded from both control groups. The flow diagram of patient recruitment is presented in Fig. 1.

We recorded the demographic data, clinical/laboratory findings, and current medications from electronic patient medical files and evaluated the weight and height of the patients according to the Turkish children's growth standards.[19] For the FMF group, the disease severity was calculated by the International Severity Scoring System for FMF (ISSF) which was validated for use in children and adults with FMF. A score of 3 or higher represents moderate-to-severe disease.[20]

Complete blood cell count with differential was tested in all study participants. We obtained serum APRs (C-reactive protein (CRP, N <5 mg/L), amyloid-A (SAA, N <0.5 mg/dL), fibrinogen (N <4.2 g/L), and erythrocyte sedimentation rate (ESR, N<15 mm/h)) for the FMF group at two visits (G1-V1 and G1-V2) and for the febrile control group (G2). We measured serum IL-1β and IL-18 levels with enzyme-linked immunosorbent assay (ELISA) and GSDMD-Δ by real-time polymerase chain reaction (RT-PCR).

We obtained written informed consent from all participants. The study complied with the Declaration of Helsinki, and the Clinical Research Ethics Committee of Hacettepe University Faculty of Medicine approved the study protocol. This work was supported by the Scientific Research Projects (BAP) Coordination Unit of Hacettepe University.

Measurement of serum IL-1β and IL-18

Serum was extracted from the peripheral blood by centrifugation at 3500 g for 15 minutes at +4 °C, subsequently transferred to sterile tubes and stored at -20 °C. The tubes were stored at -80 °C if the laboratory analysis was planned for later than three months.

Serum IL-1β and IL-18 were analyzed using human IL-1β and IL-18 sandwich ELISA kits

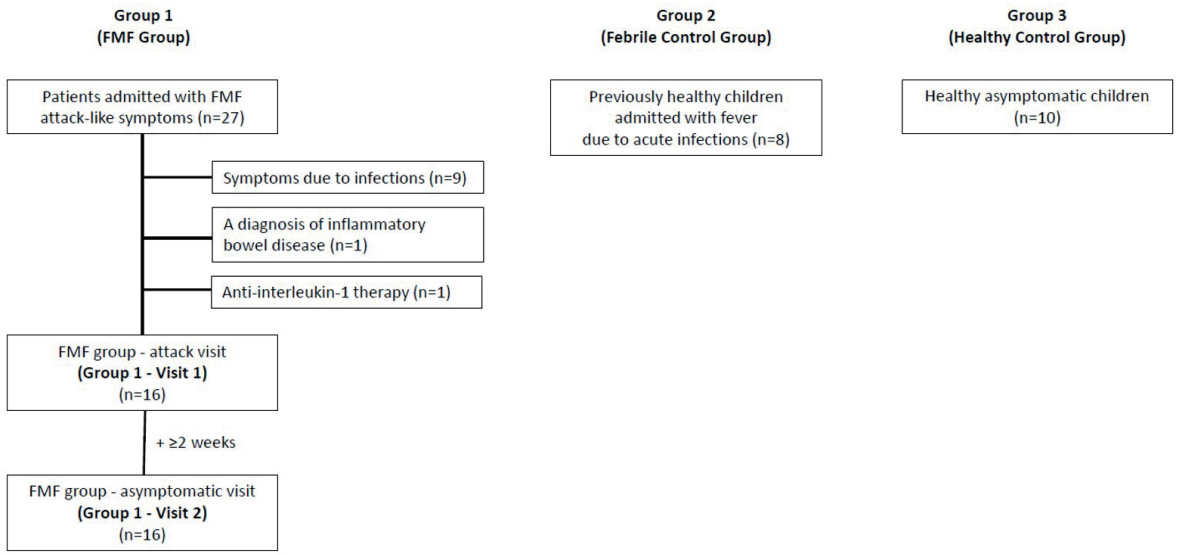

Fig. 1. Flow diagram of patient recruitment.
FMF: familial Mediterranean fever.

(BT Lab, Shanghai Korain Biotech Co., Ltd., China). We used the manufacturer's instructions and tested all samples twice. We measured the optical density (OD) spectrophotometrically at 450 nm in a microplate reader (SPECTROstar Nano, BMG LABTECH GmbH, Germany). Serum IL-1β (N <12 pg/mL) and IL-18 (N <120 pg/mL) levels were compared and quantified with the standard curves as pg/mL.

Measurement of GSDMD gene expression

We collected the peripheral whole blood into the PAXgene Blood RiboNucleic Acid (RNA) Tubes (Qiagen, Germany). The tubes were gently inverted, and stored at room temperature for 2-6 hours and then moved to +4°C for 1-2 days. Subsequently, we stored the tubes at -20°C if we planned to do the RT-PCR for less than three months. The samples were transferred to -80°C if we planned to perform RT-PCR for later than three months. We used a reference gene (glyceraldehyde-3-phosphate dehydrogenase, *GADPH*) to normalize GSDMD-Δ levels.

Ribonucleic acid was extracted according to the manufacturer's protocol from the peripheral blood mononuclear cells using the PAX gene Blood RNA System Kit. We measured the extracted deoxyribonucleic acid (DNA) using a NanoDrop ND-1000 spectrophotometer (Labtech International, UK). The average RNA concentration was 173.1 ng/μL. The quality output was evaluated with 260/280 and 260/230 ratios of purity estimations.

We used Quantitech Reverse Transcription Kit (Qiagen, Germany) for ribonucleic acid reverse transcription following the manufacturer`s protocol. We used specific primers and TaqMan probes for the target and housekeeping genes. Amplification occurred on the RT-PCR Corbett Rotor-Gene 6000. We tested each sample twice.

The threshold value was 10^{-3}, and the cycle threshold value (Ct) was 40 cycles. To evaluate the GSDMD-Δ level in the FMF and febrile control groups, we used the median fold change of GSDMD-Δ in the healthy control group. We performed the delta-delta Ct method to analyze RT-PCR data as a relative quantification method.

Statistical analysis

IBM SPSS Statistics (SPSS v21.0, IBM Corp, NY, USA) was used for statistical analysis. Descriptive statistics were presented with continuous variables with mean ± standard deviation (SD) or median (minimum-maximum) and categorical variables with frequency (n (%)). The distribution of variables was evaluated by distribution graphs and the Shapiro-Wilk test. One-way ANOVA was used for parametric data and the Mann-Whitney U test for nonparametric data. Fisher's exact test was used to analyze differences between categorical variables. Spearman's rho correlation test analyzed the correlation between clinical and laboratory data. The correlation coefficient (r) classified correlation significance as weak if r=0.2-0.4, moderate if r=0.4-0.6, and strong if r≥0.6.[21] The statistical significance level was accepted as $p<0.05$.

Results

Study participants

Sixteen patients with FMF (mean age 8 years and 56% female) evaluated both during a disease attack (G1-V1) and an attack-free visit (G1-V2) were recruited to the study. Demographic, clinical, and laboratory findings of the patients with FMF are presented in Table I. The mean age at the diagnosis of FMF was 3.3 ± 2.2 years. Parental consanguinity was present in 37.5% of the patients. The mean standard deviation scores of weight and height were within normal ranges (0.2 ± 1.0 and 0.6 ± 1.0, respectively).

The most common clinical findings of FMF were fever (100%) and abdominal pain (81.3%). Disease severity, as defined by the ISSF score, was categorized as moderate-to-severe in 50% of the patients while the remaining had mild disease. Eleven patients had a new diagnosis of FMF and two patients with a prior diagnosis were noncompliant with colchicine.

Table I. Demographic, clinical, and laboratory findings of patients with familial Mediterranean fever.

Demographic, clinical, and laboratory findings	Results (n=16)
Age in years, mean ± SD	8.0 ± 3.8
Female:Male	9:7
Consanguinity, n (%)	6 (37.5)
Family history, n (%)	
Familial Mediterranean fever	8 (50.0)
Secondary amyloidosis	1 (6.3)
Recurrent clinical findings of familial Mediterranean fever, n (%)	
Fever	16 (100)
Abdominal pain	13 (81.3)
Chest pain	4 (25.0)
Arthritis	1 (6.3)
Erysipelas-like erythema	1 (6.3)
Total ISSF score, mean ± SD	3.0 ± 1.8
Serum acute phase reactants, median (min-max)*	
Attack visit	
CRP, mg/L	45.1 (9.1-108.0)
SAA, mg/dL	44.1 (2.9-127.0)
Fibrinogen, g/L	3.9 (2.4-5.8)
ESR, mm/h	25.0 (7.0-43.0)
Attack-free visit	
CRP, mg/L	1.8 (0.4-6.6)
SAA, mg/dL	0.4 (0.2-7.9)
Fibrinogen, g/L	2.6 (2.0-3.8)
ESR, mm/h	10.5 (4.0-41.0)

*Normal ranges for serum acute phase reactants: CRP <5 mg/L; SAA <0.5 mg/dL; fibrinogen <4.2 g/L; ESR <15 mm/h.
CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; ISSF: International Severity Scoring System for Familial Mediterranean Fever; SAA: serum amyloid-A; SD: Standard Deviation.

Half of the FMF group (n=8) had biallelic 'exon 10' mutations in the *MEFV* gene, whereas seven patients had monoallelic 'exon 10' mutation either alone (n=5) or compound heterozygous with an 'exon 2' (E148Q) mutation (n=2) (Supplementary Table S1). One patient had heterozygous `exon 2` mutation (E148Q/-) in the *MEFV* gene. The expanded periodic fever syndrome panel resulted negative in this patient who was excluded from the group comparisons. The clinical and laboratory findings did not differ in FMF patients carrying biallelic and monoallelic `exon 10` mutations in the *MEFV* gene (Supplementary Table S2).

The febrile control group (G2, n=8) was found to have acute upper respiratory tract infection (n=5), gastroenteritis (n=2), and lower respiratory tract infection (n=1). Microbial cultures revealed Group A beta-hemolytic streptococcus in two patients with upper and *Streptococcus pneumoniae* in one patient with lower respiratory tract infection. The mean duration of fever at blood collection was 28.5 ± 16.5 hours for the febrile control group (G2), whereas it was 22.6 ± 12.7 hours for the FMF group-attack visit (G1-V1), and it was not statistically different between G1-V1 and G2 (p=0.343). The maximum duration of fever at blood drawn was 48 hours in both groups.

Serum acute phase reactants, serum IL-1β and IL-18, and GSDMD gene expression

Table II shows the comparison of serum APRs, serum IL-1β and IL-18, and GSDMD-Δ levels between the groups (G1-V1, G1-V2, G2, G3). All patients with FMF had significantly increased serum APRs during the attack visits compared to their levels at attack-free visits (all p≤0.005). The most significant increase was observed in SAA (39.0 [2.9-127.0] vs. 0.5 [0.2-7.9] mg/dL, p<0.001) and CRP (41.2 [9.1-99.6] vs. 1.9 [0.6-6.6] mg/L, p<0.001). The comparison of the FMF group-attack visit (G1-V1) with the febrile control group (G2) demonstrated increased APRs without statistical significance (all p>0.05).

The median of serum IL-1β and IL-18 were measured within normal ranges at both visits of the FMF group (G1-V1 vs. G1-V2) without statistical significance. We found that `G1-V1 vs. G3`, `G1-V2 vs. G3`, and `G2 vs. G3` differed in terms of the serum IL-1β and IL-18 where G1 and G2 had significantly higher levels compared to G3.

The distribution of GSDMD-Δ across study groups is provided in Fig. 2. `G1V1 and G3` differed significantly in terms of GSDMD-Δ levels (p<0.001), whereas GSDMD-Δ levels were similar in G1-V2, G2, and G3. The median GSDMD-Δ increased more than 10 times in the FMF group-attack visit (G1-V1) compared to the attack-free visit (G1-V2) (p<0.001). On the other hand, when compared to the febrile control group (G2), a more than four times significant increase in GSDMD-Δ levels were detected in the FMF group-attack visit (G1-V1) (p=0.026).

Correlation between serum acute phase reactants, serum IL-1β and IL-18, and GSDMD gene expression

The correlation analysis between serum APRs, serum IL-1β and IL-18, and GSDMD-Δ is shown in Table III for all participants and the FMF group, separately. The GSDMD-Δ demonstrated a moderate correlation with serum CRP, SAA, and fibrinogen in all participants, whereas a

Table II. Comparison of serum acute phase reactants, serum IL-1β and IL-18, and GSDMD gene expression in groups*.

	G1-V1 (n=15)	G1-V2 (n=15)	G2 (n=8)	G3 (n=10)	G1-V1 vs. G1-V2#	G1-V1 vs. G2#	G1-V2 vs. G2#	G1-V1 vs. G3#	G1-V2 vs. G3#	G2 vs. G3#
CRP, mg/L	41.2 (9.1-99.6)	1.9 (0.6-6.6)	41.7 (4.2-144.2)	NA	0.000	0.872	0.000	NA	NA	NA
SAA, mg/dL	39.0 (2.9-127.0)	0.5 (0.2-7.9)	24.0 (5.6-94.0)	NA	0.000	0.561	0.000	NA	NA	NA
Fibrinogen, g/L	3.9 (2.4-5.8)	2.6 (2.0-3.8)	4.0 (1.9-4.5)	NA	0.000	0.583	0.013	NA	NA	NA
ESR, mm/h	24.0 (7.0-43.0)	10.0 (4.0-41.0)	36.0 (7.0-55.0)	NA	0.005	0.258	0.016	NA	NA	NA
IL-1β, pg/mL	2.3 (1.4-10.2)	2.5 (1.6-10.0)	4.0 (1.4-10.0)	1.6 (1.2-1.9)	0.885	0.220	0.333	0.013	0.006	0.019
IL-18, pg/mL	42.0 (21.8-165.3)	39.6 (29.5-158.9)	70.5 (25.4-164.8)	27.0 (18.4-36.4)	0.787	0.197	0.220	0.006	0.008	0.013
GSDMD-Δ**	8.2 (2.1-61.7)	0.8 (0-6.5)	2.0 (0.7-21.3)	1.00 (0.4-2.1)	0.000	0.026	0.061	0.000	0.632	0.100

Normal ranges for serum acute phase reactants and interleukins: CRP <5 mg/L; SAA <0.5 mg/dL; fibrinogen <4.2 g/L; ESR <15 mm/h; IL-1β <12 pg/mL; IL-18 <120 pg/mL. CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; G1-V1: FMF group-attack visit; G1-V2: FMF group-attack-free visit; G2: febrile control group; G3: healthy control group; GSDMD: gasdermin-D; GSDMD-Δ: gasdermin-D gene expression; NA: not applicable; SAA: serum amyloid-A. *Median (min-max); **mean 2^(-delta delta Ct); #p-value.

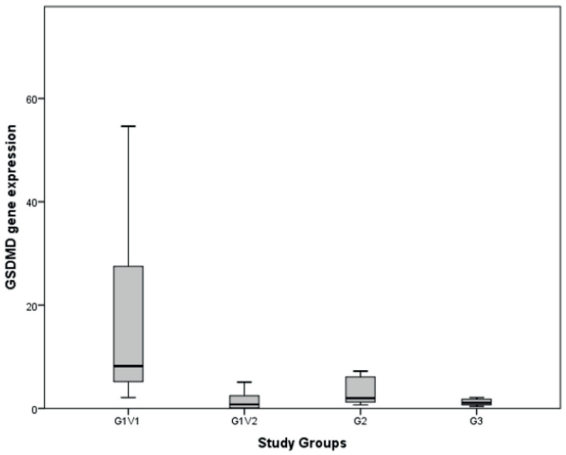

Fig. 2. Gasdermin-D gene expression across study groups.

Gasdermin-D (‡mean 2^(-delta delta Ct)); boxplots represent medians with standard errors.
G1-V1: FMF group-attack visit, G1-V2: FMF group-attack-free visit, G2: febrile control group, G3: healthy control group.

strong correlation of GSDMD-Δ with serum CRP and SAA was observed in the FMF group. The GSDMD-Δ did not correlate with serum IL-1β and IL-18, whereas serum IL-1β and IL-18 strongly correlated with each other. Furthermore, GSDMD-Δ did not correlate with the duration of fever in the G1-V1 and G2 groups (p>0.05).

Discussion

To our knowledge, this is the first human study looking into changes in GSDMD-Δ during disease attacks and attack-free intervals in FMF. Our findings revealed a significant upregulation in GSDMD-Δ during FMF attacks. More than 10-fold increase in median GSDMD-Δ was observed during FMF disease attacks compared to attack- and symptom-free periods. Asymptomatic patients exhibited very low GSDMD-Δ levels, akin to healthy children. On the other hand, significantly higher GSDMD-Δ was detected in FMF attacks compared to otherwise healthy children with acute infections.

Gasdermin-D is a crucial molecule in inflammation by its direct and indirect effects on pyroptosis, significantly contributing to both acute and chronic inflammatory processes. Gasdermin-D deletion in vivo prevented spontaneous autoinflammatory attacks and systemic effects of chronic inflammation such as diminished growth, anemia, and tissue damage in the FMF knock-in mouse model.[9] In this study, GSDMD-Δ significantly correlated with serum APRs in FMF patients admitted with attack symptoms. However, serum inflammatory cytokines, IL-1β and IL-18, did not differ

Table III. Correlations between serum acute phase reactants, serum IL-1β and IL-18, and GSDMD gene expression*.

	All Participants			FMF Group		
	IL-1β, pg/mL	IL-18, pg/mL	GSDMD-Δ**	IL-1β, pg/mL	IL-18, pg/mL	GSDMD-Δ**
WBC, *10^9/L	0.085	0.119	0.264	0.121	0.195	0.338
CRP, mg/L	0.143	0.171	0.540^^	-0.017	0.059	0.611^^
SAA, mg/dL	0.049	0.094	0.570^^	-0.093	0.002	0.622^^
Fibrinogen, g/L	-0.102	-0.098	0.504^^	-0.244	-0.222	0.523^^
ESR, mm/h	0.151	0.164	0.332^	-0.099	-0.066	0.369^
IL-1β, pg/mL	1.000	0.935^^	-0.012	1.000	0.912^^	-0.101
IL-18, pg/mL	0.935^^	1.000	0.007	0.912^^	1.000	-0.059

Normal ranges for serum acute phase reactants and interleukins: CRP <5 mg/L; SAA <0.5 mg/dL; fibrinogen <4.2 g/L; ESR <15 mm/h; IL-1β<12 pg/mL; IL-18<120 pg/mL; *Spearman's Rho correlation (r for weak correlation: 0.2-0.4, moderate correlation: 0.4-0.6, strong correlation: ≥0.6; ^p<0.05; ^^p<0.01); GSDMD: gasdermin-D; FMF: familial Mediterranean fever; GSDMD-Δ: gasdermin-D gene expression (**mean 2^(-delta delta Ct)); WBC: white blood cell count; CRP: C-reactive protein; SAA: serum amyloid-A; ESR: erythrocyte sedimentation rate.

according to the presence of FMF attack and were measured within normal ranges at both visits of the FMF group. These findings show the distinct role of GSDMD for FMF attack periods compared to serum inflammatory cytokines IL-1β and IL-18. Furthermore, GSDMD-Δ was the only laboratory test in the study that could differentiate FMF attacks from otherwise healthy children with febrile acute infections. Thus, we suggest that GSDMD be used as a biomarker of disease attacks. Although testing GSDMD-Δ can be impractical and expensive in clinical practice, further research at its protein level and cleavage can help to identify an affordable and feasible biomarker. The results of this study underline the importance of GSDMD in FMF pathogenesis.

The crucial role of GSDMD-mediated pyroptosis in the clearance of bacterial infections, particularly in sepsis, has been well-established.[22,23] Gasdermin-D promotes cellular death of the infected cells, increases mucosal inflammation, and captures bacteria in pore-induced intracellular and extracellular traps.[24,25] Additionally, GSDMD has been shown to restrict pathogen load in tissues and organs, control inflammation kinetics, and prevent epithelial disruption during acute *Salmonella* gut infection. Other gasdermins appeared dispensable for these protective functions.[26] The importance of GSDMD in viral infections remains elusive although the pore-forming activity of GSDMD has been thought to be a potential therapeutic target in viral infections.[27] In the present study, previously healthy children with acute infections demonstrated about a two fold increase in GSDMD-Δ compared to healthy children which was about one-fourth of the levels detected in FMF disease attacks. This finding also underpins the critical role of GSDMD in FMF.

FMF is classically accepted as an autosomal recessively inherited disease. There are few reports suggesting autosomal dominant pattern of inheritence in some patients.[28] The expression of similar disease phenotypes in heterozygotes proposes a different inheritance pattern such as pseudo-dominant inheritance. Population-based clinical studies indicate no clear genotype-phenotype correlation in FMF.[15,29,30] The most common *MEFV* gene mutations in the Turkish population are 'exon 10' mutations; M694V, M680I, and V726A.[14] 'Exon 10' mutations in the *MEFV* gene encode the B30.2 domain of pyrin protein and lead to severe disease phenotype.[31] The M694V mutation, the most common mutation found in Turkish population, is related to a severe disease phenotype and secondary amyloidosis; however, its biallelic or monoallelic presence has not been clearly linked to the severity of the disease phenotype.[32,33] An 'exon 2' mutation, E148Q, is accepted as a polymorphism in some populations, nevertheless, it has been shown as a disease-causing mutation in the Turkish population.[34,35] A recent study indicated that E148Q could induce inflammasome activation increasing the disease risk and severity rather than acting as a disease-causing monogenic mutation.[36] Because of the dilemma on E148Q mutation, the only patient carrying monogenic E148Q mutation was excluded from group comparisons. In the present study, patients with biallelic and monoallelic 'exon 10' mutations had similar ages of disease onset, disease severity, clinical features, serum APRs, GSDMD-Δ, and serum IL-1β and IL-18 levels in disease attacks and in-between attacks.

Major limitations of the study are the limited number of participants and the lack of homogeneity within the groups. Different types of infections were present in the febrile control group. Although the genetic heterogeneity among FMF patients existed, there was no difference between patients carrying biallelic and monoallelic 'exon 10' mutations in the *MEFV* gene regarding clinical, laboratory findings, and ISSF score. Furthermore, the timing for blood draw was heterogeneous for the FMF group-attack visit and the febrile control group, although it was between 8-48 hours of fever. A major strength of this study is the inclusion of pediatric FMF patients with highly penetrant *MEFV* mutations. The evaluation of the patients

both during attack and asymptomatic visits was another important strength. Comparison with febrile and healthy controls leads to the characterization of the distinct role of GSDMD-Δ for FMF attack periods compared to APRs and serum inflammatory cytokines, IL-1β and IL-18.

Conclusion

In conclusion, this study showed the distinct role of GSDMD in FMF attacks for the first time in humans. The significant GSDMD-Δ increase during FMF attacks relative to febrile but otherwise healthy patients highlights the functional significance of GSDMD-Δ in FMF pathogenesis. We propose that it has a potential utility as a biomarker of FMF attacks. This study also supports findings that phenotypic variations are not directly related to the genotype of FMF at a molecular level, also in terms of GSDMD-Δ. Further studies in larger and more homogeneous FMF cohorts and inclusion of different control groups such as patients with acute appendicitis are needed to validate these results on GSDMD that are also needed at the protein level and its cleavage.

Supplementary materials

Supplementary materials for this article are available online at https://doi.org/10.24953/turkjpediatr.2025.5389

Ethical approval

The study was approved by Clinical Research Ethics Committee of Hacettepe University Faculty of Medicine (date: 05.10.2021, number: GO21/1062).

Author contribution

The authors confirm contribution to the paper as follows: Study conception and design: PÖAA, ZBÖ, DÇ; data collection: PÖAA, İY, and DI; analysis and interpretation of the results: PÖAA, İY, Dİ, ZBÖ, SOH, DÇ; draft manuscript preparation: PÖAA. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Original Article

Disruptive behaviors in early childhood: the influence of family practices and functionality in a Turkish sample

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ABSTRACT

Background. Disruptive behaviors (DB) are common problems in young children. The aim of the current study was to highlight the effect of disruptive behaviors on functionality in preschool children and their families and identify factors that may be related to functionality.

Materials and Methods. A total of 223 patients were included in the current study from the Turkish Validity and Reliability Study of Preschool Age Psychiatric Assessment (PAPA). The disruptive behavior problems group (n=93) was selected according to PAPA and consisted of patients who had more than 3 conduct problem symptoms, with these symptoms leading to impairment. The control group (n=130) was selected from patients with no disruptive behavior disorder and 3 or fewer conduct problem symptoms . Preschool Age Psychiatric Assessment and Child Behavior Checklist for Ages 1.5-5 (CBCL/1½–5) were used for assessment.

Results. We found that spanking with the hand, verbal dispraise, and selective negative view to child were more frequent in the DB group than in the control group. DB symptoms were found to have a negative impact both on the child's functioning in several areas and on the parent's life in specific areas. Additionally, most of the CBCL scores were significantly higher in the DB group. Finally, it was shown that not only disruptive symptoms but factors such as the presence of attention deficit hyperactivity disorder, parental psychopathology, and the age of the child predicted impairment in this functioning.

Conclusion. These findings emphasize that parents' and child's functionalities can be highly affected by disruptive problems even in an early period such as preschool and that this area should not be ignored in evaluation and interventions.

Key words: disruptive behaviors, preschool, children, functionality, parenting.

Disruptive behaviors (DB), such as aggression, defiance, and temper tantrums, are among the most commonly reported types of behavioral difficulties in early childhood.[1] This early clinical phenomenology reflects deviation from normative developmental patterns within an age period.[2] Although some DB are considered a normal part of the developmental process of preschool children, they are the most common complaints among parents of young children in multiple settings.[3,4] According to previous studies, severe DB in early-life is predictive of a child's performance as they grow into school age, adolescence, and adulthood. Furthermore, early childhood diagnosis of disruptive behavior disorders (DBDs) is a strong predictor

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of long-lasting disruptive psychopathology and adverse life outcomes.[5-7]

Earlier research has highlighted various risk factors linked to disruptive behaviors, such as parental depression[8], conflict and maltreatment in the family[9], and poverty.[10] Parenting styles have been among the most commonly studied risk factors.[11-13] Most observational studies have consistently shown that children whose parents have negative parenting styles are more likely to develop DB problems. In contrast, parents who employ positive parenting styles are less likely to have children displaying disruptive behaviors.[11,12] In particular, exposure to physical and verbal punishment during preschool years is found to be associated with high levels of DB, which persists or increases across development.[14,15]

Studies conducted in different cultures have shown similar results. For example, a recent study involving a large cohort from United States with low-income urban families found that maternal spanking at age three predicted externalizing behaviors at age five, independent of other parental factors.[16] A study of clinic-referred children in Belgium found that high levels of maternal-reported coerciveness, including physical punishment, were associated with an increase in externalizing behaviors over time.[17] Considering the long-term negative consequences of conduct problems, it is important to recognize the associated factors in the early period. Although there are some studies investigating parenting practices in preschool children in Turkey[18,19], to our knowledge there is no study in the Turkish sample examining the relationship between behavioral problems and parenting attitudes, including functionality in preschool-aged children through the Turkish adaptation of the Preschool Age Psychiatric Assessment (PAPA).

Although many studies assume parents have a one-directional influence on their children's behaviors, findings suggest a reciprocal relationship, such that children's behaviors may have an impact on their parents too.[13,20,21] There have however been a few studies that have examined this bidirectional relationship between various externalizing behavioral problems and parenting styles, yet findings have been inconsistent.[22-24] This bidirectional relationship has been mostly studied in attention deficit hyperactivity disorder (ADHD), which often accompanies conduct problems and has been shown to be bidirectionally impaired in relation to parental functionality.[25,26] The results support both parent and child effects in the relation between child ADHD symptoms and family functioning.[25] Moreover, existing research and cross-cultural theory suggest that children's socialization, developmental patterns, and parent-child interactions may vary across cultures.[26] Therefore, necessitating an investigation into this bidirectional relationship in our own culture in a clinical preschool sample.

In the current study, we hence aimed to evaluate parenting characteristics (i.e. emotional warmth, control attempts, discipline) family functionality, and disruptive problems in a sample of Turkish preschool children. We hypothesized that children with more DB symptoms would have more problematic parental attitudes and poorer family functioning than children with fewer DB symptoms. Additionally, we expected DB symptoms and other factors such as ADHD diagnosis and parental psychopathology to affect family functioning in a negative way.

Materials and Methods

Participants

The participants of the study were selected from the Turkish Preschool Age Psychiatric Assessment (PAPA) Reliability and Validity Study. Parents of all children who applied to our university's Department of Child and Adolescent Psychiatry outpatient clinic, between the ages of 2 and 6 years, were administered the structured interview PAPA. The Turkish PAPA study involved 300 patients. Because behavioral problems are often accompanied

by other disorders, patients diagnosed with neurodevelopmental disorders such as autism spectrum disorders and global developmental delay were excluded so as to not confound the results. Diagnostic assessments for autism spectrum disorder and global developmental delay were performed by experienced clinicians based on the criteria outlined in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5). A total of 223 patients were included in the current study.

The high DB group (n=93) included children who had more than 3 conduct problem symptoms whose symptoms were related to functional impairment due to disruptive problems and had no chronic medical conditions. Patients with 3 or fewer conduct problem symptoms, who had no functional impairment due to disruptive problems and no chronic medical condition were considered as the Low DB group (n=130). Since oppositional defiant disorder (ODD) in the PAPA includes the same diagnostic criteria as DSM-5, and the diagnosis requires meeting at least four criteria and impaired functioning, this was accepted as the threshold. Other psychiatric disorders (ADHD, anxiety disorders etc.) were also assessed using PAPA and were not exclusion criteria for either group.

Measures

Sociodemographic data form:

The form was created by researchers based on the original sociodemographic form of the PAPA and included questions to gather information about the age, gender, education level of the child and parents, family structure, and number of siblings.

The 'Child behavior checklist for ages 1.5-5' (CBCL/1½–5):

The CBCL/1½–5 is a parent-rated questionnaire designed to assess children's problem behaviors and consists of 100 items about children's behaviors and emotional issues.[27] The CBCL/1½–5 consists of seven subscales into which the items can be clustered: emotionally reactive, anxious/depressed, somatic complaints, withdrawn, sleep problems, attention problems, and aggressive behavior. The Turkish standardization study was conducted by Erol et al.[28] This scale was used in this study to assess and compare behavioral and emotional problems.

Preschool age psychiatric assessment (PAPA):

PAPA, developed in 1999, is an interviewer-based, caregiver-reported diagnostic assessment method. This structured interview evaluates the symptoms in four main areas: (1) diagnostic criteria of all diagnoses in DSM-5 and ICD-11, (2) all of the Research Diagnostic Criteria-Preschool Age (RDC-PA) items, (3) all of the Diagnostic Classification of Mental Health and Developmental Disorders of Infancy and Early Childhood 5 (DC: 0-5), (4) potential behaviors and symptoms that are not merely diagnostic criteria like sleeping rituals and peer relationships. In addition, the interview assessed the family environment and relationships, family psychosocial status, and functional impairments. It covered not just disorders and problems, but all aspects that affect young children's mental health.[29]

The reliability study of PAPA was conducted by Egger et al., and no significant difference was found in reliability according to age, gender, and race. The interview has been shown to be a valid tool for children aged between 2 and 6 years, although its use up to age 8 years has been established.[30,31] The psychometric study was conducted in Turkey, and reliability and validity were demonstrated in the Turkish population.[32] The PAPA conduct section covers all DSM-5 criteria for ODD and conduct disorder (CD). The PAPA was used in the current study to evaluate conduct problems, family demographics, and family functionality. For this assesment, Family section, Conduct Problems section, Incapacity section and Child and Adolescent Impact Assessment sections were used. In the interview, the interviewer asked about symptoms related to each relevant module. Following the guidelines in the PAPA

dictionary, each symptom was assessed on a three-point scale — absent (0), sometimes present (1), or definitely present (2) —. The symptom scales provided a continuous measure of observed symptoms. These scores were summed to obtain the total score. Similarly, a numerical variable was obtained by summing the scores of the variables related to functionality. Higher scores indicate more severe impairment in functioning across more areas.

Procedure

Patients aged between 2 and 6 years who applied to our outpatient clinic were evaluated, and written and verbal consent was obtained from the parents who agreed to participate in the study. Following this, a child psychiatrist interviewed the parents and children. After the interview, the clinician completed the sociodemographic data form and PAPA short forms, and the CBCL/1½–5 was completed by the parents. Of the parents interviewed, 215 (96%) were mothers, 5 were fathers (2.2%), and in 3 cases (1.34%) were both mothers and fathers. The study was approved by the Ankara University Faculty of Medicine Non-Invasive Clinical Research Ethics Committee on October 10, 2019 (Decision No: I4-151-19).

Statistical analysis

For evaluating the tests and scales, IBM SPSS Statistics for Windows 30.0 software was used. Descriptive statistics are given as mean ± standard deviation or median and interquartile range (IQR) for continuous variables. Frequencies (percentages) are given for categorical variables. The Kolmogorov-Smirnov test is used to check whether continuous variables follow a normal distribution. For comparisons of groups, the independent sample t-test was used for independent samples of normally distributed data. The Mann-Whitney U test was performed to test the significance of pairwise differences. The chi-square or Fisher's exact test was used for comparison of categorical variables. A multiple linear regression model was used to identify independent predictors of functionality. Goodness-of-fit statistics were used to assess model fit. The significance level for this study was set to 5%. The $p<0.05$ was considered statistically significant.

Results

A total of 223 children, with a mean age of 45.74 months (standard deviation: 12.93), were included in the study. Among the children, 116 (52.07%) were boys. The sociodemographic characteristics of the groups are shown in Table I.

We assessed maternal and paternal attitudes that could be related to DB. Spanking with the hand, verbal dispraise, and selective negative view were more common in the DB group (Table II).

We also analyzed parents' relationship, perceptions about their partners, and mental health status, as factors that could be related to higher DB. Both mothers and fathers have more psychopathology in the high DB group. Parental arguments were seen as more frequent in the high DB group. Socioeconomic levels were similar in the groups (Table III).

Another aspect that we evaluated was the areas of impairment in the PAPA. There was a serious deterioration in the child's functionality in almost all areas (Table IV). When the correlation between CBCL and impairment in child's functioning was evaluated, it was found that the domains of relationships with parents, helping in cooperation, leisure time activities, problems with adults outside the home/nursery and ability to act appropriately outside home or daycare/school were positively correlated with the CBCL domains of ADHD (relationship with mother: $r=0.24$, $p<0.001$; relationship with father: $r=0.24$, $p<0.001$; helping in cooperation: $r=0.2$, $p=0.002$, problems with adults outside the home/nursery: $r=0.21$, $p=0.001$; ability to act appropriately outside home or daycare/school: $r=0.21$, $p=0.001$), aggressive behaviors (relationship with mother: $r=0.4$, $p<0.001$;

Table I. Sociodemographic characteristics of the groups .

Sociodemographic characteristics	Low DB group n=130	High DB group n=93	p
	M±SD / n (%)	M±SD / n (%)	
Age (months)	45.8±14.48	44.91±12.13	0.86[a]
Gestational age at birth (week)	38.2±1.7	38.43±1.26	0.53[a]
Maternal age (years)	34.16±5.68	33.29±6.18	0.32[a]
Years of maternal education	12.59±4.07	12.75±4.51	0.75[a]
Paternal age (years)	37.81±7.58	36.36±6.8	0.38[a]
Years of paternal education	12.85±4.31	12.6±4.8	0.39[a]
Gender			
Female	65 (50)	42 (45.2)	0.47[b]
Male	65 (50)	51 (54.8)	
Who child lives with			
Mother and father	121 (93)	89 (94.6)	0.43[c]
Only the mother or father	9 (7)	4 (5.4)	
Psychopathologies			
No	84 (64)	0 (0)	<0.001***
ADHD	17 (13)	45 (48)	<0.001***
Anxiety disorders	31 (23.8)	35 (37.6)	0.02[c]*
Mood disorders	1 (0.7)	5 (5.37)	0.08
Other (eating disorders, tic, etc)	6 (4.6)	9 (9.6)	0.3

ADHD, attention deficit hyperactivity disorder; DB, disruptive behavior; M, mean; SD, standart deviation; [a]independent sample t-test; [b]chi-square test; [c]fisher's exact test; *p<0.05, **p<0.01, ***p<0.001

relationship with father: r=0.44, p<0.001; helping in cooperation: r=0.28, p<0.001, problems with adults outside the home/nursery: r=0.19, p=0.002; leisure time activities: r=0.25, p<0.001; ability to act appropriately outside home or daycare/school: r=0.21, p=0.001; peer relationships: r=0.19, p=0.003), somatic (helping in cooperation: r=0.23, p<0.001, problems with adults outside the home/nursery: r=0.17, p=0.008; leisure time activities: r=0.27, p<0.001; ability to act appropriately outside home or daycare/school: r=0.22, p<0.001), sleep (relationship with mother: r=0.24, p<0.001; relationship with father: r=0.22, p<0.001; sibling problems: r=0.24, p<0.001; helping in cooperation: r=0.25, p<0.001, problems with adults outside the home/nursery: r=0.22, p<0.001; leisure time activities: r=0.19, p=0.003), anxious/depressed (relationship with mother: r=0.24, p<0.001; relationship with father: r=0.18, p=0.007; helping in cooperation: r=0.18, p=0.005, problems with adults outside the home/nursery: r=0.26, p<0.001; peer relationships: r=0.2, p=0.002), withdrawn (problems with adults outside the home/nursery: r=0.29, p<0.001; leisure time activities: r=0.25, p<0.001; ability to act appropriately outside home or daycare/school: r=0.19, p=0.004; peer relationships: r=0.27, p=0.001), emotional reactivity (relationship with mother: r=0.25, p<0.001; relationship with father: r=0.22, p<0.001; helping in cooperation: r=0.21, p=0.008, problems with adults outside the home/nursery: r=0.22, p<0.001).

We also assessed the specific impact of symptoms on aspects of the parent's life. We found that DB symptoms had a more significant negative impact on the parent's relationship with other children, relationship with other family members, and participation in social and personal activities, in the high DB group compared to the low DB group (Table V).

Table II. Comparison of maternal and paternal disciplinary practices between groups, n (%).

Variables	Low DB group n=130	High DB group n=93	p
Maternal Discipline			
Time out	11 (8.5)	9 (9.7)	0.46[a]
Spanking with hand	18 (13.8)	31 (33.3)	0.001**[b]
Spanking with object	3 (2.3)	3 (3.2)	0.49[a]
Marks or bruises	1 (0.8)	1 (1.1)	0.66[b]
Sent to room	32 (24.6)	26 (28)	0.64[b]
Loss of privileges	63 (48.5)	55 (59.1)	0.18[b]
Verbal dispraise	10 (7.7)	18 (19.4)	0.01*[b]
Verbal rejection	8 (6.2)	12 (12.9)	0.08[b]
Selective negative view	3 (2.3)	9 (9.7)	0.01*[b]
Disciplinary style			
Normal	34 (26.2)	18 (19.4)	0.45[b]
A bit angry, but controlled	91 (70)	70 (75.3)	
Cold, out of control	5 (3.8)	5 (5.4)	
Paternal Discipline			
Time out	5 (3.8)	3 (3.2)	0.71[b]
Spanking with hand	7 (5.4)	24 (25.8)	<0.001***[b]
Spanking with object	5 (3.8)	4 (4.3)	0.73[b]
Marks or bruises	2 (1.5)	0 (0)	0.35[b]
Sent to room	14 (10.8)	19 (20.4)	0.11[b]
Loss of privileges	36 (27.7)	34 (36.6)	0.31[b]
Verbal dispraise	9 (6.9)	15 (16.1)	0.04*[b]
Verbal rejection	3 (2.3)	6 (6.5)	0.19[b]
Selective negative view	2 (1.5)	10 (10.8)	0.002**[b]
Disciplinary Style			
Normal	40 (30.7)	21 (22.6)	0.09[b]
A bit angry, but controlled	86 (66.2)	64 (68.8)	
Cold, out of control	4 (3.1)	8 (8.6)	

DB, disruptive behavior; [a]Chi-square test; [b]fisher's exact test; *p<0.05, **p<0.01, ***p<0.001

In line with our hypothesis and correlation analysis, multiple linear regression analysis was performed to investigate the factors that affected children's functionality (Table VI). We used the backward linear regression method. First, we included the child's age, gender, maternal psychopathology, paternal psychopathology, presence of ADHD, and number of symptoms of DB in the model. Then, the maternal psychopathology and gender of the child, which was found to be unrelated, were excluded from the model. It was found that the gender of the child, presence of paternal psychopathology, presence of ADHD, and number of symptoms of DB were associated with deterioration of child's functionality, as shown in Table VI.

We also performed correlation analyses to investigate the influence of factors related to parental impact. There was a weak positive correlation between impact on parents and symptom number of DB (r=0.16, p=0.01 and presence of ADHD (r=0.19, p=0.004). In the multiple regression analysis, no significant effect of these factors was found.

Table III. Comparison of maternal and paternal perceptions, mental health, and relationship problems between groups, n (%).

Variables	Low DB group n=130	High DB group n=93	p
Maternal perceptions and mental health			
Dissatisfaction with partner's help	40 (30.7)	41 (44)	0.23[a]
Dissatisfaction with communication and decision-making	26 (20)	31 (33)	.12[a]
Psychopathology	28 (21.5)	33 (35.5)	0.01[b]*
Received therapy from mental health professional	24 (18.5)	23 (24.7)	0.58[a]
Problems related to alcohol/drugs	0 (0)	1 (1.1)	0.24[a]
Paternal perceptions and mental health			
Dissatisfaction with partner's help	10 (13)	16 (17)	0.12[a]
Dissatisfaction with communication and decision-making	14 (10.8)	21 (22.5)	0.09[a]
Psychopathology	9 (6.9)	17 (18.3)	0.03*[a]
Received therapy from mental health professional	8 (6.2)	11 (11.8)	0.32[a]
Problems related to alcohol/drugs	1 (0.8)	5 (5.4)	0.24[a]
Parents relationship problems			
Parental arguments	88 (67.7)	76 (81.7)	0.03*[a]
Interparental physical violence	9 (6.9)	12 (12.9)	0.32[a]
Involvement of child in arguments or violence	24 (18.5)	25 (26.9)	0.4[a]
Financial Coverage			
Very well	21 (16.2)	17 (18.3)	0.85[a]
Fairly well	88 (67.7)	63 (67.7)	
Poorly	21 (16.2)	13 (14)	

DB, disruptive behavior; [a]chi-square test; [b]fisher's exact test; *p<0.05, **p<0.01, ***p<0.001

Table IV. Comparison of impairment areas between groups, n (%).

Variables	Low DB group n=130	High DB group n=93	p
Parental relationships - mother	21 (16.2)	51 (54.8)	<0.001**[a]
Parental relationships - father	9 (6.9)	31 (33.3)	<0.001**[a]
Sibling relationships- in the home	11 (8.5)	27 (29)	<0.001**[a]
Sibling relationships- out of home	0 (0)	5 (5.4)	0.01*[a]
Cooperative helping	6 (4.6)	20 (21.5)	<0.001**[a]
Daycare/school performance	5 (3.8)	16 (17.2)	0.003*[a]
Suspended from daycare/school	0 (0)	4 (4.3)	0.02*[a]
Daycare provider /teacher relationship	6 (4.6)	13 (14)	0.03*[a]
Peer relationships at daycare/school	12 (9.2)	18 (19.4)	0.09[a]
Play (outside of daycare/school)	9 (6.9)	22 (23.7)	<0.001***[a]
Relationships with adults outside the home or daycare/school	11 (8.5)	15 (16.2)	0.12[a]
Relationships with peers	21 (16.2)	31 (33.3)	0.002*[a]
Ability to act appropriately outside of home or daycare/school	13 (10)	33 (35.5)	<0.001***[a]
Treatment	22 (16.9)	22 (23.7)	0.14[a]
Medication	4 (3.1)	5 (5.4)	0.44[a]

DB, disruptive behavior; [a]chi-square test; *p<0.05, **p<0.01, ***p<0.001

Table V. Comparison of the impact of child's problems on family dynamics, relationships, and other activities between groups, n (%).

Variables	Low DB group n=130	High DB group n=93	p
Negative impact on parent's current partnership			
Some negative effect	8 (13.8)	9 (9.7)	
Severe negative effect	5 (3.8)	12 (12.9)	0.06[a]
Child's problems contributed to marital breakdown	0 (0)	1 (1.1)	
Impact on parent's relationship with other child(ren) in the household			
Less time for other child(ren), but not otherwise affected	10 (7.7)	16 (17.2)	0.07[a]
Worsening of the relationship	1 (0.8)	1 (1.1)	
Impact on the relationship of other child(ren) in the household			
Some conflict	6 (4.6)	13 (14)	0.04*[a]
Major disruption	1 (0.8)	2 (2.2)	
Impact on the behavior of other child(ren) in the household			
Some problems	7 (5.4)	13 (14)	0.05[a]
Negative impact on relationships with other family members	12 (9.3)	22 (23.7)	0.01*[a]
Impact on relationships with friends	13 (10)	17 (18.3)	0.18[a]
Restrictions on family's social activities	13 (10)	27 (29)	0.001**[a]
Restrictions on parents' personal activities	15 (11.6)	25 (26.9)	0.02*[a]
Stigmatization	11 (8.5)	15 (16.2)	0.26[a]

DB, disruptive behavior; [a]chi-square test; *p<0.05, **p<0.01, ***p<0.001

Table VI. Multiple linear regression analysis for children's functionality.

Variables	B	SE	Beta	t	p
Intercept	-1.01	1.09		-0.92	<0.001***
Number of DB symptoms	0.28	0.04	0.43	7.07	<0.001***
Presence of ADHD	2.27	0.2	0.18	3.03	0.003**
Paternal psychopathology	0.09	0.03	0.16	3.07	0.002**
Age of child	0.04	0.02	0.11	1.99	0.04*

ADHD, attention deficit hyperactivity disorder; B, estimated coefficient; DB, disruptive behavior; SE, standard error; *p<0.05, **p<0.01, ***p<0.001

Discussion

The current study investigated the relationship between familial factors, family functioning and disruptive behavior problems in preschool children in a Turkish clinical sample. Our hypothesis was that children exhibiting more behavioral problems were more likely to have parental problems and worse familial functioning than the control group. It was found that spanking with hand, verbal dispraise, and selective negative view of the child were more frequent in the DB group than in the control group. Additionally, although problems such as physical violence between parents were not more frequent, arguments between parents and parental psychopathology were found to be reported more frequently in the DB group. Finally, in the current study, DB symptoms were shown to have a negative impact in several areas on both the child's functioning and the parent's life. We also showed that not only disruptive symptoms but also various factors such as the presence of ADHD, parental psychopathology,

and the age of the child predicted impairment in their functionality.

The first finding of the current study was that sociodemographic characteristics were similar in both groups. Previous research has shown that families of children with ODD/CD are more likely to experience socioeconomic disadvantage, including lower parental educational status.[33] According to the current study, the DB group showed no significant differences compared with the control group. In previous studies conducted in our country, socioeconomic status was associated with DB problems.[34] The difference in our study was attributed to the clinical sample. It was also thought that the similarity in these characteristics may have minimized confounding factor in terms of the results.

Coercive, harsh, and conflictual parenting practices are known risk factors for developing of clinically meaningful DB problems.[35] Consistent with previous studies, we found that spanking by hand, verbal dispraise and selective negative view of the child were more common in the DB group. We also hypothesized that difficulties in parents' marital relationships may affect their parenting and thereby constitute a risk to their child's DBs, as shown in previous studies.[36] However, we did not find any significant differences in parental relationship dissatisfaction. We thought that we did not find a difference between the two groups, because both groups were clinical samples. Additionally, temperamental traits influenced by genetic factors also play a role in conduct disorders, including genetic variants that affect emotional reactivity and social affiliation.[37] Therefore, we may find the impact of environmental factors as more limited.

Parental psychopathology, especially depression, is one of the best known risk factors for child mental health problems.[36] We found that parental psychopathology was more common in both mothers and fathers, consistent with the findings reported in existing literature regarding familial mental health trends. Parental psychopathology appears to be both an important risk factor for DB and a potential area for various interventions.

A mother's negative perception of her child has been examined as a possible risk factor for child conduct problems. However, it was also suggested that the strong associations between parental perceptions and child conduct problems cannot confirm a causal connection between the variables or the direction of any such association if there is a causal link. It is highly likely that child conduct problems also promote negative parental cognitions and vice versa.[38] The cross-sectional structure of our study also does not allow such a casual inference.

When the functioning of these children was evaluated, we showed that although they were at a very early age (mean 45.74 months), DB negatively affected their functioning in many areas, especially in family relationships. While a large body of parenting research exists, fewer works has concentrated on exploring family functioning. In a study conducted with boys aged 11 to 16 years, it was shown that families with children with conduct problems had poorer affective involvement, general family functioning, and more poorly defined family roles than families with typically developing children.[39] To the best of our knowledge, we have not found a study investigating the effect of DB on family functioning in the preschool period. The findings of our study are consistent with those of previous studies in older children.[40]

We have shown that DB has a very negative impact not only on the child's functioning but also on the parents' lives. We have shown that it negatively affects their relationships with other children, other family members, their family social activities, and their personal activities. These factors may contribute to the bidirectional relationship shown in previous studies.[41] Given that both parenting and home environment contribute to the development of DB, exploring the relationship between family functioning and DB in prospective studies could provide

further insight into how the family functions as a whole.

Finally, we showed that the number of DB symptoms, presence of ADHD, paternal psychopathology and the child's age may be predictive factors for functioning. The deterioration in functioning as the number of DB symptoms increased was a generally anticipated result. ADHD and ODD frequently co-occur during preschool years [42], and there is a correlation between the symptoms of these two disorders at this developmental stage.[43] It is also known that ADHD affects the functioning of both children and their families through many mechanisms.[36] Therefore, when evaluating and monitoring a child with one of these conditions, the effect on functionality should not be ignored.

A surprising finding of the study was that maternal psychopathology had no predictive effect on functioning, while only the father's psychopathology had an effect. A different pattern for mothers and fathers has been shown in an ADHD study, although the study reported findings opposite to our findings.[25] We thought that the reason for this might be that fathers had more severe disorders compared to mothers. Additionally, similar to the disruptive problems seen in children, the father may also have issues related to emotional dysregulation, which could affect the functioning of both the family and the child. Similarly, some of the previous research suggested that fathers' psychological health has a significant impact on children's externalizing behaviors especially, and fathers play a crucial role in shaping their children's development.[36] It would be useful to perform a detailed assessment of the psychopathologies of the parents in order to make sense of this relationship.

Follow-up and evaluation of these patients will continue. However, even in this form, the findings of our study have important practical implications for family assessments and interventions. First, interventions that focus solely on parents or children may have limited effectiveness in breaking the feedback loop between poor parenting styles, family functioning, and children's disruptive behaviors. Second, both parents appear to be significantly impacted by their children's disruptive problems, so family-based interventions should involve both parents and address their functioning. Third, early intervention for young children is crucial, as our findings show clear and severe impairments in children around the age of 4 years.

There are several limitations that should be noted. First, in this article, preliminary findings are presented cross-sectionally. This prevents the establishment of a causal relationship in the child-parent relationship loop. Second, the study relied largely on PAPA validity and reliability study variables, future research is needed to replicate the findings using multimethod assessment. Third, in the present study, we did not have detailed information about the parents' psychopathology. Future studies should also use collateral information to assess parent psychopathology. Fourth, although a longitudinal design provides stronger support for a causal link than a cross-sectional design, it is important to consider potential confounding variables. Fifth, this sample included only two-parent families, which limits generalizability. Finally, the study sample is a clinical sample and many other psychopathologies may be confounding factors.

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Ethical approval

The study was approved by Ankara University Ethics Committee (date: 10.10.2019, number: İ4-151-19).

Author contribution

The authors confirm contribution to the paper as follows: Study conception and design: MCU, DBÖ; data collection: MCU, EY, GYE and DBÖ; analysis and interpretation of results: MCU, HKÜ, SAA ; draft manuscript preparation: MCU, EY, DBÖ, HKÜ, SAA. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Original Article

Evaluation of mid- and long-term quality of life in patients operated on for esophageal atresia

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ABSTRACT

Background. This study aimed to evaluate general and condition-specific quality of life in esophageal atresia (EA) patients, identifying risk factors such as associated anomalies and chronic diseases, as well as examining their impact on life quality.

Methods. Patients operated on for EA between 2004 and 2020 comprised the study population. Pediatric Quality of Life (PedsQOL 4.0) and the Esophageal Atresia Quality of Life (EA-QOL) questionnaires were administered to parents of 2-7 year old children as well as both patients aged 8-17 year and their parents. Results of the PedsQOL 4.0 scale were compared with 43 healthy children.

Results. The study included 66 patients (40 aged 2-7 years, 26 aged 8-17 years), with 45.5% females and 54.5% males. The mean age was 7±4.4 years. Quality of life measured by EA-QOL and PedsQOL 4.0 questionnaires showed no significant differences based on patient sex, gestational age or having an anastomotic stricture. In the 8-17 age group, EA patients demonstrated significantly higher emotional scale quality of life than the healthy group (p=0.001) according to parent and child PedsQOL 4.0 questionnaire scores.

Conclusions. The better emotional functioning in the 8-17 age group supports their enhanced anxiety management. Sex, gestational age, or presence of an anastomotic stricture did not impact quality of life. While differences existed between patient age groups in the questionnaires administered, factors like anatomical EA type, repair mode, low birth weight, tracheomalacia, frequent lung infections, presence of associated vertebral, anorectal, cardiac, renal, limb anomalies and/or hydrocephalus (VACTERL-H), gastrostomy placement, and surgical interventions other than EA significantly influenced patients' quality of life. These findings may guide implementing measures to enhance quality of life in EA patients.

Key words: PedsQOL 4.0, EA-QOL, esophageal atresia, VACTERL-H, quality of life.

Esophageal atresia (EA) occurs at a rate of 2.4 per 10,000 live births, often accompanied by tracheoesophageal fistula (TEF) or without fistula, and involves esophageal discontinuity.[1,2] Surgical repair remains the definitive treatment, with advancements in neonatal intensive care and surgical techniques leading to improved survival rates and decreased mortality rates since the first successful primary repair in 1941.[3,4]

Mortality rates, once high in both preoperative and postoperative periods, have now decreased to single digits.[3] However, concerns have shifted towards long-term morbidity and quality of life issues persisting into adulthood.[2] These include esophageal, gastrointestinal,

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pulmonary diseases, and developmental problems, significantly impacting patients and their families.[5-7]

Analyzing the relationship between postoperative physical development and their quality of life can significantly enhance the understanding of factors influencing the developmental process, the nature, and importance of the disease, thereby improving appropriate care and treatment outcomes.[8] Due to the limited applicability of questions used to assess health-related quality of life (HRQOL) in EA patients, there has been a need to develop a condition-specific quality of life questionnaire. In a study conducted in 2018 in Sweden and Germany, specific HRQOL questions under the name Esophageal Atresia Quality of Life (EA-QOL) questionnaire[9] tailored for EA patients was developed and the validity was demonstrated. In a study conducted in Türkiye in 2021, the Turkish feasibility and validity of both age- and disease-specific EA-QOL questionnaire items, originated from Sweden and Germany, were demonstrated.[10]

The quality of life of patients who have undergone surgery for EA can be improved through early intervention, effective treatment methods, and surgical procedures, which enhance the physical health, nutritional status, and social development of children. Such a hypothesis can be further tested through more detailed research to examine the impact of the postoperative recovery process on the general health and quality of life of these children.

As a rare disease, long-term quality of life in EA patients has not been studied extensively in the medical literature. Furthermore, the degree of development of a country and the sociocultural background of its people have an impact on the perceived quality of life, with culture explaining 15.9% of the variance obtained in one study.[11] This study aims to evaluate the overall and condition-specific quality of life in EA patients who are under follow-up in a single unit in Türkiye. By, including accompanying anomalies and chronic illnesses as potential risk factors on life quality, the study seeks to enhance our understanding of EA's impact and improve treatment outcomes

Materials and Methods

On 09/07/2022, an institutional ethics committee approval was granted under the decision number 2022/0507. The study included EA patients who were born and surgically treated between October 2004 and October 2020, and whose esophageal definitive surgery was performed at our pediatric surgical clinic. Demographic data, presence of VACTERL-H (vertebral anomalies, anorectal malformation, cardiac anomalies, TEF, renal anomalies, limb anomalies, and hydrocephalus) association and other anomalies, complications, surgical procedures performed, and radiological imaging data were retrospectively collected from hospital databases and patient records. EA is classified according to the Gross classification as follows: *Type A:* Isolated EA, in which both the proximal and distal esophageal segments end blindly without any tracheoesophageal fistula. *Type B:* EA with a proximal TEF and a blind-ending distal esophagus. *Type C:* EA with a blind-ending proximal esophagus and a distal TEF (the most common type). *Type D:* EA with both proximal and TEF. *Type E:* TEF without EA, also referred to as an "H-type" fistula.[12]

The study was conducted in accordance with the declaration of Helsinki. The legal guardians of the patients were informed of the purpose and content of the research. Written informed consent for participation in the study was obtained from patients aged >7 years as well as their parents by face-to-face intervention during regular outpatient clinic follow-up visits. This was followed by administration of the questionnaire. For patients who did not present to the outpatient clinic during the study period, verbal consent was obtained via telephone and the questionnaire was conducted remotely by the principal author.

Additionally, The Pediatric Quality of Life (PedsQOL 4.0) questionnaire was administered to healthy volunteers. These were children and the relevant proxies of volunteering hospital staff who were initially matched according to patient age groups. The questionnaires were conducted either face-to-face after obtaining written informed consent or by telephone interviews. The results were then statistically compared with the patient group.

The pediatric quality of life (PedsQOL 4.0)

The scale is designed to assess the health-related quality of life (HRQOL) of children and adolescents aged 2-17 years and consists of four main domains: Physical (8 items), Emotional (5 items), Social (5 items), and School Functioning (5 items), totaling 23 items. For children, the scale is divided into two age groups: 8-12 and 13-18 years. Additionally, there is a parent form that covers the age ranges of 2-4 and 5-7 years. Scoring is conducted through a linear conversion, where responses are converted to points as follows: 0=100, 1=75, 2=50, 3=25, and 4=0. Higher scores indicate better quality of life.[13]

The esophageal atresia quality of life (EA-QOL)

For children aged 2-7 years, there is a parent form, while for adolescents aged 8-17 years, both parent and child forms are available. The parent form for children aged 2-7 consists of a total of 17 questions, divided into three main categories. These include nutritional status (7 questions), physical health status (6 questions), and relationships with others (4 questions). For patients aged 8-17 years, there are a total of 24 questions, with both parents and the patient themselves providing answers. These questions are divided into four main categories. These include nutritional status (8 questions), relationships with others (7 questions), thoughts on body and scar (5 questions), and physical health status (4 questions). Responses are scored linearly on a scale of 0-100 (0-25-50-75-100). The score for each subcategory is the arithmetic average of the total scores divided by the number of items in that subcategory. The total score is obtained by dividing the total score of all items by the total number of items. The total score ranges from 0 to 100, with higher scores indicating better quality of life.[14,15] The Turkish validity and reliability study of this questionnaire, developed by Dellenmark-Blom[9] was conducted by Soyer et al.[10] demonstrating its feasibility.

Statistical analyses

The relationship between two independent continuous variables, not normally distributed, was assessed using the Mann-Whitney U test. Categorical variables were examined using the chi-square test, with Yates continuity correction or Fisher's exact test where applicable. Significance level was set at 0.05. Spearman rank correlation coefficient (rho) was used to analyze the correlation between non-normally distributed continuous variables. Statistical analyses were performed using MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013).

Results

During the included time interval, 155 patients underwent surgery for EA at our clinic. Lost-to-follow-up, inaccessible (n=55) or deceased (n=30) patients were excluded. Parents of 4 patients (2.6%) refused participation. Sixty-six (42.6%) patients were included, with 40 aged 2-7 years and 26 aged 8-17 years (Fig. 1). The responses to the questionnaires were obtained during outpatient clinic visits in 34 patients and via telephone interview in 32. The demographic characteristics of the patients and their parents are summarized in Table I. Gross classification revealed 75.8% had type C EA, 15.2% type A, and others types B, D, and E. Concomitant anomalies included urinary system anomalies (25.8%), anorectal malformations (21.2%), vertebral anomalies (19.7%), limb anomalies (9.1%), major cardiac disease (7.6%), hydrocephalus (3%), and

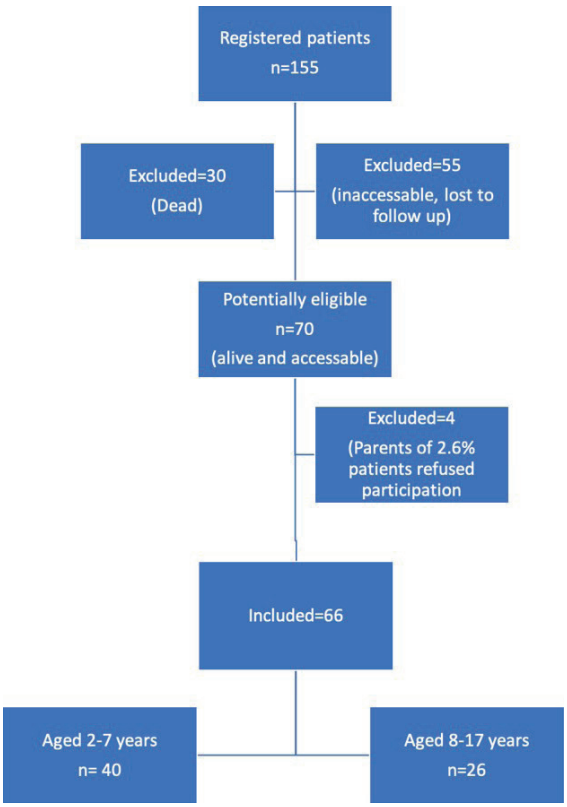

Fig. 1. The flow diagram of the study participants.

minor anomalies (21.2%). Five patients (7.6%) received special education for developmental delays. VACTERL-H association was present in 17 (25.8%) patients, and 2 (3%) had components of CHARGE (Coloboma, Heart disease, Atresia of the choanae, Retarded growth and mental development, Genital anomalies, and Ear malformations and hearing loss) syndrome. Genetic disorders were present in 2 (3%) patients, specifically homozygous mutations causing Fanconi anemia. Clinical follow-up and treatment details are summarized in Table II.

The results of the EA-QOL questionnaire

The proxy report results of the EA-QOL questionnaire for children aged 2-7 years

The proxy report of the EA-QOL questionnaire for children aged 2-7 years (n=40) showed no significant differences based on Gross classification, birth weight (<2500 g, n=24), or gestational age (preterm n=16, term n=24) (Supplementary Table S1). Similarly, no significant differences were observed between

Table I. Demographic characteristics of patients, parents and control participants (N=66).

	Patients	Controls
Sex, n (%)		
Female	30 (45.5)	21(48.8)
Male	36 (54.5)	22 (51.2)
Age, years (Mean±SD)	7±4.4	8.9±4.1
Height, cm (Mean±SD)	118.8±25	130±26.2
Weight, kg (Mean±SD)	25.4±15.1	30.6±14.8
BMI, kg/m^2 (Mean±SD)	16.9±6.9	17.3±2.9
Birth weight, g (Mean±SD)	2503±707.8	
Gestational age, weeks (Mean±SD)	36.5±3	
From twin pregnancy, n (%)	3 (4.5)	
Has sibling, n (%)	45 (68.2)	
Single parent, n (%)	3 (4.5)	
Mother's age, years (Mean±SD)	36.5±7.2	
Mother's education level, n (%)		
Elementary and middle school	36 (55)	
High school and beyond	30 (45)	

Sex distribution and ages were not significantly different between the patients and controls (p=0.881 and p=0.766, respectively).
BMI, body mass index; SD, standard deviation.

Table II. Clinical follow-up and treatment information (N=66).

	n (%)
The center where first surgery was performed	
Our center	56 (84.8)
Other center	10 (15.2)
Esophagostomy	5 (7.6)
Placement of a gastrostomy	25 (37.9)
Preoperative gastrostomy placement for EA	17 (25.8)
Closure of gastrostomy	21 (31.8)
Primary repair	55 (83.3)
If there is primary repair	
Early	47 (85.2)
Late	8 (14.8)
Replacement surgery	11 (16.7)
Postoperative intubation[1]	45 (78.9)
Refistula repair	7 (10.9)
Extraesophageal surgery	40 (60.6)
Fundoplication	17 (26)
PSARP or anoplasty	13 (20)
Colostomy	8 (12.1)
Colostomy closure	7 (11)
Major cardiac operation	2 (3)
Aortopexy	1 (1)
Duodenal atresia	3 (4)
Excision of esophageal diverticulum	1 (1)
Scoliosis surgery	1 (1)
Nephrectomy	2 (3)
Other	6 (9.1)
Tracheostomy	1 (1.5)
Tracheomalacia	7 (10.6)
Neurogenic bladder	5 (7.6)
Anastomotic stricture	40 (60.6)
Congenital esophageal stenosis	2 (3)
Dilation[2]	41 (62.1)
Steroid administration	7 (10.6)
Resection anastomosis	1 (1.5)
Eosinophilic esophagitis	6 (9.1)
Recurrent lung infections	16 (24.2)
Scoliosis	6 (10.3)
Winged scapula	2 (3.8)

[1]45 patients were intubated for a median of 4 days (min-max: 1-115).
[2]Patients underwent anastomosis dilations for a median of 3 times (min-max: 1-16).
EA, esophageal atresia; PSARP, posterior sagittal ano-rectoplasty

those having VACTERL-H association (n=9), gastrostomy (n=15), non-EA surgeries (n=22), and those that did not (Supplementary Tables S1-S2). Patients with tracheomalacia complaints (n=7), had significantly lower scores in physical health status (median: 50, min–max: 33.3–100; p=0.019), relationships with others (median: 50, min–max: 25–83.3; p=0.001), and total (median: 60.2, min–max: 30.9–82.8; p=0.011) compared to patients without tracheomalacia. Patients experiencing frequent lung infections (n=9) had lower quality of life in the physical health status domain (median: 54.1, min–max: 33.3–79.2; p=0.002). No significant differences were observed in patients diagnosed with anastomotic stricture (n=24) or eosinophilic esophagitis (n=5) compared to those without these (Supplementary Table S3). Patients' sex, parental education level, and presence of siblings did not create a difference (Supplementary Table S4).

The proxy report results of the EA-QOL questionnaire for children aged 8-17 years

Patients with birth weights below 2500 grams (n=15) had lower quality of life in nutritional status (median: 81.3, min–max: 59.4–100; p=0.038), and body scar (median: 81, min–max: 80–100; p=0.047) domains. No significant difference was found based on preterm (n=11) or term birth. Type C EA patients (n=20) had significantly higher quality of life in nutritional status (median: 89.1, min–max: 71.9–100; p=0.019) and total scale (median: 94.2, min–max: 79.2–100; p=0.041) compared to Type A (n=5) (Supplementary Table S6). No significant difference was observed between patients with VACTERL-H association (n=8) or anastomotic strictures (n=16) compared to those without. Patients who had a gastrostomy (n=10) had lower quality of life in nutritional status (median: 76.6, min–max: 59.4–100; p=0.002), body scar (median: 80.5, min–max: 55–100; p=0.048), and total scale (median: 81.8, min–max: 53.3–100; p=0.019). Primarily repaired patients (n=19) scored higher in nutritional status (median: 87.5, min–max: 59.4–100; p=0.018) compared to replacement surgery (Supplementary Tables

S6-S7). Patients with a history of frequent lung infections (n=7) had lower quality of life in relationships with others (median: 78.6, min–max: 57.1–100; p=0.006), body scar (median: 81.3, min–max: 62.5–100; p=0.037), physical health status (median: 81.3, min–max: 62.5–100; p=0.035), and total scale (median: 81.3, min–max: 58.3–99; p=0.035) (Supplementary Table S8). Sex, parental education level, and presence of siblings did not have significant effects (Supplementary Table S9-S10).

The child report results of the EA-QOL questionnaire for children aged 8-17 years

No significant difference was observed based on birth weight (< 2500 g, n=15) or gestational age (term, n=14). No significant difference was observed in any subscale of the questionnaire between patients with Gross Type C (n=20) and Type A (n=5) (Supplementary Table S11). Presence of VACTERL-H association (n=8) or anastomotic strictures (n=16) did not create a significant difference. Patients who underwent non-EA surgical procedures (n=18) had significantly lower scores in the physical health status (median: 93.7, min–max: 56.3–100; p=0.040), and total (median: 83.8, min–max: 72.9–100; p=0.026) subscales (Supplementary Tables S11-S13). Sex, parental education level, and presence of siblings did not significantly affect the quality of life as assessed by this questionnaire (Supplementary Table S14).

The results of the PedsQOL 4.0 questionnaire

The proxy report results of the PedsQOL 4.0 questionnaire for children born with EA aged 2-7 years

Patients with a birth weight below 2500 grams (n=16) had significantly lower quality of life in terms of physical functioning (median: 68.7, min–max: 12.5–100; p=0.005), social functioning (median: 77.5, min–max: 40–100; p=0.042) and total score (median: 74.5, min–max: 48.9–97.8; p=0.037). The patients who were preterm (n=24) did not show a significant difference compared to those who were not. There was no significant difference observed based on the EA Gross classification (type C, n=30 and type A, n=5). No significant difference was found between those with and VACTERL-H association (n=9) or anastomotic strictures (n=24) compared to those without.

There was no statistically significant difference between patients who underwent gastrostomy (n=15) and those who did not. The social functioning score (median: 80, min–max: 40–100; p=0.018) of patients (n=22) who underwent non-EA surgical procedures was significantly lower. No significant difference was found in quality-of-life scales based on complaints of tracheomalacia (n=7) or frequent lung infections (n=9). The patients' sex and parental education level did not affect the quality of life measured by this questionnaire.

The proxy report results of the PedsQOL 4.0 questionnaire for children born with EA aged 8-17 years

For EA children aged 8-17 years (n=26), according to proxy reports on the PedsQOL 4.0 questionnaire, no significant difference was found in any of the scales based on birth weight (n=15, birth weight <2500 grams) or gestational age (n=11, preterm). Presence of VACTERL-H association (n=8) yielded a lower quality of life in terms of physical functioning (median: 77.5, min–max: 31.3–100; p=0.010), social functioning (median: 80, min–max: 40–100; p=0.033), and total score (median: 74.5, min–max: 48.9–97.8; p=0.037). No significant difference was found between patients who underwent non-EA surgical procedures (n=18) or primary esophageal repair (n=19) compared to those who did not. The patients' sex, parental education level, and the presence of siblings did not affect quality of life. There was no significant difference observed between patients with anastomotic stricture (n=16) and those without.

The child report results of the PedsQOL 4.0 questionnaire for children born with EA aged 8-17 years

No significant difference was found in any of the scales based on birth weight (n=15, birth weight <2500 grams) or gestational age (n=11, preterm). However, those with VACTERL-H association (n=8) were found to have significantly lower quality of life in terms of emotional functioning (median: 82.5, min–max: 60–100; p=0.037), social functioning (median: 95, min–max: 75–100; p=0.049), and total score (median: 75.5, min–max: 66.3–91.3; p=0.042).

There was no significant difference observed between patients who underwent non-EA surgical procedures (n=18) or primary repair (n=19) compared to those who did not. The patients' sex, parental education level, and the presence of siblings did not affect the quality of life in this questionnaire.

Parameters resulting in statistically significant differences from both surveys are summarized in Table III.

Comparison of PedsQOL 4.0 child and parental reports with a healthy volunteer group

A comparison of the PedsQOL 4.0 patient and healthy groups was conducted. Among the healthy volunteers, 21 (48.8%) were females, and 22 (51.2%) were males. There was no statistically significant difference in sex (p=0.881) or age group distributions (p=0.766) when patients and healthy children were compared. In both parental and child reports for ages 8-17 years, EA patients scored higher on the emotional subscale (median: 90, min–max: 60–100; p<0.001) compared to the healthy group (Table IV).

Discussion

The question of how EA and its associated morbidities affect long-term quality of life has been addressed previously. In 5 out of 7 evaluated studies in a review, the quality of life of EA patients was found to be impaired compared to healthy references.[16]

In a study conducted in Sweden and Germany, when evaluating PedsQOL 4.0 proxy reports, lower total scores were obtained compared to the healthy population. However, interestingly, EA children obtained significantly better results in the emotional and social subscales.[17] In our study, when comparing the reports of children and parents aged 8-17 years with those of the healthy population, the patient group obtained better scores in the emotional subscale. The challenging medical experiences that EA patients undergo from childhood and the frequent hospital visits may result in increased anxiety and depression.[18] On the other hand, children with chronic illnesses may also learn to cope better with daily stressors compared to healthy references.[19] Patients with EA probably adapt to their condition at an earlier age and employ coping strategies, supporting our findings.[20,21]

Sex may have an effect on the results obtained. In some studies in EA patients, girls scored higher on the school scale of the PEDQOL 4.0 questionnaire compared to boys.[17,22] In another study, the proxy report obtained when EA patients were 8 years old, the school functional score of female children was low.[23] Male patients on the other hand in both the 8-year-old and 12-year-old groups scored higher in emotional functioning compared to females.[23] In our study, no significant differences were found between sexes in any of the questionnaires.

In an assessment conducted 20 years after EA repair, the quality of life of patients who underwent primary repair was better than those who underwent colon interposition.[24] Patients with colon interposition suffered more from various gastrointestinal and respiratory symptoms.[24] In a pilot study, it was noted that patients with Gross type C EA who underwent primary repair had milder feeding difficulties, while those who underwent complex and complicated surgeries experienced significant feeding difficulties.[25] In our study, in the proxy report of the EA-QOL questionnaire for patients aged 8-17 years, patients who underwent replacement surgery had lower scores for

Table III. Parameters resulting in statistically significant differences from EA-QOL questionnaire and PedsQOL 4.0 questionnaire.

Significant parameters creating differences	Evaluated categories
EA-QOL questionnaire	
Proxy report for ages 2-7	
Tracheomalacia	Lower physical health status [50 (33.3-100) vs. 83.3 (37.5-100); p=0.019], Interpersonal relationships [50 (25-83.3) vs. 100 (0-100); p=0.001], Total score [60.2 (30.9-82.8) vs. 84.4 (25-100) p=0.011]
Frequent respiratory infections	Lower physical health status [54.1 (33.3-79.2) vs. 83.3 (37.5-100): p=0.002]
Proxy report for ages 8-17	
Birth weight below 2500 grams	Lower nutritional status [81.3 (59.4-100) vs 89.1 (81.3-100); p=0.038]
Isolated EA with replacement surgery	Lower nutritional status [75 (62.5-100) vs 87.5 (59.4-100); p=0.018]
Gastrostomy placement	Lower nutritional status [76.6 (59.4-100) vs. 92.2 (75-100); p=0.002], Body scar [80.5 (55-100) vs.100 (80-100); p=0.048], Total score [81.8 (58.3-100) vs. 94.7 (79.2-100); p=0.019].
Frequent respiratory infections	Lower total score [81.3 (58.3-99) vs. 93.8 (72.7-100); p=0.035]
Child self-report for ages 8-17	
Patients who underwent surgeries other than EA	Lower physical health status [93.7 (56.3-100) vs. 100 (87.5-100); p=0.040] Total score [83.8 (72.9-100) vs. 95.8 (83.3-100);p=0.026]
PedsQOL 4.0 questionnaire	
Proxy report for ages 2-7	
Birth weight below 2500 grams	Lower physical functioning [68.7 (12.5-100) vs. 96.9 (56.3-100): p=0.005], Social functioning [77.5 (40-100) vs 100 (60-100); p=0.042] Total score [70.1 (47.8-100) vs 89.5 (60.8-100); p=0.006)
Patients who underwent surgeries other than EA	Lower social functioning score [80 (40-100) vs.100 (40-100); p=0.018]
Proxy report for ages 8-17	
VACTERL-H association	Lower physical functioning [77.5 (31.3-100) vs. (98.4 (56.3-100); p=0.010] Social functioning [80 (40-100) vs. 100 (25-100); p=0.033] Total score [74.5 (48.9-97.8) vs. 92.3 (34.8-100); p=0.037]
Child self-report for ages 8-17	
VACTERL-H association	Lower emotional functioning [82.5 (60-100) vs. 100 (60-100); p=0.037] Social functioning [95 (75-100) vs. 100 (25-100); p=0.049] Total score [75.5 (66.3-91.3) vs. 92.9 (55.4-100); p=0.042]

Scores presented as median (min-max). EA-QOL, esophageal atresia quality of life; PedsQOL, pediatric quality of life; VACTERL-H, vertebral anomalies, anorectal anomalies, cardiac anomalies, tracheoesophageal fistula, renal anomalies, limb anomalies and hydrocephalus; EA, esophageal atresia.

feeding status and total quality of life. A similar finding was reported before where parental reports of patients aged 8-17 years had lower quality of life associated with nutrition when primary anastomosis was not performed.[14]

The overall quality of life of EA patients showed that patients with distal fistula (Type C) scored higher in the proxy report at age 2-7 years compared to those with isolated (Type A) atresia, while no significant difference was found in the child report for patients aged 8-17

Table IV. Comparison of patient and control group's PedsQOL 4.0 questionnaire results.

	Patients, median (min-max)	Controls, median (min-max)	P
PedsQOL 4.0, 8-17 years, children	n=26	n=19	
Physical functioning	85.4 (54.1-100)	84.4 (50-93.8)	0.239
Emotional functioning	90 (60-100)	65 (35-85)	<0.001
Social functioning	100 (25-100)	100 (55-100)	0.297
School functioning	80 (40-100)	80 (40-95)	0.729
Total	85.3 (55.4-100)	81.5 (52.2-91.3)	0.144
PedsQOL 4.0, 2-7 years, proxy	n=40	n=24	
Physical functioning	96.8 (53.1-100)	98.4 (32.3-100)	0.730
Emotional functioning	82.5 (35-100)	80 (25-100)	0.566
Social functioning	100 (40-100)	90 (55-100)	0.951
School functioning	90 (45-100)	91.6 (35-100)	0.828
Total	87.5 (48.6-100)	85.2 (45.7-100)	0.451
PedsQOL 4.0, 8-17 years, proxy	n=26	n=19	
Physical functioning	84.4 (53.1-100)	75 (46.9-93.8)	0.106
Emotional functioning	90 (60-100)	70 (15-90)	<0.001
Social functioning	100 (25-100)	95 (40-100)	0.072
School functioning	75 (40-100)	80 (40-100)	0.419
Total	85.3 (55.4-100)	79.3(59.8-90.2)	0.056

Mann-Whitney U test. min: Minimum, max: Maximum
PedsQOL, pediatric quality of life.

years.[17] Our study partly supports this finding because distal fistula patients had higher scores in feeding and total scores in the parental report of the EA-QOL questionnaire for ages 8-17 years. However, the type of EA did not give a significant result in the same age group for the child report or in the parental report for patients aged 2-7 years. Although the reasons for the differences between studies are not entirely clear, it is possible that different levels of expectations regarding the quality of life of the family or the child could be an explanatory factor.

In cases where EA is too complex to be operated on in the neonatal period or becomes complicated due to postoperative complications, resulting in an inability to orally feed, a gastrostomy is performed. It was found that 2-7 years old patients who underwent gastrostomy scored lower on feeding status scales and their quality of life in terms of their social life was adversely affected.[14] In our study, however, there was no difference in quality of life between those who underwent gastrostomy before or after EA surgery and those who did not in the proxy reports of the same age group. However, proxy reports for patients aged 8-17 years showed those who underwent gastrostomy had significantly lower scores in feeding status, body scar, and total score quality of life. Although the expected results may vary depending on age and scales, the use of gastrostomy can be considered a factor negatively affecting quality of life.

One of the most common complications following EA repair is anastomotic stricture. The treatment involves dilating the stricture by using a balloon or a bougie.[26,27] While the need for anastomotic dilation is observed in approximately 36% of patients within the first 2 years after Gross type C EA surgery, there is a decrease in the need for dilation over time.[28] In our study, quality-of-life scales did not differ between patients who experienced anastomotic strictures and underwent dilation compared to

those without strictures. In one study, the need for dilation at least once in children aged 8-17 years was reported as a risk factor in the feeding status scale.[14] Our contradicting results can be due to differences in expectations of quality of life between parents and children, inadequacy in understanding the questions, and/or less scrutiny of the problems. Furthermore, esophageal dilatations alleviate the disturbing stricture symptoms which may positively affect life quality as reported in one other study.[29]

In our study, presence of VACTERL-H association negatively affected the quality of life in emotional functioning, social functioning, and total subscales in the 8-17 age group according to the child report of the PedsQOL 4.0 questionnaire but not on the EA-QOL scale. A similar finding was previously attributed to the fact that the focus of EA-QOL questionnaire questions are on the upper gastrointestinal system and respiratory problems and the prioritization of the severity of anorectal or cardiac malformations rather than the number of VACTERL-H components.[14]

In EA patients, respiratory disorders, impairs quality of life and respiratory symptoms are found to be more common compared to healthy groups.[7] Approximately 50% of EA patients are hospitalized due to respiratory tract diseases, which can have a significant impact on both children and parents, mostly in early childhood.[30] Respiratory symptoms were reported to negatively affect physical health status, relationships with others, and total scores in the 2-7 age group EA-QOL questionnaire.[14] Similarly, in our study, in the 8-17 age group EA-QOL parent report, patients with frequent lung infections and those with tracheomalacia findings in the 2-7 age group were observed to report negative effects on their physical health status, relationships with others, and total scores. We can conclude that respiratory tract disorders affect EA patients more in terms of physical and social aspects. Additionally, respiratory system symptoms are more commonly seen at younger ages.[14] In accordance with this, tracheomalacia was not observed in the 8-17 age group in our study.

In our study, patients who underwent non-EA surgical procedures like colostomy creation and closure, surgery for anorectal malformation, fundoplication, and cardiac surgery could not be subdivided for individual assessment due to the small number of cases within groups. According to parental responses, patients were adversely affected by non-EA surgeries in social functioning in the 2-7 years PedsQOL 4.0 questionnaire and in physical health status and overall quality of life in the 8-17 years EA-QOL questionnaire. Fundoplication surgery was previously reported to have positive effects on patients' overall quality of life.[31] Surgical interventions can improve an individual's nutrition and respiratory issues. However, complications that may arise after surgical procedures can have the opposite effect. Conditions such as incontinence or constipation that may develop after anal atresia surgery can negatively affect the patient's quality of life socially and psychologically.[32] We cannot make strict comments about the impact of individual non-EA surgical procedures because of small patient numbers.

Our study has some limitations. Due to being a single-center, the number of patients was limited, and the groups were not homogeneously distributed. Therefore, some important comorbidities like individual components of VACTERL-H association cannot be analyzed in a more detailed manner. Some others like presence of neurogenic bladder or type of cardiac anomaly could not be statistically analyzed. On the other hand, all patients in our study were followed up and treated at a single center in a similar protocol which is important for consistency in obtaining and evaluating patient data.

Conclusions

This is the first study in Türkiye which applied an evaluated both the PedsQOL 4.0 and the specific EA-QOL tools to EA patients, to our

knowledge. Previous studies on the quality of life related to EA were mostly conducted in high income, western countries. The expectations regarding quality of life may be higher or at least different in such countries, potentially leading to lower perceived quality of life outcomes in patient groups. Our study is a step to improve knowledge about the perceived quality of life in individuals originating from a unique sociocultural background and afflicted with a rare disease.

Supplementary materials

Supplementary materials for this article are available online at https://doi.org/10.24953/turkjpediatr.2025.5627

Ethical approval

The study was approved by İstanbul Medeniyet University Faculty of Medicine Ethics Committee (date: 07.09.2022, number: 2022/0507).

Author contribution

The authors confirm contribution to the paper as follows: Study conception and design: SA,CUD; data collection: SA,GE; analysis and interpretation of results: SA, CUD, AIA, GE; draft manuscript preparation: SA, CUD. All authors reviwed the results and approved the final version of the article.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Original Article

Association between C677T variant of methylene tetrahydrofolate reductase and hypospadias risk in Algeria

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ABSTRACT

Background. Hypospadias, a congenital condition characterized by the urethral opening being on the underside of the penis, has received limited attention in its association with the *MTHFR* C677T variant. Given the crucial role of folate metabolism in embryonic development, and the involvement of the *MTHFR* C677T polymorphism in folate metabolism, this study aims to investigate whether this variant contributes to the risk of hypospadias in an Algerian population.

Methods. This case-control study included 105 patients with hypospadias and 125 controls. Genotyping of the *MTHFR* gene C677T variant was performed using polymerase chain reaction-restriction fragment length polymorphism.

Results. A statistically significant difference in the genotype distribution of the *MTHFR* C677T variant between patients with hypospadias and controls was revealed. The significance was observed in the codominant genetic model CT vs. CC (p=0.034, odds ratio [OR]: 1.89, 95% CI: 1.04-3.44) and TT vs. CC (p=0.042, OR: 2.15, 95% CI: 1.02-4.53), as well as in the dominant model CC vs. CT+TT (p=0.010, OR: 1.98, 95% CI: 1.17-3.35).

Maternal periconceptional folic acid supplement intake showed a significant association with the anatomical types of hypospadias in relation to the *MTHFR* C677T genotypes when folic acid was taken (p=0.006). Furthermore, a significant association was observed with the TT genotype in isolated hypospadias cases (p=0.038, OR=3.47, 95% CI: 1.03-11.68), suggesting a potential role of folic acid in modifying hypospadias risk.

Multiple logistic regression analysis identified intrauterine growth restriction, gestational hypertension, residency, and the *MTHFR* C677T variant as independent potential risk factors for hypospadias development (p-values: 0.030, 0.016, 0.040, and 0.045, respectively).

Conclusions. This study reports, for the first time, an association between the *MTHFR* gene C677T variant and hypospadias in the Algerian population. The findings suggest a strong association between the *MTHFR* C677T variant and susceptibility to hypospadias. Identified risk factors such as intrauterine growth restriction, gestational hypertension, rural residency, and the *MTHFR* C677T variant contribute valuable insights into the multifaceted etiology of hypospadias in this population.

Key words: hypospadias, *MTHFR*, C677T, risk factors, folic acid, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

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Hypospadias, a congenital malformation affecting the male genitourinary tract, results from partial urethral fold fusion during early fetal development.[1] It is among the most prevalent congenital anomalies, affecting approximately one in 200–300 newborn boys.[2] The condition's severity stratifies it into three degrees: milder anterior forms present with the urethral opening at the glandular or subcoronal position of the penis, while more severe middle hypospadias has the meatus opening at the midshaft of the penis, and the most severe posterior forms display penoscrotal, scrotal, or perineal openings.[3] Although most cases of hypospadias occur independently, associated abnormalities include unilateral or bilateral cryptorchidism and micropenis.[4] While the precise causes of hypospadias remain incompletely understood, it is believed that a confluence of environmental and genetic factors contributes to its development.[5]

Folic acid plays an essential role in DNA (deoxyribonucleic acid) methylation processes, which are crucial for harmonious embryonic development. Adequate maternal folic acid supplementation, especially during the periconceptional period, is paramount for preventing congenital anomalies.[6,7] This supplementation is associated with a lower risk of neural tube defects, as well as cardiac, urinary tract, and limb defects.[8] Folic acid supplementation increases the activity of methylenetetrahydrofolate reductase (MTHFR), an enzyme involved in folate metabolism, which helps to reduce the risk of birth defects by ensuring proper folate metabolism.[9]

The *MTHFR* C677T gene variant is a functional variant in the *MTHFR* gene, leading to reduced enzymatic activity and elevated blood homocysteine levels.[10] Research investigating the role of *MTHFR* C677T in DNA methylation has yielded varying results, suggesting that the *MTHFR* C677T variant may have diverse effects on DNA methylation depending on the cell type, folate availability, and other factors.[11,12] Additionally, the *MTHFR* C677T variant has been extensively studied in relation to various conditions, including cardiovascular ischemic risk, neural tube defects, and cleft lip and palate.[13-15] However, only one study has investigated the relationship between the *MTHFR* C677T variant and hypospadias, indicating a significant gap in the literature on this topic.

A case-control study involving 855 hypospadias cases and 713 controls found that hypospadias was not generally associated with folic acid use, the *MTHFR* C677T polymorphism, or their interaction. However, the study identified an association between middle hypospadias and the absence of folic acid supplement use, especially in infants carrying the CT/TT genotype.[16] Understanding the broad impact of this variant highlights the importance of studying its potential contribution to non-syndromic hypospadias.[17]

Given the limited research on the relationship between the *MTHFR* C677T variant and hypospadias, with only one study currently available, this study aims to explore the association between the *MTHFR* C677T polymorphism and hypospadias, taking into account maternal periconceptional use of folic acid supplements. We hypothesize that the C677T variant is associated with an increased risk of hypospadias and that folic acid supplementation may modulate this effect. To investigate this hypothesis, our study intended to evaluate the connection between the *MTHFR* C677T variant and hypospadias in Algerian children.

Materials and Methods

Study population

The study comprised 230 participants, categorized into two groups: 105 patients with a diagnosis of hypospadias and 125 control subjects. Patient recruitment occurred across two locations: the endocrinology-diabetology service of the University Hospital Center in Constantine and the pediatric surgery

department specialized in mother and child care at El Eulma-Setif.

For the case group, inclusion criteria were: male patients diagnosed with hypospadias, aged under 15 years, with available biological samples and complete clinical data. All patients included had a confirmed 46,XY karyotype.

Exclusion criteria were the presence of syndromic features, chromosomal abnormalities, ambiguous genitalia, or incomplete medical and/or genetic data. Ambiguous genitalia were defined as the presence of external genitalia that do not allow an assignment as male or female at birth. Although endocrine testing was not systematically performed, all patients underwent a detailed clinical examination by experienced pediatric urologists, and none showed signs of genital ambiguity or endocrine disorders.The case group included both isolated hypospadias and hypospadias associated with non-syndromic anomalies such as intrauterine growth restriction (IUGR), micropenis, cryptorchidism, and penile curvature.

For the control group, inclusion criteria were: male children without any congenital malformations, especially no history of hypospadias or other urogenital anomalies, matched in age range with the case group, and with available biological samples and clinical information. Exclusion criteria included a family history of hypospadias or congenital anomalies, known or suspected genetic disorders, or incomplete medical records.

Detailed medical records were thoroughly examined for all cases to identify familial history, evaluate the severity of hypospadias, assess clinical characteristics, and precisely locate the urethral opening. These aspects were determined by experienced pediatric urologists during a pre-surgery physical examination, along with a questionnaire filled out by us in collaboration with the child's parents.

The control group was recruited from the same healthcare institutions where the hypospadias cases were collected. These were male children who were visiting for conditions unrelated to hypospadias or any genital system anomalies. A pediatric specialist conducted a thorough clinical examination to confirm that they had no personal or familial history of hypospadias and were free from other external genital deformities, including cryptorchidism, micropenis, penile curvature, or inguinal hernia.

Informed consent was obtained from the parents or legal guardians of all participants. The case-control study received approval from the Ethical Scientific Committee of the Faculty of Natural and Life Sciences, University of Constantine 1. All procedures were carried out in compliance with the World Medical Association's 1989 Declaration of Helsinki, which served as the protocol's guide.

Genetic analysis

Blood sampling and DNA extraction:

Ten mL of venous blood were collected in tubes containing ethylenediaminotetracetic acid (EDTA). Genomic DNA from peripheral leukocytes was isolated using the sodium chloride (NaCl) technique.[18] The purity of the DNA was verified using a nanodrop (NanoDrop 2000, Thermo, Massachusetts, U.S.A.). The isolated DNA was diluted to 20ng/μL and preserved at -20 °C.

MTHFR C677T genotyping: The *MTHFR* C677T variant was genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The primer sequences used were: forward: 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ and reverse: 5′-AGGACGGTGCGGTGAGAGTG-3′. The PCR reaction mixture (20 μL) comprised template DNA, MgC_{12} (50mM), deoxynucleotide triphosphate (dNTPs) mix (final concentration 0.2 mmol/L), oligonucleotide primers (100 ng/μL), and Taq DNA polymerase (5 U/μL). An Eppendorf Mastercycler was used for the amplification process, following these conditions: initial denaturation at 94 °C for 5

minutes, then 35 cycles of denaturation at 94 °C for 30 seconds, hybridization at 65 °C for 30 seconds, elongation at 72 °C for 30 seconds, and a final extension at 72 °C for 10 minutes.

The resulting PCR product (10 μL) was digested using the *Hinf1* restriction enzyme (New England Biolabs). The fragmented products were separated on a 3% agarose gel and observed under ultraviolet light.

To ensure the accuracy of genotyping, all samples were analyzed in duplicate, and both positive and negative control samples with known genotypes were included in each PCR-RFLP run.

Statistics

Statistical analysis of the data was performed using SPSS software (version 26; SPSS Inc., Chicago, IL, U.S.A.). Continuous variables are presented as mean ± standard deviation (SD), while categorical variables are shown as frequencies and percentages. Baseline characteristics, genotype, and allele distribution were compared between case children and controls using chi-square or t-tests.

The chi-square test for goodness of fit was employed to assess if the genotype distributions within the two groups conformed to the expectations of Hardy-Weinberg equilibrium (HWE). Risk-factor analysis was conducted using logistic regression analysis. Results were expressed as odds ratios (ORs) with 95% confidence intervals (95% CIs). Statistical significance was determined at $p<0.05$.

Results

Characteristics of the study population

The characteristics of 105 patients with hypospadias and 125 controls are summarized in Table I. The mean age in the hypospadias group and the control group was 4.58 ± 2.52 and 4.39 ± 2.45 years, respectively. Isolated hypospadias was observed in 55.24% of patients.

Table I. Comparison of the case and the control group's baseline characteristics.

Characteristics	Cases (N=105) n (%)*	Controls (N=125) n (%)*	χ2/t	OR (95% CI)	*P*- value
Age (years)	4.58±2.52	4.39±2.45	0.35	-	0.729
Intrauterinegrowth restrection	17 (16.19)	1 (0.8)	8.12	11.21 (1.45-16.50)	0.004**
Low birth weight (<2500 g)	23 (21.9)	8 (6.4)	1.84	1.82 (0.76-4.38)	0.175
Preterm birth (<37 weeks)	13 (12.38)	2 (1.6)	3.78	4.10 (0.89-18.82)	0.052
Advanced maternal age (>35 years at pregnancy)	71 (67.62)	47 (37.6)	2.15	0.58 (0.28-1.21)	0.142
Gestational hypertension	26 (24.76)	4 (3.2)	8.40	4.61 (1.52-13.94)	0.004**
Gestational diabetes	15 (14.29)	7 (5.6)	0.23	1.26 (0.48-3.29)	0.634
Birth order (1st)	54 (51.43)	30 (24)	0.03	1.06 (0.56-2)	0.860
Consanguinity	26 (24.76)	8 (6.4)	3.05	2.14 (0.90-5.09)	0.081
Residence (rural)	56 (53.33)	22 (17.6)	4.26	1.97 (1.03-3.78)	0.039**
Type of hypospadias		-	-	-	-
Anterior	59 (56.2)				
Middle	14 (5.2)				
Posterior	32 (30.5)				

*Age given as mean ± standard deviation; others as n (%). **$p < 0.05$; CI, confidence interval; OR, odds ratio; t, Student's t; χ2, chi-square.

Significant differences were observed between the hypospadias and control groups regarding IUGR (p = 0.004, OR = 11.21, 95% CI: 1.45–16.50), gestational hypertension (p = 0.004, OR = 4.61, 95% CI: 1.52–13.94), and rural residence (p = 0.039, OR = 1.97, 95% CI: 1.03–3.78).

Additionally, the distribution of hypospadias types among patients indicated that anterior hypospadias was the most frequent (59 cases, 56.2%), followed by posterior hypospadias (32 cases, 30.5%) and middle hypospadias (14 cases, 5.2%).

MTHFR C677T variant genotype and allele frequencies

Genotyping results for the C677T locus within the *MTHFR* gene, determined by electrophoresis, are illustrated in Supplementary Figure S1. Three different genotypes were observed: one band (198 bp), two bands (198 and 175 bp), and one band (175 bp) in the wild-type homozygote (CC), heterozygote (CT), and mutant homozygote (TT). The presence of allele T corresponds to the presence of a cutting site for *HinfI*.

The *MTHFR* gene variant's distribution did not follow the HWE in cases and control groups ($p<0.05$). The distribution of genotype and allele frequency of the *MTHFR* C677T variant in patients with hypospadias and healthy controls is presented in Table II. The genotype frequencies of the *MTHFR* C677T variant among patients were 44.76% for CC, 35.24% for CT, and 20% for TT. In the control group, the corresponding frequencies were 61.6% (CC), 25.6% (CT), and 12.8% (TT). Despite these variations, the *MTHFR* C677T variant's genotype distribution was statistically significant between the group of patients with hypospadias and the control group under codominant genetic model CT vs. CC and TT vs CC (p=0.034, OR: 1.89, 95% CI: 1.04-3.44 and p=0.042, OR: 2.15, 95% CI: 1.02-4.53, respectively). Also, significance was noted when the controls and patients were compared using the dominant model CC vs. CT+TT (p=0.010, OR: 1.98, 95% CI: 1.17-3.35). Concerning allele distribution, patients' T allele frequency was higher than controls (37.62% and 25.6%, respectively). The *MTHFR* C677T variant's allele frequency differed significantly (p=0.005, OR: 1.75, 95% CI: 1.18-2.61).

Patient's characteristics by methylene tetrahydrofolate reductase C677T gene variant in children with hypospadias

Table III summarizes data on various patients' characteristics according to different *MTHFR* C677T genotypes (CC, CT, TT, and CT or TT). No significant variations were detected in age,

Table II. Distribution of the *MTHFR C677T* genotype in cases and controls, n (%).

Genetic model	Cases (N=105)	Controls (N=125)	OR (95% CI)	*P*-value
Codominance				
CC	47 (44.76)	77 (61.6)	Ref	
CT	37 (35.24)	32 (25.6)	1.89 (1.04-3.44)	0.034*
TT	21 (20)	16 (12.8)	2.15 (1.02-4.53)	0.042*
Dominance				
CC vs CT+TT	58 (55.24)	48 (38.4)	1.98 (1.17-3.35)	0.010*
Recessive				
CC+CT vs TT	84 (80)	109 (87.2)	1.70 (0.84-3.46)	0.138
Alleles				
C	131 (62.38)	186 (74.4)	Ref	
T	79 (37.62)	64 (25.6)	1.75 (1.18-2.61)	0.005*

*$p < 0.05$; CI, confidence interval; OR, odds ratio; Ref, reference.

Table III. Patient characteristics of children with hypospadias according to the *MTHFR C677T* gene variation.

		MTHFR C677T genotypes					
		CC N (%)	CT N (%)	TT N (%)	CT or TT N (%)	*P1*	*P2*
Age	<5 yr	34 (71.74)	26 (70.27)	13 (61.90)	39 (67.24)	0.684	0.572
	>5 yr	13 (28.26)	11 (29.73)	8 (38.10)	19 (32.76)		
Intrauterine growth restriction	Yes	8 (17.02)	2 (5.41)	7 (33.33)	9 (15.52)	0.021*	0.835
	No	39 (82.98)	35 (94.59)	14 (66.67)	49 (84.48)		
Preterm birth	Yes	6 (12.77)	3 (8.11)	4 (19.05)	7 (12.07)	0.475	0.914
	No	41 (87.23)	34 (91.89)	17 (80.95)	51 (87.93)		
Birth weight	<2500 g	14 (29.79)	8 (21.62)	1 (4.76)	9 (15.52)	0.070	0.079
	>2500 g	33 (70.21)	29 (78.38)	20 (95.24)	49 (84.48)		
Maternal age	<35 yr	33 (70.21)	27 (72.97)	11(52.38)	38 (65.52)	0.240	0.609
	>35 yr	14 (29.79)	10 (27.03)	10 (47.62)	20 (34.48)		
Gestational hypertension	Yes	9 (19.15)	8 (21.62)	9 (42.86)	17 (29.31)	0.096	0.230
	No	38 (80.85)	29 (78.38)	12 (57.14)	41 (70.69)		
Gestational diabetes	Yes	5 (10.64)	5 (13.51)	5 (23.81)	10 (17.24)	0.353	0.336
	No	42 (89.36)	32 (86.49)	16 (76.19)	48 (82.76)		
Birth order	1	22 (46.81)	22 (59.46)	10 (47.62)	32 (55.17)	0.477	0.394
	>1	25 (53.19)	15 (40.54)	11 (52.38)	26 (44.83)		
Consanguinity	Yes	13 (27.66)	7 (18.92)	6 (28.57)	13 (22.41)	0.591	0.536
	No	34 (72.34)	30 (81.08)	15 (71.43)	45 (77.59)		
Residence	Rural	27 (57.45)	16 (43.24)	13 (61.90)	29 (50)	0.293	0.447
	Urban	20 (42.55)	21 (56.76)	8 (38.10)	29 (50)		
Isolated hypospadias	Yes	26 (55.32)	23(62.16)	9 (42.86)	32 (44.83)	0.364	0.988
	No	21 (44.68)	14 (37.84)	12 (57.14)	26 (55.17)		

*$p < 0.05$; CI, confidence interval; OR, odds ratio; P1, comparison between three genotypes; P2, comparison between CT+TT vs CC.

residence, birth weight, hypertensive gestation, gestational diabetes, and birth order (all $p > 0.05$) among these genotypes. However, substantial distinctions were evident in IUGR, which demonstrated a significant association with the genotypes ($p = 0.021$).

Association between maternal periconceptional folic acid supplement intake, anatomical types of hypospadias and MTHFR C677T genotypes

Supplementary Table S1 examines the relationship between maternal periconceptional folic acid supplement intake, anatomical types of hypospadias, and the *MTHFR* C677T genotypes (CC, CT, TT). A statistically significant association was found between the *MTHFR* C677T genotypes and the anatomical types of hypospadias when folic acid was taken ($p = 0.006$).

Association between maternal periconceptional folic acid supplement intake, isolated/non-isolated hypospadias and MTHFR C677T genotypes

An association between maternal periconceptional folic acid supplement intake, isolated/non-isolated hypospadias, and *MTHFR* C677T genotypes was analyzed. A significant association was observed with the TT genotype in isolated cases ($p = 0.038$, OR = 3.47 with a 95% CI of 1.03-11.68), suggesting a potential role of folic acid in modifying hypospadias risk. However, other genotypes and non-isolated

cases did not show consistent significant associations (Supplementary Table S2).

Multivariate logistic regression

The results of multiple logistic regression analyses (Table IV) were used to identify the best independent predictors. In this analysis, IUGR ($p = 0.030$, OR = 10.07, 95% CI: 1.26-80.49), gestational hypertension ($p = 0.016$, OR = 4.27, 95% CI: 1.31-13.89), rural residency ($p = 0.040$, OR = 1.97, 95% CI: 1.03-13.89) and *MTHFR* C677T variant ($p = 0.045$, OR = 2.15, 95% CI: 1.01-4.53) were found to be independent potential risk factors for hypospadias development.

Discussion

Hypospadias, a common congenital anomaly affecting the male external genitalia, is believed to arise from a complex interplay of genetic and environmental factors, presenting a diverse range of treatment approaches.[19] This study aimed to explore the potential association between the C677T variant of the *MTHFR* gene, maternal intake of folic acid supplements during the periconceptional period, and susceptibility to hypospadias in Algerian children. Our findings suggest a significant association between this genetic variant and the risk of developing hypospadias, which could have important implications for the etiological understanding and clinical management of this congenital anomaly.

Investigations into *MTHFR* gene variants and their association with congenital genital anomalies, such as hypospadias, are currently limited. The *MTHFR* gene is crucial in folate metabolism, essential for DNA synthesis and methylation, and influencing cellular division and development.[20] The C677T variant alters the enzyme's structure, reducing its activity, leading to hyperhomocysteinemia, and reducing the availability of active folate necessary for converting homocysteine to methionine. Methionine serves as a precursor for S-adenosylmethionine (SAM), a universal methyl donor in various methylation reactions, including DNA methylation.[21] Disruptions in DNA methylation, an epigenetic mechanism, are associated with various developmental disorders, including congenital anomalies.[22] Our study's findings suggest that the C677T variant of the *MTHFR* gene, through its effects on enzymatic activity and increased homocysteine levels, may disrupt critical DNA methylation processes essential for normal embryonic development. This disruption could potentially affect signaling pathways and crucial genes involved in the formation of male genital organs, thereby contributing to the development of hypospadias.

Table IV. Logistic regression analysis of linked risk factors for patients with hypospadias.

Risk factor	OR (95% CI)	*P* value
Preterm birth	3.72 (0.80-17.29)	0.094
Birth weight (<2500 g)	1.62 (0.66-3.96)	0.290
Intrauterine growth restriction	10.07 (1.26-23.49)	0.030*
Maternal age (> 35 years)	0.57 (0.25-1.26)	0.164
Gestational diabetes	0.59 (0.18-1.97)	0.387
Gestational hypertension	4.27 (1.31-13.89)	0.016*
Consanguinity	2.02 (0.84-4.90)	0.115
Maternal periconceptional folic acid supplement intake	0.98 (0.46-2.07)	0.957
Residence (rural)	1.97 (1.03-3.78)	0.040*
MTHFR C677T variant	2.15 (1.01-4.53)	0.045*

*$p < 0.05$; CI, confidence interval; OR, odds ratio.

In our study, the violation of HWE in both cases and controls could be attributed to several factors. First, the Algerian population may present genetic heterogeneity due to substructure, which could lead to slight deviations from HWE.[23,24] Additionally, given the relatively small sample size, minor violations are not uncommon due to sampling variability.[25,26] Notably, the violation of HWE in cases may reflect a genetic predisposition to certain genetic diseases, as affected individuals are often more likely to carry specific genotypes that deviate from equilibrium. These deviations can indicate natural selection or disease-related factors, where certain alleles confer an increased risk or resistance, altering genotype frequencies compared to what is expected in an equilibrium population.[27] Furthermore, we ensured the accuracy of our genotyping by including duplicates and control samples, which suggests that this deviation is more likely due to population or disease-specific factors rather than technical errors.

Our findings revealed a prevalence of 44.76% CC genotype among patients, dominating the genetic profiles, whereas controls showed a dominant 61.6% CC genotype, with 35.24% CT and 20% TT among patients and 25.6% CT and 12.8% TT among controls. Importantly, the presence of the 677T allele was notably higher in patients with hypospadias (37.62%) compared to controls (25.6%). Our study highlighted notable variations between patients and controls' genotype distribution and allele frequency (p=0.034, p=0.042, p=0.005, respectively; Table II).

These results contrast with Dokter et al.'s[16] large-scale study in the Netherlands, which included over 800 cases and found no significant association between the *MTHFR* C677T variant and hypospadias. This discrepancy may be attributed to differences in study populations, as genetic and environmental factors vary between regions. The Algerian population may have distinct genetic predispositions or environmental exposures influencing the role of the *MTHFR* C677T variant in hypospadias risk. Additionally, while Dokter et al.[16] analyzed a much larger cohort, our study provides an initial exploration within a North African population, emphasizing the need for further regional studies to confirm these findings.

A statistically significant correlation emerged in comparing patients and controls based on CC genotype versus CT+TT genotype (p=0.010). Our study represents the first exploration of the potential correlation between the *MTHFR* C667T variant and hypospadias risk in Algerian children, marking a significant discovery of an association between the *MTHFR* C677T variant and hypospadias.

However, the impact of the *MTHFR* C677T gene variant extends beyond hypospadias to various health conditions, including impaired renal function and urinary tract anomalies. This variant, associated with elevated homocysteine levels, may contribute to renal impairment in young people with high blood pressure and expectant mothers with preeclampsia.[28] Additionally, it may affect renal function in pregnant women with preeclampsia, potentially leading to increased urinary protein levels.[29] Studies have also highlighted a significant link between *MTHFR* C677T and urinary tract anomalies in girls, indicating a potential association between this gene variant and diverse urogenital development across genders.[30]

In our cases, we observed a significant correlation between IUGR and the *MTHFR* C677T variant (p=0.021; Table III). These findings align with multiple studies; for instance, Alset et al.[31] identified a connection between *MTHFR* C677T and higher risks of fetal growth impairment and susceptibility to IUGR. Global populations exhibit a connection between the *MTHFR* C677T change and a higher risk of IUGR, as reported in a systematic review and meta-analysis.[32] Furthermore, studies[33] have demonstrated the significance of the C677T (Ala222Val) variant of the *MTHFR* gene in the development of IUGR.

Additionally, IUGR remained significantly associated with hypospadias in logistic regression (p=0.030, OR: 10.07, 95% CI: 1.26-23.49). Multiple studies have investigated the potential link between IUGR and hypospadias.[34,35] The findings suggest a higher incidence of hypospadias among male infants when birth weight is below the 10th percentile and growth restriction is present, compared to those with normal birth weights. Moreover, IUGR has been linked to placental abnormalities, such as maternal vascular malperfusion, potentially contributing to hypospadias development.

Gestational hypertension and rural residence remained significantly associated with hypospadias in logistic regression (p=0.016, OR: 4.27, 95% CI: 1.31-13.89 and p=0.040, OR: 1.97, 95% CI: 1.03-3.78; respectively). Our findings align with Greenhill et al and Sherriff et al, indicating that maternal hypertensive disorders have been connected to an elevated risk of hypopadias in offspring.[36,37] Additionally, regarding rural residence as a potential risk factor for hypospadias, our findings were consistent with Moustafa et al.[38], who found that rural residence was one of the most independent predictors for hypospadias.

The significant association between gestational hypertension and hypospadias suggests that hypertensive complications could create an unfavorable intrauterine environment, influencing genital development. Additionally, rural residence, which is associated with potentially increased exposure to environmental factors or limited prenatal care, could also play a role in the increased risk.

Our study identifies, for the first time, that the *MTHFR* C677T presents a risk factor for hypospadias (p=0.045, OR: 2.15, 95% CI: 1.01-4.53). This underscores a significant correlation between a higher chance of developing hypospadias and the *MTHFR* gene's C677T variation. These findings suggest a strong link between this variant and the studied risk. However, further investigations are required to validate this association and to elucidate the underlying mechanisms influenced by the *MTHFR* gene's C677T variation.

Our study examined the association between maternal intake of folic acid supplements during the periconceptional period, anatomical types of hypospadias, and *MTHFR* C677T genotypes, as presented in Supplementary Table S1. The results indicate a statistically significant association between *MTHFR* C677T genotypes and anatomical types of hypospadias when folic acid is taken (p = 0.006). These findings suggest that the interaction between *MTHFR* C677T genotype and folic acid intake may play a role in determining specific anatomical types of hypospadias observed. Specifically, children with certain genotypes (e.g., CT and TT) may have a differential risk of developing more severe forms of hypospadias depending on whether their mothers took folic acid supplements during the periconceptional period. According to a study by Dokter et al, infants from mothers who did not use folic acid supplements appeared to have an increased risk of midshaft hypospadias (OR 1.6; 95% CI: 1.1–2.4).[16]

Folic acid is essential for DNA methylation and other critical biological processes during embryonic development.[6] The presence of the *MTHFR* C677T variant, which alters folate metabolism, could influence these processes in a way that modifies the risk of developing different forms of hypospadias. These results underscore the potential importance of folic acid as a modulating factor in the etiology of hypospadias and suggest that future studies should further explore this interaction to better understand the underlying mechanisms and develop more targeted prevention strategies.

Furthermore, the results in Supplementary Table S2 suggest a significant association between maternal periconceptional folic acid supplement intake, *MTHFR* C677T genotypes, and isolated types of hypospadias. Specifically, the TT genotype appears to be statistically significantly associated with isolated cases of

hypospadias (p = 0.038, OR = 3.47 with a 95% confidence interval of 1.03-11.68), indicating a potential role of folic acid in modifying hypospadias risk. However, other genotypes and non-isolated cases do not show consistent significant associations.

These findings collectively suggest that folic acid may play a differential role depending on specific types of hypospadias, whether anatomical or isolated. This highlights the importance of future studies to better understand this interaction and its clinical implications. Understanding the role of folic acid in relation to the *MTHFR* C677T genotype could lead to improved strategies for the prevention and management of hypospadias, particularly in populations with a higher prevalence of this genetic variant.

This study has some limitations that should be considered. The small sample size may affect the generalizability of our findings and could also contribute to the deviation from HWE observed in the case and control groups. This deviation may reflect undetected population substructure or selection bias strict inclusion criteria. Although all included patients had a confirmed 46,XY karyotype and syndromic features or ambiguous genitalia were excluded, no systematic hormonal or molecular genetic testing was conducted to identify other potential endocrine or monogenic causes of hypospadias. The absence of such investigations is mainly due to the retrospective design of the study and limited available resources. Consequently, the differentiation between isolated nonsyndromic hypospadias and cases potentially associated with subtle endocrine or genetic abnormalities may be incomplete. In addition, maternal DNA was not available for analysis, and thus maternal *MTHFR* genotype information was not assessed, despite evidence suggesting that maternal gene variants and folic acid deficiency may be associated with miscarriages and congenital anomalies. Additionally, the definition of rural and urban residency was based only on maternal residence during pregnancy, without considering environmental exposures. Despite these limitations, our study provides preliminary insights into the genetic and environmental factors of hypospadias in Algeria. Larger, prospective, and multicenter studies with more comprehensive endocrine and genetic assessments are needed to confirm these findings and better elucidate gene-environment interactions.

In conclusion, our study is the first to demonstrate an association between the C677T variant of the *MTHFR* gene and hypospadias in the Algerian population. These findings underscore the importance of genetics in the etiology of hypospadias and suggest that genetic screening could become a valuable tool for identifying at-risk children. However, further studies with larger sample sizes and functional analyses are necessary to validate these results and elucidate the underlying biological mechanisms influenced by the *MTHFR* C677T variant.

Supplementary materials

Supplementary materials for this article are available online at https://doi.org/10.24953/turkjpediatr.2025.6049

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Ethical approval

The study was approved by Ethical Scientific Committee of the Faculty of Natural and Life

Sciences at the University of Constantine 1 (date: 28.11.2019, number: EC/UMC/SNV/02/11-2019).

Author contribution

The authors confirm contribution to the paper as follows: Study conception and design: LR and DRC; Data collection: RL, MR, RA, ST, YB and KS; analysis and interpretation of results: RL and DRC; draft manuscript preparation: RL. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Short Communication

Household transmission and carriage of Shiga toxin-producing *Escherichia coli* (STEC) O145, Stx1c: a family report

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ABSTRACT

Background. Infections induced by Shiga toxin-producing *Escherichia coli* (STEC), especially non-O157 serogroups like O145, pose considerable public health risks. Household transmission is crucial in the dissemination of STEC, particularly in settings characterized by close interaction, such as extended families. This study examines a case of a 5-month-old infant with hemolytic uremic syndrome (HUS) attributed to *stx1c*-positive STEC and analyzes transmission patterns within the household.

Methods. Perianal swab samples were obtained from a 5-month-old infant diagnosed with STEC-associated HUS and six additional household members. Samples of breast milk were examined as well. Samples were inoculated into sorbitol MacConkey agar (SMAC) and cefixime tellurite sorbitol MacConkey agar (CT-SMAC). Polymerase chain reaction (PCR) was utilized to identify *stx1*, *stx2*, and O serogroups. Fecal shedding was investigated over a four-month period with repeated sampling.

Results. Six household members, including the infant, tested positive for *stx1*, although the mother and breast milk samples were negative. The detected strains were classified within the O145 serogroup and exhibited the *stx1c* variation. Fecal shedding continued for up to four months in the majority of family members, with the infant exhibiting the briefest length of shedding. The family indicated regular intake of raw meatballs (*"çiğköfte"*), a traditional Turkish food, made with raw meat, identified as a possible source of illness. None of the family members displayed any symptoms except for the infant, who had severe HUS.

Conclusion. This study underscores the critical impact of household transmission on the dissemination of STEC and the hazards associated with traditional raw meat meals such as *çiğköfte*. Non-O157 STEC serogroups, including O145, are increasingly recognized as significant agents of human infections. The results underscore the significance of monitoring, hygiene education, and preventive strategies to mitigate the dissemination of STEC in families and the wider community. Mitigating extended fecal shedding and detecting foodborne transmission sources are essential for effective public health intervention.

Key words: hemolytic uremic syndrome, shiga toxin-producing *Escherichia coli*, STEC O145, stx1c.

The dissemination of Shiga toxin-producing *Escherichia coli* (STEC) within households presents a considerable public health threat owing to its potential to cause severe illness and its ability for fast transmission in enclosed environments. Studies indicate that home transmission rates may differ, with some showing rates ranging from 4% to 15% after isolated infections.[1] Young children are especially susceptible, serving both as carriers and as potential sources for increased community transmission.[1,2]

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Dynamics of transmission for STEC are influenced by the particular strain in question. For example, in the 2011 outbreak in Germany, the STEC O104:H4 strain demonstrated prolonged shedding in specific carriers, despite the rarity of secondary transmission within households.[3] This underscores the necessity of understanding the unique characteristics of the STEC strain involved in an outbreak to develop appropriate public health policies.

STEC can spread through various routes, with foodborne transmission recognized as the primary route. The consumption of undercooked or raw meat, especially beef, significantly contributes to the occurrence of STEC infections. Engaging directly with animals, especially ruminants like cattle, which act as key reservoirs, represents a significant route of transmission.[4] Transmission from one individual to another takes place within homes or communities, especially impacting children younger than five years, who are more susceptible to disseminating the infection.[1,4] Furthermore, STEC can endure in various environments such as soil, water, and agricultural runoff, resulting in indirect transmission by contact with contaminated surfaces or water sources.[5]

The transmission of STEC within households poses a complex challenge necessitating a comprehensive approach, which includes timely detection, case isolation, and focused hygiene education, especially in homes with young children.[6,7] Family clusters of STEC infections underscore the necessity of broadening epidemiological investigations to include all household members, given that the initial case may not consistently be the primary source of infection.[8] Comprehending these dynamics is essential for developing effective public health strategies to control and prevent STEC outbreaks, both within households as well as the wider community.

This study examines the significance of household transmission of STEC through the case of a 5-month-old infant, who was exclusively breastfed and diagnosed with STEC-related hemolytic uremic syndrome (HUS). Additionally, we investigated the extended family living with the infant for STEC carriage.

Materials and Methods

Case description

A previously healthy 5-month-old infant, was brought to the hospital by the family due to vomiting and diarrhea persisting for 4 days. The physical examination showed signs of dehydration. The peripheral blood smear revealed fragmented red blood cells (helmet cells). The patient, who presented with diarrhea, thrombocytopenia, anemia, and impaired kidney function tests, was admitted to the pediatric department with a diagnosis of HUS. The diagnostic criteria for HUS included hemolytic anemia with a hemoglobin (Hb) level of <10 g/dL, thrombocytopenia (platelets <150,000/μL) and acute renal injury (serum creatinine ≥1.5 times the upper limit of normal).[9] A Doppler ultrasound of the urinary system showed mild increased echogenicity in the renal parenchyme. A perianal swab sample was cultured, and polymerase chain reaction (PCR) testing for STEC was performed on the bacteria that grew in the culture. The patient tested positive for *stx1* and was followed up in the department. During the 10-day hospital stay, the patient experienced vomiting attacks 4-5 times a day, watery diarrhea 7-8 times, anemia, leukocytosis, thrombocytopenia, oliguria, macroalbuminuria, and signs of acute renal failure. During hospitalization, the patient did not require dialysis but needed erythrocyte suspensions. No antibiotics were administered to the patient, who had no history of antibiotic use before hospitalization. The family lives in Kocaeli city center, Türkiye, and has no history of animal husbandry. Since the infant was

exclusively breastfed and there was no food source that could transmit STEC, household transmission was suspected. Upon the family's approval to investigate STEC carriage, perianal swab samples were collected from individuals living in the same household.

Study plan

This study was conducted by the Department of Medical Microbiology, Faculty of Medicine, Kocaeli University. The study was conducted with the approval of the Clinical Research Ethics Committee and informed consent forms were obtained from each participant prior to enrollment. All procedures were conducted in accordance with the ethical standards of the Declaration of Helsinki.

Sample collection, culture and DNA isolation

Perianal swab samples were taken from the 5-month-old infant diagnosed with HUS and from six individuals living in the same household. The family was an extended family consisting of the index case (5m), mother (21y), father (25y), grandmother (47y), grandfather (45y), uncle (21y), and aunt (13y), living in the same house. Additionally, a sample of breast milk was collected. The perianal swab samples were transported in a Stuart transport medium, while the breast milk sample was delivered to the laboratory in a dry tube. Samples were taken from individuals one week after the initial detection and once a month for four months. Sorbitol MacConkey (SMAC) agar and cefixime sellurite sorbitol MacConkey (CT-SMAC) agar were used for inoculating samples. The plates were incubated at 37 °C in the incubator for 24 hours. If no growth was observed, the incubation time was extended to 48 hours. STEC suspicious colonies were selected and transferred on SMAC agar and incubated at 37°C in the incubator for 24 hours. The boiling extraction method was used for DNA isolation. The NanoDrop spectrophotometer was used for DNA quantification. After DNA isolation, *stx* positive isolates were identified by PCR method.

Determination of stx genes, stx variants and O serogroups

stx1 and *stx2* genes were investigated by the conventional PCR method.[10] O26, O45, O103, O104, O111, O121, O145 and O157 gene regions were investigated by the conventional PCR method according to the study by Paddock et al.[11] Stx1 variants (Stx1a, Stx1c and Stx1d) were investigated by conventional PCR. The primers of the Stx1 variants, the mixture prepared for the reaction, and the temperature cycles were referenced according to the study by Scheutz et al.[12]

Results

Perianal swab samples taken from the infant and six individuals living in the same household were screened for *stx1* and *stx2* using PCR. All family members excluding the mother tested positive for *stx1*. The breast milk sample taken at the time of admission was found to be negative for *stx*-PCR. Perianal swab samples were taken from the individuals on the 7th day, 1st month, 2nd month, 3rd month, and 4th month after hospital admission for carrier surveillance. The *stx* PCR results of family members over time are summarized in Table I. The strains were investigated for the O serogroup and toxin variants, and all strains were found to be positive for the O145 serogroup and the *stx1c* variant.

Multiple interviews were conducted with the family to investigate the source of the STEC infection. The 5-month-old patient had been exclusively breastfed and had not received any complementary foods. The mother reported not consuming meat, as she follows a vegetarian diet. Other family members, however, regularly consumed meat products. None of the family members had experienced any clinical symptoms suggestive of STEC infection in the two weeks prior to presentation. The family reported consuming a traditional Turkish dish made with raw meat (*"çiğköfte"*) eight days before presentation and noted that they

Table I. The *stx* PCR results of family members over time.

	Stx1 / Stx2					
Date	1st day	7th day	1st month	2nd month	3rd month	4th month
Index case	+ / -	+ / -	+ / -	-/ -	- / -	-/-
Mother	-/-	-/-	-/-	-/-	-/-	-/-
Breastmilk	-/-	-/-	-/-	-/-	-/-	-/-
Father	+ / -	+ / -	+ / -	+ / -	+ / -	-/-
Grandmother	+ / -	+ / -	+ / -	+ / -	+ / -	-/-
Grandfather	+ / -	+ / -	+ / -	+ / -	+ / -	-/-
Uncle	+ / -	+ / -	+ / -	+ / -	+ / -	-/-
Aunt	+ / -	+ / -	+ / -	+ / -	+ / -	-/-

Except for the mother's breastmilk, all of the other samples were collected by perianal swabs.

frequently consume *çiğköfte*. They suggested that this food might have been the source of the STEC infection.

Discussion

Household transmission of STEC is a major concern because of its rapid spread between individuals and the potential severity of the infection. In households, children are particularly vulnerable, both to acquiring and transmitting the infection.[7] Studies have indicated that siblings and mothers are at a higher risk of contracting the infection from an infected child, with transmission rates in households varying between 0% and 34.4%.[1,6] In this study, the infant diagnosed with HUS had an extended family and the family members were very young. The detection of STEC in all household members except the mother suggests that this case was a result of household transmission. Although the infant had no siblings, the aunt, who was living in the same house, was a 13-year-old child. The infant was exclusively breastfed. Although there is no data in the literature on STEC transmission through breast milk, samples were also taken from breast milk because breastmilk was the infant's only source of nutrition. The infant's close contacts were the mother and the child's aunt. Since the mother was negative for *stx* and transmission among children is more common, we suspected that the transmission occurred from the aunt. Although adults can acquire the infection, transmission rate is generally lower than that observed in children.[8] Transmission of STEC within families is significantly influenced by closeness and hygiene practices, with children playing an important part in disseminating the illness. Preventive measures, such as isolating sick individuals and promoting hygiene education, are essential for reducing transmission rates, especially among young children.[6,13] For example, promptly separating siblings after a diagnosis has been recommended as an effective approach to limit further transmission.[2]

In the study on the outbreak of O26:H11 STEC by Brown et al., the risk of infection in children <36 months was twice the risk among children of 36 to 47 months.[14] Although STEC was detected in all family members in the study, only the infant developed HUS. We suggest the reason for that is that younger children have a less developed immune response, making them more susceptible to infections and potential complications like HUS.[14-16] In a study by Alconcher et al.[7] including 82 HUS patients, 36.6% of HUS patients had 36 STEC-positive household contacts and nearly one third of them were children. There was a high concordance (83%) between the serotype and/or *stx*-genotype of HUS patients and their household contacts.[7] Similarly, *sxt1c* and O145 serogroups were detected in all family members included in this study.

The family stated that *çiğköfte* could be the source of STEC infection. *Çiğköfte* is a traditional Turkish street food often made with raw meat, posing potential health risks due to contamination with STEC. Research has shown that STEC, particularly *E. coli* O157, might be present in *çiğköfte.* The research in Türkiye found that *E. coli* O157 was detected in 20.8% of meat-based and 14.6% of vegetarian *çiğköfte* samples.[17] This highlights the possible risk of STEC infection in both meat and vegetarian versions of the food.

While STEC O157:H7 was previously considered the most common serotype in HUS patients, the recognition of non-O157 STEC isolates has been increasing in recent years. The STEC serogroups O26, O45, O103, O111, O121, O145 and O157 represent the "top seven" STEC serogroups that are common in humans.[18,19] For this reason, this study conducted a PCR analysis encompassing the seven predominant serogroups for O serogroup detection. In the study by Carbonari et al.[20], STEC O145 was the second most common serogroup associated with HUS, following O157 and it accounted for 20.3% of HUS cases in Argentina. STEC has several variants and epidemiologic studies suggest that Stx2 variants differ in potency and cause different clinical conditions.[21] Stx1c-producing STEC strains are often eae-negative and belong to various serotypes not typically associated with severe human disease, such as O157, O26, O103, O111, or O145.[22,23] Stx1c and O145 serogroups were detected in the family members included in the study. Stx1c and O145 STEC strains are important contributors to human infections, with distinct genetic and virulence profiles. While *stx1c* is often associated with milder symptoms, O145 can cause severe disease and is a concern in outbreak scenarios.[23,24] Understanding their prevalence, identification, and transmission patterns is essential for reducing public health risks associated with these diseases.

Studies have shown that fecal shedding can be both extended and sporadic. A study conducted among children in Argentina revealed that distinct STEC strains were shed for durations ranging from 19 to 37 days, highlighting the variability in shedding duration among different serotypes.[25] In the 2011 outbreak in Germany, the median period of pathogen shedding was seen to be 17-18 days, with certain patients shedding the virus for as long as 157 days.[26] Prolonged fecal shedding of STEC was noted in the family members participating in the study. The minimal shedding duration was noted in the infant with HUS. Fecal shedding of STEC may be reduced in patients with HUS relative to those without HUS, and therapy can affect the duration of shedding.[26,27] The extended shedding of STEC has considerable consequences for managing the dissemination of infection. Infected persons, particularly in high-risk settings such as childcare facilities, may require exclusion until they test negative for STEC to prevent further cases.[25,28]

The limitation of the study is that clonal relationships between isolates could not be examined. Investigating clonal relationships and increasing the study populations are among the future goals of the researchers.

Conclusion

STEC poses a significant public health challenge, particularly in environments where close contact among family members facilitates pathogen spread. This study highlights the transmission dynamics of STEC within a household, emphasizing the role of young children in the dissemination of the infection. The detection of the same STEC strain in multiple family members, except for the mother, strongly suggests intra-household transmission as the primary route of infection. The prolonged fecal shedding observed in family members further illustrates the potential for extended transmission within households, reinforcing the need for strict hygiene measures and early detection strategies.

Ethical approval

The study was approved by Kocaeli University Non-Interventional Clinical Research Ethics Committee (date: 08.05.2019, number: 2019/289).

Author contribution

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

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Conflict of interest

The authors declare that there is no conflict of interest.

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Case Report

Neonatal-onset citrin deficiency: long-term outcomes in four cases and identification of a novel variant

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ABSTRACT

Background. Citrin deficiency (CD), caused by mutations in the *SLC25A13* gene, is a rare autosomal recessive urea cycle disorder with variable clinical presentations depending on age. These include neonatal intrahepatic cholestasis (NICCD), failure to thrive with dyslipidemia, and adult-onset type II citrullinemia. Patients with NICCD typically present with transient intrahepatic cholestasis in infancy, which often resolves spontaneously by one year of age; however, some may progress to severe complications later in life.

Case presentation. Four cases diagnosed with NICCD phenotype are presented. All patients presented with neonatal cholestasis, hypertransaminasemia, galactosuria, and elevated citrulline levels. Molecular analysis identified three disease-causing variants: two previously reported variants, c.955C>T (p.Arg319*) and c.74C>A (p.Ala25Glu), and a novel variant, c.1359G>T (p.Lys453Asn). Treatment included a galactose-free formula, medium-chain triglycerides, and nutritional supplementation, resulting in biochemical and clinical improvement. All patients in our series exhibited a milder clinical course, with no episodes of hyperammonemia or hypoglycemia, no progression to liver failure, and favorable long-term outcomes with dietary management. During a long-term follow-up period ranging from 7 to 11 years, no severe complications were observed. Notably, one patient developed a recurrence of cataract, emphasizing the importance of lifelong dietary adherence and regular eye examinations.

Conclusions. The findings in this paper further expand the genotypic spectrum and genotype-phenotype correlations of CD. Lifelong follow-up is recommended, including ocular examination.

Key words: citrin deficiency, cholestasis, *SLC25A13*, citrullinemia type 2, urea cycle disorder.

Citrin, a calcium-binding aspartate/glutamate carrier, is a component of the malate–aspartate shuttle and is closely linked to several biochemical pathways, including glycolysis, gluconeogenesis, *de novo* lipogenesis, beta-oxidation, the tricarboxylic acid (TCA) cycle, and the urea cycle. Citrin deficiency (CD), an autosomal recessive trait, is a rare disorder of the urea cycle caused by mutations in the *SLC25A13* gene. It was first described in the Japanese population and is quite common among East Asians, but it is now considered a pan-ethnic disorder.[1] The clinical spectrum of CD includes three distinct age-related phenotypes: neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD, OMIM#605814), failure to thrive (FTT) and dyslipidemia by citrin deficiency (FTTDCD), and adult-

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onset type II citrullinemia (CTLN2, OMIM #603471).[2,3] NICCD patients primarily present with transient intrahepatic cholestasis during the infantile period; additional manifestations include hepatomegaly, citrullinemia, ketotic hypoglycemia, elevated levels of certain amino acids, hypoalbuminemia, cataracts and developmental delay.[1] In most NICCD patients, symptoms resolve spontaneously by 12 months of age. However, in rare cases, severe hepatic failure may occur, necessitating liver transplantation. Following a silent remission period until after adolescence, less than 20% of patients develop a fatal metabolic disease, CTLN2, which is distinguished by recurrent episodes of hyperammonemia, hepatosteatosis, and neuropsychiatric manifestations such as disorientation, delirium, cognitive impairment, and abrupt episodes of unconsciousness.[2,4] The onset of symptoms can be sudden and is typically observed between the ages of 20 and 40 years.[4]

In this study, we present clinical and laboratory findings and outcomes of four genetically confirmed cases of NICCD patients from non-Asian origin. Our report expands the mutation spectrum of the *SLC25A13* gene with the identification of a novel c.1359G>T (p.Lys453Asn) variant and highlights clinical observations such as cataract recurrence during follow-up. This study aimed to highlight the importance of disease awareness, clinical follow-up during early infancy and childhood, and the importance of ocular examinations in the follow-up of cases with CD.

Case Presentations

We present the clinical characteristics of a case series diagnosed with NICCD, including three siblings from the same family, comprising a set of dizygotic twins, and an additional patient from a different family. All the studies were conducted in accordance with the Declaration of Helsinki and guidelines for good clinical practice. All legal guardians of the patients were informed, and their informed consent was obtained.

Case A1

A 17-day-old male infant, whose parents were second cousins, was referred to our metabolic center for abnormal newborn screening test for phenylketonuria (PKU). In medical history, the patient, born at 38 weeks of gestation, had a birth weight of 2290 g (-2.6 SD), a length of 44 cm (-2.6 SD), and a head circumference of 31 cm (-2.8 SD).

The newborn screening performed on the second day was normal (0.1 mg/dL; cut-off: 2) but repeated phenylalanine (Phe) level on the tenth day of life was 5.5 mg/dL. At our center laboratory tests had revealed normal Phe and elevated citrulline (Cit) levels (372 μmol/L, normal range: 3-57) by tandem mass spectrometry (MS). Evaluation for elevated Cit included further investigations for distal urea cycle disorders and CD. At the initial admission, icterus with pale colored stools and mild hepatomegaly were detected. No dysmorphic appearance was noted. He subsequently developed progressive cholestasis and worsening jaundice by 47 days of age. Laboratory results revealed cholestasis, elevated international normalized ratio (INR), hypertransaminasemia, significantly elevated alpha-fetoprotein (AFP), positive urine test for reducing substances, and galactosuria (Table I). Stool examination detected +3 steatorrhea. Fundus examination was normal, but cataract formation was identified. Echocardiography was normal, and neuromotor development progressed age-appropriately. Quantitative plasma amino acid analysis was consistent with CD, as characteristic alterations in blood amino acid levels were observed. These included increased levels of Cit (376 μmol/L; reference range [RR]: 6–35), arginine (Arg, 191.2 μmol/L; RR: 18–102), methionine (Met, 124.8 μmol/L; RR: 9–44), and threonine (Thr, 685 μmol/L; RR: 33–160), as well as an elevated threonine-to-serine ratio (Thr/Ser, 4; RR: <1.1) (Table II). Genetic analyses

Table I. Summary of our patients (Patients 1-4) and other reported patients with NICCD from Türkiye.

Characteristic	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11
Age	11 y	11 y	7 y	10 y	3 y	18 mn	NA	NA	1.3 y	1.5 y	6 mn
Gender	M	M	F	M	M	M	M	M	F	M	F
Gestation week, birth weight	38 w, 2290 g	38 w, 2160 g	33+5 w, 1620 g	39 w, 3200 g	39 w, 3070 g	NA	NA	NA	3000 g, term	3200 g, term	NA
Age at symptom onset	47 d	47 d	25 d	3.5 mo	1 mo	3 mo	2.5 mo	5 mo	3 mo	4 mo	2 mo
Clinical presentation	Jaundice	Jaundice	Family history	Jaundice	Jaundice	Jaundice, failure to thrive	Jaundice	Jaundice	Jaundice, hepatomegaly	Jaundice, hepatomegaly	Jaundice, hepatomegaly
Biochemical findings*											
Total bilirubin, mg/dl	8.9	5.2	6.4	6.2	8.4	6.3	11.6	21.3	4.5	4.8	NA
Direct bilirubin, mg/dl	2.0	1.3	1.4	3.5	1.9	2.7	4.6	12.3	3.45	3.7	NA
AST, U/L	94	76	53	103	90	171	94	1425	125	145	139
ALT, U/L	28	40	15	30	NA	125	25	671	214	235	46
Hyperammonemia	-	-	-	-	+	+	+	+	-	-	-
INR	1.56	1.66	2.1	1.06	1.62	N	1.8	NA	NA	NA	1.09
Hypoglycemia	-	-	-	-	-	-	-	+	-	-	-
Albumin, g/dl	2.5	2.4	2.6	4.7	3.1	NA	2.7	1.9	NA	NA	NA
AFP, ng/ml	121000	60500	NA	3416	30341	41420	326000	450000	NA	NA	NA
Galactosuria	+	+	+	+	NA	+	+	+	-	-	-
SLC25A13 genotype	c.955C>T / c.955C>T	c.955C>T / c.955C>T	c.955C>T / c.955C>T	c.74C>A / c.1359G>T	c.851-854delGTAT/ c.869T>C	c.1354G>A / c.1354G>A	c.478delC / c.478delC	c.640C > T / c.640 C > T	c.691G>T / c.28_29del	c.691G>T / c.28_29del	c.1793T>G / c.1793T>G
Outcome	SF	SF	SF	SF	SF	SF	SF	Died	SF	SF	SF
Reference	Case A1	Case A2	Case A3	Case B1	(17)	(18)	(6)	(6)	(19)	(19)	(20)

* The values represent the measurements at the time of diagnosis. ALT, alanine aminotransferase; AST, aspartate aminotransferase; AFP, alpha-feto protein; d, days; F, female; g, gram; INR, international normalized ratio; N, normal; NA, not available; NICCD, neonatal intrahepatic cholestasis by citrin deficiency; M, male; mo, month; SF, symptom free; w, week; y, year.

Table II. Plasma quantitative amino acid levels of the patients at diagnosis (μmol/L).

Plasma level (Reference range)	Patient A1	Patient A2	Patient A3	Patient B1
Citrulline (6-35)	**376**	**243.1**	**350.1**	**61.5**
Threonine (33-160)	**685**	**563.4**	**630.5**	**183.9**
Methionine (9-44)	**124.8**	**87.4**	71.6	**84.3**
Arginine (18-102)	**191.2**	**102.4**	**258.3**	88.6
Tyrosine (14-114)	69.4	107.3	**219.9**	61
Lysine (56-200)	**281.9**	**201**	**314.4**	168.8
Glutamine (368-652)	**323**	**180**	**200.6**	**230**
Isoleucine (28-92)	37.7	30	49.8	35.1
Leucine (55-149)	71.5	55.1	84.2	59.7
Phenylalanine (26-120)	31.3	**22.6**	35.6	49.7
Tryptophan (23-70)	**8.4**	**10.5**	**21.8**	47
Valine (79-267)	100.4	82.83	130.5	131.6
Histidine (30-110)	**125.6**	101.2	97.5	70.6
Threonine/Serine (< 1.1)	**4**	**7.1**	**4.3**	**1.8**

Abnormal values are in **bold.**

revealed homozygosity for the pathogenic c.955C>T (p.Arg319*) variant at exon 10 of the *SLC25A13* gene. Both parents were confirmed to be heterozygous for the variant. The patient was prescribed a galactose-free formula with medium-chain triglycerides (MCT), and Arg and fat-soluble vitamin supplements were initiated. While the clinical features improved, the elevation in Cit persisted until the age of 25 months. After achieving clinical and biochemical improvement, the galactose-free and MCT diet was discontinued at two years of age, recommending avoidance of excessive galactose content in the diet. Although previously normal, an abdominal ultrasound displayed grade 1 hepatosteatosis at the age of six years. On the latest visit to the outpatient clinic, at the age of eleven, the patient weighed 33 kg (-0.6 SD) and measured 141 cm (-0.4 SD). Physical examination findings were normal, and all biochemical parameters, including liver enzymes and synthetic functions, Cit level, lipid profiles, AFP, and ammonia, were within the normal range. Grade 1 hepatic steatosis persisted according to ultrasonography (USG). Ophthalmologic examination was completely normal, with no evidence of cataracts or other ocular abnormalities.

Case A2

Case A2, the dizygotic twin brother of Case A1, was delivered at 38 weeks of gestation, weighing 2160 g (-3.0 SD), with a length of 45 cm (-2.1 SD) and a head circumference of 31 cm (-2.8 SD). At the age of 17 days, the infant was referred for abnormal newborn screening result for PKU. Subsequent tandem MS testing revealed normal Phe and elevated Cit levels (352 μmol/L, RR: 3-57). At the age of 47 days, the patient presented with jaundice. Physical examination revealed minimal hepatomegaly. Laboratory work-up revealed cholestasis, elevated INR, and hypertransaminasemia, elevated AFP, positive urine test for reducing substances (Table I). Screening for classical galactosemia with the Beutler test was unremarkable. Quantitative plasma amino acids were consistent with CD (Table II). Ophthalmologic examination revealed a posterior subcapsular cataract. Abdominal USG was normal. The patient was prescribed a galactose-free formula with MCT, and supplemented with Arg and fat-soluble vitamin supplements as his twin brother. Sequence analysis of the *SCL25A13* gene revealed the same novel homozygous variant c.955C>T (p.Arg319*).

After 25 months, all biochemical tests were normalized, the cataract was resolved, and the galactose-free MCT-containing diet was discontinued, which was replaced by a low-galactose diet. Throughout the patient's follow-up visits, reducing substances in urine were consistently negative. Nevertheless, at the age of six, a mild cataract development was detected during the eye examination. Abdominal USG was normal. The patient was advised to resume the galactose-free dietary treatment. His current diet includes a protein: fat: carbohydrate ratio of 16%: 48%: 36%, along with the appropriate energy intake, in addition to the galactose-free diet with MCT oil. At the age of eight and a half years, the Wechsler Intelligence Scale for Children – Revised (WISC-R) disclosed an intelligence quotient (IQ) of 104 (verbal IQ, 95; performance IQ, 114). He was 11 years old at the last outpatient evaluation and his weight was 33 kg (-0.7 SD), and his height measured 143 cm (-0.1 SD). Physical examination findings were normal, and all biochemical parameters, including liver enzymes (aspartate and alanine aminotransferases) and synthetic functions (albumin level and coagulation parameters such as INR), Cit level, lipid profiles, AFP, and ammonia, were within the normal range. In the abdominal USG, no hepatic steatosis was detected. The cataract had not disappeared or progressed, but remained stable with dietary intervention of galactose restriction.

Case A3

This patient, the younger sister of patients A1 and A2, was delivered at 33+5 weeks of gestation due to preterm labor, with a birth weight of 1620 g (-0.5 SD), a length of 45 cm (0.8 SD), and a head circumference of 25 cm (-3.3 SD). Due to prematurity and respiratory distress, the patient was admitted to the neonatal intensive care unit. At 25 days of age, the patient remained clinically stable in terms of respiratory status; however, cholestasis was noted, and the urine test was positive for reducing substances. On physical examination, mild jaundice of the skin and bulbar conjunctiva were detected whereas no hepatosplenomegaly was noticed (Table I). The infant remained hospitalized for 37 days, with the highest recorded ammonia level reaching 96 μg/dL (normal range <110 μg/dL). The patient was prescribed a galactose-free formula and cholestasis resolved. The *SLC25A13* variant was confirmed and identified as the same novel homozygous variant, c.955C>T (p.Arg319*), as in her brothers. After achieving clinical and biochemical improvement at the age of three years, the patient's diet was eased which excluded milk and dairy products. Denver Developmental Screening Test II at age five years was age-appropriate. The patient is following a diet with a protein: fat: carbohydrate ratio of 16%: 43%: 41%, with an energy intake appropriate for her age. On the last admission to the outpatient clinic, at the age of seven years, the patient weighed 23 kg (-0.1 SD) and measured 116 cm (-1.1 SD) in length. Physical examination findings, abdominal USG, eye examination, and blood tests including liver function tests were normal.

Case B1

The patient was the first child of healthy non-consanguineous Turkish parents. The pregnancy was unremarkable. He was delivered at term via normal spontaneous delivery without asphyxia, with a birth weight of 3200 g (-0.3 SD), a length of 48 cm (-1.1 SD), and a head circumference of 33 cm (-1.5 SD). At the age of three and a half months, jaundice was observed. He was found to have cholestasis and a reducing substance was detected in his urine and was referred to our department at the age of five months. On physical examination, he weighed 6860 g (-0.68 SD) and measured 64 cm (-0.64 SD) in length. His liver was palpable 4 cm below the costal margin with no signs of splenomegaly. Laboratory evaluation showed cholestasis and mildly elevated liver transaminases (Table I). He also displayed increased AFP (3416 ng/mL; normal: <13.6). Acylcarnitines by tandem MS exhibited a free carnitine (C0) level of 56.5 μmol/L (RR: 8.6-90), tetradecanoyl carnitine (C14) of 0.99 μmol/L (RR: 0-0.8), linolenoyl carnitine (C18:2) of 1.25

μmol/L (RR: 0-0.9), and oleyl carnitine (C18:1) of 3.43 μmol/L (RR: 0-2.8). Bile acid analysis from a dried blood spot sample revealed elevated bile acids as indicative of cholestasis or liver disease (taurochenodeoxycholic acid [TCDC] 23.1 μmol/L [RR: <4 μmol/L], glycochenodeoxycholic acid [GCDC] 25.6 μmol/L [RR: <4 μmol/L], taurocholic acid [TC] 11.2 μmol/L [RR: <4 μmol/L] and glycocholic acid [GCA] 6.5 μmol/L [RR: <4 μmol/L]). Evaluation of urinary succinylacetone to screen for tyrosinemia type I was unremarkable. Plasma amino acid analysis displayed elevations in the concentrations of Cit, Thr, Met, tyrosine (Tyr), and Arg (Table II). Abdominal USG indicated normal liver echogenicity and a simple cyst was observed in the right kidney cortex. The ophthalmologic examination was normal. Genetic analyses revealed heterozygosity for a previously described variant c.74C>A (p.Ala25Glu) on exon 3 and a novel c.1359G>T (p.Lys453Asn) on exon 14 on the *SLC25A13* gene.[5] Parental Sanger sequencing confirmed that the c.74C>A (p.Ala25Glu) variant was maternally inherited, whereas the c.1359G>T (p.Lys453Asn) variant was paternally inherited, supporting a compound heterozygous state in the patient. MCT oil was administered with a galactose-free formula at the age of five months. His clinical condition and pathologic laboratory findings improved and diet was discontinued at 18 months of age when all abnormal laboratory findings had normalized. The biochemical parameters, including liver enzymes and synthetic functions, lipid profiles, carnitine and Cit levels, and ammonia remained within normal range, and he attained normal neurocognitive development at follow-up. Fig. 1 illustrates the plasma Cit values of all four patients during the follow-up period. At the latest outpatient visit at the age of 10 years, he weighed 40 kg (1.1 SD) and was 146 cm (1.42 SD) tall. Physical examination findings, abdominal USG, and blood tests including liver function tests were normal. The eye examination was completely normal.

Discussion

Here we describe four patients from two unrelated families from Türkiye; all of them had the NICCD phenotype and biallelic variants in the *SLC25A13* gene. The patients were followed for periods of 11, 11, 10, and 7 years at our center. Due to the rarity of CD deficiency in non-Asian countries, the understanding of its clinical spectrum is still evolving, encompassing both infantile and late-onset forms.

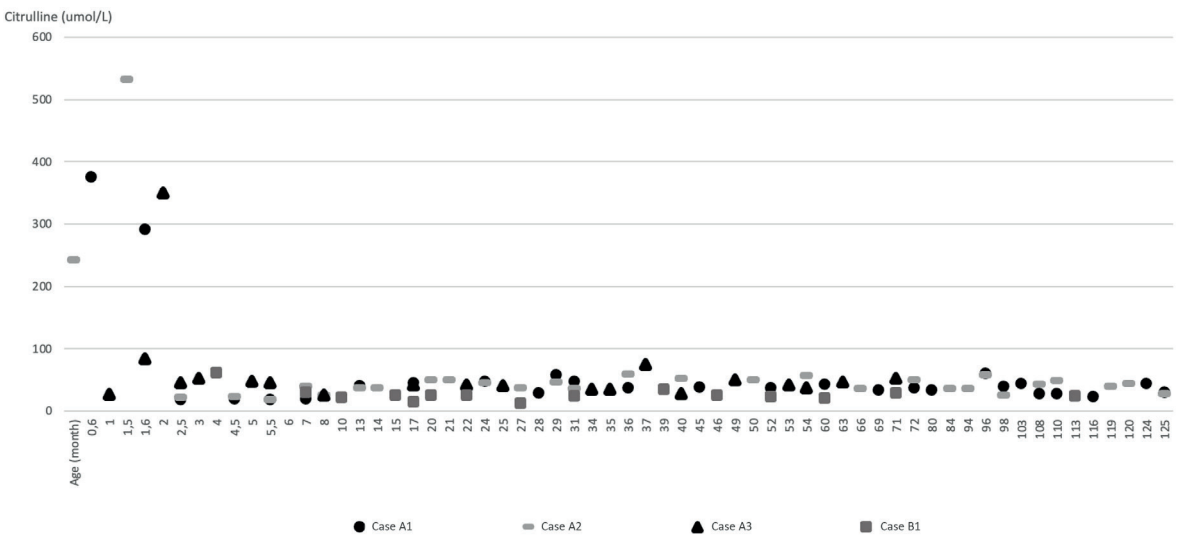

Fig. 1. Plasma citrulline values of the patients during follow up.

All our patients presented with neonatal cholestasis, hypertransaminasemia, galactosuria and elevated Cit levels. However, there were notable variations in the severity of hyperbilirubinemia, with total bilirubin levels ranging from 5.2 mg/dL to 8.9 mg/dL in our cases, whereas previously published cases showed a broader range, with some cases exceeding 21 mg/dL.[6] Direct bilirubin levels in our cohort were relatively mild (1.3–3.5 mg/dL), while published cases exhibited more severe cholestasis, reaching up to 12.3 mg/dL.[6] AFP levels also displayed significant variability. While our cases exhibited moderately elevated AFP (3,416–121,000 ng/mL), other reported cases had extreme elevations, with some exceeding 450,000 ng/mL.[6] Hyperammonemia and hypoglycemia were not observed in our cases. Despite the biochemical abnormalities, all patients showed favorable long-term outcomes following dietary intervention.

The patients with NICCD typically exhibit a mild increase in galactose and galactose-1-phosphate levels without a pronounced elevation in galactose-1-phosphate and UDP-galactose, as observed in epimerase deficiency.[4] It is assumed that the inhibition of UDP-galactose epimerase by NADH, a competitor of NAD^+ bound to the enzyme, may be the cause of secondary galactosemia in NICCD. Markers of galactosemia such as galactose, galactitol, and galactonate are frequently detected in urine samples of NICCD cases. The accumulation of galactitol may be implicated in the pathogenesis of clinical manifestations including jaundice, hepatosplenomegaly, hepatocellular insufficiency, and cataracts.[7] All of our patients had elevated galactose levels at the time of diagnosis. We determined urinary-reducing substances while monitoring our patients instead of galactose levels as it was more easily accessible. With a galactose-restricted diet, urinary galactose excretion normalized. Despite negative results in patients during follow-up, one patient developed cataracts. Tazawa et al.[8] reported that hypergalactosemia was detected in 6 out of 9 patients with NICDD, and 4 of these patients had also developed cataracts. Dimmock et al.[5] reported a case who presented with an elevated Phe and total galactose the upper limit of normal on newborn screening with positive reducing substances in the urine. They reported detecting arginosuccinate synthetase (ASS) enzyme deficiency on skin fibroblast culture in NICDD patients.

In the reported cases from our country, patients presented with symptoms consistent with the literature, such as jaundice, hepatomegaly, and FTT (Table I). Clinically, our cases did not develop hepatic failure, and all had favorable long-term outcomes with dietary management. In contrast, some published cases demonstrated progressive hepatic dysfunction, with one requiring liver transplantation.[2] Except for one patient who passed away shortly after the diagnosis, the other patients have remained asymptomatic. The deceased patient exhibited direct hyperbilirubinemia, a 20-fold elevation in liver transaminases, prolonged coagulation, and hypoglycemia. A reductant agent was detected in the urine, and galactose levels were found to be elevated. Despite the administration of MCT supplementation and a galactose-free formula, the patient succumbed to a sepsis attack during the observation phase.[6]

In CD, diet therapy is the only treatment option, if patients do not adhere to the lifelong diet, it may lead to a series of complications, such as cataracts, FTT, dyslipidemia, liver failure, and hyperammonemic encephalopathy.[4] Patient 2 was advised to discontinue the dietary treatment, and the development of cataracts was observed during follow-up. While this finding does not necessarily predict the occurrence of other clinical manifestations, it may serve as an early indicator of disease progression and warrant closer monitoring of metabolic and hepatic parameters. The mechanisms leading to this transition have yet to be defined as these findings attributed to carbohydrate toxicity serve as predictive markers that can help to anticipate the initiation of CTLN2. In contrast, a high-protein/high-fat/low-carbohydrate diet prevents the onset of CTLN2. Some

patients exhibit specific clinical features such as growth retardation, fatigue, weight loss, fatty liver, hyperlipidemia, hyperammonemia, and citrullinemia shortly before the onset of CTLN2. It is crucial to monitor patients for these pre-CTLN2 symptoms.[4] External factors such as sugar intake, alcohol consumption, and specific medications like acetaminophen, as well as high-sugar solutions and/or glycerol, and surgical procedures, can aggravate these manifestations.[9] Care should be provided especially to NICDD patients for the onset of CTLN2 with weight loss, height stagnation, easy fatigability, and high blood levels of ammonia, Cit, Thr/Ser ratio, and pancreatic secretory trypsin inhibitor (PSTI).[4] Additionally, we recommend evaluating patients for galactose levels and cataracts.

To our knowledge, a total of four Turkish patients with CTLN2 have been reported in the literature to date. Köse et al.[6] first reported three siblings with CTLN2 carrying the homozygous c.1478 G>A (p.Asp493Gly) variant in the *SLC25A13* gene. The proband, a 15-year-old male, presented with intellectual disability, generalized tonic-clonic convulsions, and autism. He experienced delirium, and laboratory data showed hyperammonemia and elevated Cit levels. Ateş et al.[10] reported a 30-year-old male patient with CTLN2 who developed hyperammonemic encephalopathy induced by sodium valproate. His blood ammonia level was normal at admission, but it increased to 339 μmol/l (RR: 9-97 μmol/l) after the initiation of multiple antiepileptic drugs due to seizures. Plasma amino acid analysis revealed moderately elevated levels of Cit (142.2 μmol/L [normal range: 17–46 μmol/L]) and Thr (312 μmol/L [normal range: 60–200 μmol/L]), with an increased Thr/Ser ratio. Molecular analysis revealed a homozygous c.848 G>T (p.Gly283Val) variant in the *SLC25A13* gene. There have been no reported cases with the FTTDCD phenotype to date in our population.

The nonsense c.955C>T (p.Arg319*) variant in the *SLC25A13* gene was first identified in Chinese NICCD patients and subsequently reported in a Japanese NICCD patient. This variant, located in exon 10, introduces a premature stop codon, predicted to lead to the early termination of the citrin protein.[11,12] This variant occurs between the linker loop domain and the carrier domain, truncating the protein before it can form the full carrier domain required for mitochondrial transport. As this domain is essential for aspartate-glutamate transport, the mutation likely leads to a complete loss of citrin function (https://www.uniprot.org/). According to the American College of Medical Genetics and Genomics (ACMG) criteria, this variant is classified as pathogenic.[13] The c.74C>A (p.Ala25Glu) variant in the *SLC25A13* gene was first identified in an NICCD patient of Arabic descent. This variant, located in the N-terminal domain, predicted not to affect the transport activity of citrin, but it is expected to impair calcium regulation.[5] Based on available data, this variant is classified as likely pathogenic according to ACMG criteria.[13] There is also a Caucasian case presenting with the NICCD phenotype, who was compound heterozygous for a missense variant, c.74C>A (p.Ala25Glu), which produces an Ala-to-Glu substitution at position 25, and a nonsense variant, c.1081C>T (p.Arg361*).[14] These publications have shown that the same variants are observed in different ethnic groups.

The c.1359G>T (p.Lys453Asn) variant has not been previously reported in the literature and was identified as very rare (0.0008%) in the gnomAD population. According to the ACMG criteria, this variant has received scores for PM2 and PP3 and was determined as a variant of uncertain significance (VUS). The functional effect was predicted to be disease-causing by MutationTaster with a score of 1, deleterious by Sorting Intolerant From Tolerant (SIFT) with a score of 0.00 (<0.05 is predicted to be deleterious), and harmful by PolyPhen-2 HumVar with a score of 1 (1.0 is predicted to be deleterious).[13]

A recent report identified a novel c.1610_1612delinsAT (p.Leu537TyrfsTer2) variant in an Indian patient, presenting

with recurrent encephalopathy and hyperammonemia, underscoring this mutation's potential to induce severe neuropsychiatric manifestations. The study also analyzed 79 citrin deficiency cases from 24 studies to establish genotype-phenotype correlations. The c.851_854del4 (p.Met285Profs*2) mutation demonstrated positive associations with alpha-fetoprotein, ammonia, and Tyr levels, while showing an inverse correlation with Thr. The IVS16ins3kb mutation was linked to elevated total and conjugated bilirubin, along with increased aspartate transaminase, whereas Cit levels were lower in these patients. Additionally, c.674C>A (p.Ser225*) and c.1645C>T (p.Gln549*) mutations were associated with a milder hepatic phenotype, suggesting a potential role of these mutations in modulating the severity of liver impairment in CD.[15]

A review of 138 Chinese NICCD patients revealed that homozygous mutations of 1638_1660dup, IVS6+5G>A, and IVS16ins3kb were exclusively observed in the acute liver failure (ALF) and liver dysfunction groups, suggesting a strong association with severe liver impairment. The high-frequency c.851_854delGTAT (p.Met285Profs*2) mutation was detected across acute liver failure, liver dysfunction, and non-liver dysfunction groups, indicating that this mutation may contribute to varying degrees of hepatic injury.[16]

The initial biochemical findings of NICCD may overlap with PKU or galactosemia, making early diagnosis challenging. In NICCD, an elevation in phenylalanine levels can be observed due to liver damage. However, unlike PKU, not only phenylalanine but also other amino acids that increase in liver dysfunction are elevated. Additionally, similar to galactosemia, galactosuria and the presence of reducing sugars in urine may suggest an impairment in galactose metabolism. However, classical galactosemia testing does not reveal a significant enzyme deficiency. Therefore, expanded metabolic screening and genetic analysis are crucial for distinguishing NICCD from other metabolic disorders.

According to our latest information, very few patients have been reported from Türkiye so far.[6,17-20] The diagnosis of some patients may have been missed. Newborn screening for CD based on Cit level measurement by tandem MS has low sensitivity. Therefore, Kido et al. proposed that a new scoring system based on tandem MS, including levels of Arg, Cit, isoleucine+leucine, Tyr, and the C0 to glutarylcarnitine (C5-DC) ratio, was more sensitive.[3] Implementing this enhanced screening method in our country could facilitate the diagnosis of new cases through newborn screening programs.

This study reported the diagnosis and the clinical course in four new genetically confirmed cases of NICCD patients of non-Asian origin. Information on the variant spectrum of *SLC25A13* in the Turkish population is limited. The findings in this paper further expanded the genotypic spectrum and genotype-phenotype correlations of CD. Although cases are presenting with cataracts in cases of CD, a case of cataract recurrence during follow-up has not been reported.[7,8] Therefore, it is important to follow patients throughout childhood, adolescence, and adulthood, including eye examination.

Ethical approval

Informed consent was obtained from all individuals included in this study, or their parents / legal guardians.

Author contribution

The authors confirm contribution to the paper as follows: Study conception and design: AS, GG; data collection: AS, ŞK, ADA; analysis and interpretation of results: AS, MK, MCB, ADA, ZOU, GG; draft manuscript preparation: AS, ADA, GG, ZOU. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Case Report

A pediatric case of cat scratch disease, complicated by meningitis, diagnosed by metagenomic next-generation sequencing

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ABSTRACT

Background. Cat scratch disease (CSD) presents with diverse symptoms; however, meningitis as a complication is rare, and effective treatment strategies remain underexplored.

Case Presentation. An 11-year-old girl presented with a prolonged fever of unknown origin, mild cough, and headache. Metagenomic next-generation sequencing (mNGS) identified *Bartonella henselae* in the bloodstream, and cerebrospinal fluid analysis confirmed meningitis. The patient was diagnosed with CSD complicated by meningitis and demonstrated a successful recovery following treatment with doxycycline, rifampicin, and prednisone.

Conclusions. In CSD patients presenting with headaches and persistent fever, the possibility of meningitis should be considered. mNGS is a valuable diagnostic tool for CSD, especially in cases of fever of unknown origin. The combination of doxycycline, rifampicin, and prednisone proved effective in managing CSD with meningitis.

Key words: cat scratch disease, *Bartonella henselae*, meningitis, pediatric, metagenomic next-generation sequencing.

Cat scratch disease (CSD), a zoonotic bacterial infection mainly caused by *Bartonella henselae (B. henselae)*, exhibits a global prevalence. CSD is characterised by fever, erythematous papules at the site of scratches or bites, and regional lymphadenopathy.[1] Atypical manifestations may involve various organs, including the eyes, nervous system, heart, liver, spleen, musculoskeletal system or present as prolonged fever of unknown origin.[2,3] The primary neurological manifestations of CSD include neuroretinitis, encephalopathy, spinal radiculitis, and cerebellar ataxia.[4,5] Nonetheless, meningitis remains a rare occurrence in CSD patients.[5]

Metagenomic next-generation sequencing (mNGS) has emerged as a valuable tool in clinical infectious disease diagnosis, enabling rapid and accurate identification of multiple pathogens from diverse sources.[6] Here, we report a pediatric case of CSD complicated by meningitis diagnosed using mNGS. This case highlights the importance of considering meningitis in CSD patients and underscores the role of mNGS in diagnosing CSD, particularly in cases of fever of unknown origin.

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Case presentation

An 11-year-old girl developed a fever 15 days before admission to our hospital. The patient's personal and family histories were unremarkable, and he had no history of prior blood transfusions.

Initially, the patient experienced a low-grade fever, which gradually escalated to a high fever of 40°C, occurring four times daily, accompanied by a slight cough and headache. The physical examination revealed no abnormalities at a local hospital. A routine blood test revealed elevated white blood cell counts (WBC: 11.53 × 10^9/L, normal range: 4–10 × 10^9/L) and C-reactive protein levels (CRP: 18.4 mg/L, normal range: < 0.5 mg/L). The chest computed tomography scan revealed small patchy opacities in the right upper lobe and bilateral lower lobes, most suggestive of chronic inflammation, along with a ground-glass opacity nodule in the posterior segment of the right lower lobe. After treatment with oral cough medication, a four-day course of oral cefdinir, and subsequent intravenous piperacillin-tazobactam (112.5 mg/kg every 8 hours) for four days, the patient's cough improved. However, due to persistent fever, she was referred to our hospital for further evaluation.

Upon admission, physical examination revealed no positive signs. The patient was alert, with no meningeal signs or pathological reflexes. Blood tests showed a normal white blood cell count (10.5 × 10^9/L) but elevated CRP levels (36.4 mg/L). The erythrocyte sedimentation rate was significantly increased at 68 mm/hour (normal range: <26 mm/hour). Comprehensive laboratory investigations, including renal and hepatic function tests, ferritin levels, serological testing for Epstein-Barr virus and cytomegalovirus, assessment of cellular and humoral immunity, rheumatoid factor, antinuclear antibodies, anti-mitochondrial antibodies, and bone marrow aspiration with culture, all yielded normal results. Cardiac evaluation via echocardiography and immunological screening with purified protein derivative and interferon-gamma release assay were unremarkable. The respiratory multiplex nucleic acid test was negative, including influenza virus, parainfluenza virus, adenovirus, rhinovirus, coronavirus. Imaging studies, including chest computed tomography and cranial magnetic resonance imaging, revealed no structural or pathological abnormalities.

Diagnosis and treatment

The patient was initially diagnosed with fever of unknown origin and acute bronchitis. Despite receiving six days of intravenous cefoperazone sodium and sulbactam sodium (50 mg/kg every 8 hours), along with oral azithromycin (10 mg/kg once daily for the first day, followed by 5 mg/kg once daily for the next 4 days), the fever persisted. Additionally, her headaches worsened, particularly during febrile episodes, with no abnormalities on neurological examination. Subsequent cerebrospinal fluid (CSF) analysis revealed an elevated nucleated cell count (50×10^6/L; reference range <15×10^6/L) with lymphocytic predominance (78%), while biochemical parameters were within normal limits. However, broad-spectrum quantitative PCR assays targeting 23 bacterial and viral pathogens (including herpes simplex virus types 1 and 2, but excluding *B. henselae*) returned negative results.

Suspecting meningitis associated with viral or atypical bacterial infection, the therapy was adjusted to intravenous acyclovir (10 mg/kg every 8 hours), ceftriaxone (50 mg/kg every 12 hours), and vancomycin (15 mg/kg every 6 hours), considering the possibility of methicillin-resistant *Staphylococcus aureus* infection. However, the fever persisted. Consequently, a blood sample was collected for pathogen detection via mNGS, using an Illumina NextSeq 550 sequencer with a single-end 75-base-pair sequencing strategy. A total of 36,744 reads were ultimately analyzed, revealing four specific sequences corresponding to *Bartonella henselae*. The patient also reported a history of

a cat scratch on her left wrist two months prior, which had not been treated.

CSD complicated by meningitis was considered. The treatment regimen was modified to oral doxycycline (2.2 mg/kg every 12 hours) and rifampin (10 mg/kg every 12 hours). By the second day, the patient's temperature had normalized (36.5–36.8 °C), and the headache had alleviated. Prednisone (1 mg/kg) was initiated three days later. Blood tests, CRP levels, and biochemical markers returned to normal ranges after one week of treatment. The patient received six weeks of rifampin and doxycycline therapy in total. Concurrently, the prednisone dosage was gradually tapered over one month. During follow-up interviews conducted by phone at one and three months post-discharge, the patient reported no discomfort or adverse symptoms.

Written informed consent was obtained from the parents for this publication.

Discussion

CSD is the primary clinical manifestation of *B. henselae* infection, most commonly transmitted through percutaneous inoculation via scratches, bites, or contact with flea-infested cats. While CSD symptoms vary, meningitis as a complication is exceedingly rare.[7] In this case, the child presented with a prolonged fever of unknown origin and headaches. CSF analysis confirmed meningitis. Subsequent detection of *B. henselae* in the blood and targeted antibiotic therapy led to resolution of the fever and headaches, clinically supporting the association between meningitis and *B. henselae* infection. However, the absence of CSF mNGS testing precluded differentiation between bacterial and aseptic meningitis.

CSD is typically diagnosed clinically and confirmed serologically, with cat contact or scratches serving as important diagnostic clues. Immunofluorescence antibody tests and enzyme immunoassays are commonly used for detecting *B. henselae* antibodies, although their specificity and sensitivity vary.[8] *B. henselae* culture is challenging due to its facultative and fastidious growth characteristics. PCR assays, while highly specific, exhibit variable clinical sensitivity and are costly, limiting their availability to specialized laboratories.[9] mNGS has been applied in diagnosing CSD.[10] As a rapid and unbiased diagnostic methodology, mNGS enables researchers to explore pathogen-related inquiries without inherent biases. It facilitates earlier diagnosis and initiation of targeted antibiotic therapy by simultaneously and promptly detecting multiple pathogens, particularly beneficial for identifying rare, atypical, and complex infectious diseases.[11] However, mNGS is relatively expensive and less effective for determining drug resistance, with a high risk of microbial contamination during testing. Additionally, interpreting mNGS results also poses challenges.[12]

Therapies of CSD are tailored according to the severity of clinical symptoms. Typical or milder cases of CSD are often self-limited and resolve spontaneously within 2–4 months.[3] However, severe forms of CSD necessitated therapeutic intervention.[13] Therapies for complicated CSD mainly consist of azithromycin, rifampin, ciprofloxacin, trimethoprim/sulfamethoxazole, and/or gentamicin, either as monotherapy or in combination. However, guidelines for paediatric patients or no standardized treatment protocols for CSD complicated by meningitis are limited. However, a regimen of doxycycline and rifampin was suggested for children older than eight years, administered for a duration of 4–6 weeks.[14] Additionally, oral prednisone for six weeks was suspected to be beneficial in cases involving neurological manifestations or severe complications.[14-16] Despite receiving five days of azithromycin in the initial phase, the patient in this case exhibited no improvements, which may be related to the severity of CSD characterized by prolonged fever and meningitis. However, the patient responded well to a regimen of doxycycline, rifampin, and

oral prednisone, confirming the diagnosis and effectiveness of the treatment strategy.

Conclusions

In patients with CSD presenting with headaches and persistant fever, consideration of meningitis is crucial. mNGS proves beneficial for diagnosing CSD, particularly in cases of fever of unknown origin, facilitating accurate diagnosis and prompt initiation of treatment.

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Ethical approval

Publication of this case report followed the regulations of Hospital and was conducted according to the latest version of the Helsinki Declaration. Written informed consent for publication was obtained from the patient's guardian. All identifiable patient information was omitted during the manuscript's development.

Author contribution

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

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The authors declare that there is no conflict of interest.

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Case Report

Successful viral suppression in a two-year-old child with human immunodeficiency virus infection treated with bictegravir/emtricitabine/tenofovir alafenamide

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ABSTRACT

Background. Adherence to antiretroviral therapy (ART) is a major challenge in pediatric human immunodeficiency virus (HIV) management, especially in young children due to medication formulation, administration difficulties, and psychosocial barriers. Single-tablet regimens (STRs) have been shown to improve adherence and viral suppression in adults and adolescents, yet their use in younger children remains limited. Bictegravir/emtricitabine/tenofovir alafenamide (BIC/FTC/TAF) is an STR with a high genetic barrier to resistance, making it a promising option for pediatric patients with adherence difficulties.

Case Presentation. We report a case of a 2-year-old girl with perinatally acquired HIV who experienced persistent viremia despite multiple ART regimens. The mother received zidovudine prophylaxis during delivery, and the infant was started on zidovudine (AZT) prophylaxis on the first day of life. The patient's ART history included AZT monotherapy at birth, followed by combination therapy with lamivudine (3TC), lopinavir/ritonavir (LPV/r), and later tenofovir/emtricitabine (TDF/FTC) with dolutegravir (DTG). Despite these regimens, poor adherence related to medication administration difficulties and caregiver challenges contributed to persistent viremia. A multidisciplinary team approach was implemented to address adherence barriers. Given the patient's ongoing virological failure and resistance mutations (L76V and V179E), off-label use of BIC/FTC/TAF (50mg/200mg/25mg) was approved. The dosage was adjusted based on weight, and medication administration was closely monitored. Within one month of treatment, HIV RNA levels significantly declined from 1,800,000 to 207 copies/mL. Viral suppression was maintained over subsequent three-month intervals, with HIV RNA levels of 35, 40, and 43 copies/mL, alongside immune recovery as indicated by increased CD4 counts.

Conclusion. The successful off-label use of BIC/FTC/TAF in a treatment-refractory pediatric HIV case highlights its potential efficacy in young patients facing adherence challenges. Its high genetic barrier to resistance and favorable tolerability make it a promising option when standard therapies fail. Further research is needed to optimize pediatric ART strategies and expand access to STRs globally.

Key words: pediatric HIV, antiretroviral therapy, adherence, single-tablet regimen, bictegravir/emtricitabine/tenofovir alafenamide, viral suppression.

Adherence to antiretroviral therapy (ART) is crucial for achieving and maintaining viral suppression in individuals living with human immunodeficiency virus (HIV). In pediatric populations, ensuring consistent adherence presents unique challenges due to factors

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such as medication formulation, palatability, dosing frequency, side effects, and the child's developmental stage. Psychosocial and behavioral barriers, including family dynamics and caregiver involvement, further complicate adherence efforts. Studies have shown that adherence rates among children and adolescents can vary widely, with nonadherence rates ranging from 15% to 93%.[1]

The complexity of ART regimens, especially those requiring multiple tablets or doses per day, has been associated with lower adherence rates. Single-tablet regimens (STRs) have emerged as a strategy to simplify treatment, reduce pill burden, and improve adherence. In adult and adolescent populations, STRs have been linked to higher adherence rates, decreased hospitalizations, and a greater likelihood of achieving undetectable viral loads.[2] However, the availability and approval of STRs for younger children have lagged, limiting their use in this vulnerable population.

Bictegravir/emtricitabine/tenofovir alafenamide (BIC/FTC/TAF) is an STR that has demonstrated efficacy and safety in adults and adolescents. Recent studies have extended these findings to pediatric populations. For instance, a study involving children aged 2 years and older, weighing between 14 kg and 25 kg, showed that a low-dose co-formulated BIC/FTC/TAF regimen was well-tolerated and effective in maintaining viral suppression.[3] The high genetic barrier to resistance offered by BIC/FTC/TAF makes it particularly advantageous in pediatric patients, who are at a higher risk for poor treatment adherence and subsequent virological failure.[4]

Despite these advancements, challenges remain in implementing STRs like BIC/FTC/TAF in younger children, particularly in regions where such formulations are not yet approved or available. This case report discusses the successful use of BIC/FTC/TAF in a 2-year-old child with HIV, highlighting the considerations and outcomes associated with off-label use to achieve viral suppression.

Case Presentation

A 2-year-old girl was referred to a tertiary healthcare center due to persistently high plasma viral load despite multiple ART regimens. She was born via cesarean section at 39 weeks, with a birth weight of 2960 grams. The mother's HIV infection status was unknown until routine prenatal screening shortly before delivery, at which time she was found to be HIV-seropositive and had not received ART during pregnancy. The mother received zidovudine prophylaxis during delivery; however, the maternal HIV RNA viral load was positive after delivery, but the exact level at delivery was not available. Due to the limited availability of antiretroviral drugs at the initial healthcare center, the infant was started on zidovudine (AZT) prophylaxis on the first day of life until referral to a tertiary center within 48–72 hours postpartum. Upon admission to the tertiary center, triple ART was promptly initiated given the high-risk status of the mother-infant pair.

During the first month, triple ART consisting of AZT, lamivudine (3TC), and lopinavir/ritonavir (LPV/r) was initiated for the infant; however, the family reported intermittent difficulties in maintaining a consistent medication supply, which likely contributed to persistently high viral loads. The infant's HIV RNA was 249,000 copies/mL and the CD4 count was 3561/mm^3 during this period. Baseline genotypic resistance testing revealed no resistance mutations against protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs), or non-nucleoside reverse transcriptase inhibitors (NNRTIs). At two months of age, combination therapy with AZT, lamivudine (3TC), and lopinavir/ritonavir (LPV/r) was initiated. However, by the fourth month, viral suppression was not achieved (HIV RNA >10,000,000 copies/mL, CD4 count: 1351/mm^3, stage 2 immunosuppression). Consequently, trimethoprim/sulfamethoxazole (TMP/SMX) and fluconazole prophylaxis were started, and the ART regimen was modified to tenofovir/emtricitabine (TDF/FTC), dolutegravir (DTG), and LPV/r.

Despite multiple ART adjustments, long-term viral suppression was not achieved. Reports from the previous treatment center indicated that the child was not consistently brought to follow-up visits, and medication adherence was poor. Family history revealed that the parents had ongoing conflicts, leading to the grandmother becoming the primary caregiver. The grandmother had limited knowledge about ART and HIV management, and she reported frequent difficulties in administering medications. Additionally, the child exhibited strong resistance to taking medications, frequently spitting them out or vomiting, particularly with the more complex multi-pill regimens.

At 24 months of age, the child was referred to a tertiary healthcare center for further evaluation and management. Upon admission, she was in good general condition with normal physical examination findings. Laboratory tests revealed HIV RNA of 1,800,000 copies/mL and a CD4 count of 1373/mm³. Additional evaluations included Quantiferon (IFN-ɣ) positivity, prompting further assessments with thoracic computed tomography (CT) and gastric aspirates. No evidence of *Mycobacterium tuberculosis* infection was found (negative acid-fast bacilli staining, polymerase chain reaction, and culture results). Isoniazid (INH) prophylaxis was initiated and completed over nine months.

The ART regimen of TDF/FTC, DTG, and LPV/r was continued, and a repeat resistance analysis was performed. Sequencing identified a newly acquired L76V mutation, conferring intermediate resistance to LPV/r, as well as the persistence of NNRTI resistance with V179E mutation. These findings suggested ongoing viral replication under suboptimal ART adherence, increasing the risk of developing further resistance.

Given the challenges in medication adherence, the pill burden and administration difficulties, and the risk of further resistance mutations, STR was considered. The child's mother had achieved viral suppression on BIC/FTC/TAF (50mg/200mg/25mg), leading to the decision to explore this regimen for the child. However, the pediatric formulation (BIC/FTC/TAF 30mg/120mg/15mg) was not available in Turkey, necessitating off-label approval for the adult formulation. Approval was granted, and BIC/FTC/TAF (50mg/200mg/25mg) was administered in a dose adjusted to body weight (12.5 kg) by crushing one adult tablet in 5 mL of water and administering 3 mL once daily.

Within the first month of BIC/FTC/TAF initiation, HIV RNA levels significantly decreased to 207 copies/mL, indicating rapid virologic response. Subsequent viral load measurements at three-month intervals showed continued suppression, with values of 35, 40, and 43 copies/mL respectively, confirming sustained viral control. The CD4 count at the latest follow-up was 1261/mm³ (Fig. 1).

Additionally, structured counseling sessions were provided to the child's caregivers to improve treatment adherence and understanding of ART importance. These included one-on-one sessions with a pediatric HIV specialist, a clinical psychologist, and a pharmacist to optimize medication administration strategies.

Written informed consent was obtained from the patient's legal guardian for publication.

Discussion

This case highlights the importance of simplified regimens in pediatric HIV patients with adherence challenges and demonstrates the successful off-label use of BIC/FTC/TAF to achieve viral suppression in a previously treatment-refractory child. Pediatric HIV management presents significant challenges, particularly regarding medication adherence and the development of drug resistance. Younger children often struggle with adherence due to factors such as medication formulation, caregiver involvement, psychosocial barriers, and pill burden. This case highlights the importance of STRs) with high genetic barriers

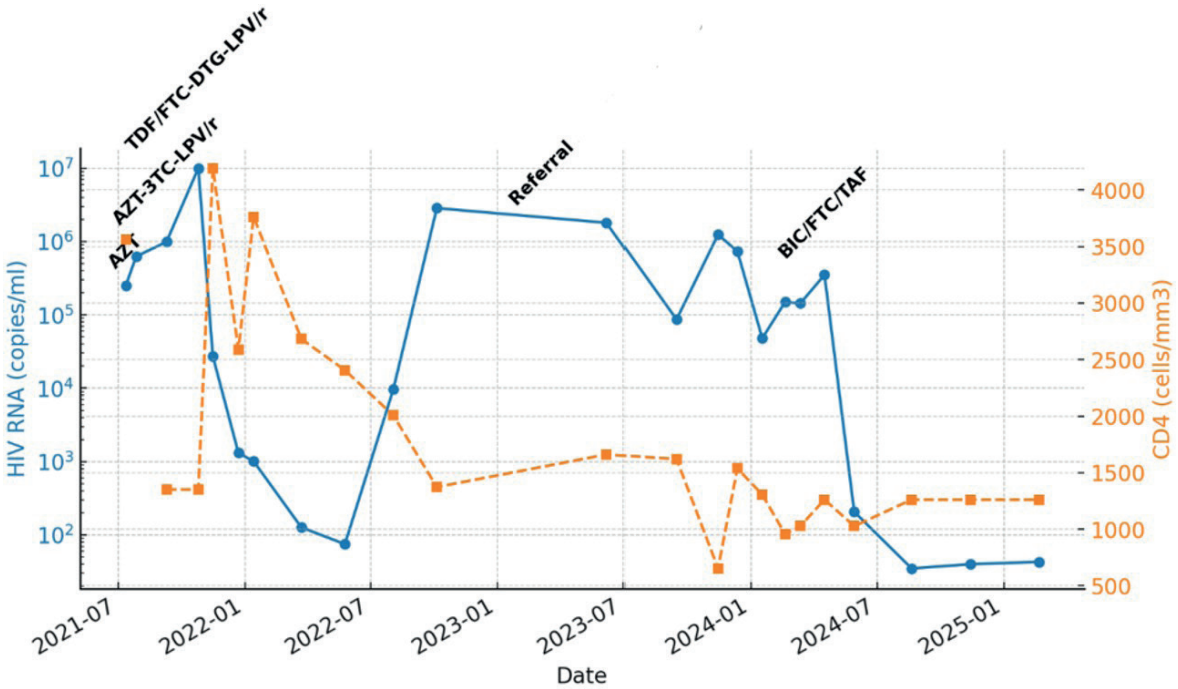

Fig. 1. HIV laboratory follow-up results and management.

3TC: lamivudine; AZT: zidovudine; BIC: bictegravir; CD4: cluster of differentiation 4 T cells; DTG: dolutegravir; FTC: emtricitabine; HIV RNA: human immunodeficiency virus ribonucleic acid; LPV/r: lopinavir/ritonavir; TAF: tenofovir alafenamide; TDF: tenofovir disoproxil fumarate.

to resistance and the role of multidisciplinary approaches in improving adherence.

Pediatric administration of fixed-dose combination ARTs often requires formulation adjustments, such as tablet crushing or dissolving, to facilitate swallowing. Recent pharmacokinetic studies have highlighted the impact of these modifications on drug bioavailability. The SOLUBIC randomized crossover trial demonstrated that dissolving BIC/FTC/TAF tablets in water maintains comparable bioavailability to whole-tablet ingestion, whereas crushing the tablet significantly decreases exposure to emtricitabine and tenofovir alafenamide.[5] Similarly, the HIV.gov pediatric treatment guidelines and recent reviews caution against tablet crushing due to potential reductions in drug absorption and therapeutic efficacy.[6,7] Moreover, pediatric pharmacokinetic and safety data confirm the suitability of dissolved formulations for children aged 2 years and older, supporting the practice used in this case.[8] These findings underscore the importance of careful consideration of formulation strategies to ensure effective viral suppression in pediatric HIV patients.

According to the European AIDS Clinical Society (EACS) guidelines, the recommended first-line ART regimen for children under two years of age typically consists of NRTIs (such as abacavir/lamivudine or zidovudine/lamivudine) in combination with a boosted PI (e.g., LPV/r) or an integrase inhibitor (INSTI) (e.g., raltegravir [RAL]).[9] However, real-world applications often reveal suboptimal adherence to these regimens due to poor taste, high pill burden, and gastrointestinal side effects. The patient initially received AZT+3TC+LPV/r as per guidelines but failed to achieve viral suppression, requiring multiple regimen changes and ultimately necessitating an alternative, off-label approach.

Studies have demonstrated that children on multi-pill regimens have lower adherence rates compared to those on simplified regimens.[1] A

meta-analysis investigating ART adherence in pediatric populations found that nonadherence rates vary from 15% to 93%, with the most common barriers including caregiver-related difficulties, pill burden, and side effects.[10] Simplified antiretroviral regimens like BIC/FTC/TAF have been associated with low discontinuation rates and high virologic suppression in both treatment-naive and treatment-experienced individuals, according to a systematic review and meta-analysis.[11]

A recent systematic review analyzing real-world outcomes of BIC/FTC/TAF therapy in diverse HIV populations found that treatment discontinuation rates were significantly lower in individuals on BIC/FTC/TAF compared to other ART regimens.[11] This finding is particularly relevant for pediatric populations, as treatment retention is often compromised by medication side effects and regimen complexity. The study also emphasized that BIC/FTC/TAF use was associated with high rates of virologic suppression, reinforcing its potential role as an alternative therapy in children who struggle with adherence to traditional regimens. The patient's case aligns with these findings, as the transition to BIC/FTC/TAF resulted in a rapid decline in viral load from 1,800,000 copies/ml to 207 copies/ml and sustained suppression over time.

Another phase 2/3 study investigating the pharmacokinetics and safety of co-formulated BIC/FTC/TAF in children aged 2 years and older reported that BIC/FTC/TAF maintained therapeutic drug concentrations, was well-tolerated, and achieved high virologic suppression rates.[8] Notably, the study demonstrated that pediatric patients receiving BIC/FTC/TAF exhibited minimal adverse effects, which is crucial for maintaining adherence and treatment continuity. In our patient, despite previous poor adherence to multi-pill regimens, BIC/FTC/TAF administration resulted in significant clinical improvement, sustained virologic suppression, and an increase in CD4 count to 1261 cells/mm^3. This case supports the potential for broader use of STRs in pediatric populations, particularly in those with complex adherence barriers.

Children with poor adherence are at increased risk of developing drug resistance, which can significantly limit future treatment options. BIC is particularly advantageous in such cases due to its high genetic barrier to resistance.[4] Our patient developed the L76V mutation, conferring intermediate resistance to LPV/r, along with the NNRTI-associated V179E mutation. Despite these mutations, BIC/FTC/TAF achieved and maintained viral suppression, emphasizing the importance of high-barrier regimens in treatment-experienced children.

Multidisciplinary interventions play a crucial role in pediatric HIV management.[12] A study on children living with HIV found that psychosocial support, caregiver education, and pharmaceutical counseling significantly improved ART adherence and virological outcomes.[10] In our case, a multidisciplinary approach was implemented, including one-on-one caregiver education on ART adherence, structured counseling sessions with a psychologist to address the child's medication refusal, and pharmacist-led medication administration training. These strategies helped stabilize adherence, leading to sustained viral suppression (final HIV RNA: 43 copies/mL) and improved immune response (final CD4 count: 1261 cells/mm^3).

The off-label use of BIC/FTC/TAF in pediatric HIV remains an area of active investigation. Our case contributes to growing evidence that simplified regimens may serve as an effective alternative in children with adherence difficulties and drug resistance. However, further research is needed to assess the long-term safety and pharmacokinetics of BIC/FTC/TAF in younger children, its impact on adherence and clinical outcomes, and strategies to expand access to pediatric formulations in resource-limited settings.

Conclusion

This case underscores the importance of simplified ART regimens, multidisciplinary care, and individualized treatment strategies in pediatric HIV management. The successful off-label use of BIC/FTC/TAF in a treatment-refractory child highlights its potential efficacy in young patients with adherence challenges. The high genetic barrier to resistance and well-documented tolerability of BIC/FTC/TAF makes it a promising option, especially in cases where standard therapies fail due to nonadherence or drug resistance. Future research should focus on optimizing treatment strategies and expanding access to STRs for pediatric populations worldwide.

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Ethical approval

Written informed consent was obtained from the patient's legal guardian for publication.

Author contribution

The authors confirm contribution to the paper as follows: Study conception and design: GA, CE, ZŞB, EÇÖ, AA; data collection: SE, GA, CE, AA; analysis and interpretation of results: GA, CE; draft manuscript preparation: GA, CE. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

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Comments on the relationship between microRNA-155-5p and postoperative inflammatory markers in children with acute suppurative appendicitis, and its role in predicting postoperative complications

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Dear Editor,

I read with interest the study by Dr. Duan et al.[1] titled "The relationship between microRNA-155-5p and postoperative inflammatory markers in children with acute suppurative appendicitis and its role in predicting postoperative complications" published in The Turkish Journal of Pediatrics. This study makes an important contribution to the field by demonstrating the potential role of miR-155-5p in predicting postoperative complications. The large patient cohort (n=316), the use of advanced techniques such as quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA), and the comparison of laparoscopic and open appendectomy groups are methodological strengths. The demonstration of correlation between miR-155-5p levels and inflammatory markers and visual analog scale (VAS) score contributes to biomarker-based clinical decision-making. I congratulate the authors for this valuable work. However, I believe some aspects of the study could be further improved.

The follow-up period of postoperative complications, such as wound infection and intraabdominal adhesion were not clearly specified in the study. Furthermore, the lack of late wound healing parameters, such as incisional hernia or scar quality does not fully reflect the impact of surgical techniques on patient comfort.

The study did not address metabolic comorbidities (e.g. obesity, diabetes) or nutritional status, which are known to affect systemic inflammation and surgical outcomes. Childhood obesity increases the risk of postoperative infection, prolongs the duration of surgery and prolongs hospitalization.[2] Similarly, low preoperative albumin levels are associated with increased inflammation and complications.[3] The exclusion of these factors may limit the assessment of miR-155-5p as an independent variable and reduce the generalizability of the results by ignoring the potential modulation of its expression by chronic conditions. Expanding the study's comparison between laparoscopic and open appendectomy within a broader framework could also be beneficial. While the VAS score is an effective method for measuring pain severity, the lack of objective parameters such as analgesic consumption limits a comprehensive assessment of patient comfort. Additionally, although the return of gastrointestinal function

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was measured via 'time to first flatus,' other parameters—such as quality of fecal output or time to resume oral intake—could provide further insights. Investigating whether the early postoperative advantages of laparoscopic surgery are linked to miR-155-5p levels could significantly enhance biomarker-based surgical decision-making.

In order to increase the prognostic power of miR-155-5p, comparison with inflammatory markers such as neutrophil-to-lymphocyte ratio (NLR), Systemic Inflammatory Response Index (SIRI) and Systemic Immune-Inflammation Index (SII) may provide a broader clinical perspective. Furthermore, risk prediction can be made more precisely by using machine learning and artificial intelligence-based analyses to create models that predict miR-155-5p levels and the development of postoperative complications.

Finally, in future studies, the molecular mechanisms through which miR-155-5p affects inflammation processes by using mouse appendicitis models or in vitro macrophage cultures may increase the value of the study in terms of translational medicine. This study highlights the importance of biomarker-based approaches in the management of pediatric appendicitis. Further research on molecular mechanisms, long-term follow-up and additional clinical parameters could enhance its impact. I congratulate the authors and the journal for their scientific contribution.

Author contribution

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

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Conflict of interest

The authors declare that there is no conflict of interest.

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LETTER TO THE EDITOR

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Nurcan Çoşkun

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