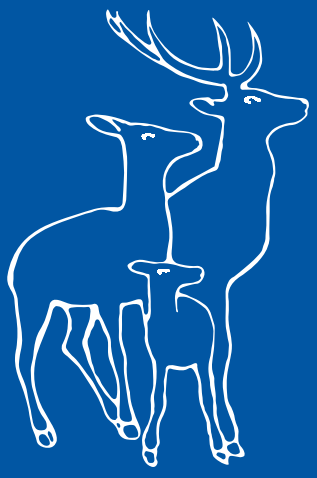

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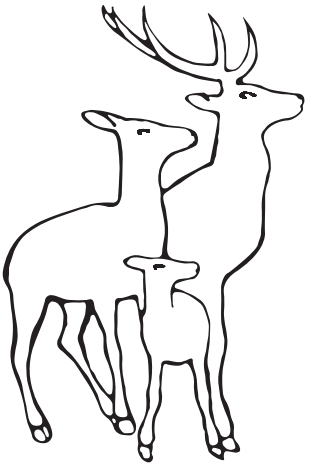

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Original Article

Quality of life and the psychological status of the adolescents with asthma and their parents during the COVID-19 pandemic

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ABSTRACT

Background. The new coronavirus pandemic affected many people's both physical and mental health around the world, psychiatric problems and anxiety was common during the pandemic. We aim to evaluate the anxiety and quality of life (QoL) of children and their parents who were followed up with the diagnosis of asthma during the COVID-19 pandemic comparing with an age-matched control group.

Methods. This study was conducted after the first lockdown of the coronavirus pandemic, namely the new normalization period. Demographic features were noted, all adolescents completed the State-Trait Anxiety Inventories for Children (STAI-C) state and trait scales, and Pediatric QoL inventories (PedsQL). Parents also filled PedsQL parent version, STAI state and trait scales. Asthma control test (ACT) was completed only by the asthma group.

Results. Totally 121 adolescents [61 asthma group (59% female); 60 control group (73.8% female)] were included in the study. The mean age of the patients was 15.4 ± 1.69 years and their parents was 41.52 ± 6.04 years. In the asthma group 65.6% of the patients used asthma medications regularly and 73.8% of them continued asthma follow-up during the pandemic. There was no significant difference in terms of PedsQL from both the child's and parent's perspective, STAI-C and STAI scores between study groups. The QoL was associated with asthma severity, all of the PedsQL scores were significantly lower in the uncontrolled asthma group. Asthma severity was also correlated with anxiety, as the uncontrolled asthma group reached the highest STAI-C trait scores. Girls with asthma had significantly lower physical PedsQL and ACT scores than boys.

Conclusions. Although the quality of life and anxiety scores of children with asthma did not differ from the control group, good asthma control in adolescents with asthma may improve QoL. Adolescence is a sensitive age group, and requires meticulous consideration by caregivers. The parents' awareness of anxiety and other psychological symptoms may help them to cope with the challenges during the pandemic.

Key words: adolescent, anxiety, asthma, coronavirus pandemic, COVID-19, psychological, quality of life.

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The new coronavirus disease namely COVID-19 caused a wide spectrum of clinical findings ranging from asymptomatic infection or with mild clinical symptoms to pneumonia or severe respiratory distress and even death.[1] Initially COVID-19 infection was thought to be asymptomatic or with mild clinical presentation

in children[2], but later a new phenomenon multisystem inflammatory syndrome in children (MIS-C) was reported in relatively older children mimicking Kawasaki Syndrome.[3]

Asthma is one of the most common chronic lower airway diseases among children[4] and has a potential risk of severe COVID-19 infection similar to other underlying chronic diseases.[5] Previous studies about severe SARS-CoV-2 infection in patients with asthma had controversial results, Guan et al.[6] reported the presence of comorbidities in a considerable number of patients with COVID-19 and there were no patients diagnosed with asthma. Contrastingly, in a recent study from United Kingdom (UK), asthma was reported as a risk factor of death from COVID-19.[7]

Adolescence is a special period in which significant physical, psychological and social developments are seen during the transition from childhood to adulthood. During adolescence, an individual acquires the physical, cognitive, emotional, social, and economic resources that are the foundation for later life, health and wellbeing.[8]

Asthma treatment compliance is essential for optimal asthma control, and better asthma control is associated with good quality of life.[9,10] Studies have shown that poor asthma control is associated with puberty and health related quality of life (HRQoL) of adolescents are negatively affected by inadequate asthma control.[10,11] Moreover, adolescents with asthma reported poorer HRQoL scores than their peers without asthma.[12]

Anxiety is one of most prevalent psychiatric disorder among Turkish children.[13,14] Stressful life events, health concerns and excessive use of internet can affect the mental health of adolescents.[15] Since the pandemic has begun, the lives of many people around the world have been affected socially and economically as well as their mental health. Social interactions have also been interrupted due to the pandemic, for instance social distancing, school closures and lockdowns have also had psychosocial effects on mental health causing anxiety and depression symptoms.[16]

In addition, the current epidemic situation causes fear of viral contamination during hospital admissions in patients, especially those with chronic diseases. Patients and families worry about their health conditions and COVID-19, which may cause negative psychological effects and anxiety.[17] In the present study, we aimed to evaluate the quality of life of adolescents with asthma and the anxiety that may occur due to COVID- 19 pandemic compared with an age-matched control group during their hospital visits. A further aim was to assess the asthma control status and its effect on quality of life of adolescents with asthma during the COVID-19 pandemic.

Material and Methods

Study participants

This was a population-based, observational and cross-sectional study. This study was conducted after the first lockdown of the coronavirus pandemic, namely the new normalization period. Sixty-one adolescents aged between 12 and 18 years, who were followed by a diagnosis of asthma based on Global Initiative for Asthma (GINA) report[18] at least six months in our outpatient pediatric allergy and immunology clinic and their parents were prospectively recruited to the study throughout 15 May-15 November 2020. Sixty children without a previous history of atopic or any other chronic disease and without any sign or complaints of COVID-19 infection in the same age group who were admitted to the adolescent outpatient clinic and their parents were also included as a control group for the study. Demographic features (age, gender, parental gender, age and education, family income etc.) of the participants were recorded. And, also a follow-up form was filled in the asthma group, including asthma medications, symptoms, atopy and control asthma status.

The study was approved by the institutional ethics committee of Ankara City Hospital (approval no. E1-20-689) and the study protocol was approved by the Turkish Ministry of Health. Written informed consent was obtained from the study participants.

Measurements

The State-Trait Anxiety Inventories for Children (STAI-C)

This is a self-report scale composed of two subscales each with 20 items, and it rates state and trait anxiety levels of children.[19,20] State anxiety refers to anxiety symptoms under certain circumstances and trait anxiety to permanent individual features creating a tendency for anxiety perception. Higher scores indicate a high anxiety level.

Pediatric Quality of Life Inventory (PedsQL)

These were developed to rate health-related quality of lives of children and adolescents aged 2-18 years. It questions the domains of physical health, emotional and social functionality, which are the properties of the state of healthiness as defined by the World Health Organization. This scale is used in children with a variety of chronic medical conditions. It contains two subscale scores, namely psychosocial health (PSH) and physical health (PH), and a total health (TH) score. The scale contains both parent and child versions for physical and psychosocial functioning, and a higher PedsQL total score indicates a better QoL. In this study, the parent version PedsQL scale was administered to the caregiver. Memik et al. established the validity and reliability study in Turkish.[21,22]

State-Trait Anxiety Inventory (STAI)

The STAI-A is a 40-item scale. The scale can be used to measure both trait anxiety (STAI-T: how dispositionally anxious a person is across time and situations) and state anxiety (STAI-S: how anxious a person is feeling at a particular moment) as it consists of two separate subscales and each has 20 items.[23,24] Higher scores indicate a high anxiety level.

Asthma Control Test

The questionnaire consists of 5 items which evaluates self-reported asthma symptoms, the impact of asthma on daily life, and the need of rescue medications in the past 4 weeks. The items response range from 1 to 5, and lower scores are compatible with poor asthma control.[25,26]

Data analysis

Data analysis was performed by SPSS® version 22.0 for Windows (IBM SPSS, Chicago, IL, USA). The definitions were fulfilled as numbers and proportions for distinct variables; means and standard deviations or as medians with 25th or 75th percentile values in case skewed distribution. for continuous variables. Categorical variables were analyzed by using chi square test. Independent t-test was used for parametric tests and Mann-Whitney U test was used for nonparametric tests to comparison of independent groups. One-way ANOVA with post hoc Tukey HSD test or Kruskal-Wallis test were used to compare more than two independent groups according to the skewed distribution. Pearson and Spearman correlation analysis were used for correlation analysis according to the data distribution. ANCOVA test was used to remove gender effect on scores. A p value of less than 0.05 was considered statistically significant.

Results

Characteristics of the whole population

Totally 121 adolescents [61 asthma group (59% female); 60 control group (73.8% female)] were included into the study. The mean age of the patients was 15.4 ($\pm$1.69) years, and the mean age of the parents were 41.52 ($\pm$6.04) years. The comparative demographic and general characteristics of the patients are shown in Table I.

Table I. Demographic characteristics of patients with asthma and the control group.

	Study population (n=121)		
	Asthma group (n=61)	Control group (n=60)	*P* value
Age(year) †	15.26 ± 1.90	15.55 ± 1.45	0.35
Gender, (%)			0.022
Male	25 (41)	13 (21.7)	
Female	36 (59)	47 (78.3)	
Parent's age †	41.36 ± 6.49	41.68 ± 5.6	0.77
Parent's gender, (%)			0.2
Male	16 (26.2)	14 (23.3)	
Female	45 (73.8)	46 (76.7)	
Parental chronic disease, n (%)	16 (26.2)	18 (30)	0.64
Parental psychiatric disorder, n (%)	5 (8.2)	4 (6.7)	0.74
Mother's education , n (%)			0.32
Primary school	26 (42.6)	22 (36.7)	
Secondary school	14 (23)	10 (16.7)	
High school	16 (26.2)	16 (26.7)	
Undergraduate	5 (8.2)	10 (16.7)	
Postgraduate	0	2 (3.3)	
Father's education, n (%)			0.13
Primary school	15 (24.6)	11 (18.3)	
Secondary school	12 (19.7)	9 (15)	
High school	24 (39.3)	19 (31.7)	
Undergraduate	10 (16.4)	21 (35)	
Monthly income, n (%)			0.38
<1500 TL	6 (9.8)	4 (6.7)	
1501-3000 TL	28 (45.9)	22 (36.6)	
>3001 TL	27 (44.3)	34 (56.7)	
Owns a pet, n (%)	11 (18)	14 (23.3)	0.55

† mean ± standard deviation; TL: Turkish Lira

There was no significant difference between the asthma and control group in terms of child's and parent's proxy PedsQL PH, PSH and TH scores (Table II). And, also no significant difference was found in the comparison of children's STAI-C (state and trait), and parents' STAI (state and trait) scores (Table II). The gender effect on the scores was removed with ANCOVA test and re-analyzed, there was no difference in the clinical scores of the asthma and control group (p> 0.05).

Characteristics of the asthma group

The mean age of the adolescents with asthma was 15.26 ± 1.9 years (male/female=25/36) and the median time for asthma follow-up time was 48 (IQR: 12-96) months. In the asthma group 65.6% of the patients used asthma medications regularly and 73.8% of them continued asthma follow-up during the COVID-19 pandemic. None of the patients with asthma had a history of SARS-CoV-2 infection neither in themselves nor in their families. 54.1% asthmatic adolescents had no history of emergency admission and none of the patients required intensive care admission due to asthma in the last year.

Asthma group was subdivided into 3 groups according to asthma symptom control based on GINA asthma report[18], and 52.5% of them were classified as well-controlled asthma. Children's

Table II. The comparison of the clinical scores of the children and parents.

	Study population (n=121)		
	Asthma group (n=61)	Control group (n=60)	*P* value
PedsQL Physical Health Score			
Child self-report	71.26 ± 20.04	70.46 ± 19.79	0.83
Parent proxy-report	68.53 ± 2 0.03	68.85 ± 19.58	0.93
PedsQL Psychosocial Health Score			
Child self-report	75.77 ± 14.13	72.39 ± 15.72	0.21
Parent proxy-report	76.89 ± 16.05	72.55 ± 15.31	0.13
PedsQL Total Score			
Child self-report	74.2 ± 14.62	71.72 ± 15.35	0.36
Parent proxy-report	74 ± 15.85	71.52 ± 14.86	0.37
Children STAI-C			
State anxiety score	35.11 ± 5.89	35.07 ± 6.6	0.96
Trait anxiety score	36.89 ± 6.46	37.45 ± 6.99	0.64
Parent STAI			
State anxiety score	38.31 ± 8.13	39.25 ± 9.48	0.56
Trait anxiety score	42.16 ± 9.27	41.63 ± 10.67	0.77

All data expressed as mean ± standard deviation; PedsQL: Pediatric Quality of Life Inventory; STAI: State-Trait Anxiety Inventory

STAI-C trait scores were significantly different according to asthma symptom control groups (p=0.006), the uncontrolled asthma group had the highest anxiety scores (Fig. 1A). A statistically significant deterioration in terms of asthma control status was observed with increasing age in adolescents (p=<0.001). PedsQL PH, PSH and TH scores were also significantly different according to the asthma control status, p value is <0.001, 0.023 and 0.008, respectively. The uncontrolled asthma group had the lowest PedsQL scores (Fig. 1B-D).

There were 36 (59%) girls and 25 boys (41%) in the asthma group. The age of the girls was significantly older than boys (p <0.001), and when we compare the ACT, STAI, STAI-C and PedsQL PH, PSH and TH scores by gender, the PedsQL PH and ACT scores were significantly lower in girls than boys, p value was 0.011 and 0.021, respectively.

Correlation analysis

The PedsQL scores of the adolescents with asthma had a significant correlation with parents' proxy PedsQL scores, and there was a significant, but weak, reverse correlation between the PedsQL scores and parent's STAI (state and trait) scores (Table III).

The PedsQL scores of the adolescents with asthma had a significant correlation with each other and ACT scores. Moreover, there was a significant, but reverse correlation between the PedsQL scores and children's STAI-C (state and trait) scores (Table IV).

Discussion

Asthma is one of the most common chronic lung disease that requires regular treatment and follow-up.[18] Asthma has considerable physical and psychological effects not only on children but also on their parents.[27] In our study we evaluate the impact of the COVID-19 pandemic on quality of life and anxiety in adolescents with asthma and their parents compared with a control group. In the comparison of PedsQL both child's and parent's perspective, and anxiety levels we couldn't find any significant difference between study groups. In the asthma group the quality of life was associated with

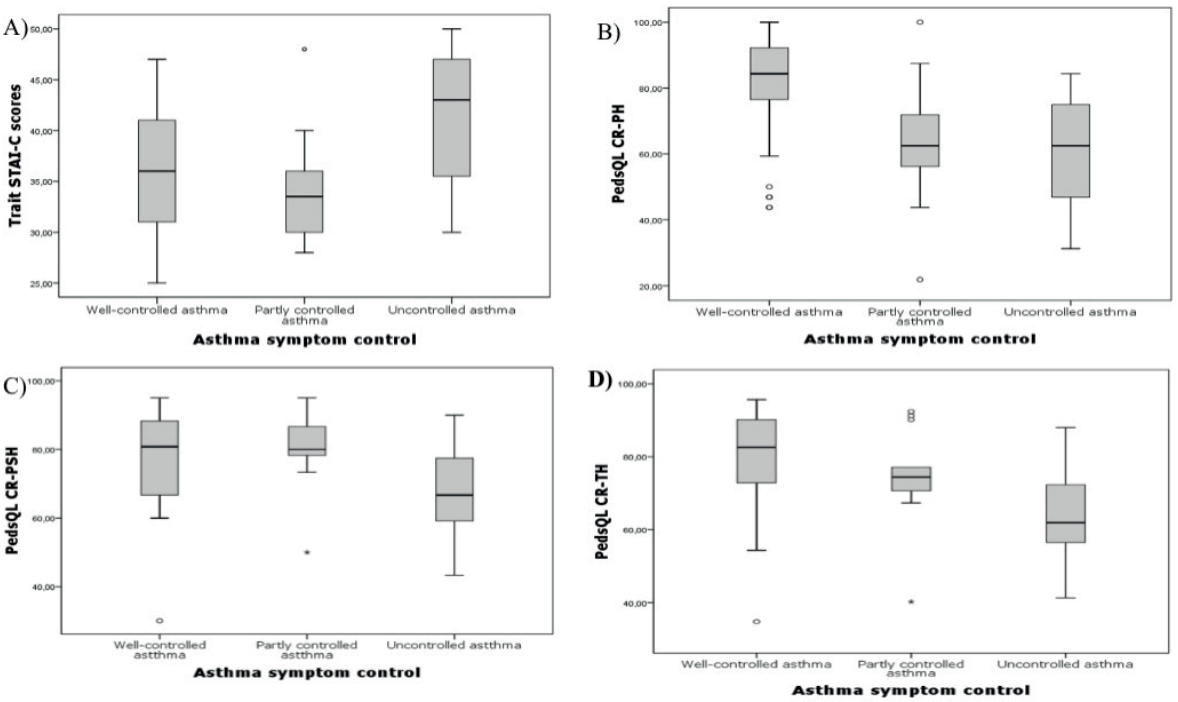

Fig. 1. Clinical scores of the adolescents with asthma according to their asthma control status: **A)** State-Trait Anxiety Inventory for Children (STAI-C) trait scores, **B)** Pediatric Quality of Life Inventory (PedsQL) child self-report physical health scores (CR-PH), **C)** PedsQL child self-report psychosocial health scores (CR-PSH); and **D)** PedsQL child self-report total health scores (CR-TH).

Table III. Bivariate correlation analysis between child self-report PedsQL scores and parent proxy-report PedsQL and parent STAI scores.

	PedsQL PR-PH	PedsQL PR-PSH	PedsQL PR-TH	STAI-State	STAI-Trait
PedsQL CR-PH	r=0.630**	r=0.452**	r=0.591**	r=-0.235	r=-0.249
	p<0.001	*p*<0.001	*p*<0.001	*p*=0.068	*p*=0.053
PedsQL CR-PSH	r=0.455**	r=0.736**	r=0.686**	r=-0.390**	r=-0.300*
	p<0.001	*p*<0.001	*p*<0.001	*p*=0.002	*p*=0.019
PedsQL CR-TH	r=0.587**	r=0.679**	r=0.714**	r=-0.358**	r=-0.308*
	p<0.001	*p*<0.001	*p*<0.001	*p*=0.005	*p*=0.016

PedsQL: Pediatric Quality of Life Inventory; CR-PH: Child self-report physical health score; CR-PSH: Child self-report psychosocial health score; CR-TH: Child self-report total health score; PR-PH: Parent proxy-report physical health score; PR-PSH: Parent proxy-report psychosocial health score; PR-TH: Parent proxy-report total health score; STAI: State-Trait Anxiety Inventory

asthma severity, all of the PedsQL scores were significantly lower in the uncontrolled asthma group. Asthma severity was also correlated with anxiety, as the uncontrolled asthma group reached the highest STAI-C trait scores. Moreover, in the asthma group when we compared the clinical scores by gender, girls with asthma had significantly lower physical PedsQL and ACT scores than boys.

The SARS-CoV-2 infection is a highly contagious virus which has led to a dramatic increase of cases in a short while throughout the world.[28] The spread of the COVID-19 pandemic has not only impacted the physical but also mental health by causing negative emotions. Mandatory stay-at-home, economic burden, school and business closures contributed to negative emotions and cognition.[29] The obscurities at the beginning

Table IV. Bivariate correlation analysis between child self-report PedsQL scores, STAI-C scores, and ACT score.

	PedsQL CR-PH	PedsQL CR-PSH	PedsQL CR-TH	STAI-C State	STAI-C Trait	ACT score
PedsQL CR-PH	-	r=0.624**	r=0.870**	r=-0.498**	r=-0.555**	r=0.499**
		p<0.001	*p*<0.001	*p*<0.001	*p*<0.001	*p*<0.001
PedsQL CR-PSH	r=0.624**	-	r=0.928**	r=-0.488**	r=-0.714**	r=0.299*
	p<0.001		*p*<0.001	*p*<0.001	*p*<0.001	*p*=0.021
PedsQL CR-TH	r=0.870**	r=0.928**	-	r=-0.545**	r=-0.715**	r=0.441**
	p<0.001	*p*<0.001		*p*<0.001	*p*<0.001	*p*<0.001

PedsQL: Pediatric Quality of Life Inventory; CR-PH: Child self-report physical health score; CR-PSH: Child self-report psychosocial health score; CR-TH: Child self-report total health score; ACT: Asthma control test; STAI: State-Trait Anxiety Inventory

of the pandemic caused health concerns, fear and anxiety in people especially those with underlying chronic diseases. Many patients with chronic diseases postponed hospital admissions and routine health controls due to risk of contamination. A new healthcare system telemedicine consultation came into our lives during the pandemic which prevents the risk of disease transmission to patients.[30]

Lung involvement is common during the SARS-CoV-2 infection due to the relatively large number of angiotensin converting enzyme (ACE2) receptors in the respiratory epithelium.[31] Therefore, chronic lung diseases have a potential predisposition to the SARS-CoV-2 infection. But the psychological impact of COVID-19 pandemic on children with chronic lung diseases had disputable results. Senkalfa et al.[32] reported that COVID-19 had no effect on the anxiety of children with cystic fibrosis (CF). In contrast, in another study which evaluated psychiatric and general health effects of COVID-19 in children with chronic lung diseases in which majority of them were diagnosed with CF, both children and their parents had more anxiety due to the COVID-19 pandemic comparing with healthy peers.[33] In our study, we couldn't find any significant difference in health quality and anxiety scores between adolescents with asthma and the control group. This may be attributed to none of the adolescents with asthma having a history of COVID-19 infection in our cohort.

The COVID-19 pandemic is challenging for the asthmatic patients, as asthma is the most widespread chronic lung disease.[18] The clinical course of SARS-CoV-2 infections still remains unclear in asthmatic patients. There are some studies including pediatric COVID-19 patients that have reported no association with asthma and COVID-19.[2,34,35] Long-lasting school closures, reduced air pollution and wearing masks may prevent the transmission of viral infections which may trigger asthma exacerbations.[36] Furthermore, a recent study including adults and children with asthma, depicted the lower expression of ACE2 receptors in those with allergic sensitization. These findings may suggest the reduced risk of severe COVID-19 infection in patients with respiratory allergies.[37] These preventive effects may be related to similar QoL and anxiety asthmatic adolescents with the control group.

Conversely, Choi et al.[38] reported 2.9% prevalence of asthma among 7590 COVID-19 patients, and they also indicated high mortality rate among the patients with asthma. Williamson et. al.[7] also emphasized the link between asthma and higher risk of hospital death from COVID-19. This difference may be related to the diversity in the control status of asthmatic patients. In our study, the control status of asthma was negatively associated with QoL of asthmatic adolescents. Careful management of asthma may be especially important during the pandemic and uncontrolled patients may need

special care both for physical and psychosocial wellbeing. Further studies are required to determine the relationship between COVID-19 and asthma.

Separation from classmates due to school closures, prolonged exposure to internet, online distance learning, health and future concerns led anxiety during the pandemic.[15] In previous studies, well-controlled childhood asthma was associated with no increased risk of anxiety and depression comparing with healthy controls.[39] In our study, most of the patients in the asthma group were classified as well-controlled and partly controlled asthma, this may have had an effect on the absence of difference in anxiety scores between the asthma and control group.

The QoL of adolescents with asthma is a substantial health outcome which is affected by various factors. COVID-19 pandemic has had an impact on mental health and may cause psychological distress.[16] To our knowledge there is no study evaluating QoL of adolescents with asthma during the COVID-19 pandemic. Several studies have been conducted on assessing the quality of life in children with asthma before the COVID-19 pandemic.[11,40] Similar to our findings Stridsman et al.[11] reported the female gender and poor asthma control to be associated with low QoL scores.

Although, sometimes there may be differences in perception of QoL between parents and adolescents, in our study group, QoL results of adolescents and parents were correlated with each other.

STAI-C trait and STAI-C state scores of our patients showed an inverse correlation with PedsQL scores. This shows us that asthmatic adolescents with lower QoL may experience higher anxiety and thus physicians must be aware of this possibility to evaluate the presence and help management of anxiety among this patient group.

Asthma control test results of our patients were positively correlated with PedsQL scores, showing that asthma control was correlated with QoL. The effect of disease control has been previously reported in the literature, for increasing the QoL of asthmatic adolescents and the first step is to take measures for providing asthma control.[41]

Asthma control and QoL are important disease outcomes for asthmatic patients. Illness perceptions (cognitive and emotional representations of the illness) and medication beliefs are essential determinants of medication adherence, and subsequently disease control and QoL in adolescents. Kosse et al.[41] have found a strong positive correlation between disease control and QoL in 243 adolescents with asthma. They also mentioned that all illness perceptions items were correlated with disease control and QoL, and medication adherence was correlated to medication beliefs, disease control, and QoL.

In our study population, the PedsQL PH and ACT scores were significantly lower in girls than boys. This gender difference in QoL is in accordance with the literature. Asthmatic girls are reported to have a worse perception of the disease in spite of similar or even better pulmonary function tests and similar medications. This effect of gender is not fully understood, however there have been some probable explanations. Female sex hormones are hypothesized to affect asthma outcomes. Furthermore, females are shown to have lower threshold for healthcare contact and they seem to need more encouragement and education for correct use of inhalers. Caregivers are reported to help their male children better than girls and this may also have an impact.[11,42,43]

Moreover, studies have shown the high rate of mental problems in adolescents during the pandemic, and female gender was related with high risk for anxiety and depression symptoms.[44] Whatever the underlying reason, adolescent girls have more severely affected quality of life and this indicates to the need for more attention for this patient group during the management of asthma.

In our study, asthma control was declining with increasing age. This may be the effect of adolescent behavior getting more obvious with increasing age and getting more freedom from parent control for older adolescents.

The most important limitation of our study is that although we excluded atopic, chronic diseases and the patients with COVID-19 infection related symptoms in the control group, there may be other contributing factors which may affect the clinical scores that we could not predict. However, there was no significant difference in terms of parents' chronic or psychiatric diseases between groups which may have an impact on clinical scores. Another limitation of our study is that we could not take all independent variables that can affect the quality of life of our patients, however we believe we evaluated the most important factors that were directly related to the subject.

The most important strength of our study is that we evaluated the anxiety and QoL of adolescents with asthma and age-matched controls during their hospital visits with face-to-face questionnaire which helps to determine real scores.

In conclusion, the impact of COVID-19 pandemic on adolescents, in terms of QoL and anxiety did not differ between the asthma and control groups. The QoL scores and trait anxiety scores were related with asthma control status in adolescents with asthma. Girls had poor asthma control with lower ACT and physical QoL scores than boys.

Adolescence is a sensitive age group, and requires meticulous consideration by caregivers. Adolescents need the feeling of support and unconditional love from their parents. Optimizing asthma treatment adherence, being aware of the difficulties that adolescents experience during the pandemic and improving coping styles may help to promote both physical and mental health of adolescents during the COVID-19 pandemic.

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Ethical approval

The study was approved by the institutional ethics committee of Ankara City Hospital (approval no. E1-20-689) and the study protocol was approved by the Turkish Ministry of Health.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: BK, EDM, OSU; data collection: BK, AS, ZSE, AOA, GSD; analysis and interpretation of results: BK, EC, EC, MT, EDM; draft manuscript preparation: BK, MT, EDM. 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

The relationship between smoking, alcohol, and substance abuse and psychiatric diseases among adolescents treated in a child and adolescent psychiatry inpatient unit

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ABSTRACT

Background. This study aimed to investigate the prevalence of smoking, alcohol, and substance abuse disorders among adolescents hospitalized in a university hospital child and adolescent psychiatry inpatient unit with different diagnoses, and to determine the rates of these disorders according to the mental illness diagnosis groups.

Methods. The study was conducted with 346 adolescents aged 12–18 who had been hospitalized with any psychiatric diagnosis between September 2016 and January 2020 in the child and adolescent psychiatry inpatient unit. The study considered the psychiatric diagnoses, based on the results of the DSM-5-based psychiatric interview; sociodemographic and clinical characteristics; the psychopathology history of first-degree relatives; comorbidities; length of hospital stay; income levels, and smoking, alcohol, and substance abuse.

Results. Twenty-four percent (n=83) of the participants had been smoking for 18 months or longer, 6.9% (n=24) were using alcohol, and 1% (n=28) were substance abusers. When the diagnosis distributions were examined, smoking was found to be higher in those with depressive disorders and trauma and related disorders, while smoking, alcohol, and substance use were found to be higher in the disruptive behavior disorder group. Smoking was found to be significantly lower in the obsessive-compulsive disorder group.

Conclusions. Smoking, alcohol, and substance use among inpatient children and adolescents may worsen their existing psychopathology, so health professionals working in this field should consider this situation.

Key words: smoking, alcohol, substance, adolescent, psychiatry clinic.

Smoking, alcohol, and substance abuse disorders are recognized as important public health problems, and this situation has increased over the years both in our country and globally.[1] One of the most critical periods in the development of a substance use disorder is adolescence,[2] when first contact with addictive substances and harmful usage occur most frequently.[3] In a study investigating the prevalence of smoking and substance abuse among 1235 adolescents in our country, the frequencies for smoking and use of other addictive substances were found to be 15.8% and 5%, respectively. The rate of smoking was 24.1% in male students and 7.7% in female students, while the rate of use of other addictive substances was 8.2% in male students and 9% in female students. It has been emphasized that harmful behaviors such as smoking and substance abuse were high in adolescents in that study.[4] The high prevalence of those behaviors is worrying because of the potential adverse effects on social, emotional, cognitive, physical, and academic outcomes.[5]

Among the risk factors for smoking, alcohol, and substance abuse in adolescents, individual factors, family and peer influence, and genetic

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and environmental factors are often cited.[3] Many previous studies have shown that alcohol, substance, and cigarette use are higher among psychiatric patients.[6] Adolescents may be more prone to substance abuse if they suffer from disruptive disorders, such as oppositional defiant disorder (ODD), conduct disorder (CD), and attention deficit hyperactivity disorder (ADHD), depression, or another psychiatric disorder.[1] Over half of the young people diagnosed with ADHD also have a substance abuse disorder.[7] In ODD and CD, the child or adolescent may be angry, may be in conflict with authority, may experience exclusion, and may be inclined to make friends with adolescents who exhibit similar behaviors. This situation poses a serious risk for criminal and substance abuse behaviors. In addition, substance abuse has been found to be more frequent in those exposed to trauma and neglect in childhood.[8] According to Karakuş et al.[9] in a study conducted with adult patients hospitalized in a psychiatric clinic, alcohol and substance use ranged between 25% and 75%. In that study, alcohol and substance use disorders were found to occur in 55% of those with psychotic disorders, 61% with mood disorders, and 81% with anxiety disorders.

Smoking, alcohol, and substance abuse disorders in children and adolescents under psychiatric treatment in an inpatient service may adversely affect the disease and treatment process by worsening the existing psychopathology. The failure to treat smoking, alcohol, and substance abuse disorders during or after treatment of the primary disorder may aggravate the disease process, create resistance to treatment, and make the treatment more difficult. For this reason, it is important for physicians to know the relationship between cigarette, alcohol, and substance abuse and psychiatric disorders, especially in adolescents receiving inpatient psychiatric treatment.

Although there are various studies with different methodologies on smoking, alcohol, and substance use disorders among adolescents, the number of these studies is small compared to studies conducted in adults. In addition, no study has been found in the literature on smoking, alcohol, and substance use disorders among adolescent psychiatric patients treated in the inpatient service. Considering the limitations in the literature on smoking, alcohol, and substance abuse data among child and adolescent psychiatric patients, this study aimed to determine the prevalence of smoking, alcohol, and substance abuse disorders among adolescents hospitalized with different diagnoses in a university hospital child and adolescent psychiatry inpatient unit; it also aimed to determine the rates of these disorders according to the diagnostic group.

Material and Methods

The study was conducted with adolescents who had been hospitalized with any psychiatric diagnosis between September 2016 and January 2020 in our child and adolescent psychiatry inpatient unit. Patients younger than 12 years of age were excluded from the study. A total of 384 inpatients were reached, and 38 patients with missing data were excluded; thus, files of 346 adolescents aged 12–18 were analyzed retrospectively. The study considered the psychiatric diagnoses, based on the results of the DSM-5-based psychiatric interview; sociodemographic and clinical characteristics; the psychopathology history of first-degree relatives; comorbidities; length of hospital stay; income levels, and smoking, alcohol, and substance abuse. Adolescents who had smoked cigarettes daily for more than 1 year and used alcohol or another substance for at least 2 days a week were defined as the "user group." Ethical approval was obtained for this study from the Malatya İnönü University Health Sciences Non-Interventional Clinical Research Ethics Committee (Protocol no: 2021/2327, Date: 27.07.2021).

Statistical Analysis

The SPSS 17.0 (Statistical Program in Social Sciences) package program was used for the statistical analysis. Data on quantitative variables were expressed as mean (x) ± standard

deviation (ss), while data on qualitative variables were expressed as numbers and percentages. Pearson and Fisher's chi-square test was used to evaluate qualitative variables, with $p < 0.05$ considered statistically significant.

Results

Two hundred and thirty-two female (67.1%) and 114 male (32.9%) hospitalized adolescent patients were included in this study. The mean age of the participants was 15.42 ±1.38 years (min–max=12–18). Of the patients, 77.7% (269) lived with nuclear families, 5.2% (18) with extended families, and 17.1% (29) with divided families. The sociodemographic variables are shown in Table I.

Of the participants, 24% (83) used cigarettes, 6.9% (24) used alcohol, and 8.1% (28) had been using substances for more than 1 year.

On the basis of the DSM-5-based psychiatric interview, the diagnoses of the participants were as follows: 22.8% (79) schizophrenia and other disorders with psychosis, 34.7% (120) depressive disorder, 21.4% (74) disruptive behavior disorder, 7.5% (26) trauma and related disorder, 7.8% (27) bipolar disorder, 6.1% (21) anxiety disorder, 7.2% (25) eating disorder, 6.1% (21) somatic disorder, 13.0% (45) neurodevelopmental disorder, and 5.5% (20) obsessive-compulsive disorder (OCD) (Table II).

In the analysis made in terms of demographic variables (gender, family type, living with, psychopathology in the family, income levels), no statistically significant difference was found between those who use cigarettes, alcohol, and drugs and those who do not (Table III).

Table I. Sociodemographic characteristics of the participants.

Characteristics		n	%
Gender	Female	232	67.1
	Male	114	32.9
Family type	Nuclear	269	77.7
	Extended	18	5.2
	Divided	59	17.1
Living with	Family	325	93.9
	Social service	13	3.8
	Relative	8	2.3
Income level	Low	169	48.8
	Middle	124	35.8
	High	10	2.9
Psychopathology in first-degree relatives	Yes	152	43.9
	No	194	56.1
Smoking	Yes	83	24.0
	No	263	76.0
Alcohol use	Yes	24	6.9
	No	322	93.1
Substance abuse	Yes	28	8.1
	No	318	91.9
Age	Mean±SS	Min-Max	
Length of stay (days)	15.42±1.38	12-18	
	25.30±24.32	1-175	

Table II. Psychiatric diagnoses received by the participants.

Primary Psychiatric Diagnosis		n	%
Schizophrenia and related disorders	Yes	79	22.8
	No	267	77.2
Depressive disorder	Yes	120	34.7
	No	226	65.3
Disruptive behavior disorders	Yes	74	21.4
	No	272	78.6
Trauma and related disorders	Yes	26	7.5
	No	320	92.5
Bipolar disorder	Yes	27	7.8
	No	319	92.2
Anxiety disorders	Yes	21	6.1
	No	325	93.9
Eating disorder	Yes	25	7.2
	No	321	92.8
Somatic disorder	Yes	21	6.1
	No	325	93.9
Neurodevelopmental disorders	Yes	45	13.0
	No	301	87.0
Obsessive-compulsive disorder	Yes	20	5.8
	No	326	94.2
Secondary psychiatric diagnosis	Yes	199	57.5
	No	147	42.5

Table III. Smoking, alcohol and substance abuse rates regarding demographic values.

Features		Smoking				Alcohol				Substance			
		Yes n (%)	No n (%)	X^2	p	Yes n (%)	No n (%)	X^2	p	Yes n (%)	No n (%)	X^2	p
Gender	Female	51 (22.0)	181 (78.0)	1.553	0.229	14 (6.0)	218 (94.0)	0.887	0.372	18 (7.8)	214 (92.2)	0.106	0.834
	Male	32 (28.1)	82 (71.9)			10 (8.8)	104 (91.2)			10 (8.8)	104 (91.2)		
Family type	Nuclear	66 (24.5)	203 (75.5)	0.199	0.906	19 (7.1)	250 (92.9)	0.806	0.668	21 (7.8)	248 (92.2)	0.262	0.877
	Extended	4 (22.2)	14 (77.8)			2 (11.1)	16 (88.9)			2 (11.1)	16 (88.9)		
	Divided	13 (22.0)	46 (78.0)			3 (5.1)	56 (94.9)			5 (8.5)	54 (91.5)		
	Family	80 (24.6)	245 (75.4)			20 (6.2)	305 (93.8)			26 (8)	299 (92)		
Who lives with?	Relative	2 (25.0)	6 (75.0)	1.968	0.374	2 (25.0)	6 (75.0)	5.789	0.055	0 (0)	8 (100.0)	1.638	0.441
	Social service	1 (7.7)	12 (92.3)			2 (15.4)	11 (84.6)			2 (15.4)	11 (84.6)		
Psychopathology in the relatives	Yes	38 (25.0)	114 (75.0)	0.152	0.395	11 (7.2)	141 (92.8)	0.038	0.504	10 (6.6)	142 (93.4)	0.835	0.239
	No	45(23.2)	149 (76.8)			13 (6.7)	181 (93.3)			18 (9.3)	176 (90.7)		
Income level	Low	36 (21.3)	133 (78.7)			8 (4.7)	161 (95.3)			16 (9.5)	153 (90.5)		
	Middle	36 (29)	88 (71.0)	2.425	0.297	10 (8.1)	114 (91.9)	1.595	0.451	9 (7.3)	115 (92.7)	1.997	0.368
	High	2 (20.0)	8 (80.0)			1 (10.0)	9 (90.0)			2 (20.0)	8 (80.0)		

In terms of psychiatric disorders, smoking, alcohol, and substance abuse were statistically similar for those experiencing schizophrenia, bipolar disorder, anxiety, eating disorders, somatic disorders, and neurodevelopmental disorders. Smoking was found to be significantly higher in those with depressive disorder and with trauma and related disorders (p=0.001, p=0.023). Smoking, alcohol, and substance abuse were found to be significantly higher in those with disruptive, impulse-control, and CDs (p=0.002, p=0.005, p=0.007). In the OCD group, smoking was found to be significantly lower (p=0.040) (Table IV).

Discussion

In the current study, the prevalence of smoking, alcohol, and substance abuse disorders was investigated by including 346 adolescents between the ages of 12 and 18 who had been hospitalized in a child and adolescent psychiatry inpatient unit, and their smoking, alcohol, and substance abuse was compared according to the diagnosed psychiatric disorder. When the data were analyzed, it was determined that 24% of the participants had used cigarettes, 6.9% had used alcohol, and 8.1% had used other substances for more than 1 year. It was found that smoking was higher in the depressive disorder and the trauma and related disorders group, and that cigarette, alcohol, and substance abuse were higher for disruptive, impulse-control, and conduct disorders. In the OCD group, smoking was found to be significantly lower.

According to the World Health Organization, 24 million adolescents aged 13–15 (17 million boys and 7 million girls) globally were reported to be current cigarette smokers. This averages out at 6.5–9% of boys and 4% of girls in this age group.[10] In a study investigating smoking and substance use among 1235 young people between the ages of 14 and 18 in 2020, the prevalence of smoking was found to be 15.8%, and the frequency of other addictive substances was 5%.[4] Dikeç et al.[11] found that the rate for smoking was 16.2%, alcohol use was 2.8%, and

Table IV. Relationship between psychiatric diagnoses and smoking, alcohol and substance abuse of the participants.

Diagnosis		Smoking		X^2	p	Alcohol		X^2	p	Substance		X^2	p
		Yes n (%)	No n (%)			Yes n (%)	No n (%)			Yes n (%)	No n (%)		
Schizophrenia and related disorders	Yes	14 (17.7)	65 (82.3)	2.205	0.177	5 (6.3)	74 (93.7)	0.058	0.809	7 (8.9)	72 (91.1)	0.081	0.776
	No	69 (25.8)	198 (74.2)			19 (7.1)	248 (92.9)			21 (7.9)	246 (92.1)		
Depressive disorder	Yes	41 (34.2)	79 (65.8)	10.438	**0.001**	12 (10.0)	108 (90.0)	2.671	0.102	14 (11.7)	106 (88.3)	3.156	0.076
	No	42 (18.6)	184 (81.4)			12 (5.3)	214 (94.7)			14 (6.2)	212 (93.8)		
Disruptive behavior disorders	Yes	29 (37.2)	49 (62.8)	9.610	**0.002**	11 (14.1)	67 (85.9)	8.011	**0.005**	12 (15.4)	66 (84.6)	7.200	**0.007**
	No	54 (20.1)	214 (79.9)			13 (4.9)	255 (95.1)			16 (6.0)	252 (94.0)		
Trauma and related disorders	Yes	11 (42.3)	15 (57.7)	5.174	**0.023**	2 (7.7)	24 (92.3)	0.025	0.875	3 (11.5)	23 (88.5)	0.449	0.503
	No	72 (22.5)	248 (77.5)			22 (6.9)	298 (93.1)			25 (7.8)	295 (92.2)		
Bipolar disorder	Yes	4 (14.8)	23 (85.2)	1.352	0.245	1 (3.7)	26 (96.3)	0.474	0.491	2 (7.4)	25 (92.6)	0.018	0.892
	No	79 (24.8)	240 (75.2)			23 (7.2)	296 (92.8)			26 (8.2)	293 (91.8)		
Anxiety disorders	Yes	4 (19.0)	17 (81.0)	0.299	0.584	2 (9.5)	19 (90.5)	0.232	0.630	1 (4.8)	20 (95.2)	0.333	0.564
	No	79 (24.3)	246 (75.7)			22 (6.8)	303 (93.2)			27 (8.3)	298 (91.7)		
Eating disorder	Yes	4 (16.0)	21 (84.0)	0.943	0.331	1 (4.0)	24 (96.0)	0.360	0.549	1 (4.0)	24 (96.0)	0.607	0.436
	No	79 (24.6)	242 (75.4)			23 (7.2)	298 (92.8)			27 (8.4)	294 (91.6)		
Somatic disorder	Yes	3 (14.3)	18 (85.7)	1.154	0.283	0 (0.0)	21 (100.0)	1.666	0.197	1 (4.8)	20 (95.2)	0.333	0.564
	No	80 (24.6)	245 (75.4)			24 (7.4)	301 (92.6)			27 (8.3)	298 (91.7)		
Neurodevelopmental disorders	Yes	8 (17.8)	37 (82.2)	1.094	0.296	2 (4.4)	43 (95.6)	0.498	0.481	1 (2.2)	44 (97.8)	2.397	0.122
	No	75 (24.9)	226 (75.1)			22 (7.3)	279 (92.7)			27 (9.0)	274 (91.0)		
Obsessive-compulsive disorder	Yes	1 (5.0)	19 (95.0)	4.197	**0.040**	0 (0.0)	20 (100.0)	1.582	0.208	0 (0.0)	20 (100.0)	1.896	0.172
	No	82 (25.2)	244 (74.8)			24 (7.4)	302 (92.6)			28 (8.6)	298 (91.4)		

substance use was 2.8% in adolescents who applied to a child psychiatry outpatient clinic. In the present study, the overall rate for smoking was found to be 24.0%, alcohol was 6.9%, and substance abuse was 8.1%, results higher than in the literature. These results are important in terms of showing that adolescents treated in an inpatient psychiatry clinic have a higher rate of smoking, alcohol, and substance use disorders. These high rates emphasize that it is necessary for clinicians to intervene in smoking, alcohol, and substance use disorders as well as treating the existing psychiatric disease. Our results concern adolescents receiving inpatient treatment, and there is no study in the literature with a similar methodology for comparison of the results. We think that it would be inappropriate to compare the data from this study on the frequency of smoking, alcohol, and substance use with the general population or outpatients in similar age groups. It is known that the frequency of cigarette, alcohol, and substance use is higher in individuals with existing psychiatric disorders than in the general population. In addition, considering that adolescents receiving tertiary health care and inpatient treatment are resistant to treatment, it is to be expected that the frequency of smoking, alcohol, and substance use is higher than in the general population and in patients followed in an outpatient clinic.

Previously, the rates of using addictive substances, both cigarettes and those other than cigarettes, were found to be significantly higher in male students than female students.[4] On the other hand, there are also studies showing that there is no gender difference regarding smoking among adolescents.[12] Smoking, alcohol, and substance abuse were found to be similar in all demographic variables, including gender, which may be related to the study group with existing psychiatric disorders.

The association of psychiatric disorders with smoking, alcohol, and substance use disorders has been increasing in recent years.[6] Although the etiology of this association is not clearly known, it is thought that either a causal relationship or a common etiological factor underlying both disorders may be responsible. Although information on smoking, alcohol, and substance abuse in psychiatric patients is included in the literature, studies investigating inpatients are limited. In a study investigating alcohol and substance abuse disorders in adult patients hospitalized in a psychiatric clinic in 2012, addiction or abuse problems were found at rates of 57.4% for nicotine, 21.9% for alcohol, and 18% for other substances.[9] In this study, smoking was found to be higher than alcohol and substance abuse, and the data support the literature.

Smoking was found to be higher in patients with a depressive disorder and with trauma and related disorders. In many studies, smoking is high in adolescents with previous depressive symptoms.[13,14] The results of a longitudinal epidemiological study of a population of 10,800 (adolescence to early adulthood) in the USA showed that the link between depressive symptoms and substance abuse was bidirectional. Another study found that higher levels of depression in adolescence were associated with more frequent abuse of psychoactive substances in early adulthood; it emphasized that more frequent substance abuse was also associated with an increase in the intensity of depressive symptoms after a few years.[15,16] In addition, nicotine dependence may be associated with anhedonia in patients with depression, and nicotine may have an effect on the hedonic system.[16]

Previous studies have shown a positive relationship between trauma and related disorders and smoking.[17,18] For example, daily cigarette use and nicotine addiction among people with post-traumatic stress disorder (PTSD) were found to be higher than among those without PTSD.[19] Breslau et al.[20] showed that individuals who had a traumatic event also increased their smoking after the trauma and PTSD symptoms. Yaşan et al.[18] found that the experience of trauma before the age of 16 in a smoker group was twice as high as in a non-smoker group, and this situation was associated with individuals being more

open to experiencing traumatic experiences in developing countries due to family relationships and social gender roles. The higher rate of smoking in trauma-related disorders in our study is also consistent with this literature.

In disruptive, impulse-control, and conduct disorders such as ODD and CD, the child or adolescent may be angry and in conflict with authority, may experience exclusion, and may be inclined to make friends with adolescents displaying similar behaviors. This situation poses a serious risk for delinquency and substance use behavior.[21] Longitudinal studies have found a strong positive relationship between the presence of externalizing behaviors in childhood and subsequent substance use in adolescence.[22] In a study that investigated the clinical characteristics of adolescents who were hospitalized in a child and adolescent substance treatment center, it was found that 80% of the cases had at least one comorbid psychiatric disorder, and of these, 46.3% were diagnosed with a conduct disorder.[23] A meta-analysis showed that individuals with ODD and CD were at high risk for substance-related disorders (the odds ratios were for alcohol: 1.73; for nicotine: 4.22; and for other substances: 4.86). In this study, as in the literature, cigarette, alcohol, and substance use were found to be significantly higher in the disruptive behavior disorder group.[24]

In the present study, smoking was found to be lower in the OCD group than in those without OCD. Although smoking is more common in psychiatric disorders than in the general population, may not apply to OCD. Some studies have shown that individuals with OCD have a significantly lower rate of smoking than the general population.[25,26] There are different opinions regarding the low smoking rate in these patients. Bejerot et al.[27] reported that non-smoking was mostly associated with an obsessive-compulsive personality disorder among individuals with OCD. In addition, these people do not like to take risks and do not exhibit impulsive behaviors related to smoking; it is also suggested that OCD-specific symptoms, such as fear of starting a fire, harm to the body, and fear of getting sick, can discourage smoking behavior.[28] Otherwise, one of the studies examining the relationship between OCD and smoking in adolescents did not find a significant difference in smoking status among adolescents with OCD, but it was suggested that this may have been due to the small sample size.[29]

In the adult population, smoking, alcohol, and substance use increase in disorders such as psychotic disorders, bipolar disorder, and anxiety disorders.[30-32] However, no difference was found among the groups in this study: the relatively small number of participants and the fact that the study was not a follow-up study may be the reasons for this result.

To the best of our knowledge, this is the first study to investigate the relationship between smoking, alcohol, and substance use and psychiatric disorders in adolescents receiving inpatient treatment in a psychiatry inpatient unit. The relatively large sample size and the inclusion of comorbid psychiatric diagnoses are strengths of our study. However, the fact that it is a retrospective study and that a structured scale was not used when evaluating smoking, alcohol, and substance use can be limitations.

In the current study, smoking was higher in the depressive disorder and the trauma and related disorders groups, and smoking, alcohol, and substance abuse were higher for disruptive, impulse-control, and conduct disorders. It should be kept in mind by health professionals working in this field that smoking, alcohol, and substance use in children and adolescents under psychiatric treatment in an inpatient service may increase their existing psychopathology. For this reason, awareness of the accompanying psychopathologies will contribute to the treatment of primary psychopathologies as well as smoking, alcohol, and substance use. To define the factors related to substance use in adolescents with a psychiatric diagnosis more clearly, studies beyond cross-sectional studies and with larger samples are needed.

Ethical approval

Ethical approval was obtained for this study from the Malatya Inonu University Health Sciences Non-Interventional Clinical Research Ethics Committee (2021/2327).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: AÇD, YED; data collection: AÇD, YED, GK analysis and interpretation of results: AÇD, GK, MEB, GT; draft manuscript preparation: AÇD, YED, ÖÖ. 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

Phenotypic and genotypic characteristics of children with Bartter syndrome

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ABSTRACT

Introduction. Bartter syndrome (BS) is a group of autosomal-recessive tubular disorders and it is classified into five genetic subtypes. BS can also be classified by phenotype (antenatal, classic). Patients with mutations in the same gene can present different phenotypes. In the present study, target gene sequencing was performed to evaluate the genotype-phenotype relationship.

Methods. Biochemical, clinical and renal ultrasonography results were collected at presentation and the last clinic visit. Genetic analyses were performed. The findings of patients with classical BS (cBS) and antenatal BS (aBS) at presentation and the last visit were compared.

Results. Our study included 21 patients (12 female, 57.1%) from 20 families with BS. The median age at diagnosis was 8 months and the median follow-up period was 39 months. The most frequent complaint was growth failure. We have found 18 different types of mutations in four genes, including nine in the *CLCNKB* gene, seven in the *SLCA12A1* gene, one in the *KCNJ1* gene and one in the *BSND* gene. In ten patients, nine different types of CLCNKB gene mutations were detected, five of them were novel. Seven different mutations in the SLC12A1 gene were detected in eight patients, five of them were novel. Compared to patients with aBS and cBS, prematurity was significantly higher in the group with aBS. Nephrocalcinosis was present in only one patient with cBS, all the ten hypercalciuric patients with aBS had nephrocalcinosis at the time of diagnosis and the last visit. The mean height standard deviation score (SDS) of patients with aBS were significantly lower than the cBS group at the time of presentation. The mean weight SDS at the time of presentation was worse in patients with aBS than in patients with cBS. The mean plasma potassium and chloride concentrations were significantly lower in the patients with cBS at the time of diagnosis.

Conclusions. This investigation revealed the mutation characteristics and phenotype-genotype relationship of our patients and provided valuable data for genetic counseling.

Key words: Bartter syndrome, phenotype, genotype, mutation.

Bartter syndrome (BS) is a group of autosomal recessive tubular disorders characterized by hypochloremic, hypokalemic metabolic alkalosis with hyperreninemic hyperaldosteronism but normal blood pressure with a prevalence of 1 in 100,000.[1,2] BS is a heterogeneous disorder both clinically and genetically, that can be classified into five genetic subtypes (Suppl. Table I). Loss of function mutations in five genes: *SLC12A1*, *KCNJ1*, *CLCNKB*, *BSND* and *MAGE-D2* encoding proteins involved in ions transportation in the thick ascending limb of loop of Henle (TAL) and distal convoluted tubule (DCT) result in BS type I, type II,

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type III, type IV and type V, respectively.[3] *SLC12A1* encodes the apical sodium-potassium-chloride cotransporter, NKCC2. *KCNJ1* gene encodes the apical voltage-dependent potassium channel, ROMK. *CLCNKB* gene encodes the basolateral chloride channel protein CIC-Kb. BSND gene encodes barttin, a β-subunit for CIC-Ka and CIC-Kb, expressed in the basolateral membrane of the TAL and cochlea. Loss of functions in alleles of both *CLCNKA* and *CLCNKB* are classified as BS type IVb, which results in a phenotype indistinguishable from that of BS type IVa. Recently, a novel transient form of aBS has been found to be caused by melanoma associated antigen-D2 (*MAGE-D2*) mutation, which is characterized by complete resolution of symptoms after birth, defined as BS type V[4] (Suppl. Table I). Gain of function mutation in the *CASR* gene encoding the basolateral calcium-sensing receptor, CASR, has been defined as BS type V in some reports.[5-8] But is nowadays classified as a Bartter like subform of familial hypocalcemia. BS type III is known as classical BS (cBS) whereas other types of BS are known as antenatal BS (aBS).

Typical findings of children with BS are growth retardation, polyuria, polydipsia, hypercalciuria and nephrocalcinosis. Different types of BS can be distinguished by different clinical manifestations. For example, type I BS presents with features of typical triangular facies, polyhydramnios, prematurity and hyperparathyroidism whereas patients with type II BS can have transient hyperkalemia. BS type III presents during early childhood with milder symptoms and generally without nephrocalcinosis, while type IV BS presents with sensorial deafness and mild hypochloremic alkalosis. Massive salt wasting and severe but transient hypochloremic metabolic alkalosis are characteristics of BS type V.[4,9] However, clinical findings may not always allow differentiation between subtypes, and even patients with mutations in the same gene may present different phenotypes.[10] For this reason, a gene-based classification is important for the definitive diagnosis.

In this study, we collected clinical, laboratory and renal ultrasonography results from patients with BS. We aimed to detect new pathogenic mutations by target gene sequencing. We planned to evaluate the genotype-phenotype correlation, especially the effects on growth, in the presence of newly identified mutations. The presentation and follow-up findings of our patients with classical and antenatal BS were also compared.

Material and Methods

The study was approved by the Institutional Ethics Committee of Marmara University Hospital (Protocol number: 09.2021.156). A written informed consent was obtained from all individual participants older than 18 years of age and the parents of all children included in the study. This study included 21 patients from 20 families with BS who were followed up in the Pediatric Nephrology Department of Marmara University Hospital. All patients in this study were hypokalemic and hypochloremic and had metabolic alkalosis, high renin and aldosterone levels with normal blood pressure. We classified BS into four types (I-IV). Patients with secondary BS or pseudo-BS were excluded

Genetic analysis

After detailed pedigree analyses and written informed consents were obtained, all patients' and their parents' total genomic DNA was extracted from peripheral blood using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). *CLCNKB* (NM_000085), *CLCNKA* (NM_001042704), *BSND* (NM_057176), *SLC12A1* (NM_000338), *KCNJ11*(NM_000220) genes were sequenced using Sophia Nephropathies Solution (NES) kit via Next-Generation Sequencing (NGS) (Illumina Nextseq 500). Since the used kit (NES) did not contain the MAGE-D2 gene, this gene could not be analyzed in patients. Single nucleotide polymorphisms (SNPs) and Copy number variations (CNVs) were analyzed through Sophia-DDM-v4 platform. Variants with minor allele frequency

(MAF) <1% according to population studies [ESP, ExAC, 1000 Genome (1000G), and Genome aggregation database (gnomAD)] were filtered and retained variants were searched in the Human genome mutation database (HGMD), Clinvar and Varsome databases. Pathogenicity scores were predicted using Mutation taster, Provean, Polyphen, Human Splicing Finder (HSF) and Sorting Intolerant From Tolerant (SIFT) in silico tools. Segregation analyses for detected mutations were performed and exon deletions were confirmed via ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Patients and biochemical analysis

Biochemical results, presenting symptoms and medical treatments were recorded at presentation and the last visit (respectively). Hypomagnesemia was defined as a serum magnesium level below 1.7 mg/dl and hypokalemia as a serum potassium level below 3.5 mEq/L. Hypochloremia was defined as a serum chloride concentration below 98 mEq/L. Hypernatremia was defined as a serum sodium level greater than 145 mEq/L. Normocalciuria was defined with the normal ranges for the patient's age.[11] Estimated glomerular filtration rate (eGFR) was calculated using the modified Schwartz formula, with a k-value of 0.413 and with serum creatinine measured by enzymatic method.[12]

Classical BS and aBS and also patients with missense and truncating mutations were compared in terms of demographic, biochemical characteristics, anthropometric data, urine examinations and renal ultrasonography (US) results. Renal US was performed at presentation in all children and repeated in follow-up.

Assessment of growth

Body height, weight and body mass index (BMI) standard deviation scores (SDS) were recorded at the presentation and the last visit according to the anthropometric references in Turkish children.[13] Frequency of height SDS <-2 and BMI SDS <-2 were also recorded. An increase in SDS more than +1 SDS was defined as improvement; a decrease in SDS more than -1 SDS was defined as deterioration; cases in between were defined as stable.

Statistical analysis

All data were analyzed using the Statistical Packages for the Social Sciences (SPSS Inc., Chicago, IL, USA) 21.0 package. Categorical variables were expressed as numbers and percentages. The normality of distribution for continuous variables was confirmed by the Shapiro-Wilk test. Results are expressed as mean with standard deviation (mean ± SD) in case of normal distribution and median (IQR: Q1-Q3; min-max) in case of non-normal distribution. Comparisons of numerical variables between two independent groups were evaluated using t-test and Mann-Whitney U test in normal distribution and non-normal distribution, respectively. In related samples of groups, the paired samples t-test was used in case of normal distribution and Wilcoxon test in case of non-normal distribution. Chi-square test was used for comparison of the categorical data. A p-value of <0.05 was considered statistically significant.

Results

Genetic analysis results; known and novel mutations

The detected mutations determined by the molecular genetics studies are shown in Table I. In the entire cohort, a compound heterozygous mutation was detected in only one patient (P10), with a novel mutation affecting one allele and a known mutation affecting the other. Homozygous mutations were detected in all other patients. The most common mutated gene was *CLCNKB*. In ten patients, nine different types of *CLCNKB* (NM_000085) gene mutations were detected, five of them were novel (there were one compound heterozygous and four homozygous patients for novel mutations).

Table I. Eighteen variants with 10 novel variants (showed in bold) in Bartter syndrome-related genes.

Patients	Clinical diagnosis	Gene	Status	Mutation	Position	Type of mutation	Reference
Type III+	Classical BS	*CLCNKB*	Homozygous	c.371C>T (p.Prol24Leu)	Exon 5	Missense	Simon et al., 1997
Type III+	Classical BS	*CLCNKB*	Homozygous	c.371C>T (p.Prol24Leu)	Exon 5	Missense	Simon et al., 1997
Type III	Classical BS	*CLCNKB*	Homozygous	**c.867-2delA**	Intron 8	Splice-site	Novel
Type III	Classical BS	*CLCNKB*	Homozygous	Exon 2-20 deletion	Exon 2-20	Gross deletion	Simon et al., 1997
Type III	Classical BS	*CLCNKB*	Homozygous	**c.499-2insG**	Intron 4	Splice-site	Novel
Type III+	Classical BS	*CLCNKB*	Homozygous	c.910C>T (p.Arg304*)	Exon 10	Nonsense	Messa et al., 2020
Type III+	Classical BS	*CLCNKB*	Homozygous	c.910C>T (p.Arg304*)	Exon 10	Nonsense	Messa et al., 2020
Type III	Classical BS	*CLCNKB*	Homozygous	**c.1930-2A>C**	Intron 18	Splice-site	Novel
Type III	Classical BS	*CLCNKB*	Homozygous	**c.499G>A (p.Gly167Ser)**	Exon 5	Missense	Novel
Type III	Classical BS	*CLCNKB*	Compound heterozygous	**c.66G>A (p.Trp22*)**	Exon 2	Nonsense	Novel
				c.865G>C (p.Gly289Arg)	Exon 9	Missense	Sahbani et al., 2020
Type IV	Antenatal BS	*BSND*	Homozygous	Exon 2-4 deletion	Exon 2-4	Gross deletion	Bircan et al., 2009
Type II++	Antenatal BS	*KCNJ1*	Homozygous	c.365T>A (p.Val122Glu)	Exon 5	Missense	Károlyi et al., 1997
Type II++	Antenatal BS	*KCNJ1*	Homozygous	c.365T>A (p.Val122Glu)	Exon 5	Missense	Károlyi et al., 1997
Type I	Antenatal BS	*SLC12A1*	Homozygous	c.596G>A (p.Arg199His)	Exon 4	Missense	Acar et al., 2019
Type I	Antenatal BS	*SLC12A1*	Homozygous	**c.2572C>T (p.Arg858*)**	Exon 21	Nonsense	Novel
Type I	Antenatal BS	*SLC12A1*	Homozygous	**c.1034C>A (p.Thr345Asn)**	Exon 8	Missense	Novel
Type I	Antenatal BS	*SLC12A1*	Homozygous	**c.2584A>T (p.Lys862*)**	Exon 21	Nonsense	Novel
Type I	Antenatal BS	*SLC12A1*	Homozygous	**c.2276G>A (p.Gly759Asp)**	Exon 18	Missense	Novel
Type I	Antenatal BS	*SLC12A1*	Homozygous	c.348dupT (p.Asn117*)	Exon 2	Nonsense	Adachi et al., 2007
Type I++	Antenatal BS	*SLC12A1*	Homozygous	**c.2485+5G>A**	Intron 20	Splice-site	Novel
Type I++	Antenatal BS	*SLC12A1*	Homozygous	**c.2485+5G>A**	Intron 20	Splice-site	Novel

+: unrelated patients, ++: related patients

Novel mutations were one nonsense (c.66G>A, p.Trp22*), one missense (c.499G>A, p.Gly167Ser) and three splice-site (c.867-2delA; c.499-2insG; c.1930-2A>C) mutations (Fig 1). Recurrent mutations were whole gene deletion (exon 2-20) and missense c.865G>C (p.Gly289Arg) mutation detected in one patient each, and missense c.371C>T (p.Prol24Leu) mutation and nonsense c.910C>T (p.Arg304*) mutation detected in two patients each. Seven different mutations in the *SLC12A1* (NM_000338) gene were detected in eight patients. Two missense (c.1034C>A, p.Thr34Asn; c.2276G>A, p.Gly759Asp) mutations, two nonsense (c.2572C>T, p.Arg858*; c.2584A>T, p.Lys862*) mutations and one splice-site (c.2485+5G>A) mutation were novel (Fig 2). *KCNJ1* (NM_000220) mutation (c.365T>A,p.Val122Glu) was detected in two patients who were related. One known gross deletion (exon 2-4 deletion) in the *BSND* (NM_057176) gene was detected in one patient. In the entire cohort, 9 (9/21) missense non-truncating mutations and 12 (12/21) truncating mutations were detected.

Patients' characteristics and biochemical analysis

Our study included 21 patients (12 female, 57.1%) from 20 families with BS. Fourteen consanguineous marriages were found in 20 families. The patients' demographic findings, biochemical parameters, urinary Ca/Cr ratios and renal US results at the time of presentation are summarized in Supp. Table II. The median age at diagnosis was 8 months (IQR: 4-18.5 months; min-max: 1-139 months). The most frequent complaint was growth failure (11; 52.3%) at diagnosis. Other frequent symptoms were vomiting (4; 19%) and polyuria-polydipsia (4; 19%). Four patients were diagnosed incidentally, one of them was diagnosed before an inguinal hernia operation, and three of them were diagnosed while being investigated for fever. Hypercalciuria was present in 16 patients (76.1%) and 11 patients had nephrocalcinosis (52.3%) at the time of presentation (Supp. Table II). Demographic, biochemical characteristics, medication at the last visit, and initial and final eGFRs of patients are given in Supp. Table III.

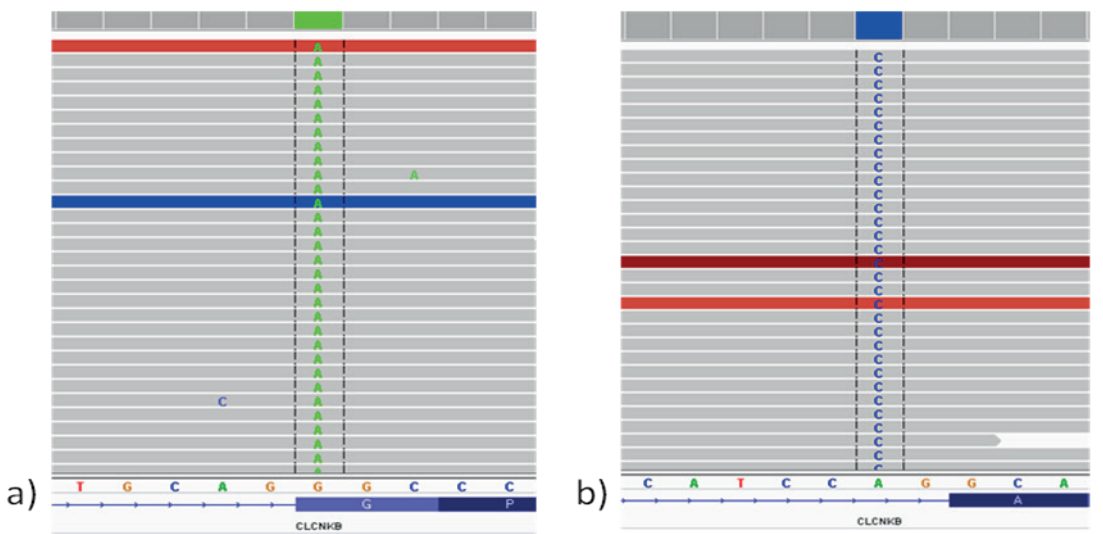

Fig. 1. The Integrative Genomic Viewer (IGV) sequence data of some novel mutations detected in *CLCNKB* gene (*NM_000085*) **a)** c.499G>A (p.Gly167Ser) **b)** c.1930-2A>C

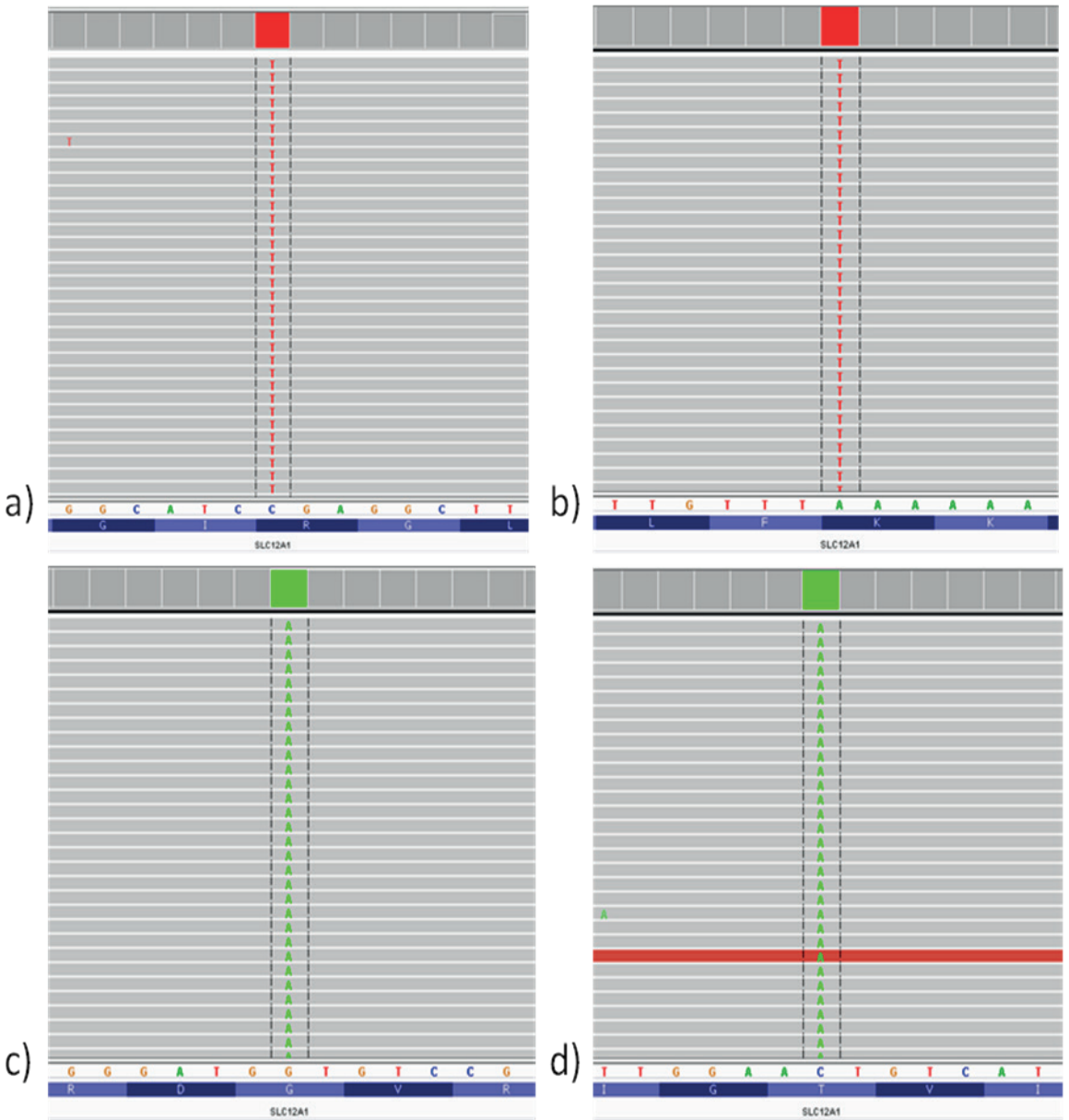

Fig. 2. The Integrative Genomic Viewer (IGV) sequence data of some novel mutations detected in *SLC12A1* gene (NM_000338) **a)** c.2572C>T (p.Arg858*) **b)** c.2584A>T (p.Lys862*) **c)** c.2276G>A (p.Gly759Asp) **d)** c.1034C>A (p.Thr345Asn)

The median age at the last visit was 48 months (IQR: 25-149 months; min-max: 14-245 months). The median follow-up period was 39 months (IQR: 17-105.5 months; min-max: 3-236). Medical treatment at the last follow-up visit included potassium supplementation (18; 85.7%), sodium supplementation (4; 19%), indomethacin (12; 57.1%) and spironolactone (10; 47.6%). The median eGFR was 79 ml/min/1.73m^2 (IQR: 56-113.5 months; min-max: 31-172 ml/min/1.73m^2) at presentation and 101 ml/min/1.73m^2 (IQR: 77-117 months; min-max: 8-192 ml/min/1.73m^2) at the last clinic visit.

Assessment of growth

At the time of presentation, the mean height SDS, weight SDS and BMI SDS were -2.32±1.39; -3.54±2.10 and -3.44±3.02, respectively. At the last visit the mean height SDS, weight SDS and BMI SDS were -2.00±2.03; -1.91±2.27 and -0.64±1.79, respectively. The improvements in the last weight SDS and BMI SDS according to the first presentations of the patients were found to be statistically significant (p=0.003; p<0.001, respectively), but there was no statistically significant difference in height SDS. Similarly, when the aBS and cBS groups were evaluated separately, there was an improvement in weight and BMI SDS in both groups at the initial and final visits, but no difference was found in height SDS (Table II). At the time of presentation, the percentage of patients with height SDS <-2 and BMI SDS <-2 were 57.1% (12/21) and 61.9% (13/21), respectively. At the last visit the percentage of patients with height SDS <-2 and BMI SDS <-2 were 47.6% (10/21) and 23.8% (5/21), respectively. The percentage of patients with a final BMI SDS <-2 according to the first presentation was found to be significantly higher (p=0.008). There was no difference in the percentage of those with height SDS <-2. On the other hand, according to the changes in height SDS over time, ten patients (47.6%) remained stable, eight patients improved (38%) and only three patients deteriorated (14.2%).

Comparison of the initial and final findings of patients with classical BS and antenatal BS

The presenting and follow-up findings of 10 patients with cBS and 11 patients with aBS were compared (Table II). There was no difference between the two groups in terms of age of diagnosis and follow-up time. Prematurity was significantly higher in the patients with aBS, compared to the patients with cBS (p=0.017). As a presenting complaint, growth failure was more frequent in the cBS group (7/10; 70%) than aBS group (4/11; 36.3%); but polyuria-polydipsia was more frequent in the aBS group (1/10; 10% vs. 3/11; 27.2%). The mean plasma potassium and chloride concentrations were significantly lower in the patients with cBS at the time of diagnosis (p=0.009 and p=0.020, respectively). Hypercalciuria was non-significantly lower in patients with cBS than patients with aBS at the presentation (6/10; 60% vs. 10/11; 91%) and the last visit (1/10; 10% vs. 6/11; 57.5%) Nephrocalcinosis was present in only one patient with cBS, this patient had a truncating mutation, on the other hand, all the ten hypercalciuric patients with aBS had nephrocalcinosis at the time of diagnosis and the last visit. We had only one patient in the aBS group who was normocalciuric and did not have nephrocalcinosis. (Table II, Suppl. Table II). The mean height SDS at the time of presentation was significantly worse in patients with aBS than in patients with cBS (p=0.03). Similarly, at the last visit the mean height SDS of patients with aBS was lower than patient's with cBS with a border p-value (p = 0.051). The percentage of patients with height SDS <-2 at the presentation and the last visit were higher in aBS group than cBS (p=0.03 and p=0.08, respectively). The mean weight SDS at the time of presentation was worse in patients with aBS than in patients with cBS, very close to statistical significance (p=0.06). Other initial and final anthropometric values (weight SDS at the last visit, BMI SDS and percentage of patients with BMI SDS <-2) of both groups were not statistically different (Table II). Although the difference was statistically not significant, eGFR at presentation and the last visit was worse in the antenatal group than in the classical group (Table II). In our study, the only patient who had chronic kidney disease (CKD) stage V at the last visit and underwent hemodialysis was in the aBS group.

Comparison of the initial and final findings of patients with classical BS

The median age was 11.5 months (IQR: 6.2-18 months; min-max: 1-76 months) and 49 months (IQR: 25-77.5 months; min -max:18-196 months) at diagnosis and the last visit, respectively.

With the effect of treatment, the mean plasma sodium, potassium and chloride concentrations were significantly higher at the last visit (p=0.008, p=0.00, p=0.12, respectively). The mean BMI SDS at presentation was significantly higher than the mean BMI SDs at the last follow-up (p=0.032) (Table II).

Comparison of the initial and final findings of patients with antenatal BS

The median age was 5 months (IQR: 3-20 months; min-max: 2-139 months) and 47 months (IQR: 23-189 months; min-max: 14-245 months) at diagnosis and the last visit respectively. The mean plasma potassium concentrations were

Table II. Comparison of the findings of patients at presentation and at last visit according to clinical groups: classical Bartter syndrome (cBS) and antenatal Bartter syndrome (aBS)

Parameters	At presentation		p	At last visit		p	p*	p**
	Classical BS (n=10)	Antenatal BS (n=11)		Classical BS (n=10)	Antenatal BS (n=11)			
Female/male	5/5	7/11	0.520				-	-
Age at presentation, months	17.3±21.4	25.4±44.5	0.387				-	-
Age at the last visit, months				61.7±52.9	103.9±93	0.756	-	-
Follow-up, months				44.4±35.9	78.4±72.6	0.189	-	-
Prematurity, n (%)	3 (30.0)	10 (90.9)	**0.017**				-	-
Polyhydramnios, n (%)	6 (60.0)	9 (81.8)						
Growth failure, n (%)	7 (70.0)	4 (36.3)	-			-	-	-
Polyuria-Polydipsia, n (%)	1 (10.0)	3 (27.2)	-	1 (10.0)	3 (27.2)	-	-	-
Vomiting, n (%)	3 (30.0)	0 (0)	-	3 (30.0)	0 (0)	-	-	-
Incidental, n (%)	1 (10.0)	3 (27.2)	-	2 (20.0)	3 (27.2)	-	-	-
Hearing loss, n (%)	0 (0)	1 (9.1)	-	0 (0)	1 (9.1)	-	-	-
Blood pH	7.53±0.8	7.48±0.09	0.219	7.48±0.06	7.45±0.06	0.263	0.086	0.379
Blood HCO^3 (mmol/L)	36.6±9.5	30.6±5.7	0.104	33.4±5.4	33.6±9.7	0.970	0.267	0.371
Plasma sodium (mEq/L)	133.6±5.1	138.6±6.8	0.072	138.7±2.8	140.2±5.9	0.482	**0.008**	0.540
Plasma potassium (mEq/L)	2.5±0.4	3.1±0.5	**0.009**	3.4±0.5	3.6±0.6	0.490	**0.000**	**0.011**
Plasma chloride (mEq/L)	84.4±10.03	93.2±7.73	**0.020**	93.8±3.76	95.3±10.66	0.656	**0.012**	0.283
Plasma calcium (mg/dl)	10.6±0.81	10.8±0.81	0.654	10.6±0.53	10.1±0.62	0.052	0.798	**0.027**
Plasma magnesium (mg/dl)	2.2 ± 0.5	2.4 ± 0.4	0.352	1.9±0.3	2.2±0.5	0.173	0.059	0.319
Hypercalciuria, n (%)	6 (60.0)	10 (90.9)	0.121	1 (10.0)	6 (54.5)	0.063	0.063	0.25
Nephrocalcinosis, n (%)	1 (10.0)	10 (90.9)	**0.001**	1 (10.0)	10 (90.9)	**0.001**	1	1
eGFR (ml/min./1.73m^2)	102.3± 40	77.4±37.6	0.159	119.9±40.7	83.2±34.7	0.173	0.203	0.624
Height SDS	-1.64±1.16	-2.9±1.3	**0.03**	-1.2±1.4	-2.8±2.3	0.051	0.354	0.803
Height SDS<-2, n (%)	3 (30.0)	9 (81.8)	**0.03**	2 (20.0)	6 (54.5)	0.08	1	0.5
Weight SDS	-2.6±1.6	-4.4±2.2	0.06	-1.5±1.5	-2.3±2.8	0.973	0.056	**0.026**
BMI SDs	-2.5±1.9	-4.3±3.6	0.314	-1.1±1.5	-0.3±1.9	0.291	**0.032**	**0.003**
BMI SDs <-2, n (%)	7 (70.0)	7 (63.6)	0.659	3 (30.0)	2 (18.1)	0.635	0.125	0.125
CKD stage, n (%)								
Stage II	3 (30.0)	3 (27.2)	-	2 (20.0)	3 (27.2)	-	-	-
Stage III	1 (10.0)	5 (45.5)	-	0 (0)	2 (18.1)	-	-	-
Stage V	0 (0)	0 (0)	-	0 (0)	1 (9.1)	-	-	-

BS: Bartter syndrome, CKD: chronic kidney disease, eGFR: estimated glomerular filtration rate, BMI: body mass index, SDS: standard deviation score.
Stage II: e GFR 60-90 ml/min./1.73m^2; Stage III: eGFR 30-59 ml/min./1.73m^2; Stage V: eGFR <15 ml/min./1.73m^2
P *: comparison of findings at the time of presentation and at the last visit in cBS group.
P** : comparison of findings at the time of presentation and at the last visit in aBS group.

significantly higher at the last visit (p=0.011) and plasma calcium concentrations were significantly lower at the last visit (p=0.027). The mean weight SDS and BMI SDS were significantly higher at the last visit (p=0.026 and p=0.003, respectively) (Table II).

Comparison of the findings of patients with missense and truncating mutation

The median age of patients with a missense mutation was 7 months (IQR: 4-10.5 months; min-max: 3-15 months) at diagnosis. The median age of patients with a truncating mutation was 14 months (IQR: 3.5-62.25 months; min-max:1-139 months). The difference was statistically significant (p=0.034). BMI SDS were significantly higher at the last visit both in patients with missense mutation and truncating mutation (p=0.006 and 0.006, respectively). BMI SDS at the last visit were also significantly higher in patients with truncating mutation than missense mutation (p=0.021) (Suppl. Table IV).

Discussion

The present study describes the clinical features and genotypes of 21 children with BS from a single center and it also evaluates the clinical, radiological and biochemical data of patients with type I-II-IV BS known as aBS and patients with type III BS known as cBS.

The most commonly affected gene was *CLCNKB* and this result was compatible with previous studies.[3] c.867-2delA mutation was not reported in population studies. The mutation taster predicted this variant as pathogenic, but according to HSF, it had no significant impact on splicing signals. c.499-2insG and c.1930-2A>C mutations which were not reported in population studies, were predicted as pathogenic according to mutation taster and HSF. The c.499G>A (p.Gly167Ser) mutation was not reported in gnomAD, 1000G and exAC databases and was predicted as pathogenic according to in silico tools. The c.1930-2A>G mutation at the same position was previously reported.[14] The c.66G>A (p.Trp22*) variant was considered pathogenic because it caused premature protein termination and was not reported in public databases.-

Previous studies from different countries had shown that whole gene deletion was the most common mutation in *CLCNKB* gene, this mutation was detected in only one patient in our cohort.[3,15-17] This finding indicates that Turkish patients may have different ancestry. Although the c.1830G>A (p.Trp610*) mutation in the *CLCNKB* gene was one of the common mutations in Japanese and Korean patients[3,18], the absence of this mutation in all of our patients supports this hypothesis. In addition, the fact that we could not detect any hot spot mutations in our cohort also showed the genetic diversity in Turkish patients.

Seven different *SLC12A1* gene (NM_000338) mutations, five of which were novel, were detected in eight patients. The allele frequency of the novel c.2572C>T (p.Arg858*) and c.2584A>T (p.Lys862*) mutations were 0.00000797 and 0.00000398 in gnomAD, respectively. Both variants were extremely rare and were considered pathogenic because they had a truncated effect on the protein. c.1034C>A (p.Thr34Asn) mutation's allele frequency was 0.00003185 in GnomAD. Provean, SIFT, Polyphen and Mutation taster in silico tools predicted this mutation as pathogenic. The c.2276G>A (p.Gly759Asp) mutation was not reported in GnomAD, ExAC and 1000G databases and was predicted as pathogenic via in silico tools. Both novel missense mutations altered the conserved amino acid residues. c.2485+5G>A mutation's allele frequency was 0.000004013 in GnomAD and predicted as pathogenic according to Mutation taster and HSF in silico tools.

Among our patients, a known gross deletion (exon 2-4 deletion) in the *BSND* gene (NM_057176) was found in one patient. Mutations in the *BSND* gene cause type IV BS. Our patient presented with severe clinical symptoms. Hearing loss is the most important

characteristic of this type of BS and hearing loss was one of the presenting symptoms of our patient. Remarkable biochemical and clinical features of our patient were extremely low levels of serum chloride and the lowest initial eGFR with 31 ml/min./1.73 m^2. *BSND* mutations are one of the factors that have been reported to influence renal survival.[3] Bircan et al.[19] reported the same mutation in a 2-month-old boy. There were some similarities between the case presented in this report and our patient. Both patients needed a large amount of fluid and electrolytes, which could only be provided through a nasogastric tube and gastrostomy. Our patient was the only patient in the antenatal group who did not have hypercalciuria or nephrocalcinosis. In the patient reported by Bircan et al.[19], transient hypercalciuria was present. But Spanish, Turkish, Italian and Portuguese reports have described different mutations with hypercalciuria or normocalciuria.[20-23] Case presentations and series do not agree on hypercalciuria. In fact, inhibition of NaCl reabsorption in cBS causes hypercalciuria, whereas decreased NaCl reabsorption in the DCT is associated with hypocalciuria as in Gitelman syndrome.[22] The absence of hypercalciuria in BS type IV unlike other antenatal types can be explained by the fact that these two opposite effects are present in BS type IV because of the extent of the functional loss of barttin in the kidney tubules.[22]

In BS type II with *KCNJ1* mutations, transient hyperkalemia and also metabolic acidosis can be seen. In this study, *KCNJ1* (NM_000220) mutation (c.365T>A, p.Val122Glu) was detected in two patients (P12, P13) who were related. They were neither hyperkalemic nor acidotic, however, the bicarbonate values of these patients were not very high and even close to normal (36.6 mEq/L and 27.2 mEq/L, respectively) and the potassium values were also not low enough to need replacement and were even normal (3.9 mEq/L and 3.6 mEq/L, respectively).

An earlier age of diagnosis is mostly typical for aBS[9] and, in our cohort, the age at diagnosis was lower in the aBS group with 5 months compared to the cBS group with 11.5 months; but it was not statistically significant. This finding was consistent with other studies[3,15,24] but note that, at 84 months and 139 months, the two eldest children of our cohort were in the aBS group. A novel homozygous splice-site mutation (c.2485+5G>A) in *SLC12A1* gene was detected in these two patients who were cousins. It is not yet known whether this new mutation will affect the age at diagnosis in patients with type I BS.

Recently, it has been shown that patients with BS may be complicated by a nephrogenic diabetes insipidus (NDI) like phenotype.[25] Although most of our patients had normal serum sodium levels, we had two patients, who had remarkable hypernatremia (P15, P16). These two patients were diagnosed with BS type I with no identifiable mutations in AVPR2 or AQP2. Also, the serum sodium levels of those patients were not as high as they might be in patients with NDI. Secondary NDI seen in BS patients has been reported to be specific for BS type I and II, but it is unlikely to be a mutation specific complication.[25] Serum sodium levels were not statistically different in patients with cBS and aBS. But note that, hyponatremia was detected and treated only in the cBS group and in one patient with BS type IV in our cohort. Our patients with BS type I and II were normonatremic or hypernatremic. It is well known that hypochloremia and hypokalemia are more severe in patients with cBS.[9] In our study, plasma potassium and chloride concentrations were significantly lower in cBS group, as expected.

Growth retardation seen in BS is known to be the result of volume depletion.[26] In our cohort, the most common presenting complaint in the aBS group was polyuria-polydipsia and growth retardation was more common in the aBS at the last visit (Suppl. Table II). All findings in our study show that while there was an improvement in weight and BMI SDS in our patients at follow-up, there was no significant improvement in height SDS. Growth hormone (GH) deficiency has been reported in children

with BS.[27] Hochberg et al.[28] showed GH and insulin-like growth factor-1 did not stimulate longitudinal growth unless hypokalemia was corrected. In our study, GH was used in only one patient (P14), who was diagnosed with BS type I. She had one of the lowest initial eGFR among all patients and needed hemodialysis during follow-up.

The mechanism of CKD development is multifactorial. Nephrocalcinosis, chronic hypokalemia, long-term treatment with NSAIDs, damaging effect of elevated aldosterone levels on podocytes and BSND mutations are some of the possibilities for CKD development in patients with BS.[24] Prematurity is also thought to be a cause of CKD development.[24] It is assumed that patients with aBS are often born prematurely.[29-31] Similarly, prematurity was seen more commonly in our patients with aBS than cBS (90% vs 30%) (Table II). The fact in our study that eGFR values were lower in the antenatal group, also supports the relationship between prematurity and CKD.

One of the reasons for CKD is nephrocalcinosis and it is less frequently seen in the cBS group than in the aBS group. Hypercalciuria and nephrocalcinosis are more common in BS Type I and II due to the defective cotransporter NKCC2 as in BS type I and the defective ROMK activity as in BS type II.[32] Similarly, we detected hypercalciuria and nephrocalcinosis in almost all (10/11; 90.9%) patients with aBS in our study. Also, consistent with previous studies, in our cohort, nephrocalcinosis was detected in only one patient with cBS who had hypercalciuria. But note that, this patient had the highest level of calciuria among patients with cBS and one of the highest levels in the entire cohort. It has been reported that impaired salt reabsorption in TAL can lead to an impaired paracellular cation uptake, mostly manifesting as hypercalciuria as in *CLCNKB* mutations.[33]

Although few studies[15,17] have shown that there is a genotype-phenotype correlation in patients with cBS, many studies[34,35] have not found a significant genotype-phenotype correlation. One recent study[34] covering 30 patients with cBS detected no genotype-phenotype association, whereas Seys et al.[17] reported an association between complete loss of function (CL/CL) mutations of *CLCNKB* and severe phenotypes. Although we did not demonstrate by functional analysis, there was only one large deletion (Exon 2-20 deletion, P4) in our cohort that could be predicted to cause complete loss of function. This patient had the lowest eGFR at presentation and one of the lowest eGFR at the last visit among patients with *CLCNKB* mutation.

Similar phenotypic characteristics were not found even among patients harboring the same mutation. Additionally, intrafamilial variability in clinical manifestations was also present. This poor genotype-phenotype correlation may be associated with modifier genes, environmental factors, or epigenetic mechanisms. However, when aBS and cBS patients were compared, significant phenotypic differences were found between them, (even between types of antenatal groups) as mentioned above. In addition, we have not found any significant difference between patients with truncating mutation and missense mutation except in terms of presenting age and final BMI SDs (Suppl. Table IV). In addition, the only patient with nephrocalcinosis and cBS also had a truncating mutation. This makes us think that the localization of the mutation and the protein it affects are more related to the phenotype than whether the mutation is truncating or not.

The most important limiting factor of our study is the relatively low number of patients. Nonetheless, the strength of our study is that the diagnosis of our patients was confirmed by a very detailed genetic analysis. And it revealed the long-term outcome with respect to growth and CKD and phenotype-genotype relationship of our patients. We were also not able to investigate the *MAGE-D2* mutation because the kit (NES) we used did not contain the MAGE-D2 gene so this gene could not be

analyzed. However, further studies are planned to design specific primers analyzing this gene in panel negative patients. In addition, it would have been much better if we could have looked at the difference in findings of patients with truncating and non-truncating mutations in the aBS and cBS groups, and even in patients with a single type of mutation. Unfortunately, our patient numbers were not sufficient for these comparisons. For all these reasons, to explain the genotype-phenotype correlation with all its clarity, there is a need for prospective studies with longer follow-up periods, in which all genes that may cause BS are studied and more patients can participate.

In conclusion, we present the clinical features, molecular diagnosis and the prognosis of 21 children with BS. We found ten novel mutations and this investigation further expanded the mutation spectrum of BS and provided valuable data for genetic counseling. Although CLCNKB gene mutations were the most common type of mutation seen in our patients, NGS panels are pivotal when searching for disease-causing genes, especially in populations having genetic diversity like Turkish people.

Ethical approval

The study was approved by the Institutional Ethics Committee of Marmara University Hospital (Protocol number: 09.2021.156).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: SG, IG, CA; data collection: SG, CA, EDB, MS, SP, ONT, PA; analysis and interpretation of results: SG, NC; draft manuscript preparation: SG, IG, NC, CA. 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.

Supplementary information is available at: http://www.turkishjournalpediatrics.org/uploads/turkjped.2021.4697.S1.pdf

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Original Article

Complicated acute appendicitis in children: the importance of stewarding antibiotic prescriptions

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ABSTRACT

Background. The aim was to assess the success of a three-drug regimen, consisting of cefazoline, metronidazole and gentamicine, for the antimicrobial treatment of complicated appendicitis and to investigate predictors of failure.

Methods. This retrospective study included patients who had undergone appendectomy for complicated appendicitis from 2013 to 2018. The shift to second-line antibiotics was considered a failure. The choice was based upon clinical deterioration. Patients were grouped into 2 groups: localized complicated appendicitis (LCA) and extensively complicated appendicitis (ECA) for the study purpose. Univariate and multivariate analysis were performed to identify predictors of failure.

Results. Ninety patients (65.2%) with LCA and 48 patients (35%) with ECA were included. Three-drug regimen failed in 50 patients (36%) with a higher rate in the ECA group (50%, p=0.017). In a multivariate analysis, this failure was found to be associated with ECA (adjusted OR 3.00 [1.2-7.4], p=0.041). Children with ECA experienced a longer hospital stay (median length 8 days, p<0.001) and antimicrobial therapy (median length 8 days, p<0.001). However, no difference in the rate of surgical site infections was found (p=0.514).

Conclusions. The institutional antibiotic stewardship program highlighted a high failure rate for the old three-drug regimen. A new protocol should be recommended, especially for the patients affected by ECA.

Key words: complicated acute appendicitis, three-drug regimen, antibiotics, antibiotic stewardship, surgical site infections.

Acute appendicitis is one of the most common urgencies in pediatric surgery.[1] The disease might be complicated in 25-39% of pediatric cases.[2] The definition of complicated acute appendicitis (CAA) might be controversial because it is currently based on intra-operative findings. Nevertheless, a recent multicenter study by Cameron et al.[3] identified four intraoperative features pathognomonic for CAA: visible perforation, purulent exudate, free fecalith and intra-abdominal abscess. It is crucial to formulate the correct diagnosis and to describe the extension of peritoneal contamination because CAAs are associated with worse clinical outcomes, higher rates of complications and higher costs.[4,5] Recently, the number of abdominal quadrants involved in the infectious process has been described as a predictor of the outcome.[6]

Furthermore, CAAs required a more demanding antibiotic therapy to control the source of infection. This aspect was remarked to the international guidelines for the treatment of complicated intra-abdominal infections.[7]

For many years, a three-drug regimen, consisting of aminoglycoside together with a first-generation cephalosporine and metronidazole, has been the first choice in the treatment of

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complicated intra-abdominal infections. This empirical triple association provided broad coverage for the commonest pathogens.[8]

Antimicrobial - resistant strains increased from 25.2% to 42.1% over the last decade, mainly due to the growth of extended spectrum beta-lactamase (ESBL) producing *E. coli* and the emergence of *Pseudomonas aeuroginosa*.[9] For this reason, broad-spectrum beta-lactamase and carbapenemes were successfully introduced for the treatment of CAAs. Moreover, these latter drugs might be used as monotherapy providing further benefits in terms of compliance and costs.[10,11]

The significant variability in the choice of antibiotics together with the increase of antimicrobial resistance and the potential side effects related to antimicrobial misuse required the establishment of antimicrobial stewardship. These programs are extremely important to optimize patients' clinical outcomes and minimize exposure to unnecessarily broad-spectrum drugs.[12]

The primary aim of this study was to describe our experience with the use of a three-drug regimen in the treatment of CAAs and to assess its rate of success. The secondary aim was to investigate potential predictors of treatment failure, especially the extension of peritoneal contamination. This process should help clinicians in the choice of the proper antibiotic regimen and in the identification of patients who required an escalation of antibiotics.

Material and Methods

Study Design

Our Institutional Review Board was notified. This was a retrospective and observational study and exempted from approval.

The 6-year period of the study ranged from January 2013 to December 2018. All children aged <18 years, who had undergone an appendectomy for CAA within 48 hours of admission to Padua University Hospital (Italy), were included. The diagnosis of CAA was based on the intra-operative findings of a necrotizing or visible perforated appendix, the presence of purulent exudate, extraluminal fecalith or intra-abdominal abscess.[3] Abdominal ultrasound was performed only in the case of doubtful clinical diagnosis of acute appendicitis.

As of November 2017, conservative therapy, and delayed appendectomy for CAAs with abscess were proposed, but these patients were excluded from the study. Among the cohort of patients affected by CAA, we excluded the patients who did not receive the standard three-drug regimen at the time of diagnosis.

Demographic data, blood test results (white blood cells (WBC) count, C-reactive protein (CRP) value, operative findings from standardized surgical reports and antibiotic therapy administered were collected. Patients included were further divided into two groups according to the severity of the abdominal infection. As previously reported in the literature, CAAs were categorized as localized complicated appendicitis (LCA) or extensively complicated appendicitis (ECA).[4-6] The ECA subgroup was characterized by the involvement of two or more abdominal quadrants in the infectious process.

Preschool age (<6 years), WBC > 18 x 10^9/L and CRP value > 100 mg/L were considered as potential predicting factors of severity according to literature and clinical experience.[13,14]

As to the primary outcome, the assessment of the treatment failure of the three-drug regimen was based on the rate of shift to a broader antimicrobial regimen. The escalation was indicated in the case of persistent fever together with worsening of clinical symptoms (i.e., abdominal tenderness, feeding intolerance, diarrhea and dysuria) after 72 hours from the beginning of the antibiotics. The length of antibiotic therapy and the length of hospital stay, the rate of surgical site infections (SSI) and organ/space surgical site infections

(OSSI), according to the CDC definition[15], were considered as a measure of success of the treatment.

Electronic medical charts were searched for long-term complications related to the CAA that occurred after the patient was discharged from the hospital.

Treatment protocol

Once the diagnosis of CAA was intra-operatively established by the surgeon, a three-drug regimen was administered. This protocol was established during previous meetings with infectious diseases specialists from our Department and consisted of intravenous cefazoline 30 mg/kg/dose every 8 hours (maximum dose 1 g), metronidazole 7.5 mg/kg/dose (maximum dose 500 mg) every 8 hours and gentamicin 5 mg/kg/dose. In most cases, gentamicin was added after the intraoperative confirmation of a CAA. The interval between the last shot of antibiotics and the surgical procedure should be no more than 60 minutes. The therapy continued for at least 5 days after the intervention. No intraoperative specimen of purulent fluid was routinely collected for microbiological culture.

The placement of an abdominal drain depended on the senior surgeon's decision.

If the patient was still febrile with clinical signs of abdominal involvement (i.e. abdominal tenderness, intestinal obstruction, diarrhea, vomits) after 72 hours from the intervention, that is to say half-course of the antibiotic therapy, blood tests were repeated. Urinary tract infection was excluded by performing a urine analysis. In case of respiratory symptoms, a chest X-ray was performed to exclude pneumonia or pleural effusion. After that, a shift to another regimen was taken into account. Our practice consisted in introducing a third-generation cephalosporin (ceftazidime 25 mg/kg/dose every 8 hours) to replace cefazolin, whilst maintaining the other two antibiotics unaltered. Ceftriaxone 75 mg/kg/day or broad spectrum beta-lactams (amoxicillin-clavulanate 30 mg/kg/dose every 8 hours, piperacillin-tazobactam 100 mg/kg/dose every 6 hours) were used in a few cases, after consultation with the infectious diseases specialist. In both cases, the second-line regimen continued for 5 days or longer, according to the clinical response.

Statistics

Categorical variables were reported as numbers (%) and they were compared by using Fisher's exact tests. Continuous variables were reported as median (M) and inter-quartile range (IQR). Odds-ratio of the risk factors for treatment failure were calculated by logistic regression analysis.

A p-value ≤ 0.05 was considered statistically significant.

Logistic regression and multivariate analysis were performed using the Windows version 9.4 of the program SAS (SAS Institute Inc., Cary, NC, USA).

Results

During the 6-years, 143 children underwent appendectomy for CAA in our tertiary-care hospital. Five patients (two ECAs and three LCAs) were excluded because they did not receive the standard three-drug regimen. Most of the patients were referred from other hospitals.

One hundred and thirty-eight children were eligible for the study. At the time of the diagnosis, the median age was 9.1 years (IQR 6.0-11.6 years), the median value of the WBC count was 17 x 10^9/L (IQR 14-21 x 10^9/L), and the median value of CRP was 61 mg/L (IQR 28-134 mg/L). The median hospital stay was 7 days (IQR 6-8 days) similar to the median duration of the antibiotic therapy (7 days, IQR 5-8 days).

We identified 90 LCAs (65%), including 38 appendicular abscesses (28%) that were treated by surgery in first instance. The rest of the population (48 children, 35%) was affected by

ECA (Table I). None of them presented with signs of septic disease at diagnosis.

Eleven (8.0%) patients developed SSI. There were five OSSIs (two patients with ECA) among them. A laparotomy was required in one case, and it was due to the presence of abdominal collection together with intestinal occlusion. The other four patients were admitted to the hospital for clinical observation and administration of parenteral antibiotic therapy.

The median follow-up was 2.4 years, ranging from 0.2 to 5.9 years. No episodes of intestinal occlusion or late re-admission were reported in this span of time.

The standard three-drugs antibiotic regimen was shifted to a second-line regimen in 50 cases (36%) (Table II). Moreover, four patients were not responsive to the second-line regimen either. These children presented with persistent fever, increased CRP-values and altered WBC count after 72 hours after the first shift of the antibiotic therapy. Three of them were in the ECA group. A further antimicrobial escalation was required, consisting of piperacilline-tazobactam in three of them. Meropenem (40 mg/kg/dose) was given only in one case.

Table II. Antibiotic agents used in second line.

Ceftazidime, n (%)	38 (76)
Piperacillin-tazobactam, n (%)	7.0 (14)
Amoxicillin-clavulanate, n (%)	3.0 (6.0)
Ceftriaxone, n (%)	2.0 (4.0)

Among the preoperative variables, the age at intervention was higher in the LCA group (p=0.016). Indeed, these children presented with a median age of 9.6 years, while the median age of those affected by ECA was 7.6 years. However, WBC count (p=0.617) and CRP value (p=0.197) were similar before the intervention. Furthermore, it is relevant to report that WBC-count and CRP-value were poor predictors of severity.

Six patients (6.7%) affected by LCA suffered from SSI, whilst 5 patients (10%) in the ECA group suffered from SSI. Nevertheless, there was no difference between the two groups (p=0.514).

The therapy in the ECA group needed to be changed more frequently (p=0.017) with an overall longer treatment duration (p<0.001). Consequently, the hospital stay was at least one day longer (p<0.001) (Table I).

Table I. Characteristic of children affected by CAAs.

	LCA n (%) 90 (65)	ECA n (%) 48 (35)	p-value
Age at intervention years (M, IQR)	9.6 (7.0-12)	7.6 (4.8-11)	*p=0.016
WBC count 10^9/L (M, IQR)	17 (14-20)	18 (14-22)	p=0.617
CRP mg/L (M, IQR)	57 (27-130)	62 (36-146)	p=0.197
Laparoscopic appendectomy, n (%)	32 (36)	21 (44)	p=0.364
Abdominal drain, n (%)	34 (38)	15 (31)	p=0.463
SSI, n (%)	6.0 (6.7)	5.0 (10)	p=0.514
Length of antibiotic therapy days (M, IQR)	6.0 (5.0-8.0)	8.0 (7.0-9.0)	*p<0.001
Length of hospital stay days (M, IQR)	7.0 (5.0-8.0)	8.0 (7.0-9.0)	*p<0.001
Shift to 2nd line therapy, n (%)	26 (29)	24 (50)	*p=0.017

CAA: complicated acute appendicitis
LCA: localized complicated appendicitis
ECA: extended complicated appendicitis
WBC: white blood cell
SSI: surgical site infection
*Statistically significant (p-value <0.05)

Furthermore, the outcomes of the patients with an abdominal drain were compared to those without it in the ECA group. As to the failure of the first-line regimen, seven patients (47%) with a drain required a shift to second-line drugs and 17 patients (52%) without a drain required a shift. This rate was similar (p=0.755). As to the occurrence of SSI, the rate was higher in the patients with a drain (p=0.013). Indeed, four patients with a drain (27%) presented with an SSI, whilst only one patient (3.0%) without a drain experienced this adverse event.

Twenty-four patients (48%) that failed the first-line therapy presented with ECA (Table III). Indeed, a univariate analysis, including several demographic and clinical factors (age at intervention, the technique of appendectomy, the presence of abdominal drain, the value of CRP, WBC count, and the severity of abdominal contamination), found that only the extension of the abdominal contamination was associated with an increased risk of failure for the first-line treatment. The estimated OR was 3.0 (95CI 1.3-7.0; p=0.036) (Table III).

The same results were replicated in a multivariate analysis adjusted for the values of CRP and for the age at intervention. The extension of the abdominal contamination was still associated to the failure of the first-line therapy. The adjusted OR was 3.0 (95CI 1.2-7.4; p=0.041) (Table IV).

Table IV. Results of the multivariate logistic regression on severity, age and CRP adjusted.

		OR (95%CI)	p-value
Age <6 years		1.1 (0.5-2.6)	p=0.817
CRP ≥100 mg/L		1.4 (0.6-3.0)	p=0.424
Severity	Localized	1	
	Abscess	1.3 (0.5-3.4)	*p=0.041
	Extended	3.0 (1.2-7.4)	

*Statistically significant (p-value <0.05)

Discussion

The three-drug antimicrobial regimen used for the treatment of pediatric CAAs presented a relevant rate of failure that occurred in one-third of the patients. When the patients affected by ECA were separately considered, the rate of failure reached 50%, regardless of the age at intervention and CRP values. Moreover, patients suffering from ECA were usually younger and required a longer hospital stay, even though an increased number of SSIs did not occur. These aspects proved once again that CAAs concerned a wide range of clinical situations.

Our antibiotic protocol required the administration of the three-drug regimen, which was well-consolidated for decades.[8,16] However, surveillance of the outcomes was required by the occurrence of several cases of failure, even though the data of our Service

Table III. Results of the univariate logistic regression analysis.

		First line therapy n (%) 88 (64)	Second line therapy n (%) 50 (36)	OR (95%CI)	p-value
Age <6 years, n (%)		21 (24)	16 (32)	1.5 (0.7-3.3)	p=0.284
Laparoscopic appendectomy, n (%)		30 (34)	23 (46)	1.7 (0.8-3.4)	p=0.154
Abdominal drain, n (%)		31 (35)	18 (36)	1.1 (0.5-2.2)	p=0.890
WBC count ≥18 x 10^9/L†, n (%)		37 (42)	24 (48)	1.5 (0.7-3.1)	p=0.265
CRP ≥100 mg/L‡, n (%)		30 (34)	21 (42)	1.4 (0.7-2.9)	p=0.334
Severity, n (%)	Localized	39 (44)	13 (26)	1	
	Abscess	25 (28)	13 (26)	1.6 (0.6-3.9)	*p=0.036
	Extended	24 (27)	24 (48)	3.0 (1.3-7.0)	

†Frequency missing=5.0
‡Frequency missing=3.0
*Statistically significant (p-value <0.05)

of Microbiology reported that the presence of ESBL bacteria was less than 10% (data not shown). Conversely, a recent survey by Kwok et al.[9] reported an increased rate of resistances, especially caused by ESBL strains. The authors found that gentamicin provided coverage only in 45.3% of the children affected by CAA. Similarly, in our series, the overall rate of failure was 35.6%. This rate was largely above 10%, namely the threshold established in literature in order to define the lack of efficacy of an antimicrobial therapy.[7]

It is relevant to underline that our measure of failure was the shift to a second-line regimen. This was based on the worsening of clinical conditions, rather than the results of intraoperative cultures which were not performed routinely. In the previous years, we found no relationship between the intraoperative cultures after appendectomy and those taken from abdominal collections secondary to CAA. Most of the strains that grew after the appendectomy were polymicrobic or sensible to the first-line regimen (data not shown). This might be a further reason to abandon the intraoperative cultures during an appendectomy.

Moreover, since no randomized trial has demonstrated the superiority of intraoperative cultures, an approach based on clinical conditions was preferred to avoid an escalation of antibiotics, that was not supported by clinical deterioration.[17] It is well known that there is not a cause-effect association between the intraoperative samples and the occurrence of postoperative abdominal abscesses. Indeed, an escalation of the antimicrobial therapy, tailored to the microbiological results, did not prevent abscess formation in both the adult and pediatric population.[18,19]

This consideration was further supported by our findings. In the ECA group, the presence of the abdominal drain did not reduce the rate of failure of the first-line therapy. However, the rate of SSIs was surprisingly higher in the patients with the abdominal drain. This might be due to the retrospective design of the study. Indeed, the abdominal drain might have been used for the worst ECAs that presented a higher risk of complications.[4,5]

As to the potential factors associated with the failure of the standard regimen, several aspects were investigated. Nevertheless, the only significant variable turned out to be the severity of the abdominal contamination. This result was independent from the CRP values and the age at intervention in a multivariate analysis. This was more evidence of how the extension of the infection could be determinant in the workload of the postoperative management of CAAs, confirming the findings reported by Feng et al.[5]

Pediatric CAAs might present a heterogeneous spectrum of severity, that might not be pre-operatively predicted by blood tests or clinical variables.[4,5] However, in our series, the age of the children affected by ECA was sensibly lower. This was consistent with the previous literature, as younger children with acute appendicitis were more prone to present with more extended abdominal infections.[14]

Finally, patients affected by ECA might develop SSIs more frequently, requiring more aggressive treatment.[20] In our population, even though quite a higher rate of SSI was found in the ECA group, there was no significant difference with the LCA group. However, the patients affected by more severe abdominal infections had longer hospital stays and required a shift of antibiotic therapy more frequently. Moreover, the duration of the antibiotic therapy was quite longer compared to the current trends reported in the literature. The introduction of broad-spectrum beta-lactamase allowed to reduce the scheduled course of post-operative antibiotic therapy to three days.[21]

The outcomes of the children affected by CAA were directly influenced by the antimicrobial therapy.[8,11] Only a periodic review might assess the real efficacy of the antibiotic protocols in use. For this reason, the establishment of an antibiotic stewardship program has

been highly recommended to improve antibiotic prescriptions and develop new treatment protocols. To reach this goal, the local epidemiology of bacterial infections, the periodic monitoring of the outcomes related to antibiotic therapy and the clinicians' compliance with the prescription protocols should be taken into account. A well-established antibiotic stewardship program might allow identifying potential antibiotic misuse and in-house protocol inefficacies. These considerations were recently reported by Wakeman et al.[11] The authors measured the success of an antibiotic stewardship program by finding a decrease from 35% to 15% in the rate of complications after appendectomy for CAA.

Given the high rate of failure for the three-drug regimen reported in our series, a revision of the antimicrobial protocol is necessary. First, the current epidemiology and the local microbiological reports should be considered for the choice of antibiotic spectrum. Abdominal infections in children presented a high frequency of polymicrobial specimens. Coliform Gram negative, anaerobes and *Streptococci spp* were the most common pathogens. Only about 5% of the cultures identified *Pseudomonas aeruginosa*, but without any evidence of its pathogenicity.[22] However, a recent update reported an increased occurrence of *Pseudomonas aeruginosa*, reaching 10% of the cultures.[9]

Secondly, treatment-related stress among children and caregivers should be reduced by the introduction of a simplified empirical antibiotic regimen.[23] Indeed, a decrease in intravenous infusions per day might avoid the necessity of central line insertion and multiple venous cannula replacements. For these reasons, new single-drug protocols consisting of a broad-spectrum beta-lactamase or a carbapenem have been successfully introduced.[10,17]

On the other hand, several studies recommended a two-drug regimen. A third-generation cephalosporin associated with metronidazole has been recommended and no antipseudomonal coverage has been indicated in the first instance.[24,25] Indeed, a narrow-spectrum regimen presented an equivalent rate of OSSI when compared to a regimen with anti-pseudomonas activity. This activity should be reserved for second-line therapy, sparing broad-spectrum drugs, such as piperacillin-tazobactam, for a potential escalation.[26] The efficacy of this regimen, including ceftriaxone as a third-generation cephalosporin, might be chosen, was proven when dealing with CAAs in children.[24]

As to the potential side effects, the utilization of this protocol might avoid the prescription of aminoglycosides, aiming to spare an antimicrobial agent with current low coverage and reduce potential toxicity.[9] Moreover, the majority of the patients treated by our second-line regimen had positive outcomes and the rate of complications seemed to be comparable to the series that used broad-spectrum beta-lactams without the administration of aminoglycosides.[21,25]

The monitoring provided by our antibiotic stewardship program reported a high rate of failure for the standard three-drug regimen. For this reason, the implementation of a new antimicrobial protocol for the treatment of CAAs in children should be recommended. According to the current evidence, a regimen with ceftriaxone and metronidazole should be considered, avoiding aminoglycosides and the drugs with anti-pseudomonas activity in the first instance. Currently, the outcomes of this new protocol are under assessment at our institution.

Nevertheless, this study presents some limitations. The single-center and retrospective design of the study limited the sample size. This might affect the statistical power and the generalization of the results. Another bias concerned the identification of the cut-offs for the statistical analysis. Finally, the classification of CAAs relied on the surgeon's discretion, as it is well known in the literature.[27]

The extension of abdominal contamination might influence the response to the medical treatment, leading to the identification of a subset of children poorly responding to standard antimicrobial treatment.

In addition, even though the evaluation of the length of antibiotic therapy and hospital stay were not the primary aims of this study, the program might also help in reducing the rate of OSSI and the overall length of hospital stay together with its related costs.

Ethical approval

Our institutional board was notified but as this was a retrospective observational study it was exempted from approval.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: FG, CV; data collection: FG; analysis and interpretation of results: ACF, FG, CV, DD, FFL; draft manuscript preparation: FG, CV, DD, FFL, PG. 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

Clinical findings of methicillin-resistant *Staphylococcus aureus* in cystic fibrosis

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ABSTRACT

Background. Methicillin-resistant *Staphylococcus aureus* (MRSA) rates have increased in cystic fibrosis (CF) patients.This study aimed to determine the rate of MRSA, define risk factors, and clarify the effect of MRSA on pulmonary functions, annual pulmonary exacerbation (aPEx) in children with MRSA positive CF.

Methods. This was a retrospective case control study. CF patients who had ≥1 MRSA (+) respiratory culture between September 2016-2019 were included. MRSA growth rate, colonization status, clinical characteristics, hospitalization rates, FEV1 %predicted, and z-score one year prior to the MRSA isolation, at MRSA growth and one year after MRSA growth were recorded. The aPEx rate changes before-after MRSA growth were evaluated.

Results. Sixty-one subjects who had ≥1MRSA growth and 66 controls were enrolled. There was no statistically significant difference between the spirometry indices at first, and 12th month after MRSA acquisition. The mean aPEx was 0.6 one year prior to MRSA acquisition and this rate significantly increased to 1.2 one year after MRSA growth($p<0.05$). The mean hospitalization rate before and after one year of MRSA acquisition significantly increased from 0.17(±0.12) to 0.48 (±0.3)(p:0.008) admissions per year.

Conclusions. MRSA growth was related to increased aPEx. Increased aPEx and hospitalization rates after MRSA acquisition suggest MRSA should be eradicated when detected.

Key words: cystic fibrosis, methicillin resistant *Staphylococcus aureus*, pulmonary exacerbation, colonization, treatment.

Pulmonary involvement is the major cause of morbidity and mortality in cystic fibrosis (CF). Methicillin-resistant *Staphylococcus aureus* (MRSA) is the third most common microorganism detected in CF lungs in the USA.[1] Persistent MRSA infection contributes to CF morbidity and mortality by accelerating pulmonary function decline, impairing lung function recovery after pulmonary exacerbations and requiring increased antibiotic therapies and in some cases hospitalizations.[2-8]

The aim of this study was to determine the frequency of MRSA detection in patients with CF and describe the clinical characteristics, risk factors and clarify the effect of MRSA on pulmonary function, body mass index (BMI) and annual pulmonary exacerbation (aPEx) in children with MRSA acquisition and chronic MRSA compared to methicillin-susceptible *Staphylococcus aureus* (MSSA) positive patients. The second aim of this study was to determine the effect of chronic MRSA colonization on the long-term outcome parameters.

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Material and Methods

Patients

This was a retrospective case-control study. Patients between 0-18 years with confirmed CF diagnosis who had MRSA growth in sputum or deep oropharyngeal cultures between September 2016 and June 2019 were enrolled. The study was approved by our local Hacettepe University Ethics Committee (15/09/2021, GO20-639). A control group was recruited from the same pool of CF patients with methicillin-susceptible Staphylococcus aureus (MSSA), and matched with each MRSA patient for gender age, *Pseudomonas aeruginosa* (PA) colonization, other comorbidities related to CF, and CF medications.

Patients' demographic data including age, sex, body mass index (BMI) z-score, age at CF diagnosis, CF mutations, presence of other pathogens, pulmonary function measured at baseline, and related diseases such as pancreatic insufficiency, CF-related diabetes, concurrent allergic bronchopulmonary aspergillosis and inhaler treatments used, age at the time of MSSA and MRSA acquisition were recorded. BMI z-score and spirometry indices one year prior to MRSA, at the time of the MRSA acquisition, and 12 months after MRSA acquisition were recorded. Pulmonary exacerbation rates and hospitalization frequency before and 12 months after MRSA acquisition were calculated for all the patients and patients with chronic MRSA. Symptoms, physical examination findings, and microbiological status (PA, *B.cepacia, non-tuberculous mycobacteria* (NTM), *and Achromobacter*) at the time of the MRSA acquisition and at the 12-month visit were documented. Antibiotics used, route of administration, duration of therapy, and hospitalization status for initial MRSA growth were also recorded. The number of clinic visits, PA colonization status in the last twelve months before MRSA acquisition were evaluated.

Measurements

In our center, CF patients are routinely evaluated every three months. During these visits, airway samples are obtained from all CF patients, and for patients older than 5 years of age spirometry is performed.

All the samples of *S. aureus* isolates were subjected to cefoxitin disc diffusion testing using a 30 μg cefoxitin disc. The results were interpreted according to EUCAST guidelines. MRSA growth was defined if it was stated in the microbiologic culture report as phenotype resistant to cefoxitin. A new MRSA growth was defined as at least one positive respiratory tract (RT) culture in patients who were negative during the previous 12 months. The duration for the development of methicillin resistance was calculated as the period between the MSSA positive culture and the initial MRSA growth.

A child with an initial positive RT culture for MRSA who never grew MRSA again in the following twelve months was characterized as having one MRSA growth. Among these patients who received antibiotic treatment specific for MRSA and three negative respiratory samples for MRSA taken at least one week apart showed no MRSA growth following treatment were specified as MRSA eradicated. The success of the given antibiotic to eradicate MRSA was calculated by proportioning the patients whose MRSA was eradicated among all patients receiving the same eradication therapy. Patients who had ≥3 MRSA cultures in the past 6 months were defined as chronic MRSA colonization.[9] MRSA prevalence was calculated by dividing the number of MRSA positive cultures detected during the study period to the total number of RT cultures of CF patients obtained during the study period.

Spirometry test was performed with the Vyntus PC Spirometer (Carefusion; Höchberg, Germany) and in accordance with the American Thoracic Society and European Respiratory

Society (ATS/ERS) standards by the same certified spirometry technician.[10] Spirometry indices were analyzed using the reference values of Quanjer et al.[11] Spirometry curves were re-evaluated by a single senior pediatric pulmonologist following the recently published update of the ATS/ERS standardization of spirometry.

BMI-for-age z-scores were calculated using the World Health Organization (WHO) anthropometric calculator (AnthroPlus v.1.0.4) which is based on WHO Child Growth Standards and Growth Reference data. Acute pulmonary exacerbation was defined according to criteria described by Fuchs et al.[12]

Infection Control Policy

As of February 2019, strict infection control practices have been implemented in our clinic. A brochure has been prepared to inform patients from this day on. Patients are encouraged to put on surgical masks throughout their hospital stay. Hand disinfectants have been placed in the waiting room and in the examination rooms and patients are asked to use them. No other CF patient or family member is permitted to be in another child's area at any time. Doctors wash their hands and clean their stethoscopes between patients. Children colonized with MRSA, PA, *Burkholderia cepacia* and NTM attend the outpatient clinic on a different day then other CF patients. Between patient examinations desktops, chairs, surfaces are thoroughly cleaned.

Statistical Analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 23.0(SPSS Inc,Chicago,IL). To find a significant difference between 2 groups with a big effect size (cohen d=0,95), the minimum required sample size was estimated as 126 (α=0.05-1-β=0.80).[13] The Kolmogorov-Smirnov test was used to determine whether variables had a normal distribution. Patient characteristics and pulmonary function variables were presented by means (SD), median (IQR) or numbers and percentages of total, as appropriate. The Wilcoxon signed-rank test was used for comparing repeated measurements that were not normally distributed. Differences between group characteristics were assessed by independent samples T-test or Mann–Whitney test. Differences for dependent variables were assessed by paired sample T-test. Chi-square or Fisher exact test was used to analyze group differences for categorical variables. The difference for previous, initial and last FEV_1 values, and difference of these values in different groups (one growth and chronic MRSA) were calculated with the generalized linear model. P values <0.05 were considered statistically significant.

Results

During the study interval, a total of 2734 RT cultures of CF patients were examined. Eighty-eight subjects had at least one MRSA growth in which 44 of them had MRSA growth for the first time. The MRSA prevalence in CF patients in our center during the study interval was 11.8%. During the same time interval, the MRSA prevalence of our center was 18.5%.

Patient characteristics

A total of 360 CF patients were followed at our clinic between the years 2016-2019. We only enrolled 61 CF patients between 0-18 years of age and (17%) who had ≥1 MRSA growth. The control group consisted of 66 patients between 0-18 years of age with chronic MSSA growth. The demographic data of subjects and clinical findings at MRSA acquisition are summarized in Table I. The median follow-up time for patients after MRSA acquisition was 14 months (IQR:12-20). The median MRSA acquisition after the first MSSA positive culture was 42.6 months (IQR: 24.4-89.3).

Table I. Demographic characteristics of subjects at time of positive MRSA growth, and control group and clinical findings and treatment data of subjects at MRSA acquisition.

	Patients with positive MRSA growth	MSSA positive group	p
Patients, n	61	66	
Male/female	32/29	33/33	
Mean age at study enrollment yr (SD)	8.2 (3.9)	9.4 (4.3)	0.2
Mean age for first MRSA positive culture yr (SD)	8.2 (3.9)	n.a.	n.a.
Mean age for first MSSA positive culture yr (SD)	3.8 (3.5)	3.2 (3)	0.8
Mean culture frequency per year (SD)	2.6 (0.9)	2.7 (0.8)	0.9
Sample collection, n (%)			0.3
Deep oropharyngeal culture	25 (41)	21 (31.8)	
Sputum culture	36 (59)	45 (68.2)	
Chronic *Pseudomonas aeruginosa* colonization, n (%)	15 (24.6)	14 (21.2)	0.7
Chronic *Achromobacter* colonization, n (%)	1 (1.6)	-	n.a.
Genotype, n (%)			0.5
ΔF508/ΔF508	11 (18)	14 (21.5)	
ΔF508/other	10 (16.4)	15 (23.1)	
Other/other	40 (65.6)	37 (56.1)	
Pancreatic insufficiency, n (%)	58 (95.1)	61 (92.4)	0.5
Chronic liver disease, n (%)	17 (27.9)	13 (19.7)	0.3
Altered glucose tolerance/ CF related diabetes, n (%)	6 (9.8)	3 (4.5)	0.3
Allergic bronchopulmonary aspergillosis, n (%)	1 (1.6)	2 (3)	0.6
Inhaled Medications, n (%)			
Dornase-alpha	57 (93.4)	66 (100)	0.2
Corticosteroids	4 (6.6)	4 (6.1)	0.9
Hypertonic saline	10 (16.4)	8 (12.1)	0.5
Mannitol	6 (9.8)	1 (1.5)	0.06
Tobramycin	12 (19.7)	10 (15.2)	0.5
Colistin	9 (14.8)	4 (6.1)	0.1
Clinical findings at MRSA acquisition		n.a.	n.a.
Symptomatic, n (%)	28 (45.9)		
Cough	28 (100)		
Increased sputum	8 (13.1)		
Fever	4 (6.6)		
Hypoxia	3 (4.9)		

IQR: interquartile range, SD: standard deviation, MRSA: Methicillin-resistant *Staphylococcus aureus*, MSSA: Methicillin-susceptible *Staphylococcus aureus*, n.a.: not applicable

Spirometry and BMI

Baseline and twelve month lung function, BMI, and frequency of pulmonary exacerbations of patients with MRSA acquisition and control group is shown in Table II. BMI z-score and spirometry indices of patients at initial MRSA growth, and 12 months after MRSA acquisition are summarized in Table III. There was no statistically significant difference between the first, and 12th month after MRSA acquisition in spirometry indices, whereas a significant increase was seen in BMI z-score at 12th month visit.

Table II. Baseline and twelve month lung function, body mass index and frequency of pulmonary exacerbations.

Characteristics		Control subjects	Patients	P value (case/ control)	One growth group	Intermitant/ Chronically infected group	P value (one growth/ inter-chronic)
Spirometry performed n (%)		48 (72.7)	41 (67.2)		10 (83.3)	32 (65.3)	
Baseline FEV1 z-score, mean (SD)		-0.41 (1.66)	-0.68 (2.00)	0.5	-0.67 (1.20)	-0.68 (1.10)	0.9
Twelve month FEV1 z-score, mean ± SD		-0.56 (1.85)	-0.91 (1.85)	0.4	-0.49 (0.74)	-1.04 (1.00)	0.2
Baseline FVC z-score, mean ± SD		-0.48 (1.49)	-0.78 (1.89)	0.4	-1.09 (1.52)	-0.68 (1.01)	0.5
Twelve month FVC z-score, mean ± SD		-0.57 (1.69)	-0.99 (1.70)	0.2	-0.53 (0.79)	-1.13 (1.17)	0.1
Baseline FEV1 %, mean ± SD		99.7 (18.9)	95.6 (23.4)	0.4	96.0 (21.2)	95.5 (21.3)	0.9
Twelve month FEV1 %, mean ± SD		97.3 (20.8)	94.5 (21.3)	0.5	100.3 (11.0)	92.7 (23.5)	0.2
Baseline FEV1 L median (IQR)		1.64 (1.35-2.32)	1.58 (1.18-2.10)	0.6	1.46 (1.18- 1.58)	1.81 (1.21-2.26)	0.2
Twelve month FEV1 L median (IQR)		1.71 (1.39-2.41)	1.81 (1.40-2.30)	0.9	1.68 (1.40-1.88)	1.93 (1.44-2.49)	0.4
Baseline FVC %, mean ±SD		95.1 (15.4)	91.3 (19.6)	0.3	89.1 (16.7)	91.9 (20.6)	0.7
Twelve month FVC %, mean ± SD		94.5 (16.4)	92.6 (18)	0.6	96.5 (10.7)	91.3 (19.7)	0.4
Baseline FVC L median (IQR)		1.83 (1.42-2.61)	1.88 (1.42-2.39)	0.8	1.50 (1.42- 1.78)	2.01 (1.36- 2.59)	0.1
Twelve month FVC L, median (IQR)		2.02 (1.58-2.80)	2.09 (1.62-2.72)	0.8	1.89 (1.69-2.10)	2.24 (1.59- 2.86)	0.3
Baseline BMI z score, mean ± SD		0.06 (1.30)	-0.05 (1.34)	0.6	0.01 (1.24)	-0.05 (1.30)	0.8
Twelve month BMI z score, mean ±SD		0.00 (1.35)	0.01 (1.36)	0.6	0.33 (1.10)	-0.08 (1.41)	0.3
Exacerbations	Year before baseline, mean (SD)	0.5 (0.1)	0.6 (0.1)	0.9	0.4 (0.1)	0.7 (0.1)	0.2
	Year after baseline, mean ±SD	0.5 (0.1)	1.2 (0.2)	<0.001	0.6 (0.1)	1.4 (0.1)	0.04*
Hospitalization	Year before baseline, mean ±SD	0.15 (0.01)	0.17 (0.02)	0.8	0 (0)	0.21 (0.01)	0.02*
	Year after baseline, mean ±SD	0.35 (0.07)	0.48 (0.03)	0.6	0.08 (0.03)	0.78 (0.10)	0.01*

BMI: body mass index; FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity; IQR: interquartile range; SD: standard deviation; L: liter

Table III. BMI z-score and spirometric data of patients with methicillin-resistant *Staphylococcus aureus* (MRSA) at the time of initial growth and twelfth month control.

	Initial	Twelfth month	p
Spirometry performed, n (%)	41 (67.2)	41 (67.2)	
FEV_1 % predicted mean (SD)	95.6 (23.4)	94.5 (21.3)	0.1
FVC % predicted mean (SD)	91.3 (19.6)	92.6 (18)	0.9
FEF_{25-75} % predicted mean (SD)	91.9 (37.7)	87.2 (32)	0.05
FEV_1 z-score mean (SD)	-0.68 (2.00)	-0.91 (1.85)	0.07
FVC z-score mean (SD)	-0.78 (1.89)	-0.99 (1.70)	0.2
FEF_{25-75} z-score mean (SD)	-0.73 (1.47)	-0.83 (1.73)	0.2
BMI z-score, mean (SD)	-0.05 (1.34)	0.01 (1.36)	0.001

Table IV. Treatment modalities given for patients with one MRSA growth and chronic MRSA, and eradication rates of the therapies.

	Patients with one MRSA growth n (%)	Patients with chronic MRSA n (%)	p
Received eradication therapy	7 (18.9)*	30 (81.1)	0.9
Eradication therapy route			
Intravenous	1 (10)	9 (90)	0.7
Oral	6 (22.2)	21 (77.8)	
Treatments			
TMP-SMX p.o., n (%)	5 (33.3)	10 (66.7)	0.5
TMP-SMX and rifampicin p.o.	1 (14.3)	6 (85.7)	n.a.
TMP-SMX and rifampicin p.o. + %2 nasal mupirosin ointment + %4 chlorhexidin BW	-	1 (100)	n.a.
Vancomycin iv.	1 (12.5)	7 (87.5)	n.a.
Linezolid iv.	-	2 (100)	n.a.
Other (p.o.)	-	4 (100)	n.a.

p.o: per oral, iv: intravenous , n.a: not applicable, TMP-SMX: Trimethoprim-sulfamethoxazole, BW: body wash
* The overall success rate for MRSA eradication was 18.9%.

aPEx and Hospitalization

The mean aPex was 0.6 one year prior to the MRSA acquisition and significantly increased to 1.2 one year after MRSA growth ($p<0.05$). The mean aPEX was significantly higher for patients one year after MRSA growth compared to the control group (1.2 versus 0.5; p: 0.001). The mean hospitalization rate before and one year after the MRSA acquisition significantly increased from 0.17 (±0.12) to 0.48 (±0.3) (p:0.008) admissions per year.

Treatment- Eradication- Chronic Colonization

Detailed antibiotic therapy regimes for 37 patients (60.7%) and eradication success are outlined in Table IV. MRSA was spontaneously cleared in 5 patients (8.2%) with only one MRSA growth without any medical treatment. When MRSA was detected for the first time, seven (58%) of the 12 patients with only one MRSA growth and 30 (61.2%) of the 49 patients with chronic MRSA colonization were treated with antibiotics which was not statistically significant (p:0.9). The overall success rate for MRSA eradication was 18.9%.

Results of patients with chronic MRSA

Among 49 patients the median MRSA colonization time was 17 months (IQR:10-29). Figure 1 shows the difference between median FEV_1 z-score values one year prior to MRSA, the initial MRSA growth and 12 months after MRSA acquisition compared for patients with and without chronic MRSA and the control group. Even though it was not statistically significant (p:0.08) the mean FEV_1 z-score one year prior to MRSA declined from -0.71 (±1.29) to -0.67(±1.20) at initial MRSA growth and increased to -0.49 (±0.74) at the one year follow up in patients with one MRSA growth, whereas FEV_1 z-score one year prior to MRSA declined from -0.31 (±1.64) to -0.68(± 1.10), and continued to decline to -1.04 (±1.00) at the one year follow up in patients with chronic MRSA.

Acute Pulmonary Exacerbations and Hospitalization

Among the patients who had one MRSA growth, the annual exacerbation rate before and after MRSA acquisition was found to be 0.4 and 0.6, respectively. This rate was 0.7 and 1.4 before and after the initial MRSA growth in patients who had chronic MRSA growth and this difference was statistically significant (p<0.05). None of the patients with one growth of MRSA were hospitalized after MRSA acquisition. However, the mean hospitalization rate was 0.2 in patients with chronic MRSA, but this difference was not statistically significant (p: 0.2).

Factors associated with chronic MRSA

Twelve patients had PA chronic colonization in the 12-month period prior to MRSA acquisition, 11 (91.7%) of them became chronically colonized with MRSA, whereas this rate was 77.3% in patients without chronic PA(p:0.2). All patients who came for routine follow up ≥3 times (n:5) during the 6-month interval before MRSA acquisition had chronic MRSA colonization, this rate decreased to 78.3% in patients who had visits 1-2 times (n:46) and to 66.7% in patients who did not come for follow up (n: 3) in the last 6 months (p:0.3). Data of eight patients could not be reached. Additionally, nine patients (14.5%) were hospitalized within 12 months before

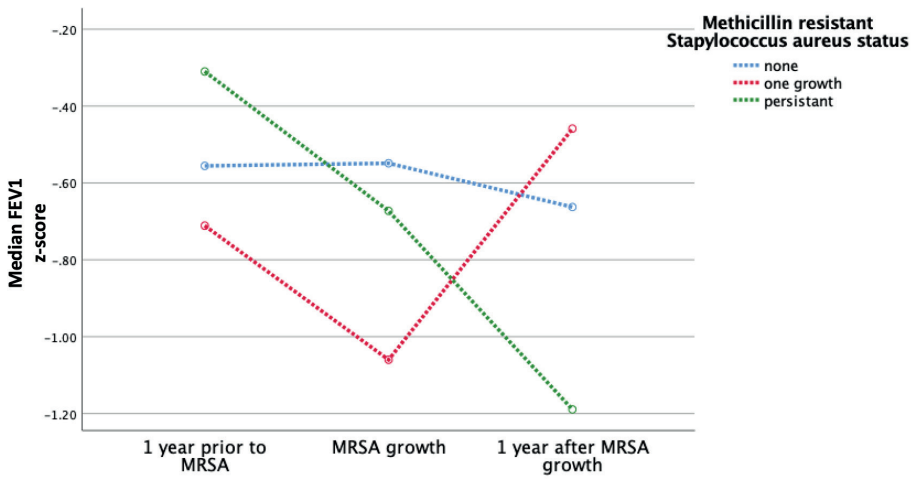

Fig. 1. Median FEV_1 z-score values one year prior to MRSA, the initial MRSA growth and 12 months after MRSA acquisition compared for patients with and without chronic MRSA and the control group. On Y-axis median FEV_1 score, on X-axis one year prior to MRSA, the initial MRSA growth and 12 months after MRSA acquisition is shown.

Table V. Logistic regression analysis for variables predicting chronic MRSA colonization.

Variables	Univariate analysis		
	Odds ratio	95% confidence interval	p
Age of encounter with MRSA	1.05	0.86-1.27	0.6
Chronic PA colonization in the 12-month prior to MRSA	0.3	0.03-2.7	0.3
Number of visits in the 6-month interval prior to MRSA	0.6	0.46-1.28	0.5
Hospitalization status in the 12-month interval prior to MRSA	0.7	0.56-1.33	0.7
Received treatment for MRSA	0.8	0.70-2.27	0.5

MRSA growth was detected. All of the patients who were hospitalized became chronically colonized with MRSA, whereas 76.7% were chronically colonized with MRSA in the non-hospitalized group (p:0.1). These factors were not found to have a significant effect on chronic colonization of MRSA in the regression analysis (Table V).

Discussion

In our study, we found that the MRSA prevalence in our center was 11.8%. Half of the patients were symptomatic and one-fourth had intermittent/persistent PA colonization when MRSA was detected for the first time. There was no significant decline in spirometry indices in the year following the MRSA acquisition, but a significant increase was found in the BMI z-score for the whole MRSA group. However, aPEx frequency one year after MRSA acquisition significantly increased compared to one year prior to the MRSA acquisition, this increase was also seen when compared with the control group. The overall success rate for MRSA eradication, and the rate of chronic MRSA in our cohort was 18.9% and 80% respectively. For the patients with chronic MRSA, the FEV1 z-score declined, and the aPEx rate increased compared to patients with one MRSA growth and the control group.

The prevalence of MRSA is reported in a wide range between countries and depends on many factors such as hospital policies for the management of MRSA, geographic differences and antibiotic susceptibility. MRSA prevalence increased from 9.2% to 25.9% between 2002 and 2017, even to 49% in some high prevalence centers in the USA.[14,15] A much lower prevalence is reported in European countries, Canada and Australia ranging between 3-18%.[9,16-19] A previous study from our center reported MRSA prevalence as 3.9% between 2003-2010.[20] The increase in MRSA prevalence in our center can be explained by several factors. First of all, the same patient population with increasing age is associated with an increased risk of antibiotic use, more frequent exacerbations and hospitalization, therefore an increase in MRSA prevalence. Besides strict infection control practices for CF were not implemented before January 2019. Therefore, children may have acquired MRSA in the clinic or hospital before that time.

Optimal treatment strategies for the eradication treatment of MRSA in patients with CF is debatable as well as the duration of the therapy and using monotherapies or combination therapies.[21-26] The treatment decision covering all of these aspects are mostly made on an individual basis combined with the patient's medical history and microbiologic test results. A recent Cochrane review concluded that due to lack of randomized controlled long-term trials, no recommendations can be made to support the eradication of MRSA, or a treatment protocol for MRSA.[27] In our study, 60% of the patients were treated when MRSA was detected for the first time yet 80% of the patients developed chronic MRSA. The success rate of MRSA eradication depends on the prevalence of MRSA in the community. It

may be successful if the household members/ community are not colonized with MRSA. However, we could not define the household members' MRSA colonization status and as such, we did not recommend any treatments to other household members for MRSA when MRSA treatment was initiated to the MRSA positive CF patients. Vallières et al.[21] examined 38 CF patients with MRSA, in their study nine different treatment modalities were used and they had an eradication success of 79%. More than one-half of the patients received a combination of rifampicin and fusidic acid treatment. This combination was not used in our study for two reasons. First of all, fusidic acid preparation is not available in our country and secondly, most patients have had difficulty attaining rifampicin which is a main anti-tuberculosis drug that can only be obtained from tuberculosis dispensaries in our country and is of limited use due to the concerns about the development of drug resistant tuberculosis. The higher rate of chronic MRSA than most of the other studies can be explained by several factors. Mainly, in the study group half of the patients were asymptomatic which caused less frequent eradication treatment for MRSA. Vallières et al.[21] treated most of the patients for at least 3 weeks and some of the patients for up to 6 months with additional nasal and skin decolonization protocol for all the patients. Even though they showed no difference in terms of eradication in patients with a longer duration of therapy, in our study the duration of the treatment was 14 days which is much lower, and only 1.6% of the patients in our study received decolonization protocol. All of these findings suggest that the combination of systemic and topical treatment strategies might enhance MRSA eradication and therefore reduce MRSA chronicity.

The rate of FEV_1 decline is still the most important outcome measure to monitor morbidity and mortality in CF. There was no statistically significant difference in terms of first and last spirometry indices, in our group. Similarly, Sawicki et al.[2] stated that MRSA detection was not associated with a significant decline in spirometry indices. On the other hand, Dasenbrook et al.[4] showed FEV_1 decline to increase in patients between 8-21 years who had chronic MRSA. Also, Vanderhelst et al.[5] reported an increased rate of decline in FEV_1. However, Sawicki et al.[2] included patients who had one positive culture for MRSA like our study, whereas the other two studies only included patients who had at least 3 cultures positive for MRSA. These findings raise the question of whether chronic MRSA state is related to FEV_1 decline in CF patients. In our study FEV_1 declined by 6% in patients with chronic MRSA, however, this decline didn't reach statistical significance. Yet the latter studies monitored their patients for 3.5-6 years, which is longer than that of the current study's follow-up period. These findings suggest that these patients in the study group should be followed closely and carefully for a significant decline in FEV_1 in the near future.

Our study has several limitations; the retrospective nature and small sample size, as well as the shorter follow up time can alter the results. Additionally, molecular analyses on the MRSA strains were not performed. The detection of MRSA carrier status of people living in the same house as the CF patient was also not obtained which may have helped to evaluate possible patient-to-patient transmission or other sources of contamination. We additionally were unable to interpret the small colony variant *S.aureus* status of our patients which is correlated with lung function decline and antibiotic resistance in CF patients.

Colonization with MRSA is an important problem for CF patients. Prevalence and chronicity rates differ between CF centers because of the lack of standardized protocols for eradication and treatment of intermittent and persistent MRSA. In our study, the increased prevalence of MRSA in CF patients, as well as chronic MRSA rates, is a warning for all the authors that strict infection control practices for CF are required, which have been implemented since January 2019. Also, even though FEV_1

decline could not be proven the increased pulmonary exacerbation and hospitalization rates after MRSA acquisition suggest that MRSA should be eradicated when detected. The failure of the MRSA eradication treatment suggests that a combination of systemic and topical treatment strategies might enhance MRSA eradication and chronicity. However larger prospective longitudinal studies are needed to focus on the benefits of eradication treatment of CF prognosis, duration of treatment, method of providing the treatment and the potential negative impacts of long-term treatments.

Ethical approval

Participation informed consent was involved and the study was approved by Hacettepe University ethics committee (GO20-639).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: BÖ, DD, NE, GH, EY, UÖ, NK; data collection: BÖ, DAT, BS, HNB, İG; analysis and interpretation of results: BÖ, DD, EY, UÖ, NK; draft manuscript preparation: BÖ, DD, NE, GH. 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

Comparison of miRNA expression in patients with seasonal and perennial allergic rhinitis and non-atopic asthma

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ABSTRACT

Background. MicroRNAs (miRNA) are small non-coding molecules that play a significant regulatory role in several allergic diseases. However, their role in allergic rhinitis is still not clearly understood. The aim of this study was to identify the candidate miRNAs that can discriminate between different forms of allergic rhinitis and also differ in and out of the allergen season.

Methods. The study included 20 healthy children, 20 patients with seasonal allergic rhinitis (SAR), 20 non-atopic asthmatics (NA-A), and 12 patients with perennial allergic rhinitis (PAR). Patients with SAR were evaluated comparatively in and outside the allergen season. The changes in the expressions of selected miRNAs (miR-125b, miR-126, miR-133b, miR-181a, and miR-206) that were found related to the allergic diseases according to the literature were determined using quantitative polymerase chain reaction.

Results. In the SAR group, expression levels of miR-125b (p=0.040) and miR181a (p=0.014) were lower than in the controls outside of the allergen season. Expression levels of miR-181a were different between patients with SAR and NA-A (p=0.003), also between the SAR and PAR (p=0.001) groups in multiple comparisons. In contrast, the expression of miR-206 was found to be decreased in patients with NA-A and PAR compared with the controls (p=0.005 and p=0.024, respectively). In correlation analysis, expression levels of miR-125b and peak expiratory flow (PEF) values were found to be negatively correlated in the SAR (p=0.013) and PAR (p=0.029) groups. The expression level of miR-206 was positively correlated with total IgE levels in PAR (p=0.007). Receiver operating characteristic analysis revealed that miR-125b and miR-181a predicted the risk of SAR (p=0.040 and p=0.014, respectively), and miR-206 for NA-A and PAR (p=0.005 and p=0.024, respectively).

Conclusions. Our study showed that expression levels of miRNAs were different according to the type of allergic diseases and the presence of allergens. miR-181a and miR-125b can be candidate biomarkers for SAR, and miR-206 for NA-A and PAR.

Key words: allergic rhinitis, asthma, epigenetics, microRNA.

The prevalence of allergic diseases such as asthma and allergic rhinitis has increased in the last few decades, especially among children. Evidence from recent studies has shown that the increase in prevalence could be explained by changing environmental conditions and some genetic factors.[1-3] Gene by environment interaction, which results in changes in the expression of genes called epigenetic changes, may contribute to this increase.[4]

Non-coding RNAs are one of the epigenetic mechanisms of the cell. MicroRNAs (miRNAs) are short, approximately 20-25 nucleotides in length, single-stranded non-coding RNA molecules, and they play a role in regulating

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gene expression by targeting mRNAs.[5] miRNAs are produced by cells in different organs and are released into the blood and other body fluids, where they can have a biologic effect. This shows that miRNAs can serve as noninvasive biomarkers.[6] miRNAs play essential roles in various disease processes. miRNAs can serve as a molecular fingerprint for the characterization and diagnosis of diseases because they are detected in the blood.[7] Recent studies have demonstrated the critical role of specific miRNAs in regulating fundamental pathogenic mechanisms of allergic inflammation and the relationship between miRNAs and allergic diseases such as asthma, eosinophilic esophagitis, atopic dermatitis, and allergic rhinitis.[8-16] Let-7 microRNAs were shown to inhibit interleukin (IL)-13 expression and have positive effects on airway inflammation, airway hyperresponsiveness, and mucus secretion and subepithelial fibrosis.[8] miR-375 has a role in the modulation of (IL)-13-driven epithelial responses.[9] Lu et al.[11] showed that miR-223 was one of the most upregulated miRNAs found in eosinophilic esophagitis. miR-374a was found correlated with lung function, especially FEV1/FVC values.[12] Panganiban et al.[5] showed that six circulating miRNAs, miR-125b, miR-16, miR-299-5p, miR-126, miR-206, and miR-133b levels were most predictive of allergic and asthmatic status. They found that the expression of miR-206 increased in plasma in patients with AR and also the expression of miR-125b increased in patients with asthma and AR.[5] In another study, 12 of the circulating miRNAs were found to be associated with exacerbations in asthma, especially miR-206.[13] miR-126 has also been reported as increased in cultured bronchial epidermal cells stimulated with IL-13.[14] A lower percentage of Treg and lower expression of IL-10 and TGF-β was shown in children with AR, and also significantly lower expressions of miR-155 and miR-181a in Treg cells in children with AR.[15] miR-21 and miR-146 were found to have a role in the polarization of adaptive immune responses and activation of T cells.[16] However, studies on this subject are quite limited.

In this study, we aimed to identify candidate miRNAs that could discriminate between different forms of allergic rhinitis, also in and out of the allergen season in children with perennial (PAR) and seasonal allergic rhinitis (SAR). Also, we tried to establish whether different miRNAs were effective in patients with non-atopic asthma and allergic rhinitis. To achieve this aim, we selected five miRNAs (miR-126, miR-125b, miR-181a, miR-133b, and miR-206) as candidate miRNAs according to literature that might play a role in the pathogenesis of the allergic disease.

Material and Methods

This study was approved by the Ethics Committee of Hacettepe University (GO 18/433) and informed consent was obtained from all individual participants before being included in the study.

Study population

Twenty children with non-atopic asthma (NA-A), 20 children with SAR, and 12 children with PAR who presented to the Pediatric Allergy and Asthma Unit, and 20 children with no history of allergy and asthmatic disease (control group) who presented to the Department of Child Health and Diseases, of Hacettepe University Faculty of Medicine between November 2018 and May 2019 were included in the study. Patients with SAR were evaluated comparatively in and out of the allergen season. In the SAR group, off-season samples were collected in November (2018), while in-season samples were collected in April-May (2019).

All the patients in SAR group had grass pollen allergy and all the patients in PAR group were positive for house dust mites, both Der p and

Table I. The demographics and clinical characteristics of the study population.

	SAR(n=20)	NA-A(n=20)	PAR (n=12)	Controls (n=20)
Age (year)	12.55 ± 2.44	10.85 ± 2.85	10.42 ± 2.15	10.98 ± 3.02
Gender (F (%))	8 (40)	7 (35)	4 (33.3)	8 (40)
FEV1	100 (93.50-106.75)	96 (84.25-102)	98.50 (95.5-100.5)	-
FVC	100 (98.20-101.50)	97.50 (87-100)	99.50 (97.75-100)	-
MEF/25-75	112 (101.50-131.70)	93 (84.25-107.50)	113 (97.25-143.50)	-
PEF	91.0 (84.8-100.1)	85.7 (74.0-97.5)	92.6 (92.3-97.3)	-
Total IgE	85.50 (33.05-333.25)	25.65 (10.84-93)	171 (94.75-391)	-
Eos number	150 (100-200)	100 (100-200)	250 (100-925)	-
Eos %	2.3 (1.6-2.9)	1.7 (1.2-2.8)	4.50 (1.83-12.78)	-

SAR: seasonal allergic rhinitis, NA-A: non-atopic asthmatics, PAR: perennial allergic rhinitis, F: Female, FEV1: Forced expiratory volume in 1 second, FVC: Forced vital capacity, MEF: maximal (mid-)expiratory flow, PEF: Peak expiratory flow, IgE: Immunoglobulin E, Eos: Eosinophils

Der f, except 2 out of 12 patients. Two patients in PAR group had only Der p allergy. The diagnosis of AR was made based on history, clinical examination, skin prick test, and specific immunoglobulin (Ig)-E measurement according to the Allergic Rhinitis and Its Impact on Asthma (ARIA) guideline.[17] The diagnosis of asthma was made based on history and lung function tests according to the Global Initiative for Asthma (GINA).[18] Eight patients in SAR group also had asthma besides AR. The characteristics of the study population are summarized in Table I.

Selection of miRNAs

We selected five miRNAs (miR-126, miR-125b, miR-181a, miR-133b, and miR-206) as candidate miRNAs that might play a role in the pathogenesis of the allergic disease according to the literature.

Sample Collection and miRNA Isolation

Blood samples were collected from the study subjects, and serum was separated and aliquoted within one hour after collection. Isolation of circulating miRNAs was performed using the miRNeasy Serum/Plasma Kit (QIAGEN, Germany) according to the manufacturer's instructions. miRNA purity and quantification were determined using NanoDrop Nucleic Acid Quantification (Thermo Scientific, Waltham, MA). miRNAs were stored at -80°C until they were translated into complementary DNA or required for further use.

Determination of miRNA levels by qRT-PCR

The obtained miRNA samples were reverse transcribed into cDNA using a miScript II RT Kit (QIAGEN, Germany). miScript Primer Assays (QIAGEN, USA) were purchased from a supplier for selected miRNAs and the control miRNA (miR-16).

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using miScript SYBR® Green PCR Kit (QIAGEN, Germany) on the Mx3005P Real-Time PCR system (Agilent Stratagene, United States). The reactions were set up according to the manufacturer's instructions. The mean value of the cycle threshold of duplicates for each sample was calculated, and the expression level of each miRNA was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to miR-16.

Statistics

The SPSS 22 for Windows program was used for statistical analysis. The comparison of numerical variables was performed using parametric or

non-parametric tests depending on whether they were normally distributed. Clinical data were compared using an independent t-test. Categorical variables were evaluated using the Chi-square test or Fisher's exact test. Expressions of target miRNAs were normalized according to the expression of control miRNA. The results were analyzed using the 2−ΔΔCt method. The Mann-Whitney U test was used to analyze miRNA expression levels. In the SAR group, the comparison of miRNA expression levels in and outside the allergen season was performed using the Wilcoxon test with two related samples. Spearman's correlation test was also used to determine correlations between miRNA expression and clinical characteristics. Receiver operating characteristic (ROC) curves were generated for miR-125b, miR-181a, and miR-206. The sensitivity, specificity, cut-off value, and 95% confidence intervals (CI) were calculated based on the ROC curves. P-values <0.05 were considered to be statistically significant.

Results

Circulating miRNAs showed altered expression levels between study subgroups

We compared the miRNA expression levels between healthy subjects, patients with SAR (out of season), PAR, and NA-A (Fig. 1). The expressions of miR-125b and miR-181a were lower in the SAR group than in the control group (p=0.039 and p=0.014, respectively) (Figs 1B and 1C). A comparison of expression levels of selected miRNAs between the NA-A and control groups revealed that miR-206 expression was lower in asthmatics than in healthy subjects (p=0.002), and the same result was found with PAR (p=0.024) (Fig. 1E). Expression levels of miR-181a were different between patients with SAR and NA-A (p=0.003), and also between the SAR and PAR (p=0.001) groups in multiple comparisons.

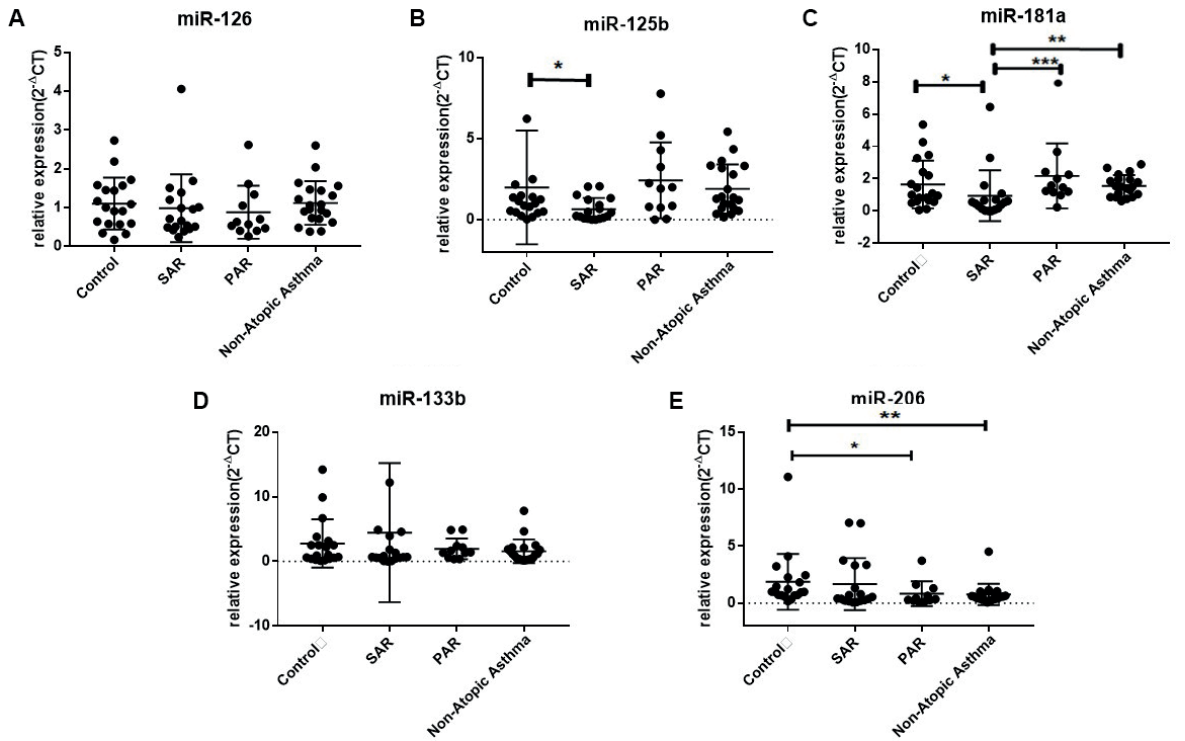

Fig. 1. Comparison of relative expression levels of serum miRNAs miR-126 (A) miR-125b (B) miR- 181a (C) miR-133b (D) and miR-206 (E) in healthy controls, SAR, PAR and asthma patients. *P<0.05 **P<0.01 ***P<0.001

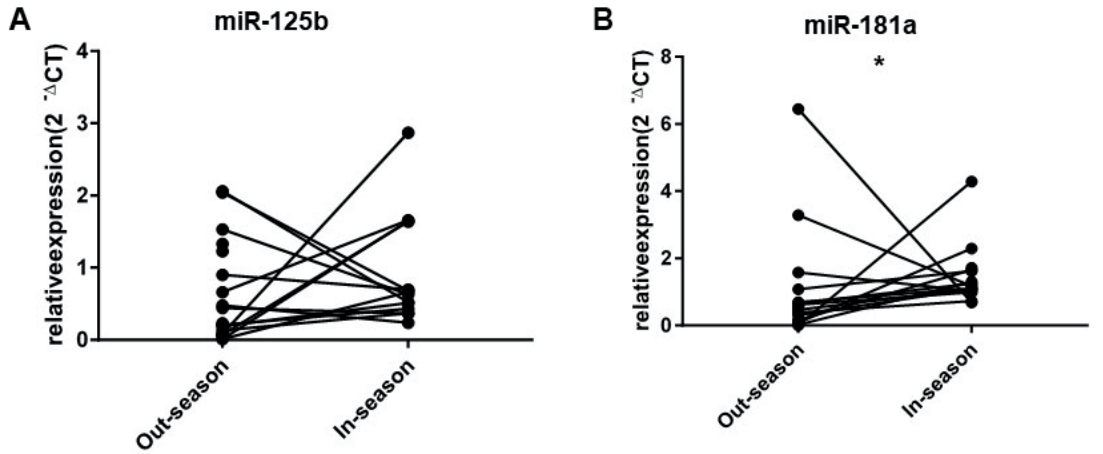

Fig. 2. Comparison of relative expression levels miR-125b (A) and miR-181a (B) between out of and in allergy seasons among SAR patients. *P<0.05

There was no difference in the expression of miR-126 and miR-133b between all study subgroups.

In the allergen season, expression levels of miR-125b and miR-181a were higher than outside of the allergen season (Fig. 2), but this increase was significant only for miR-181a (p=0.041).

Correlations between circulating miRNAs and clinical parameters

There was a negative correlation between miR-125b expression levels and PEF measurements in the SAR (r=-0.572, p=0.013) and PAR (r=-0.685, p=0.029) groups. Total IgE levels were positively correlated with miR-206 levels (r=0.817, p=0.007) in the PAR group. Although there was no differential expression level of miR-126 in the study populations, there was a positive correlation between miR-126 levels and MEF/25-75 values in patients with NA-A (r=0.491, p=0.028). Correlation analysis results are summarized in Table II.

miRNAs as a diagnostic marker

To evaluate whether miRNAs that were found differentially expressed in SAR, PAR, and NA-A could discriminate AR and NA-A, we performed a ROC curve analysis. As shown in Fig. 3, the area under the curve (AUC) of individual miR-125b and miR-181a were 0.697 (95% CI: 0.526-0.869, p=0.040) and 0.735 (95% CI: 0.567-0.903, p=0.014), respectively. The sensitivity and specificity values at the best cut-off points that yielded the maximum value of sensitivity plus specificity for miR-125b were 61.1% [positive predictive value (PPV) 68.8%] and 73.7% [negative predictive value (NPV) 66.7%], respectively. The sensitivity and specificity values for miR-181a were 61.1% (PPV=78.6%) and 84.2% (NPV=69.6%), respectively.

Table II. Correlations between clinical findings and miRNA levels.

Disease Group	miRNA	Clinical Finding	Correlation (R)	P value
SAR	125b	PEF	-0.572	0,013
PAR	125b	PEF	-0,685	0,029
PAR	miR-206	Total IgE	0.817	0.007
Non-atopic Asthmatic	miR-126	MEF/25-75	0.491	0.028

SAR: seasonal allergic rhinitis, PAR: perennial allergic rhinitis

ROC curve analysis performed to evaluate the diagnostic value of miR-206 for NA-A revealed that the AUC was 0.763 (95% CI: 0.607-0.919, p=0.005) with good sensitivity and specificity, 70% (PPV=82.4%) and 84.2% (NPV=72.7%) respectively (Fig. 4A).

The diagnostic value of miR-206 in diagnosing PAR showed an AUC value of 0.751 (95% CI: 0.548-0.954, p=0.024) and sensitivity and specificity at the best cut-off points were obtained as 72.7% and 84.2%, respectively (Fig. 4B).

Comparisons in ROC curve analyzes were made between the control group and the other groups.

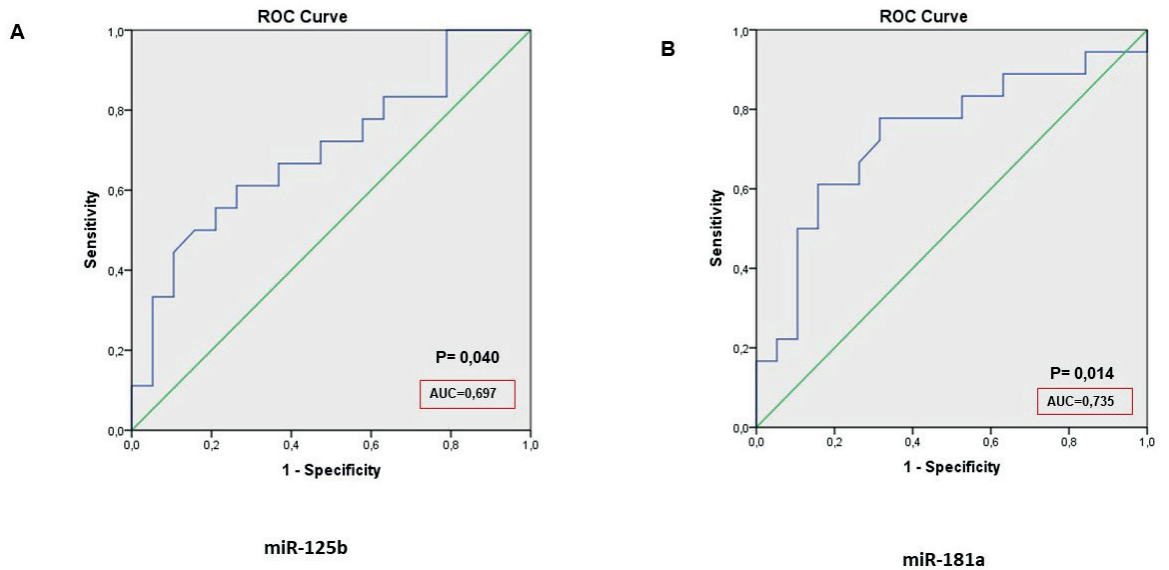

Fig. 3. ROC curve analyses of serum miR-125b (A) and 181a (B) in SAR patients.

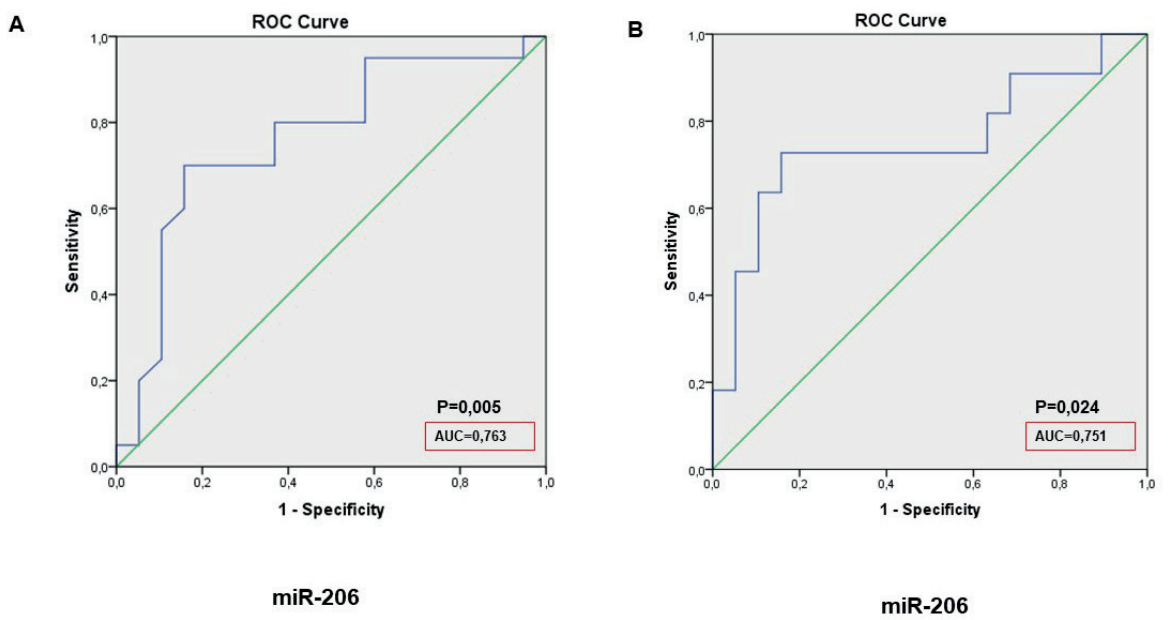

Fig. 4. ROC curve analyses of serum miR-206 in (A) NA-A and (B) PAR patients.

Discussion

To identify the candidate miRNAs that can discriminate between different forms of allergic rhinitis, that also differ in and out of the allergen season and to establish whether various miRNAs were effective in patients with non-atopic asthma and allergic rhinitis we investigated the expression levels of miR-125b, miR-126, miR-133b, miR-181a, and miR-206 that were found related to allergic diseases according to the literature. As a result of our study, we found that miR-181a and miR-125b are differentially expressed in patients with seasonal AR in and out of allergen season also compared to the patients with perennial AR and non-atopic asthmatics. Results also revealed that the expression of miR-206 is decreased in patients with NA-A and PAR compared with the controls. We also found the expression levels of miRNAs correlated to clinical parameters such as lung function and total IgE levels.

Recent studies identified that specific miRNAs had critical roles in the pathogenesis of several allergic diseases including asthma, eosinophilic esophagitis, atopic dermatitis, and AR. These studies also showed that expression levels of miRNAs are different according to the type of allergic disease and miRNAs might serve as noninvasive biomarkers for diseases. In accordance with the literature, our study showed that the expression levels of miR181a and miR-125b are different according to the type of allergic disease and the presence of allergens.

Many studies investigating the role of miR-125b in immune response and inflammation have been conducted in recent years.[19-23] Several studies have shown that miR-125b expression decreases under inflammatory conditions[19,21-23], whereas other studies found it was related to increased inflammation.[5,20] Liu et al.[16] demonstrated that the expression level of miR-125b was decreased in the sputum of asthmatics compared with controls and miR-125b could inhibit goblet cell differentiation. Tili et al.[21] reported that the expression of miR-125b decreased in macrophages of LPS-stimulated mice. In another study, miR-125b was upregulated in naïve T cell and inhibits T cell differentiation.[23] In contrast to these studies, in another study, miR-125 was shown to be effective in signaling pathways such as STAT3, MAPK, and PI3K-Akt. Quantitative RT-PCR analysis showed that the expression of miR-125b increased in patients with asthma and AR.[5] In our study, we found the expression of miR125b was significantly lower in patients with SAR than in the controls and also increased in the allergen season. Since miR-125b has been shown to inhibit T cell polarization, we can say that its lower expression in SAR compared with the controls allows for more T cell polarization, in favor of Th2-type inflammation. It is also acceptable to consider that under allergen attack during the allergen season the expression of miR125b increases to suppress polarization of naive T cell to Th2 T cells, as an immune response to prevent the development of excessive Th2 response.

Another miRNA that we found with lower expression in patients with SAR compared with controls in this study was miR-181a. However, the expression of miR-181a was found to be increased during the allergen season compared with outside the allergen season. Neilson et al. reported that miR-181a expression was elevated in immature T cells and decreased in Th1 and Th2 cells. miR-181a has been reported to suppress the expression of several genes (TCRa, CD69, BCL-2) that are effective in T cell maturation.[24] Blevins et al.[25] reported that miR-181a was a negative regulator of TCR signaling and limited T cell activation. A lower percentage of Treg and lower expression of IL-10 and TGF-β was shown in children with AR, and also significantly lower expressions of miR-155 and miR-181a in Treg cells in children with AR.[15] In another study, miR-181a was found to be inversely correlated with serum osteopontin levels, IL-4/IL-5, and total nasal symptom scores, but positively correlated with IFN-γ/IL-12 in patients with pediatric AR.[26] The increase of inflammation during the allergen season results in an increased number of Tregs and positive

regulation of the expression of cytokines IL-10, transforming growth factor (TGF)-β, and interferon (IFN)-γ, which is known as the anti-inflammatory response.[15,27,28] In our study, the increase in miR-181a levels during the allergen season is thought to be due to the anti-inflammatory response of the cell to suppress Th2 inflammation, since that miR-181a limits T cell activation and its level positively correlated with IFN-γ/IL-12 according to the literature.

miR-206 expression was found to increased in human macrophages and related to an increase in the secretion of inflammatory cytokines and MMP-9, and reduced tissue inhibitor of matrix metalloproteinase (TIMP-3) expression.[29] In a study of breast cancer, it was shown that increased expression of miR-206 significantly impaired migration and the invasive ability of cancer cells, and suppressed epithelial-mesenchymal transition (EMT) via TGF-β.[30] It is known that TGF-β acts on eosinophils, macrophages, monocytes, and neutrophils, and also reduces IgE secretion.[28] The effect of miR-206 on TGF-β and EMT is thought to be related to remodeling in asthma. In another study, 12 of the circulating miRNAs were found to be associated with exacerbations in asthma, and particularly, miR-206 had an important role in exacerbation.[13] Panganiban et al.[5] showed that six circulating miRNAs, including miR-206, were effective in allergic states, and expression of miR-206 increased in plasma in patients with AR. The expression level of miR-206 seems related to the atopic status of patients. Briefly, the role of miR-206 in the pathogenesis of diseases can change depending on whether it is suppressive or inflammatory. In our study, it was found that miR-206 expression was lower in the circulation of patients with NA-A and patients with PAR compared with healthy controls, but did not differ between SAR and controls. In addition, the expression level of miR-206 was positively correlated with the total IgE values of patients with PAR. These data are partly inconsistent with the role of miR-206 in allergic inflammation in the literature, due to lower or similar expression status of miR-206 in PAR and SAR patients compared to healthy controls.

In parallel with the expression results, the evaluation of the predictive value of miRNAs, which were found differentially expressed between the groups, as a diagnostic biomarker by using ROC curve analysis, showed that both miR125b and miR-181a for SAR, and miR-206 both for NA-A and PAR, could be used as biomarkers.

In the literature, expression of miR-126 has been reported as increased in patients with asthma and AR compared with controls, and also in cultured bronchial epidermal cells stimulated with IL-13.[5,14,31] In our study, we found no change in miR-126 expression in the study groups. However, there was a positive correlation between the expression levels of miR-126 and the MEF/25-75 values of patients with NA-A (p=0.028). We interpreted this result as follows: because the higher MEF/25-75 values are associated with better lung function, lower miR-126 levels in asthmatics are associated with worse respiratory function. Suojalehto et al.[10] reported low miR-126 expression in patients with chronic asthma, and their findings were consistent with the low MEF/25-75 values and baseline levels of miR-126 observed in our patients.

Although miR-133b has been found related to AR in both mouse models and studies with human subjects[5,32], in our study miR-133b expression was not significantly different in the study groups and was also not correlated with any clinical parameters.

Our study has some limitations. One is the low number of patients in the study groups. Secondly, the correlation between miRNA levels and inflammation markers such as cytokine levels is the missing part of the story in our study. Showing a correlation between inflammation markers and levels of circulating miRNAs, which was found significant in this study, may have supported our hypothesis.

We demonstrated that the expression of several circulating miRNAs was different between healthy individuals and allergic subjects, and between subjects with different allergic diseases. We also found the expression levels of miRNAs correlated to clinical parameters such as lung function and total IgE levels. According to our findings, we suggest that circulating miR-181a and miR-125b could be candidate biomarkers for SAR, and miR-206 for NA-A and PAR. Studies with larger numbers of patients are needed to more clearly assess the role of these circulating miRNAs as a biomarker in allergic diseases.

Ethical approval

This study was approved by the Ethics Committee of Hacettepe University (GO18/433). All procedures performed in this study were in accordance with the ethical standards of the Hacettepe University Ethical Committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: EB, FT, ÜMŞ; data collection: FT, MO, HÜ, ÖUS, BES; analysis and interpretation of results: FT, EB, ÜMŞ; draft manuscript preparation: EB, FT, ÜMŞ. 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

Pedipacks in the transfusion of pediatric patients to reduce wastage of blood components: an observational study from a tertiary center

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ABSTRACT

Background. Pedipacks prevent wastage of blood components but they are not used efficiently in pediatric clinics.

Methods. Red cell concentrate (RCC) and platelet concentrate volumes transfused in the last eight months in the pediatric clinics were screened. To calculate the wastage of blood components, the number of transfused pedipacks, whole unit RCC, and platelet units were screened from transfusion laboratory digital records to show the number of whole RCC units or platelets units used instead of pedipacks. The study results were shared with physicians and transfusion laboratory staff and they were trained on the subject in meetings. Two years later, the transfusion laboratory records were assessed again to evaluate pedipack usage. A google questionnaire was also submitted to the transfusion laboratories of other hospitals to assess the use of pedipacks.

Results. RCC and platelets were used in 82.9% of the transfusions, and 31.2% of RCC and 18.4 % of platelets were transfused to patients ≤12 months. During the study period, 569 pedipacks and 117 random donor or apheresis platelets separated into satellite packs would be required. But only 48 pedipacks of RCCs and 24 units of random donor platelets/apheresis platelets separated into satellite packs were used. After two years, in RCC transfusions of 0-12 month-old patients, the transfusion laboratory release of pedipacks increased to 67.9% from 13.5%. Other centers were not also using pedipacks efficiently. The main reasons were unawareness of the subject, the blood bank delivering two units of pedipacks even when only one unit was ordered and the risk of not using the second pedipack before the expiry date, and the short expiry date of irradiated pedipacks.

Conclusions. By increasing awareness of the subject, the collaboration of the clinic and laboratory and solving bureaucratic problems, rational use of blood components can be achieved.

Key words: transfusion, pediatric, pedipack, platelet, red cell concentrate.

The transfusion of blood and its components is frequently a life-saving procedure. Blood components are valuable and they should not be wasted. They are ready for use after many procedural steps including donor selection, donation, screening tests to detect the presence of transfusion-transmissible infection agents, blood grouping, and separation into components. Maintaining optimal storage and transport conditions is also essential. Platelets are also collected by apheresis which is a more expensive procedure and laborious for donors. It is not easy to find healthy volunteer donors; throughout the world, blood services are trying to develop strategies for donor acquisition and retention to prevent the shortage of blood components. It is reported that in our country with a population of 83,614,000, the annual blood donation requirement is about 3,000,000 units.[1] The COVID-19 (Coronavirus disease 2019) pandemic also affected blood services; donations decreased and concerns regarding the safety of blood products have emerged.[2]

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In the 21st century, neonatal and pediatric intensive care facilities increased and in these units, patients require frequent blood transfusions. In the top-up transfusion practice of newborns, 10-20 ml/kg red cell concentrates (RCC) are used.[3] If pedipacks are not used, blood components are wasted. Pedipack use may also reduce exposure to multiple donors. Exposure of a patient to multiple donors increases the risk of transmission of blood-borne infections and immunization with foreign antigens.

In newborns, 10 ml/kg platelet concentrate is transfused targeting a platelet count of at least 20 x 10^9/l for stable term infants, 30 x 10^9/l to 50 x 10^9/l for preterm infants. For infants who are bleeding or who have a consumptive coagulopathy, or undergoing invasive procedures, the target platelet count is even more than 50 x 10^9/l.[4] Random donor platelets derived from one unit of fresh whole blood are enough for non-bleeding newborns and infants. Apheresis platelets also may be divided into two or three satellite bags and may be given to newborns and infants.

RCCs derived from one unit of whole blood are generally separated into four pedipacks each with a volume of 80 ml. One pedipack is enough for all neonatal transfusions and the majority of infant transfusions. Even 30 ml packs were prepared and their transfusion was found cost-effective for some preterm babies.[5] Preterm neonates become frequently anemic partly due to diagnostic phlebotomy losses and require top-up transfusions. When pedipacks prepared from a single donation are used in repeated transfusions, this may result in the use of older blood. The age of red blood cells in the Premature Infants trial reported no effect on clinical outcomes for preterm neonates using red cells of different storage ages.[6] Although studies showed the impact of single blood donor exposure programs for infants, pediatric hematologists presumed that pedipacks were rarely used in hospitals.[7,8] Due to similar observations, this study was planned by pediatric hematologists at a single center to increase pedipacks usage. All the blood and blood components transfused in the last eight months in the pediatric clinic of the center were recorded retrospectively; the results were shared with neonatologists, pediatricians, and the transfusion laboratory staff to attract their attention to the subject. They were trained on the subject and after two years, the transfusion laboratory pedipacks released were screened again to show the outcome of the study. A google questionnaire was also submitted to some centers to investigate the pedipack usage in the country and to show reasons for limited pedipack usage.

Material and Methods

Ethical approval was obtained from Ethics Committee of Kocaeli University (No.2018/28).

The study was planned in five steps:

Step 1: This step of the study was conducted in the pediatric clinic. Transfused blood components in the last eight months (February 2018 -September 2018) were screened from patient files and electronic records. The age of the patient, transfused volume, transfused blood component and blood groups of the components were recorded. Transfused RCC and platelet concentrate volumes were classified as ≤80 ml, 81-160, and >160 ml because one pedipack contains 80 ml. The number of consumed RCC (whole units and pedipacks) and platelets were recorded from transfusion laboratory records to evaluate the wastage of blood components. Data collection by the research assistant was completed in three months.

Step 2: In 2019, the results of the study were shared with the physicians of the department of pediatrics. Contents of the pedipacks and volume wasted when whole unit RCC are used in newborn and infant transfusion, risks of multiple donor exposure, and increasing expenses when pedipacks are not used were explained. The difficulty of finding blood donors, blood component producing process, storage and logistic conditions, and expiry dates of components were emphasized. In

meetings with the transfusion laboratory staff of the center, volume transfused to newborns and infants and transfusion volume per kg in children were explained. Release of the components to the clinic considering the age and weight of the patient was recommended. Blood laboratory staff were encouraged to store enough pedipacks and always send pedipacks for the transfusion of patients younger than 12 months. In these face-to-face conversations, their awareness of the subject was increased and reasons for inefficient use were determined. They reported that there was no pedipack alternative in the software program of the hospital when physicians ordered blood components and the price of pedipacks was missing in the national health insurance system. These bureaucratic problems (adding the pedipack to hospital software, pricing the pedipacks) were solved. The training was completed in two months.

Step 3: After two years, the digital records of the transfusion laboratory were screened again (February 2020 -September 2020) to evaluate the pedipack use in patients ≤12 months.

Step 4: In 2021, a google questionnaire was submitted to transfusion laboratories of other hospitals to assess the usage of pedipacks. In addition to multiple-choice questions containing home-center problems, there were open-ended questions about the reasons for limited pedipack usage.

Step 5: Google questionnaire results were shared with directors of Red Crescent Blood Service and some recommendations were given to decrease the waste of blood components.

Statistics: Descriptive statistics (percentage) were used. (IBM SPSS 20.0- IBM Corp., Armonk, NY, USA). Frequencies were calculated as a percentage.

Results

In the pediatric clinic, 2169 transfusions were performed on 301 patients ≤18 years in the eight-month study period. Blood components were RCC (1134 units, 52.2%), platelets (668, 30.7%) fresh frozen plasma (FFP) (366 units, 16.8%), and one unit of whole blood which was used for exchange transfusion. Predominant blood groups of transfused RCC were ARh + (40.1%) and 0Rh+ (25.7%); similarly, predominant transfused platelet blood groups were A Rh + (50.6 %) and 0 Rh +(20.2%). The numbers and volumes of transfused RCCs and platelet concentrate in different age groups are shown in Tables I and II. When patients' age was evaluated, 31.2% (354/1134) of RCC and 18.4% (123/668) of platelets were transfused to patients ≤12 months.

All newborn transfusions were ≤ 80 ml. In 77% of infants, only one and in 14% two pedipacks of RCCs were required. Random donor platelets prepared from one unit of whole blood (40-80 ml) or apheresis platelets separated into three or two satellite packs (50 and100 ml) would be enough for newborns and infants. During the study period, 347 pedipacks with a volume of 80 ml RCC and 111 two pedipacks RCC (a total of 569 pedipacks) would be required whereas 117 random donor or apheresis platelets separated into satellite packs would be enough.

Table I. The numbers and volume of transfused red cells concentrate in different age groups.

Age	Transfused red cell concentrate volume		
	≤80 ml	81-160 ml	>160ml
0-1 month	219 (100%)	0	0
>1 -12 month	104 (77.0%)	19(14.0%)	12(8.8%)
>12 month - 18 year	24(3.0%)	92(11.7%)	664(85.1%)
Total	347 (30.9%)	111 (9.9%)	666 (59.2%)

Table II. The numbers and volume of transfused platelet concentrate in different age groups.

Age	Transfused platelet concentrate volume		
	≤80 ml	81-160 ml	>160ml
0-1 month	91(100%)	0	0
>1 -12 month	18(56.2%)	14(43.7%)	0
>12 month - 18 year	8(1.4%)	154(28.2%)	383(70.2%)
Total n (%)	117 (17.5%)	168 (25.1%)	383 (57.3%)

But generally whole unit RCCs were used; only 48 pedipacks of RCCs and 24 units of random donor platelets/apheresis platelets separated into satellite packs were used. For children >12 months, two pedipacks from a single donation would be enough in 92 RCC transfusions. Apheresis platelets separated into satellite packs would be enough in some > 12-month-old stable patients especially if they were not undergoing invasive procedures. FFP transfused to infants and newborns were not evaluated because no pedipacks were available for FFP in the country and due to limited indications and long half-life, there was always excess FFP in blood banks.

During the study period, the cost of one pedipack RCC was 100TRY whereas one whole RCC unit was 217 TRY. If 569 units of pedipack RCC were used instead of 48 units, the hospital budget will save 36.870 TRY. The cost of apheresis platelets was 329 TRY and one pooled random donor platelet was 379 TRY. If the apheresis platelet was split into two satellite bags each will cost about 165 TRY. If satellite bags were used in all 117 platelet transfusions <80 ml instead of only 24 random donor platelets/ half unit of apheresis platelets, about 19.500 TRY would be saved. (1Euro=10 TRY during the study period). Pooled random donor platelets were derived from 3-4 units of whole blood but for transfusion of newborns and infants 1-2 units of random donor platelet would be enough and at least 190 TRY will be saved for each transfusion.

The reasons for inefficient use of pedipacks in the center were: a) absence of pedipack alternative in hospital software program when ordering blood components b) absence of pricing of pedipacks in the national health insurance system c) Residents' and physicians' unawareness about pedipacks and waste of resources d) Lack of repeated transfusion training for rotating residents d)Unawareness of the local transfusion laboratory staff about the volume transfused to neonates and infants and waste of the components. e) Lack of communication between clinic and transfusion laboratory staff.

Study results determined the quantity of pedipack requirement of the clinic; whether the laboratory stored enough pedipacks according to these numbers and then clinicians guaranteed that they will order pedipacks. These communications eliminated the anxiety of the transfusion laboratory staff about the wastage of pedipacks due to expiry. After two years of the study, to evaluate the pedipacks usage, digital records of the transfusion laboratory were screened again. Comparison of pedipacks usage in RCC transfusions before and after training is shown in Table III. In RCC transfusions of 0-12 month-old patients, transfusion laboratory release of pedipacks was 13.5% before training whereas this increased to 67.9% after training. Due to the blood laboratory staff's awareness and organization, blood products in pedipacks were not wasted due to expiry date problems.

A Google questionnaire was submitted to the directors of transfusion laboratories of other hospitals with newborn intensive care units and pediatric inpatient units that could be reached by mobile phone or e-mail. Out of 43 directors, 41 answered the questionnaire; 46.3% (n=19) of these hospitals were Education and Research Hospitals of the Ministry of Health, 24.3% (n=10) were University Hospitals of Government, 24.3% (n=10) were Government Hospitals of the Ministry of Health and 4.8%

Table III. Comparison of pedipack usage in red cell concentrate transfusions before and after training in an eight-month period.

Age	Before Training (year 2018)		After Training (year 2020)	
	Pedipacks	Whole RCC Unit	Pedipacks	Whole RCC Unit
0-1 month n	48	171	59	13
>1 -12 months n	0	135	121	72
Total n (%)	48(13.5%)	306(86.4%)	180 (67.9%)	85(32.0%)

(n=2) were Private Hospitals. The frequency of pedipack usage in newborn units of 41 centers is shown in Figure 1: 19 (46.3%) centers never used, 10 (24.4%) rarely used, 9 (22%) always used, and 3 (7.3%) used in less than 50% of the newborn transfusions. Centers' answers for reasons of limited use of pedipacks: a) 16 centers (50%) stated ''We do not order pedipacks from blood service because we may not transfuse it until the expiry date.'' b) 10 centers (31.3%) stated ''Pediatricians and neonatologists are not aware of the pedipacks and they do not order this component. '' c) 9 centers (28.1%) stated ''We are not used to transfusing pedipacks.'' d) 7 centers (21.9%) stated ''This questionnaire increased our awareness about pedipacks and we will discuss this subject with neonatologists and pediatricians. Some directors also explained that blood services always sent a pair of irradiated pedipacks even when they ordered one for transfusion of a premature baby and the second irradiated pedipack would expire if they could not transfuse it within three days. Transfusion centers reported that they had no blood irradiator and they had to order irradiated products for premature babies.

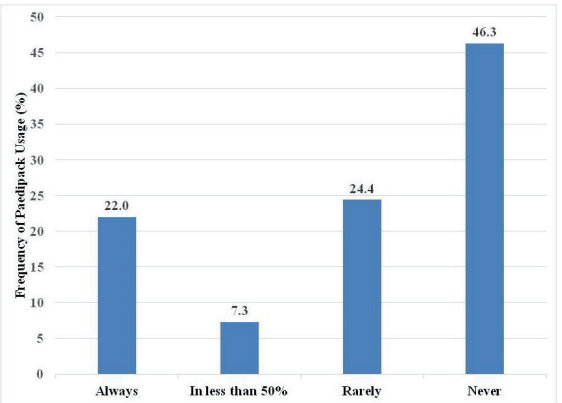

Fig. 1. Frequency of pedipack usage in newborn units of 41 centers.

Discussion

The present study showed that nearly one-third of the blood components were administered to newborns and infants in the pediatric clinic where <18-year-old patients were hospitalized. RCC in pedipacks was used in only 14.0% and random donor platelets or apheresis platelets split into satellite packs were used in only 22.0% of <80 ml transfusions of ≤12 month-old patients. In this center, about 42 units of RCC and 15 units of platelets were transfused to newborns and infants monthly. A multicenter study showed that 51.6 % of preterms had transfusions at the intensive care units.[9] Although there was controversy about the threshold of transfusion in stable premature infants, they require multiple top-up transfusions.[10] In neonatal wards and neonatal intensive care units 47.3% received one, 18.6% received two and the remaining infants received three or more RCC transfusions. Infants with a birth weight below1500 g were the group that required the highest RCC transfusions.[7]

One unit of RCC from one donation contains about 250-300 ml. Triple or double pedipacks from one donor with a storage period of 35 days would reduce wasted RCC volume and the number of donor exposure. A study showed that in a single-bag system 118.5±12.5 ml was the mean volume wasted per transfusion and the number of donor exposure was 4.4±3.5 in neonatal units. When double or triple bags were used, patients were exposed to about two donors.[7] Another study also showed that after implementing the pedipack system, red cell wastage per transfusion decreased to 24·5±10 mL.[11] During the study period, even in patients older than 12 months, 2 pedipacks RCC 80 ml each would be sufficient in 92 pediatric transfusions. In the study center, a blood irradiator was present in the transfusion laboratory and irradiation was performed just before transfusion if indicated. Indications for whole blood and cellular blood components were intrauterine transfusions, transfusions of premature infants (birth weight<1300 g), exchange transfusions, patients with leukemia and lymphoma, patients with solid tumors receiving chemotherapy, transplant patients and patients with aplastic anemia on immunosuppressive treatment.

Resource-saving is important in transfusion practice. In addition to difficulty in gaining volunteer donors, it is a subject of respect to donation. Physicians' training and awareness about transfusion practice are not always satisfying. Transfusion camps organized in Canada and United Kingdom for post-graduate training were a good solution to this problem.[12] A good collaboration between the clinical team and blood transfusion laboratory is essential for organizing transfusion practice rationally and for preventing waste of products without impairing the safety of patients.

Severe thrombocytopenia is a frequent finding in neonatal intensive care units and especially preterm neonates require prophylactic platelet transfusions to prevent intraventricular and other hemorrhages. Due to the short half-life of platelets, the prevention of waste of this blood component and reducing donor exposure will not be as easy as RCCs. The close interaction of physicians with transfusion laboratories is required to reduce wasted volume and donor exposure. In Turkey, there are regional blood banks of the Turkish Red Crescent Organization and due to logistic reasons ordered platelets arrive at the hospitals once a day. Transfusion laboratories prefer to store pooled or standard apheresis units of platelets to supply any patient, either newborn or adult. If apheresis procedures, donation, and blood component production were performed also in some hospitals as in previous years, preparing pedipacks from dedicated apheresis donors or random donor platelet production for infants would be possible. Splitting platelet units into two doses was among efforts to overcome platelet shortage during the COVID-19 pandemic in some countries.[13]

In this retrospective study, determining the reasons for ineffective use of pedipacks during face-to-face meetings, providing awareness and collaboration of clinicians and transfusion laboratory staff, and solving problems like adding pedipack alternatives to software programs increased pedipack use in pediatric patients without wastage of pedipacks due to expiry.

However newborn transfusions decreased in the last evaluation due to decreasing occupancy in the neonatal intensive care unit. This was mainly due to the establishment of new neonatal intensive care units in the district and the referral of some patients to these units. Lowering the transfusion threshold and referral of COVID-19 positive pregnant women to pandemic hospitals were also other factors for reduced transfusion numbers in the newborn intensive care unit.

The Google questionnaire was performed presuming that inefficient use of pedipacks was a general problem in the country. Answers revealed that like home-center, unawareness of the physicians and transfusion laboratory staff about the subject and lack of communication between clinicians and laboratory staff were the main reasons in addition to the anxiety of wastage of pedipacks due to expiry. Reducing expired blood components was the responsibility of the transfusion laboratory but hospital administrations were unaware of the wasted volumes in the pediatric clinic and these were never recorded. The short expiry date of irradiated blood components and always delivering two pedipacks even if one was ordered were other reported reasons.

The Google questionnaire results were shared with directors of the Red Crescent Blood Service and some recommendations were given to decrease the waste of blood components: a) supplying pedipack RCC (either irradiated or not) according to the order of the transfusion laboratories instead of routinely supplying two pedipack units, b) Preparing pooled platelets from two random donations for pediatric patients.

The limitation of the study is that the number of transfusions per patient, and the number of donor exposures of each patient were not calculated. Additionally, the Google questionnaire could not be submitted to all of the centers in the country.

Increasing the use of pedipacks and decreasing the wasted volume of blood components is possible with training and good communication among physicians, transfusion laboratory staff, and blood services.

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Ethical approval

Ethical approval was obtained from Ethics Committee of Kocaeli University (No.2018/28).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: NS; data collection: NAK; analysis and interpretation of results: NAK, EZ, SAG; draft manuscript preparation: NS, NAK. 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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Original Article

Decreased antioxidant capacity with serum native thiol and total thiol levels in children with hemophilia A: a prospective case-control study

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ABSTRACT

Background. Experimental studies have addressed the role of oxidant stress in the pathogenesis of Hemophilia A. This study aimed to determine whether dynamic thiol-disulfide exchange, a recently recognized cellular defense system against oxidative stress, is disturbed in children with hemophilia A.

Methods. This prospective case control study included male children with hemophilia A (n=62) and randomly selected healthy age and sex-matched controls (n=62). Serum native thiol, total thiol and disulfide levels were analyzed with a novel spectrophotometric method. Ratios of disulfide/total thiol, disulfide/native thiol, and native/total thiol were calculated. Statistical comparisons were made using the independent samples t-test or the Mann-Whitney U test, according to whether the data were normally distributed or not.

Results. Serum native thiol (385.0 ± 35.9 versus 418.0 ± 44.3, respectively; p<0.001) and total thiol (424.2 ± 38.7 versus 458.0 ± 46.3, respectively; p>0.001) levels were significantly lower in children with Hemophilia A compared to controls. Children with hemophilia A had significantly lower serum native thiol to total thiol ratio than controls (p=0.024). Serum disulfide levels of children with hemophilia A were close to controls (19.2 [17.6- 22.1] versus 19.8 [17.8- 21.2]), respectively; p=0.879) whereas disulfide to native thiol ratio (p=0.024) and disulfide to total thiol ratio (p=0.024) were significantly higher.

Conclusions. Decreased antioxidant capacity with levels of serum native thiol and total thiol in children with hemophilia A might be regarded as evidence for the disturbance of thiol/disulfide balance. Antioxidant treatment can be a future target of therapy in children with hemophilia A.

Key words: Hemophilia A, children, thiol, oxidative stress.

Hemophilia A is a bleeding disorder caused by Factor VIII deficiency due to an X-linked single-point mutation. Although different novel treatment options are emerging in children with hemophilia A, early initiation of bleeding prophylaxis with factor replacement remains the most widely accepted approach.[1] With the prevention of bleeding complications as well as the decrease in fatal viral transmission rates, the life expectancy of the children with hemophilia A has reached to the normal population, and the quality of life of individuals has increased significantly. In the last decade, novel therapeutic products and gene therapy have become popular research topics as novel management strategies in patients with hemophilia A.[2,3]

In-vitro studies and experimental models have suggested an important role of increased oxidative stress in the emergence and

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progression of symptoms in several single-point mutation diseases, including hemophilia A.[4] Misfolding of newly synthesized Factor VIII in the endoplasmic reticulum triggers a pathway cited as unfolded protein respond, which in turn induces apoptosis by increasing oxidative stress. The reaction could be reversed with antioxidant treatment in-vitro and in mice.[5] Immunogenicity and antibody development in patients with Hemophilia A as a complication of Factor VIII replacement were shown to be increased by ex-vivo oxidation of Factor VIII, which was resistant to in vivo treatment with N-acetylcysteine.[6] Moreover, it has been shown that the joint and bone destruction in hemarthrosis, a typical hallmark of Hemophilia A, is caused by the inability of macrophages to adequately eliminate the oxidative stress created by the proteins (but not recombinant products) in the plasma-derived Factor VIII concentrates.[7] However, the exact cellular mechanisms by which oxidative stress contributes to Hemophilia A pathogenesis has not yet been clarified.[8]

Dynamic thiol-disulfide exchange has become a common indicator in recent studies focusing on the role of oxidant stress in the pathophysiology of many diseases. Thiols, which are organic compounds, form a balancing defense against oxidative stress with the sulfhydryl (-SH) group they contain, and reversible disulfide bonds are formed as a result of the oxidation of these sulfhydryl groups.[9] The instability of Factor VIII was suggested to be due to the lack of non-covalent bonds in the A2 subunit and in-vitro and in vivo studies demonstrated that creating genetically engineered disulfide covalent bonds to this domain improved the stability of Factor VIII, indicating a novel target of therapy.[10,11] Thus, we hypothesized that children with hemophilia A may have dysregulated thiol/disulfide homeostasis associated with an increase in oxidative stress as well as a compensatory change in disulfide levels due to Factor VIII instability. The present study investigated whether serum native thiol, total thiol, and disulfide levels, as well as the balance between these parameters as an indicator of antioxidant capacity, were significantly altered in children with hemophilia A compared to healthy children.

Material and Methods

A prospective case-control study was conducted in the Department of Pediatric Hematology, Ankara Training and Research Hospital, between August 2020 and December 2020. The local ethics committee approved the study (No: E-20:378), which was performed according to the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from parents of all participating children.

A total of 62 male children aged between 2 and 18 years, diagnosed with hemophilia A were included (study group). The criterion for inclusion in the study group was that the children had received at least one Factor VIII concentrate prior to the study. One of the physicians in the pediatrics department interviewed and examined the subjects regularly at one-month intervals. The exclusion criteria were active infection, coexisting chronic hepatic, renal, cardiac, autoimmune, and rheumatological diseases. The control group consisted of randomly selected 62 healthy age- and sex-matched children who were examined in the pediatric hematology outpatient clinics. Clinical characteristics, including the duration of disease, Factor VIII levels, the frequency and the latest time of Factor VIII infusion, were recorded.

Venous blood samples were obtained from the participants, and the separated serum samples after centrifugation at 1500 x g for 10 minutes were stored at -80 °C. The measurements of native thiol (–SH) and total thiol (–SH+–S–S–) levels in serum samples of both children in the study group and the controls were performed by commercially available kits (Rel Assay Diagnostics, Turkey), using the spectrophotometric method developed by

Erel and Neselioglu.[12] As described by the developers, reducible disulfide bonds were first reduced to free functional thiol groups with sodium borohydride. The reductant sodium borohydride was removed with formaldehyde and the total thiol content of the sample was measured using Ellman reagent. Half of the value obtained after subtraction of the native thiol content from the total thiol content gave the amount of disulfide bond.

Statistical analysis

Descriptive statistics were given as mean ± standard deviation and median with interquartile range (IQR) of 25% to 75% for continuous variables depending on their distribution. Numbers and percentages were used for categorical variables. The Kolmogorov-Smirnov test was used to analyze the normal distribution of numerical variables and checked by Q-Q plots and histograms. The Levene test was used to check the homogeneity of the variances. The Independent Samples t-test was used to compare independent groups with variables with normal distribution. For variables without a normal distribution, the Mann-Whitney U test was applied. For statistical analyses, "Jamovi project (2020), Jamovi (Version 1.6.9) [Computer Software] (Retrieved from https://www.jamovi.org) and JASP (Version 0.14.1) (Retrieved from https://jasp-stats.org) were used. A p value of less than 0.05 was considered as statistically significant.

Results

Baseline characteristics are given in Table I. The mean age was similar between children with hemophilia A and controls (11.20 ± 5.40 vs. 11.16 ±5.41 years respectively; p=0.967). The median duration of hemophilia A was 11.0 years. The Factor VIII levels of 10 children (16.1%) were <2%, 15 (24.2%) were 2% - <5%, 37 (59.7%) were 5% - 20%.

Children with hemophilia A had similar hemoglobin (13.3 ± 1.8 versus 13.9 ± 1.7 g/dL, respectively; p=0.064) and hematocrit (39.5% [37.7- 44.4] versus 42.0% [37.8- 46.5], respectively; p=0.167) levels as controls (Table II). Serum native thiol (385.0 ± 35.9 versus 418.0 ± 44.3, respectively; p<0.001) and total thiol (424.2 ± 38.7 versus 458.0 ± 46.3, respectively; p>0.001) levels were significantly lower in children with Hemophilia A compared to controls. Serum native thiol to total thiol ratio was significantly lower in children with hemophilia compared to controls (0.911 [89.7 - 91.6] versus 0.914 [90.3 - 92.3], respectively; p=0.024). Serum disulfide levels of children with hemophilia A were close to controls (19.2 [17.6- 22.1] versus 19.8 [17.8- 21.2]), respectively; p=0.879) whereas disulfide to native thiol ratio (4.9 [4.6 - 5.7] versus 4.7 [4.2 - 5.4], respectively; p=0.024) and disulfide to total thiol ratio (4.4 [4.2- 5.1] versus 4.3 [3.9- 4.8], respectively; p=0.024) were significantly higher (Table II).

Discussion

The present study aimed to determine whether serum native thiol, total thiol, and disulfide levels and the ratio between these variables changed in children with hemophilia A compared to normal healthy controls, indicating an increase in oxidative stress. We found that serum native thiol and total thiol levels, as well as native thiol/total thiol ratios, were significantly lower in children with hemophilia A compared to healthy children. Our findings indicate that the

Table I. Clinical characteristics of children with hemophilia A (n=62).

Variable	Value
Duration (year)†	11.0 [6.0- 17.0]
Level of Factor VIII activity‡	
<2%	10 (16.1)
2%-<5%	15 (24.2)
5%-20%	37 (59.7)
Interval for last exposure (days)‡	
≤2	13 (21.0)
2-≤3	35 (56.5)
3-≤4	8 (12.9)
4-≤7	6 (9.7)

†: median [Q1- Q3], ‡: n (%). FVIII: clotting factor VIII.

Table II. Comparison of oxidative markers in children with hemophilia A and healthy control groups.

Variables	Hemophilia A (n=62)	Control (n=62)	p
Hemoglobin§	13.3 ± 1.8	13.9 ± 1.7	0.064
Hematocrit (%)†	39.5 [37.7- 44.4]	42.0 [37.8- 46.5]	0.167
Native thiol (μmol/L)§	385.0 ± 35.9	418.0 ± 44.3	<0.001
Total thiol (μmol/L)§	424.2 ± 38.7	458.0 ± 46.3	<0.001
Native /total thiol (%)†	0.911 [89.7- 91.6]	0.914 [90.3- 92.3]	0.024
Disulfide (μmol/L)†	19.2 [17.6- 22.1]	19.8 [17.8- 21.2]	0.879
Disulfide/native thiol (%)†	4.9 [4.6- 5.7]	4.7 [4.2- 5.4]	0.024
Disulfide/total thiol (%)†	4.4 [4.2- 5.1]	4.3 [3.9- 4.8]	0.024

§: mean ± SD, †: median [Q1- Q3]. IMA: ischemia-modified albumin

thiol/disulfide exchange may be dysregulated with a decrease in thiol levels in response to increased oxidant stress in children with hemophilia A.

We found that serum disulfide levels did not differ significantly in children with Hemophilia A, but both disulfide/total thiol and disulfide/native thiol levels were significantly higher. In case of oxidative stress, disulfide levels would be expected to be higher relative to healthy controls, and whether this is related to Factor VIII instability remains to be clarified. The stability and activation of Factor VIIIa heterotrimer, the cleaved and activated form of Factor VIII, depends on the non-covalent linkage of its A2 subunit with the A1/A3C1C2 dimer.[13,14] Genetically engineered disulfide covalent bridges between the A2 subunit and A3 extended the FVIIIa half-life[10], and the creation of an engineered disulfide interdomain bond between A2-A3 increased factor VIIIa's clotting activity by 90%.[11] It was claimed that disulfide bonds only serve to stabilize protein molecules, but over time research has proven that dynamic thiol-disulfide homeostasis acts as a defense mechanism against oxidative stress by establishing redox responsive covalent disulfide bonds between oxidation-sensitive thiol groups. The increase in oxidative stress causes the electrons transferred by the oxidative products to form reversible disulfide bonds by oxidizing the redox sensitive native thiol compounds. In this dynamic process, disulfide bonds are reversibly formed and then reduced, creating a dynamic switch between the conformational and functional states of redox sensitive molecules. However, when oxidative stress continues, the native thiol capacity decreases while the disulfide level increases linearly.[15] Disulfide levels in patients with hemophilia A did not increase proportionally with the decrease in thiol levels, we think that it may be because the disulfide formation-reduction exchange compensates for Factor VIII protein stabilization.

Inability of thiol-disulfide homeostasis to adequately cope with oxidative stress has been reported to have a role in many chronic inflammatory and hematological diseases.[16-18] Also, although thiol-disulfide homeostasis dysregulation was suggested to be associated with several protein misfolding diseases[19], no study has so far investigated whether thiol-disulfide homeostasis is dysregulated in patients with hemophilia A. There have been controversial results for the levels of native thiol, total thiol, disulfide, and IMA in various hematological diseases.[16-18,20-22] In one study on oxidative biomarkers in sickle cell disease, the authors reported decreased levels of thiol and disulfide.[21] Acute immune thrombocytopenia was another condition, in which decreased native and total thiol levels were detected.[17] Increased IMA and disulfide levels and reduced native and total thiol were reported in adult patients with myelodysplastic syndrome. Moreover, the disulfide/native thiol was found to be an independent risk factor for mortality in

myelodysplastic syndrome.[16] Beta-thalassemia and iron deficiency anemia were associated with increased levels of native thiol, total thiol, disulfide, and disulfide to total ratio.[18,20,22,] The authors proposed several mechanisms for the high levels of thiols, such as a compensatory antioxidant response against excessive oxidative stress, and transfusion-dependent elevation in a proportion of younger red blood cells.[18,20] We suggest that prospective studies are needed to clarify such controversies.

There were several limitations of our study which need to be addressed. First, the lack of power analysis for determining the sample size might cause smaller study group that was insufficient to interpret the meaning of the observed results. Second, the analysis of serum albumin levels to clarify the reciprocal relationship between serum thiol and disulfide levels and serum albumin was lacking. Third, potential complications including hemophilic arthropathy and number of annual bleeding status of the patients were not presented. Associations between theses and oxidative stress might have strengthened the message of the article. Lastly, enzymatic and non-enzymatic investigations of oxidative stress biomarkers were not used to compare thiol and disulfide levels.

Decreased antioxidant capacity with levels of serum native and total thiols in children with hemophilia A might be regarded as some evidence for the disturbance of thiol/disulfide balance. In addition to gene therapy and new therapeutic products, antioxidant therapy can be a future research topic in terms of both inhibitor development and prevention of complications in children with hemophilia A.

Ethical approval

Ethical approval was obtained from the Ethics Committee of Ankara Training and Research Hospital prior to initiation of the research work (No: E-20:378).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: ST, AG; data collection: ST, AG, AO; analysis and interpretation of results: AG, SN; draft manuscript preparation: ST, ÖE, BA. 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

Imaging spectrum of extracorporeal membrane oxygenation related neurologic events in children

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ABSTRACT

Background. Extracorporeal membrane oxygenation (ECMO) can be associated with severe neurological complications increasing morbidity and mortality. We aimed to evaluate imaging findings in patients with neurological complications associated with ECMO.

Methods. Children (<18 years) who had ECMO support and received cross-sectional imaging (cranial CT and/or MRI) were retrospectively evaluated. Age, gender, clinical and imaging findings were documented and the relation to ECMO duration and survival rates with imaging findings and imaging time (during ECMO or after weaning) were examined.

Results. Twenty children who had cranial CT/MRI during (n=6) ECMO and after weaning (n=14) were included in the study. The median duration of ECMO was 12.5 days (IQR=5-25 days) with a survival rate of 65%. Fourteen patients had positive imaging findings including ischemic stroke (n=4), hemorrhagic stroke (n=4), hypoxic-ischemic encephalopathy (n=2), posterior reversible encephalopathy syndrome (PRES) (n=3) and cerebral vein thrombosis (n=1). The duration of ECMO and survival rates did not significantly differ between patients with positive and unremarkable imaging findings. However, the survival rate was significantly higher ($p<0.001$) and the duration of ECMO was significantly lower in patients scanned after weaning compared to patients imaged during ECMO support ($p=0.033$).

Conclusions. Our series revealed PRES in ECMO-related neurologic events in addition to commonly reported thrombotic and hemorrhagic stroke in the literature. Availability of cross-sectional imaging and awareness of radiologists to these complications during ECMO or after weaning help in prompt diagnosis and treatment.

Key words: extracorporeal membrane oxygenation (ECMO), neurologic complications, imaging.

Extracorporeal membrane oxygenation (ECMO) is a set of extracorporeal life support providing cardiopulmonary bypass. Following the initial successful runs of ECMO in a posttraumatic patient in 1972 and then a newborn patient in 1976, it has been increasingly used worldwide in children and infants with severe cardiac or respiratory failure unresponsive to conventional therapies.[1-3]

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ECMO comprises a drainage cannula (either through a large central vein or right atrium) which drains deoxygenated blood from the body and passes it to the ECMO circuit. Deoxygenated blood is pumped into the ECMO circuit, where the O2/CO2 gas exchange takes place, and adjustments are done according to the metabolic needs of the patient. Then, a reinfusion cannula sends oxygenated blood backward to the body through a large central vein (venovenous - VV) or artery (venoarterial - VA) depending on the ECMO mode. Anticoagulation is used to avoid thrombosis in the circuit.[4]

According to a recent report of the Extracorporeal Life Support Organization Registry, the overall

survival of pediatric patients receiving ECMO was 61%.[3] The registry reviewed the most frequent neurologic complications among neonates and non-neonates according to ECMO indications, i.e. respiratory, cardiac and extracorporeal cardiopulmonary resuscitation (ECPR). The incidence of these neurologic complications varied according to age and ECMO indication: seizures 3-6%, central nervous system (CNS) infarction 3-11%, intracranial hemorrhage 6-14% and brain death 0.4-10%. Children with CNS hemorrhage had the lowest survival rates ranging between 21-40%, following brain death. It is reported that intracerebral hemorrhage was more common among neonates (11-14%) compared to non-neonates (5-9%), especially in patients in need of ECMO for cardiac support; but it carried a stronger association with mortality among non-neonates.[3] Additionally, these neurologic injuries during ECMO are related to an increased risk of mortality and neurologic disability among survivors.[5] Risk factors for neurological complications include indication of ECMO as ECPR, VA ECMO, carotid cannulation, younger age, renal failure, sepsis, plasma creatinine >3 mg/dL, lower pH, need for inotropic support and thrombocytopenia in children.[6-13]

Cranial imaging has a significant role in determining the precise and prompt diagnosis of neurologic injury. Most imaging studies of patients with ECMO support focused on stroke, whether hemorrhagic or ischemic and its relation to survival. On the other hand, neuroimaging spectrum of various complications, other than stroke, has not been studied and demonstrated in detail among children, yet. In this research, we aimed to determine the neuroimaging spectrum of complications during ECMO or shortly after weaning in children and its relation to factors including ECMO duration, imaging time-lapse from ECMO initiation and survival.

Material and Methods

This retrospective study was approved by the Hacettepe University institutional review board (IRB) (04.05.2021 - GO21/607), informed consent was waived by the IRB. Pediatric patients who received ECMO support during hospitalization between 01.01.2013 – 31.12.2020 were collected. Inclusion criteria were set as follows: 1) patients <18 years old 2) patients with cross-sectional cranial imaging (computerized tomography -CT- and magnetic resonance -MR-imaging) during ECMO support or within two weeks after weaning. Patients with known intracranial pathology before initiation of ECMO support were excluded from the study.

Ultrasound (US) was not included as a diagnostic imaging modality in our study, because it was not possible to review the US studies retrospectively and the inclusion of US reports would be limited to findings based on the subjective reports.

Age, gender, diagnosis of patients, indications of ECMO, type of ECMO (VA or VV), cannulation site, duration of ECMO were retrieved from the Hospital Information Systems and archives of the Pediatric Intensive Care Unit. The systemic complications that occurred prior to imaging and the time gap between cessation of therapy and cranial imaging were also noted.

Nonenhanced cranial CT (NECT) scans were obtained using 16- and 64-slice multidetector scanners (GE Optima, GE Healthcare, United States and Somatom Definition, Siemens Healthineers, Germany, respectively). All patients had axial sections with 3 mm thickness. MR studies were carried out on 1.5T scanners (Symphony, Siemens Healthineers, Germany and Achieva, Philips, Netherlands) and included axial T2 weighted image (WI), T1WI, fluid-attenuated inversion recovery (FLAIR), diffusion-weighted image (DWI) and susceptibility-weighted image (SWI). Two neuroradiologists with an experience of 5 and 21 years evaluated the cranial imaging studies in consensus.

Following documentation of clinical information and imaging findings, we searched for differences in terms of ECMO duration,

imaging time-lapse from ECMO initiation and survival when the patients were grouped according to neuroimaging findings i.e. patients with clinically important (positive) and normal/unremarkable (negative) findings and according to imaging time i.e.patients initially imaged during ECMO and after weaning. Also, the presence of renal failure, sepsis, inotrope support and thrombocytopenia were compared between patients with positive and negative imaging findings.

Statistical Analysis

Numeric variables were tested with the Kolmogorov-Smirnov test to clarify if normal distribution existed. If normal distribution was present, comparisons were made among groups using student t-test, otherwise, Mann Whitney-U test was used. Categorical variables were compared by chi-square test.

Results

One hundred and thirty-six patients received ECMO during the aforementioned period (seven years) in the pediatric intensive care unit. Cranial imaging was performed in 22 patients (16.2%). One patient had imaging four weeks after weaning and therefore was excluded from the study. Another patient with an underlying intracranial disease was also excluded from the study.

Twenty patients (female/male=5/15, median age 3.5 years with an interquartile range -IQR- 1.25-11.4) met the inclusion criteria. A chart showing the distribution of age among the study group is shown in Fig. 1. Diagnosis of the patients included congenital heart disease (CHD) (n=9), cardiomyopathy (n=5), pulmonary hypertension (n=2), Langerhans cell histiocytosis (n=1), immune deficiency (n=1), congenital diaphragmatic hernia (n=1), multisystem inflammatory syndrome in children (MIS-C) (n=1) (Table I). Indications for ECMO were cardiac support (n=13), ECPR (n=4), respiratory support (n=3) (Table I). Indications for cranial imaging were seizure (n=8), pupillary fixation (n=2), anisocoria (n=2), blurring of vision (n=1) alterations in consciousness (n=5), bradycardia (n=1) and headache (n=1) (Table I). The median duration of ECMO support was 12.5 days (IQR=5-25 days). Five patients could not be weaned from ECMO support (25%) and a total of 7 patients died (35%). Detailed information concerning clinical and imaging features of all patients are given in the Supplementary Table.

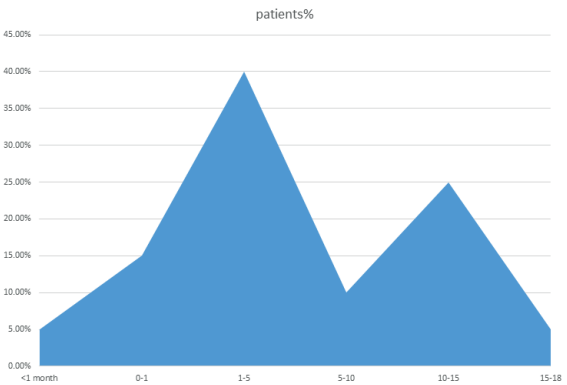

Fig. 1. Age distribution of patients.

Table I. Clinical features of patients.

Gender (M/F)	15/5
Age, years, Median (IQR)	3.5 (1.25-11.4)
Diagnosis	
Congenital heart disease	9
Cardiomyopathy	5
Pulmonary hypertension	2
Langerhans cell histiocytosis	1
Immune deficiency	1
Congenital diaphragmatic hernia	1
Multisystemic inflammatory syndrome in children	1
ECMO indications	
Cardiovascular failure	13
Respiratory failure	3
ECPR*	4
ECMO type	
Venoarterial	15
Venovenous	3
LVAD**	2

*ECPR: Extracorporeal cardiopulmonary resuscitation
**LVAD: Left ventricle assist device

Three patients were managed with VV ECMO, two patients with left ventricle assist device (LVAD) and the remaining patients had VA ECMO (Table I). The cannulation site of VA ECMO was the right atrium-aorta in all patients. Double-lumen jugular catheterization was used in 2 patients with VV ECMO, and right-left jugular vein catheterization was used for one patient with VV-ECMO.

Six patients had cranial imaging during ECMO support and all of them died (100%). The median duration of ECMO support before imaging and the total median duration of ECMO support was 5 days (IQR=3.5-17.5 days) and 19 days (IQR=13-28 days), respectively. Fourteen patients were imaged after weaning and one of them died (7.1%), and the median duration of ECMO support for these patients was 6 days (IQR=4-16 days). The survival rate was significantly higher in patients who had imaging after weaning compared to patients imaged during ECMO support ($p<0.001$). The total duration of ECMO was significantly higher in patients who were imaged during ECMO compared to those who had imaging after weaning ($p=0.033$).

Sixteen patients had CT and 8 patients had MRI scans, and 4 patients had both. To enhance understanding of neurologic events in the patients, findings were grouped as stroke (hemorrhagic/ischemic), hypoxic-ischemic encephalopathy (HIE), venous thrombosis and posterior reversible encephalopathy syndrome (PRES) following re-evaluation of the imaging studies. Ischemic stroke was grouped as a large vessel occlusion and embolic ischemia in different arterial territories. Hemorrhagic stroke was grouped as lobar parenchymal hematomas extending into the ventricles with >3cm transverse diameter and hematomas <3 cm in transverse diameter. Hemorrhagic transformation of ischemic stroke was also noted according to the Heidelberg classification.[14] The presence of microhemorrhages was also noted using SWI.

Four patients had an ischemic stroke (2.9%), one large vessel occlusion (0.7%), three embolic strokes (2.2%). The patient with a large vessel occlusion (middle cerebral artery) (Fig. 2, A) had an LVAD for cardiac support and was lost. He was imaged during ECMO and had been on ECMO support for 28 days before imaging. Two patients had embolic ischemic infarcts without hemorrhagic transformation (Fig. 2, B-C), both had VA ECMO for cardiac support, imaged after weaning and survived. One patient had a hemorrhagic transformation of embolic ischemic stroke with Type 1 a-b hemorrhage (scattered small or confluent petechial, no mass effect) according to Heidelberg classification (Fig. 2, D). The patient was on VA ECMO for

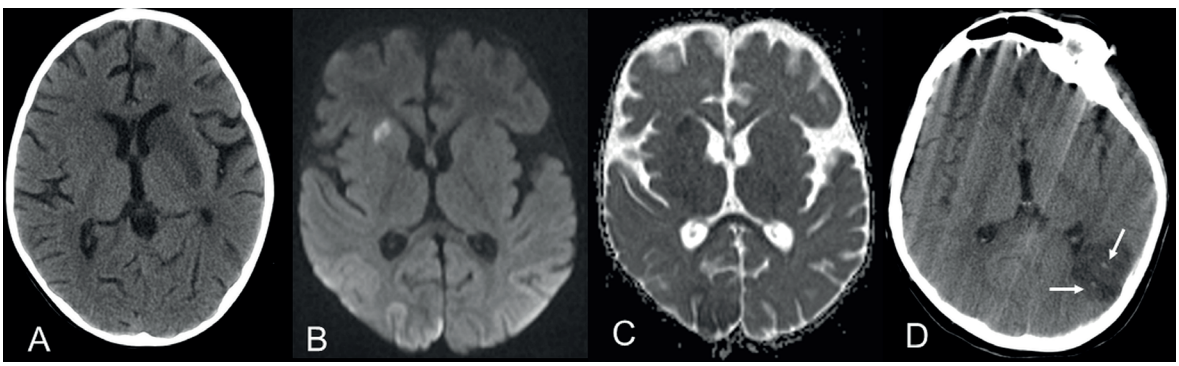

Fig. 2. (A) An acute striatal infarction with left middle cerebral artery occlusion as seen on axial non-enhanced CT (NECT) image in a 6-year-old male patient with ECMO support. Trace image (B) and ADC maps (C) of diffusion weighted imaging (DWI) detect embolic acute infarctions in the right striatum and ipsilateral occipital lobe, without hemorrhagic transformation in a 15-year-old patient after weaning. (D) Left parietooccipital acute infarction with scattered small petechial hemorrhage (Heidelberg classification type 1a) (arrows), on axial NECT image in an 11-year-old male with ECMO support.

ECPR, imaged during ECMO and was lost. Four patients had hemorrhagic stroke (2.9 %), three patients with lobar parenchymal hematomas extending to ventricles (2.2%) (Fig. 3, A), and one patient with a small hematoma (0.7%) (Fig. 3, B). Two of the patients with ventricular hemorrhage were lost (VA/VV=1/1), the others survived (VA/VV=1/1). Two patients (10%) had imaging findings consistent with HIE (1.4%), both had VA ECMO and were imaged after weaning (Fig. 4, A-B). The survival rate was 100%. Three patients had PRES (2.2%) (Fig. 4, C-D), all had VA ECMO, the patient imaged during ECMO was lost (survival rate 66.7%). Records of the patients with PRES revealed that all patients had hypertension prior to imaging, one was receiving cyclosporine and another one was receiving anakinra for their underlying diseases which were discontinued after diagnosis. One patient (0.7%) had a diffuse dural venous sinus thrombosis (Fig. 5). He had VV ECMO with right-left jugular vein catheterization and was lost. Scattered microhemorrhages were present in all MRIs on SWI. Six patients had unremarkable/normal findings on imaging. Imaging diagnosis and patients' progress are summarized in Table II. The distribution of imaging findings among different age groups is shown in Table III.

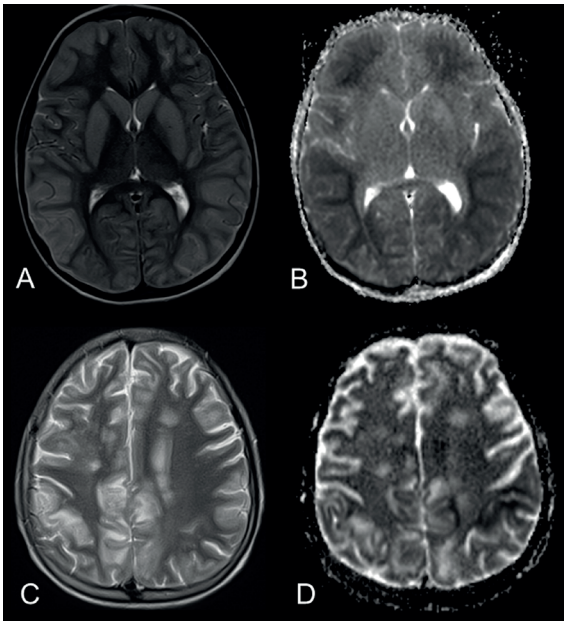

Fig. 4. MRI of a 5-year-old male patient after weaning showing diffuse cortical hyperintensity on T2-weighted MR image (A) and restricted diffusion with prominent signal loss in cortex on ADC map of diffusion weighted image (B) consistent with hypoxic ischemic encephalopathy. (C) Axial T2W MR image of a 13-year-old female after weaning demonstrating patchy hyperintense lesions in cortex and (D) subcortical white matter in both frontal and parietal lobes with heterogenous signal on ADC maps of diffusion weighted image, consistent with PRES.

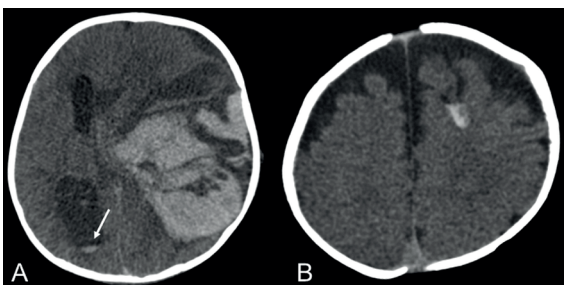

Fig. 3. (A) Axial NECT image of a 5-months-old male (A) with ECMO support, demonstrating a left parietal parenchymal hematoma (>3 cm transverse diameter) extending into ventricles resulting with a midline shift. (B) Axial NECT image of a two month-old male patient after weaning, showing a left frontal parenchymal hematoma with a transverse diameter <3 cm (B).

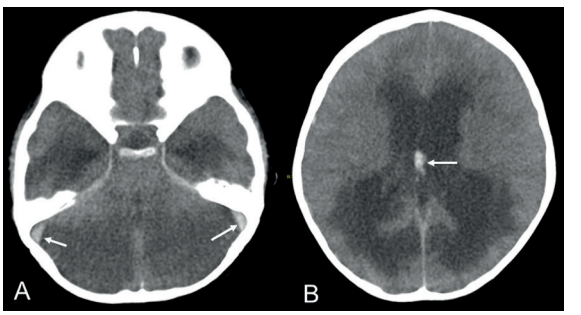

Fig. 5. (A) Diffuse cerebellar edema with hyperdensity in both sigmoid sinuses on axial NECT image of a four-year-old male patient who is on ECMO. The patient also has hydrocephalus with periventricular hypodensity and internal cerebral veins are hyperdense. Findings were suggestive of extensive cerebral venous thrombosis.

Table II. Imaging features and progress of patients.

	Numbers of patients	ECMO type (VA/VV)	Imaging time (during/ after weaning)	CT/MRI	Patient progress (survived/exitus)
Hypoxic ischemic encephalopathy	2	2/0	0/2	0/2	2/0
Posterior reversible encephalopathy syndrome	3	3/0	1/2	2/2	2/1
Venous thrombosis	1	0/1	0/1	1/0	0/1
Ischemic Stroke					
- Large vessel occlusion	1	LVAD	1/0	1/0	0/1
- Embolic infarcts	2	2/0	3/2	1/2	2/0
- Infarcts with hemorrhagic transformation	1	1/0	1/0	1/0	0/1
Hemorrhagic Stroke					
- Large	3	2/1	2/1	3/1	1/2
- Small	1	0/1	0/1	1/1	1/0
Unremarkable/Normal imaging findings	6	6/0	1/5	6/0	5/1

MRI: Magnetic resonance imaging, LVAD: Left ventricle assist device,
ECMO: extracorporeal membrane oxygenation, VA: venoarterial, VV: venovenous,
CT: computerized tomography

Table III. Distribution of imaging findings among ages.

	<1 year old	1-5 years old	>5 years old
Ischemic stroke (±hemorrhagic transformation)	-	1	3
Hemorrhagic stroke	2	2	-
Hypoxic ischemic encephalopathy	-	2	-
Posterior reversible encephalopathy syndrome	-	1	3
Cerebral venous thrombosis	-	1	-
Unremarkable imaging findings	1	2	3

The median duration for ECMO support of patients with unremarkable/normal imaging findings was 8.5 days (IQR 4.25-16.75 days). Of these patients, only one patient (16.7%) was lost due to systemic complications, i.e. lactic acidosis. Fourteen patients had positive neuroimaging findings, the median duration of ECMO was 13 days (IQR 5.5-26.5 days) and 6 patients died eventually (42.9 %). Despite the higher rate of exitus in patients with positive neuroimaging findings, the survival rate and duration of ECMO did not show a statistically significant difference among patients with unremarkable/normal and positive neuroimaging findings ($p>0.05$). Besides, renal failure, sepsis, inotrope support and thrombocytopenia were present in 3/1, 3/0, 11/4 and 9/1 patients with positive and normal/ unremarkable imaging findings, respectively. These features did not significantly differ between patients with positive and normal/ unremarkable imaging findings ($p>0.05$).

Discussion

Patients with ECMO are at risk of severe neurological injury, which in turn increases mortality and morbidity.[5] In our study, we aimed to document the imaging spectrum of ECMO related neurological injuries. In this cohort, the most common neurologic event was hemorrhagic and ischemic stroke (each 2.9%). Our results showed that PRES also occurs in

the setting of ECMO support (2.2%) besides the well-known hemorrhagic /thrombotic stroke and HIE. The major risk factor for ischemic stroke during ECMO is circuit thrombosis with a distant embolus, which might be aggravated by activating pro-thrombotic response due to exposure to foreign surfaces of ECMO and underlying severe systemic diseases.[15-18] Internal carotid artery cannulation is considered another risk factor, yet, not present in our study.[7] The latest Complication Trend Report revealed the survival rate after CNS infarction was 50% for pediatric respiratory, 45% for pediatric cardiac and 20% for pediatric ECPR in 2019.[19] However, it did not include data about hemorrhagic transformation of ischemic strokes. The survival rate of ischemic stroke was 50% in our study. Not surprisingly, occlusion of a large vessel and hemorrhagic transformation of ischemic infarct was related to poor outcome in the patients, as those who did not survive had large vessel occlusion and hemorrhagic transformation of ischemic embolic stroke.

Major risk factors for intracerebral hemorrhages in adults were shown by studies that revealed pre-ECMO cardiac arrest, sepsis, renal replacement therapy, thrombocytopenia, hemolysis, inotropic support, acute rapid increase in PaO2, and decrease in PaCO2 with the initiation of ECMO as the risk factors.[20-22] On the other hand, pediatric risk factors for hemorrhage have not been extensively studied. In our cohort, there were three patients with lobar hemorrhages extending into ventricles and only one survived. The survival rate was the lowest (33.3%) in this group in our cohort. The hematoma size, mass effect and presence of extension into ventricles in 3 of 4 patients with hematoma can explain poor survival rates herein this cohort.

The incidence of hemorrhagic and ischemic stroke (2.9%) was lower in our cohort compared to the ELSO Report which reported CNS infarction as 3-11%, and intracranial hemorrhage as 6-14%.[3] This might be due to the exclusion of US as a diagnostic tool in this study. On the other hand, the ELSO registry did not report stroke patterns in detail (i.e. ischemic vs HIE, hemorrhagic transformation of ischemia vs hematoma). While the detailed classification of neuroimaging patterns in our study fills in the lacking information, it also leads to lower rates.

Cerebral diffuse ischemia (HIE), one of the associated neurological complications of ECMO, was not listed as a discrete complication until 2016. In the pre-ECMO period, the underlying severe diseases leading to severe cardiogenic shock or hypoxia may end up with decreased cerebral blood flow or oxygen delivery, therefore it may not be directly related to ECMO. On the other hand, the pericannulation period is a risk factor for HIE.[4] Although both patients with HIE in our study survived, the survival rate from HIE was reported as 6-50 % for 2019 in the latest Complication Trend Report.[19] As HIE has been reported in Complication Trend Reports since 2016, the incidence and risk factors for HIE associated with ECMO remain to be unclear.

Impairment of cerebral autoregulation can be considered as increased vulnerability of our patients' brains to any kind of insult in the presence of ECMO as cerebral autoregulation impairment was previously shown to be correlated with the presence of abnormal and severe neuroimaging findings.[23] Animal studies showed that exposure to hypoxia affected cerebral autoregulation, which also remained impaired during the recovery phase.[24,25] Especially low flow rates (<150ml/kg/min) and loss of pulsatile flow in ECMO were shown to alter cerebral autoregulation.[26-29] Several studies in pediatric patients with ECMO supported these findings with noninvasive monitoring.[30-32] In the literature, there are only two adult case reports defining PRES in patients with ECMO.[33,34] However, in the present cohort, there were 3 patients with PRES (2.2%), who were imaged during or post-ECMO. Medical records of these patients revealed a slight increase in systemic blood pressure during ECMO and two of them were receiving medications (anakinra, cyclosporine) which were potential risk factors for PRES. Although the classical risk factors for PRES were present, impaired cerebral

autoregulation in these patients might have caused the brain microcirculation to become vulnerable to slight changes in these patients.

The studies in the literature do not differentiate ECMO related venous complications in the CNS. However, central vein catheterization is a known risk factor for jugular vein and/or cerebral sinus venous thrombosis. In a study of CT imaging after VV ECMO, 63.1% of patients with femoro-jugular catheterization demonstrated deep venous thrombosis.[35] The only patient with cerebral sinus venous thrombosis in our cohort had jugulo-jugular catheterization which might have induced and aggravated the formation of venous thrombosis and extension cranially.

The imaging findings demonstrated slight differences between different age groups as shown in Table III. Ischemic stroke and PRES tend to be seen in elder children, however, hemorrhagic stroke is more common in younger children. Due to the limited number of patients, these findings should be investigated further in larger cohorts.

The limitations of our study include retrospective design and the limited number of patients, especially infants. The transportation of patients with ECMO to imaging units is problematic. With the advances and access to portable CTs, patients can be imaged more easily which will help with the understanding of neurological complications in these patients. The limited number of infants in the present study is a consequence of our exclusion criteria. We excluded the patients having only US, which is the situation for most infants due to its bedside availability. Because of the retrospective design, it was not possible to review the US studies. We avoided including findings based on the subjective reports limited by the user's experience. As a result of the exclusion of US, the study population mostly consisted of elder children. So, we can say that our findings might not represent infants. The absence of cranial imaging prior to ECMO is another limitation, however imaging for all ECMO candidates is not practical in clinical routine especially given the current status of these patients.

Neurological complications during or after ECMO are potential risk factors for increased morbidity and mortality. Besides the commonly encountered intracranial thrombotic and hemorrhagic complications, PRES can also be seen. The patients with ECMO should receive appropriate radiological evaluation regarding the spectrum of neuroimaging abnormalities.

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Ethical approval

This retrospective study was approved by the Hacettepe University institutional review Board (04.05.2021 - GO21/607).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: EG, BK, ŞP, SK, BB, KKO; data collection: EG, BK; ŞP; analysis and interpretation of results: EG; SK; BB, KKO; draft manuscript preparation: EG, SK, BB, KKO. All authors reviewed the results and approved the final version of the manuscript.

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Original Article

Hyperprolactinemia in children and adolescents and long-term follow-up results of prolactinoma cases: a single-centre experience

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ABSTRACT

Background. Hyperprolactinaemia refers to increased circulating prolactin and is divided into functional and pathological hyperprolactinaemia. Prolactinoma is the most common cause of severe hyperprolactinaemia. Prolactinomas are rare in children. Treatment outcomes and long-term follow-up data in children are insufficient. Dopamine agonists are the first step in the treatment of prolactinomas. There are no recommendations supported by a high level of evidence regarding the dose and duration of cabergoline treatment.

Methods. Patients with hyperprolactinaemia were evaluated for etiological, clinical, and follow-up characteristics. The case files of patients with high prolactin levels who were followed up in our clinic between 2001 and 2019 were reviewed retrospectively.

Results. 27 cases (20 female, 7 male) with hyperprolactinemia were detected. The median age of the cases was 15 years (0.3–17.4). Prolactinoma was detected in 40.7% of the cases (n=11). Among these cases, six were macroadenomas. The median prolactin level was 118 ng/mL (34–4340) in those with prolactinoma and 60 ng/mL (22–200) in the hyperprolactinaemia group (p=0.007). In the prolactinoma group, the median age at presentation in macroadenoma cases (13.8 years) was lower than in microadenoma cases (17 years) (p=0.06). There was a negative correlation between prolactin level and height SDS (r=-0.770, p=0.06). In all cases, the median initial cabergoline dose was 0.5 mg/week, and prolactin levels returned to normal within an average of 2.6±2.4 months. Cabergoline treatment achieved a 50% reduction in adenoma size in the first year of treatment without high doses.

Conclusions. Prolactinoma consists of an important group among hyperplolactinemia in children. In our study, prolactinoma was detected in 40.7% of children with hyperplolactinemia, and children with prolonged use (over 4 years) tolerated cabergoline well and prolactin levels normalized without high doses. Follow-up is required for relapse after discontinuing the treatment.

Key words: hyperplolactinemia, prolactinoma, cabergoline.

Prolactin (PRL) is a hormone secreted from the pituitary gland which has been shown to affect lactation, metabolism, osmoregulation, and the immune and nervous systems.[1] Prolactin excess is defined as hyperprolactinaemia. Hyperprolactinaemia is rare in children and there are insufficient data on its frequency, aetiology, method, and duration of treatment and follow-up.[2] Hyperprolactinaemia occurs in children, adolescents, and adults for various organic and functional reasons. The frequency of prolactinoma in children with hyperprolactinaemia is also not known.[3] There are no high-level recommendations regarding diagnosing, treating, and monitoring hyperprolactinaemia and prolactinoma in children and adolescents. Similarly, there is not enough data on treatment duration, treatment

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cessation, and follow-up during the period without treatment. In children and adolescents, symptoms usually include functional alterations resulting from hyperprolactinaemia. The clinical manifestations of prolactinomas vary according to gender, age of onset, and tumour size.[4] Prolactinoma is rare in children, and there are few reports of prolactinomas in children. There are difficulties in the management of prolactinomas in children and adolescents. Dopamine agonists are the first step in the treatment of prolactinoma.[5] There is no strong evidence regarding the dose and duration of cabergoline treatment in children and adolescents. In this study, we aim to determine the frequency of prolactinoma among children and adolescents with hyperprolactinemia and long-term follow-up results.

Material and Methods

The case files of 27 patients with hyperprolactinemia who were followed up in our clinic between 2000 and 2019 were evaluated retrospectively. Using two different measurements, patients with serum PRL levels above normal (<25 g/L in girls and <20 g/L in boys) were included in the study.[2] We recorded admission characteristics, anthropometric measurements, accompanying diseases, medications, puberty stages, and physical examination findings. Neurological examination findings, visual field examination, pituitary function, and cranial imaging results were also evaluated in prolactinoma cases. This study assessed the patients in two groups: prolactinoma (PRLO) and non-prolactinoma hyperprolactinaemia (NPRLO). The given treatment (cabergoline or surgery), treatment doses, duration of treatment, clinical and laboratory evaluation, treatment side effects, and radiological follow-up of patients with prolactinoma were recorded. We recorded echocardiography results performed before and during treatment and other drug side effects in all patients who started treatment. The study protocol was approved by the Ankara University Ethics Committee (approval number: 15-638-15).

Statistical analysis

Statistical analyses were performed using SPSS v.23 for Windows (IBM Inc., Chicago, IL, USA). Normality was tested using the Shapiro-Wilk test. Data are presented as mean±SD for parametric data and median (range) for non-parametric data. Student's t-test was used to compare parametric variables, and the Mann-Whitney U test was used for non-parametric data. The Chi-squared test determined significant differences in proportions between categorical variables. The Spearman rank test was used to analyse the correlation between parameters. Crosstab and Fisher's exact tests are used to display the (multivariate) frequency distribution. A $p<0.05$ was considered statistically significant.

Results

Twenty-seven cases (20 girls and 7 boys) were diagnosed with hyperprolactinaemia during the study period. The median age (range) of the patients with hyperprolactinaemia was 15 (0.3–17.4) years, and 74% (n=20) were females. Presenting symptoms were headache in 33.3% (n=9), irregular periods in 11.1% (n=3), vision loss in 11.1% (n=3), galactorrhoea in 3.7% (n=1) and other symptoms (obesity, etc.) in 37% of the hyperprolactinaemia group.

Prolactinoma was detected in 40.7% (n=11) and macroadenoma in 22% (n=6). Other causes of hyperprolactinaemia were defined in 59.3% (n=16). Clinical features of the NPRLO and PRLO are in Table I. Other causes of hyperprolactinemia were sorted into rapid-onset obesity with hypothalamic dysfunction, hypoventilation, autonomic dysfunction syndrome (ROHHAD) (n=3), craniopharyngioma (n=2), polycystic ovarian syndrome (n=3), septo-optic dysplasia (n=2), drug-induced hyperprolactinemia (n=2), central

Table I. Presentation characteristics of cases with hyperprolactinemia.

	NPRLO (N=16)	PRLO (N=11)	*P*
Age (years) (median (min–max))	11 (0.3–17.4)	13.8 (12–15.5)	
Gender	%75 girl (n=12)	%72.7 girl (n=8)	(p>0.05)
Puberty	%57 pubertal (n=8)	%100 pubertal	
Height SDS (mean ± SDS)	-0, 77 (-4.1–0.79)	0, 67 (-2.04–1.69)	(p=0.005)
Prolactin level ng/mL (median (min–max)	38.7 (22.9–200)	118 (34–4340)	(p=0.007)

NPRLO: nonprolactinoma
PRLO: prolactinoma

neurocytoma (n=1), pituitary stalk interruption syndrome (PSIS) (n=2), and hypothyroidism (n=1).

While symptoms were observed in 89% of PRLO cases (54.5% headache, 27.3% menstrual irregularity, 9.1% galactorrhea), 37.6% of the NPRLO cases were symptomatic (headache, visual impairment). One patient presented with headache and vision loss; cranial MRI demonstrated mass lesion and PRL level was 200 ng/mL. The histopathological diagnosis was central neurocytoma (CN). Medical treatment started 3 months after surgery because PRL normalized by the third month of cabergoline treatment. Cabergoline treatment was gradually decreased and discontinued at 15 months.

The median prolactin level was 118 ng/mL (34–4340) in patients with PRLO and 38.7 (22.9–200) ng/mL in the NPRLO group (p=0.007). In the PRLO group, the median admission age in macroadenoma cases (13.8 years) was lower than in microadenoma cases (17 years) (p=0.01). In the PRLO group, there was no relationship between adenoma size and gender. The mean size of adenoma (longest axis) was 14.6±11.9 mm before treatment. Median PRLlevel in our study was higher in patients with macroadenoma (200 ng/mL; range 118–4340 ng/ml) than microadenoma (54.8 ng/mL; range 34–80 ng/ml) (p=0.006).

A statistically significant negative correlation was found between PRL level and height SDS (r=-0.770, p=0.06) in the PRLO group. Cabergoline was started as the first treatment in eight of 11 patients with PRLO. In all cases, the median initial cabergoline dose was 0.5 mg/week, and PRL levels returned to normal in an average of 2.6±2.4 months (1–9 months). The three patients with macroadenoma were treated with cabergoline with a dose of 1 mg/week. No side effects were observed at this dose. Surgery was performed as the first-line treatment in three patients with macroadenoma because of mass effect (visual impairment). Postoperative cabergoline was started in all patients due to the high PRL level. One patient received radiotherapy in addition to surgery and cabergoline treatment due to residual mass.

In patients with microadenoma (n=5), cabergoline was started at 0.5 mg/week. Normal PRL levels were achieved with this dose in all cases and no dose increment was necessary. The treatment regimen is shown in Figure 1. In our study, the PRL levels of patients were checked regularly in the first month of treatment and then at 3-month intervals. The follow-up period was 26.6±24 months (3 months–7 years) in all cases. Cabergoline treatment was discontinued in two patients who completed 4 years of therapy. One case had a 50% reduction in pituitary size (11mm to 4mm) on MRI in the 4th year of treatment and PRL level was normal in the last 2 years. The second case was a patient who underwent surgery and radiotherapy, whose MRI was normal in the 4th year of the treatment, and PRL level was normal in the last 2 years. After discontinuation of cabergoline treatment, PRL levels increased again, and cabergoline was restarted in the second month of the follow-up. One of these cases has completed 7 years in cabergoline treatment and remains on a dose of 1.5 mg/week. No side effects were observed in any case.

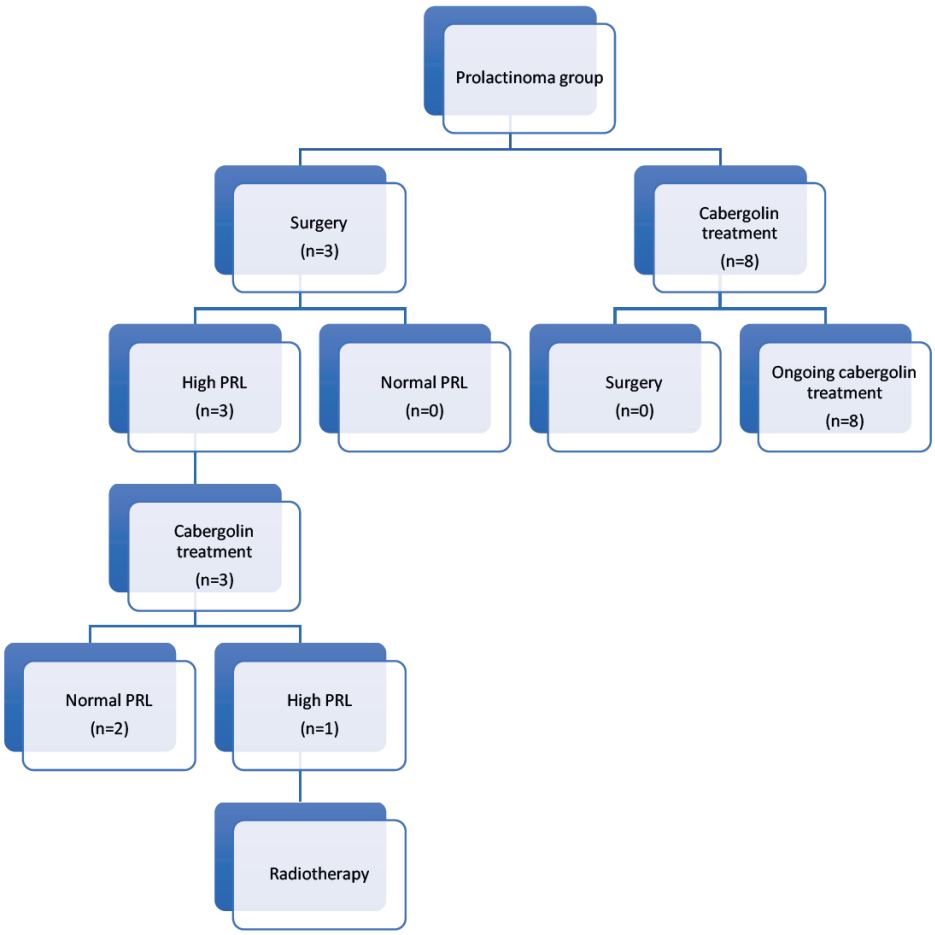

Fig. 1. Treatment regimen of prolactinoma cases.

Discussion

Hyperprolactinaemia refers to increased circulating prolactin and is divided into functional/physiological, analytical, and pathological hyperprolactinaemia. Functional hyperprolactinaemia is typically observed in pregnancy, during lactation, with high protein diets, stress (including venepuncture), and secondary hypoglycaemia due to medications.[5,6] Macroprolactinaemia is characterized by a molecular mass of PRL greater than 150 kDa as the predominant molecular form of circulating PRL.[7] There was no macroprolactinaemia in our study.

Pathologic hyperprolactinaemia is mainly due to sellar and parasellar lesions. Other reasons for organic hyperprolactinaemia are polycystic ovary syndrome (PCOS), chronic renal failure, cirrhosis, hypothyroidism, Cushing disease, and adrenal insufficiency.[3]

Hyperprolactinaemia has been reported in 7–52% of adults with PCOS.[8] One report says that PCOS with a PRL level exceeding 85.2 ng/mL should undergo pituitary MRI for prolactinoma differential diagnosis, especially with lower luteinizing hormone (LH) levels.[9] Still, there is no cut-off level for PRL in children and adolescents with PCOS with suspected prolactinoma. We had three patients with hyperprolactinaemia in PCOS, and PRL levels are 27.8 ng/mL, 32.8 ng/mL, and 37.2 ng/mL, respectively. The second had a normal pituitary MRI. More extensive studies are required to establish cut-off values for normal PRL levels

in children and adolescents that discriminate prolactinomas from hyperprolactinaemia in PCOS.

Rapid onset obesity with hypoventilation, hypothalamic dysfunction, and autonomic dysregulation (ROHHAD) syndrome is a rare disorder. Hyperprolactinaemia can be seen in this 40.2 ng/mL, and 49.6 ng/mL, respectively. None of them have neuroendocrine tumours.

Central neurocytoma (CN) is a rare brain tumour often located in the lateral ventricular region. Extraventricular neurocytoma is very rare in the paediatric population.[10] One patient has an extraventricular neurocytoma that occupied the sella area mimicking macroadenoma, WHO grade 2 without signs of malignancy. Immunohistochemical analysis demonstrated a Ki-67 index of 10%, so tumour biological behaviour was thought to be more aggressive. Therefore, histopathological diagnosis is essential in applications that mimic giant adenomas.

Idiopathic hyperprolactinaemia is considered when there are no secondary causes and normal pituitary MRI. Idiopathic hyperprolactinaemia is caused by either a microadenoma < 2mm, too small for MRI detection, or familial idiopathic hyperprolactinemia. Familial idiopathic hyperprolactinaemia is caused by a mutation in the prolactin receptor (PRLR) disrupting ligand binding.[11] There was no case with idiopathic hyperprolactinaemia in our study.

Prolactinomas are the most frequent organic cause of hyperprolactinaemia and the most common pituitary adenomas representing children and adolescents.[4] A microadenoma was defined as a pituitary tumour of less than 1 cm in diameter, and a macroadenoma was defined as a tumour above 1 cm in diameter. Serum PRL level increased in parallel with tumour size.[12]

Although they are rarely hereditary, prolactinomas can occur as part of the multiple endocrine neoplasia (MEN) type 1 syndromes, Aryl Hydrocarbon Receptor-Interacting Protein (AIP) mutations, Carney complex, and McCune-Albright Syndrome.[13,14] No risk factors have been identified for sporadic prolactinomas.[15] Mutation analysis of the *MEN* or *AIP* genes was not performed in our study group.

The clinical manifestations of PRLO vary according to gender, age of onset, tumour size, increased prolactin level, mass effect, and another accompanying pituitary hormone deficiency. In our study, PRLO mainly occurred in females (8 of 11), similar to the literature.[16] Despite a small number of patients, macroadenoma was found more often in the paediatric literature, unlike in adults.[13] In the PRLO group, the median admission age in macroadenoma patients (13.8 years) was lower than in microadenoma cases (17 years). This age difference may be related to the early symptoms of macroadenoma due to the mass tumour effect.

The first aim of PRLO treatment is to normalize PRL levels, provide normal gonadal functions, and protect other pituitary functions. It is crucial to reduce the mass tumour effect, especially in macroadenomas. There is no need for treatment in asymptomatic microadenomas, but close follow-up is recommended regarding size increases and symptoms.[17,18]

When there is no need for emergency surgery, dopamine agonists are the first treatment option in PRLO as they normalize the PRL level, reduce tumour size, and improve gonadal functions.[17,19] Although the mechanism is not precisely known, dopamine agonists also provide a reduction in adenoma size.[20] The cabergoline starting dose is 0.25–0.5 mg/week and can be increased weekly to a maximum of 3.5 mg/week until normal PRL levels are reached.[12,17] In adult studies with dopamine agonist treatment, PRL levels were normalized in 85% of patients with macroprolactinoma and tumour size was reduced by at least 25% in 80% of cases.[21] Our study achieved a nearly 50% reduction in tumour size in the first year with the mean initial dose of cabergoline in three cases for whom cabergoline treatment was preferred as the initial treatment. It has

been observed that there is no need for high-dose cabergoline in microadenoma. Control was achieved with doses of 0.5 to 1.5 mg/week in all cases.

Cabergoline has fewer side effects and better patient adherence.[22] Cardiac evaluation of patients using cabergoline for a long time is essential to evaluate cardiac valvulopathy associated with dopamine agonists.[17] Echocardiographic examinations of the cases were normal.

In our study, cabergoline treatment is well tolerated and improves clinical symptoms while reducing adenoma diameter without increasing the maximum doses. While on medical treatment, the patient's serum PRL levels should be monitored regularly.[17] Although studies have reported that the discontinuation of cabergoline treatment is safe, relapse and tumour size enlargement is common.[17] In adult guidelines, the discontinuation of cabergoline may be recommended for patients with normal PRL levels, no tumour on MRI, a 50% reduction in tumour size, and no invasion of critical structures for at least 2 years.[23] After about 4 years of dopamine agonist therapy, an attempt may be made to withdraw the drug. Cabergoline treatment should be discontinued by gradually tapering it while maintaining normal PRL levels.[17] Regular follow-up is required at 3-month intervals in the first year, then annually for at least 5 years to monitor prolactin levels and tumour recurrence. Because relapse rate is higher in macroprolactinoma cases, they should be carefully monitored with an MRI 6 months after the cessation of therapy and annually thereafter.[17,24]

Prolactin levels increased again in the follow-up of our two patients who completed 4 years of cabergoline treatment and discontinued cabergoline per the guideline suggestions, which was restarted. A study by Barber et al.[25] found recurrence rates as high as 93% for macroprolactinomas and 64% for macroprolactinomas, and hyperprolactinemia recurrence is most commonly observed during the first 6 months to 1 year following cessation. Patients with recurrence had macroadenoma and recurrence occurred in the 6th month after cabergoline discontinuation, similar to the literature. Although there is no standardization regarding the treatment dose and duration, the periods reported in the guidelines may need to be individualized.

Surgical intervention was performed in three cases. Surgical treatment is often performed for large tumours that cause a mass effect (cranial nerve palsy, visual impairment, and pituitary apoplexy), cystic tumours that do not respond to medical treatment, and intolerant patients resistant to dopamine agonists.

Radiotherapy is a tertiary treatment for prolactinomas that are not controlled by dopamine agonists or surgery, mainly to control tumour growth.[26,27] One patient received radiotherapy in addition to surgery and cabergoline treatment due to a residual tumour.

In conclusion, prolactinoma is the most common cause of severe hyperprolactinaemia. Prolactinomas are rare in children, causing difficulties in diagnosis and treatment management. Although there is not yet a standardization regarding the treatment dose and duration, the durations stated in the guidelines may need to be individualized. According to our observation, children tolerated cabergoline well in long-term use and did not need high doses to normalize the prolactin level. Treatment and follow-up results are needed in large series for paediatric and adolescent patients.

Ethical approval

The study protocol was approved by the Ankara University Ethics Committee (approval number: 15-638-15).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: MB, ZŞ; TK; data collection: TK, EÖ, EB, AC, RU; analysis or interpretation of results: TK, ZŞ, MB; draft manuscript preparation: TK, ZŞ, MB.

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Original Article

Clinical course of primary empty sella in children: a single-center experience

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ABSTRACT

Background. Various studies, mainly conducted in adults, have examined the hormonal axis in primary empty sella (PES), and reported various forms of pituitary deficiencies. We report our experience with PES in pediatric patients in terms of pituitary function, associated impairments, and responses to treatment.

Methods. We reviewed 10,560 cranial and 325 pituitary magnetic resonance imagings (MRIs) performed at our university hospital between January 2010 and December 2020 and identified patients with PES. Patients with additional abnormal MRI findings, a history of cranial surgery or radiotherapy, autoimmunity, long-term use of chemotherapeutic or immunosuppressive agents or incomplete diagnostic evaluation were excluded. Clinical, radiological and laboratory evaluations were recorded.

Results. The study included 17 patients [9 girls, 8 boys; median age 12.4 years (7.25, 4.3 - 17)]. The median size of the pituitary was 2 mm (0.7, 1.2 - 3). Based on age-dependent pituitary height measurements, fifteen (88%) patients had pituitary gland hypoplasia. Five patients presented with short stature, two had both pubertal delay and short stature, and one had pubertal delay. Nine patients presented with neurological symptoms such as headaches, tinnitus, tics, and dizziness. Five short patients had growth hormone deficiency. None of the patients had hyper- or hypoprolactinemia, adrenal insufficiency, hypothyroidism, or diabetes insipidus. There was statistically no significant association between the size of the pituitary gland and the severity of hypopituitarism ($p = 0.42$).

Conclusions. The high incidence of pituitary dysfunctions ascertain that this entity should not be considered a normal variant but, should instead be carefully evaluated with appropriate basal and dynamic hormonal testing.

Key words: primary empty sella, hypopituitarism, pituitary, magnetic resonance imaging.

Empty sella is a neuroradiological and anatomical condition, characterized by intrasellar extension of the subarachnoid space followed by flattening of the pituitary gland.[1] The term was first described in 1951, and is often regarded as an incidental finding, with a prevalence of 5.5 to 35% in the general population.[1-3] According to the underlying etiology, related pathologies or disorders can be classified as primary and secondary.[2] In primary empty sella (PES), the underlying pathophysiology remains undetermined; however, the incompetency of the diaphragma sella, which is reported in 22-77% of all previously diagnosed patients, is accepted as the major factor in the development of PES.[2,3] Further, the defects in the pituitary or upper sella, chronic increases in intracranial pressure or derangements in cerebrospinal fluid (CSF) dynamics are the other possible causative factors.[3] In contrast, among the

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responsible mechanisms in secondary empty sella are tumors, hydrocephalus, surgery, radiotherapy, trauma, autoimmunity, infection, genetic diseases and drugs.[1,4,5] PES is commonly not associated with any signs or symptoms; however, headache, obesity, menstrual irregularities, and galactorrhea may occur.[1,2,6-8] Pituitary dysfunction ranging from isolated deficiencies, such as growth hormone (GH) deficiency, to panhypopituitarism have been reported in patients with PES.[1] Various studies, mostly conducted in adults, evaluated the hormonal axis in PES, and reported different forms of pituitary deficiencies.[4,5,7,9-11] Here, we report our 10-year experience in pediatric patients with PES with particular attention to the pituitary function, associated impairments, and responses to treatment.

Material and Methods

Study design

Regardless of chief complaints, we reviewed 10,560 cranial and 325 pituitary magnetic resonance imagings (MRIs) performed at our university hospital between January 2010 and December 2020. In this retrospective chart review study for pediatric patients, 20 scans revealed empty sella. Relevant images and files were reviewed, and one case with incomplete endocrine evaluation, one with previously diagnosed Cushing's syndrome, and one with a history of chemotherapeutic agents were excluded. Eventually, our study included 17 patients (9 girls, 8 boys). MRIs were ordered for suspected endocrine dysfunctions in eight (47%), and neurological pathologies in nine (53%) of the patients. The following variables were recorded: age at diagnosis, initial complaints, period of follow-up, size of the pituitary gland on MRI, medications, physical, and laboratory findings.

Physical evaluation

Age, sex, and anthropometric parameters such as height (cm), body weight (kg), body mass index (BMI) (kg / m^2) and the respective standard deviation scores (SDS) according to the standards set for Turkish children, and pubertal staging's, were both recorded at the time of diagnosis as well as the last follow-up visit.[12,13]

Hormonal evaluation

The hypothalamic-pituitary axis was assessed using the baseline serum levels of morning cortisol, free thyroxine (fT4), thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone in boys, estradiol in girls, prolactin, insulin-like growth factor-1 (IGF-1), insulin-like growth factor binding protein-3 (IGFBP-3), sodium and urine osmolality. Central adrenal insufficiency was excluded when a morning (08:00) serum cortisol level was above 10 μg/dL or in the presence of a peak cortisol value higher than 18 μg/dL, following either an intravenous low dose (1-mcg) ACTH test or an insulin tolerance test (0.1 U / kg insulin).[14,15] The diagnosis of central hypothyroidism was based on low fT4, with low, normal, or slightly elevated TSH levels (reference ranges supplied by our central laboratory: TSH 0.38 - 5.33 mIU/L; fT4 0.5 - 1.5 ng / dL).[16] Delayed puberty, suggesting gonadotropin deficiency, was considered in the absence of breast tissue by age 13 in girls or testis development by age 14 in boys, and was excluded in the presence of a stimulated peak LH concentration above 5 IU / liter, following a LH-releasing test (gonadorelin 2.5 mcg / kg).[17] Moreover, both the spurious inhibitory effect of high levels of prolactin, defined as levels above 20 ng / mL in two consecutive measurements and low levels below the limits of detection were evaluated.[18,19] For patients with growth impairment, defined according to the Growth Hormone Research Society guidelines[20], GH deficiency was diagnosed when two different stimulation tests (clonidine, insulin tolerance or L-dopa test) resulted in a GH peak below < 7 ng / mL.[21] Central diabetes insipidus, which indicated posterior pituitary dysfunction, was diagnosed in the presence of polyuria (urinary

volume more than 4 - 5 mL / kg / h or 2 L /m^2 /d) and polydipsia (2 L / m^2 / d) along with low urine osmolality (≤ 300 mOsm / L) and high plasma osmolality (≥ 300 mosm / L).[22]

Radiological evaluation

All MRI examinations were performed with 1.5-T MR system (Ingenia; Philips Healthcare, USA) and all images were analyzed by an experienced pediatric radiologist. The characteristics of the pituitary gland and sella tursica were assessed using coronal, axial T2 images and coronal sagittal T1-weighted images. Patients with a cerebrospinal fluid filled sella and decreased pituitary gland height were evaluated. The vast majority of the measurements were performed on sagittal and coronal images at multiplanar reformatted images of the 3D T1 sequence. The measurements of the patients with pituitary MRI were made with sagittal and coronal T1 images. Measurements in the sagittal plane were made on the midsagittal section and the coronal plane were made on the section where the pituitary stalk is seen in T1 images. Pituitary gland hypoplasia was defined based on age-dependent pituitary height measurements.[23] Partial/complete PES were classified as partial when less than 50% of the sella was filled with CSF and the pituitary height was 3 mm or greater, and complete when more than 50% of the sella was filled with CSF and the pituitary height was less than or equal to 2 mm.[4]

Statistical analysis

Statistical analyses were performed using SPSS v.24 for Windows. The results of the study were presented as categorical or continuous variables. The homogeneity of the continuous data obtained in the study were tested using the Shapiro-Wilk and Kolmogorov-Smirnov test. The continuous variables were expressed as median [interquartile range (IQR), (minimum-maximum)], unless otherwise stated. Categorical variables were given as the number of patients and percentages (%). The bivariate associations between continuous variables were compared using the Mann-Whitney U test for pairwise comparisons. The Spearman rank correlation was used to discover the association between the pituitary size and auxological measurements. A p-value of < 0.05 was considered statistically significant.

Ethical approval

The research was complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration. Institutional approval was granted by the Ethics Committee of Dokuz Eylül University Faculty of Medicine. (Ethics approval number: 2021/14-2906052021). Informed consent was obtained from all individuals' parents or legal guardians included in this study.

Results

The study included 17 patients [9 girls, 8 boys; median age 12.4 years (7.25, 4.3 - 17)]. Clinical presentation, pituitary size, and treatments of the patients are presented in Table I. Brain MRI findings of two patients (#2 and #7 in Table I) as examples of the measurement methods are presented in Figures 1a, 1b, 2a, and 2b.

Brain MRIs were ordered for diagnostic evaluation of short stature in five patients, pubertal delay and short stature in two patients, pubertal delay in one patient and neurological symptoms including headache, tinnitus, tics, and dizziness in nine patients. The median duration of follow-up was 36 months (53, 9 - 70 months). The median size of the pituitary was 2 mm (0.7, 1.2 -3). Fifteen (88%) patients showed pituitary gland hypoplasia. Nine patients (53%) had complete empty sella. The median SD scores for weight, height, and BMI of the patients at the time of diagnosis were -0.19 (2.2, -2.5 - 1.9), -0.98 (2.9, -3.8 - 1.8), and 0.2 (1.6, -1.3 - 1.97), respectively.

Eight patients (47%) were evaluated with provocative tests (low dose ACTH test (n=2, 25%), insulin tolerance test (n=5, 63%), clonidine test (n=5, 63%), L-dopa test (n=3, 38%), LH-releasing test (n=3)) for pituitary

Table I. Clinical presentation, pituitary size and treatments of the patients.

Case number	Age, (years), gender	Follow-up, months	Initial complaints	Pituitary size, mm	Weight, SDS	Height, SDS	BMI, SDS	Stimulation tests	Treatment
1	11, F	11	H, T	2	1.9	1.33	1.62	LDST	None
2	12, F	36	S	1.2	-0.73	-3.06	1.01	C, ITT	GH
3	6, M	11	H	2	-0.12	-1.36	0.92	N/A	None
4	15, F	36	H, I	1.9	1.77	1.26	1.08	N/A	None
5	10, M	70	S	3	0.18	-2.38	1.56	C, ITT	GH
6	17, F	70	H	1.7	-0.19	-0.63	0.09	N/A	None
7	13, M	70	H	2	0.66	1.78	-0.17	N/A	None
8	7, F	12	H, N	2.8	1.23	-0.78	1.83	N/A	None
9	16, M	36	S	2.8	-2.41	-3.75	-0.35	C, ITT	GH
10	12, F	32	S	1.8	-0.93	-2.83	0.67	C, D, LHRH	None
11	5, M	48	S	2	-1.85	-2.11	-0.81	N/A	None
12	12, M	70	S	2	-2.41	-2.62	-1.27	ITT, D	GH
13	17, F	32	P	2.5	-2.53	-0.98	0.16	LHRH, LDST	E
14	13, F	60	S	2	0.93	-1.8	1.97	D, ITT, LHRH	GH
15	17, M	12	H	3	-0.52	-0.46	-0.5	N/A	None
16	4, M	9	H	2.1	0.49	1.61	-0.4	N/A	A
17	8, F	9	H	2.2	-0.44	-0.48	-0.2	N/A	None

F: female, M: male, H: headache, T: tinnitus, N:tics, S:short stature, I:menstrual irregularity, P: pubertal delay, LDST: Low-dose synacthen test, LHRH: luteinizing hormone releasing test, ITT: insulin tolerance test, C:clonidine test, N/A: not applicable, D:L-Dopa Test, GH: growth hormone, E:estradiol, A: acetazolamide

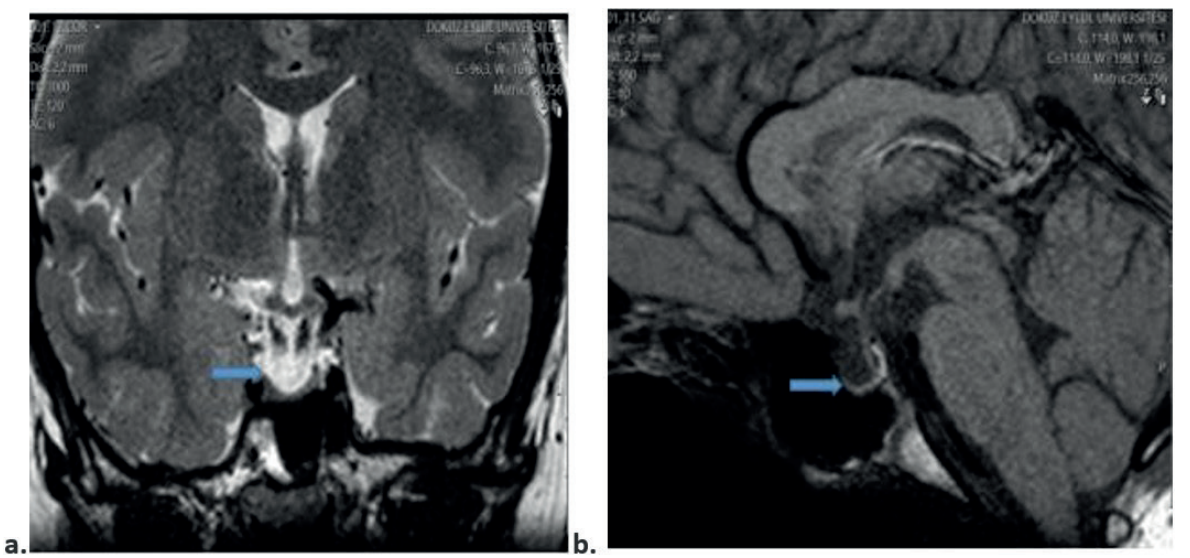

Fig. 1. MRI brain scans of patient number 2 confirming the diagnosis of empty sella. **a.** T2weighted (arrow: fluid attenuated) coronal section shows the increased signal intensity of the CSF occupying the sella turcica **b.** T1-weighted mid-sagittal section that demonstrates a fluid filled sella turcica with a flattened pituitary gland (arrow) at the base of the pituitary fossa.

dysfunctions. Five short patients (#2, #5, #9, #12 and #14) had growth deceleration and/or target height discrepancies with low to normal IGF-1 / IGFBP-3 levels [SD scores –1.2 (2, -2 - 1.2); 0.2 (3.4, -2.4 - 1.8), respectively]. All these five patients failed to achieve adequate GH

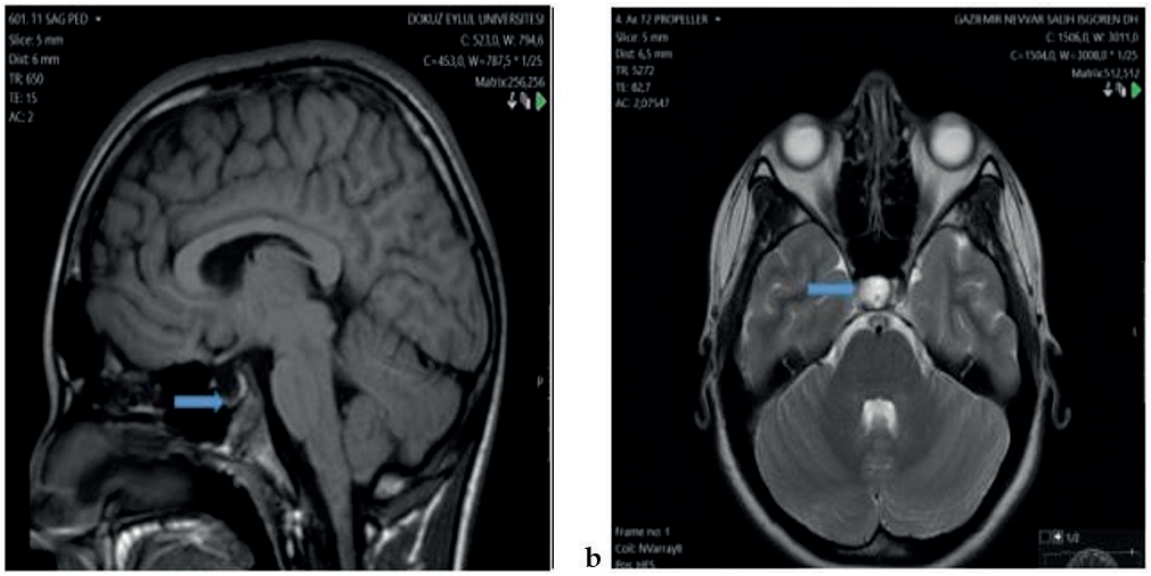

Fig. 2. MRI brain scans of patient number 7. **a.** Midsagittal T1-weighted image through the sellar region shows decreased pituitary gland (arrow) height. **b.** T2-weighted axial image shows the increased signal intensity (arrow) of the CSF at sella turcica.

responses with two GH stimulation tests; thus, GH therapy at conventional doses (25 - 35 mcg / kg / day) was initiated. The SD scores for median height at baseline and at final evaluation were -2.6 [1.3, -1.8 - (-3.75)] and -1.9 [1.3, -0.6 - (-2.6)], respectively.

Two of the six short patients (#10 and #11) lacked predisposing factors for short stature, such as a history of small-for-gestational-age births or syndromic traits. The target height SD scores of patients #10 and #11 were -1.4 and -0.5, respectively. Both patients demonstrated delayed skeletal maturation, which was consistent with their family history of constitutional delay of growth and puberty in both parents. With IGF-1 / IGFBP-3 values above the 0 SD threshold, they exhibited normal growth rates for their ages. Consequently, no GH therapy was initiated throughout the time of follow-up. At the last visit, the SD scores for height and the predicted adult heights of patients #10 and #11 were -1.8, -0.8 and -1.7, -0.5, respectively.

Two short patients (#10 and #14 in Table I) complained of delayed puberty at follow-up and one (#13 in Table I) showed no pubertal signs at 17 years of age. Their basal LH and FSH levels were 0.07, 0.8, 0.19 mIU/mL and 4.9, 4, 0.7 mIU/mL, respectively. All patients showed normal pubertal responses to GnRH test. To stimulate pubertal development in patients #10 and #13, a low dose of estrogen (5 μg/kg/day of 17-β oestradiol) was administered for a short period of time (three months). On the last examination, at age 14, with a bone age of 12, and without therapy in the previous year, patient #10 presented as tanner stages 2-3, with pubertal hormone levels and pelvic ultrasonography. Yet, one case (#13 in Table I), had no sign of breast development and her estrogen levels were below detection limits, thus low dose of estrogen was gradually increased over 3-6 months and progesterone was added to estrogen after one year. Combined estrogen and progesterone therapy facilitated her menstrual cycles. She later had normal physiological menstrual cycles. All three were considered to have transient pubertal delays.

The median cortisol levels of the patients were 10 μg/dL (9.4, 5.4 - 27). Five of the patients had a baseline cortisol levels below 10 μg/dL. All five of them showed normal cortisol responses to stimulation tests. Two patients underwent

low-dose ACTH testing and, to avoid multiple testing, three short patients underwent insulin tolerance testing to simultaneously rule out GH deficiency. None of the patients had hyper- or hypoprolactinemia [median prolactin level 7 ng/mL (8.6, 3.5 - 20)] or central hypothyroidism [fT4, 0.9 ng/dL (0.06, 0.7 - 1.7); TSH, 2.6 mIU/L (2, 0.5 - 5.6)]. Diabetes insipidus was also not observed in any of the patients.

Six patients (35%) had clinical conditions requiring pharmacological treatment. Five patients (29 %) were diagnosed with endocrine dysfunctions; all five were diagnosed with isolated GH deficiency, and GH replacement treatment was initiated. No further endocrine deficiencies were detected. One patient (#16 in Table I) with a severe headache, had papilledema without visual loss and increased intracranial pressure on lumbar puncture was detected; after acetazolamide treatment, his symptoms disappeared and surgical treatment was not required. However, on follow-up examinations, he later developed cervical lymphadenitis, and was diagnosed with Burkitt lymphoma. Control MRIs were not ordered for the other patients, since none of them developed new complaints or showed clinical progression during follow-ups.

There was no significant correlation between the size of the pituitary gland and the SD scores of weight, height, and BMI of our patients (***Spearman's Rho*** (r_s)= -0.08, p = 0.8; r_s = 0.036, p = 0.9; r_s = -0.045, p = 0.87, respectively). Also, the size of pituitary gland was similar between patients with GH deficiency and normal growth velocity (p = 0.42).

Discussion

PES is often considered an incidental finding and a normal variant, but our study showed that it may also be associated with different important neurological and endocrine problems. In the present study, empty sella was diagnosed either during the evaluation of neurological complaints or hormonal dysfunctions. Similar to prior studies[1,2], the early neurological symptoms of our patients included headache, tinnitus, tics, vertigo, and depression. As for endocrine functions in PES, endocrinological evaluation may be completely normal; however, various degrees and forms of pituitary dysfunctions, including hyperprolactinemia (4 - 37.5% of patients), GH deficiency (4 to 57% of patients), secondary defects in ACTH, TSH or gonadotropins (2.3 - 32% of patients), panhypopituitarism (2% of patients) and isolated or combined hypopituitarism (28 - 53% of patients) have also been reported.[1,4,10,24,25]

Overall, pituitary disturbances have been observed in 8 to 60% of all patients.[4,7,10,26] In our study, 29% of all our patients had isolated GH deficiency, indicating that somatotroph cells are the most vulnerable site, and so probably the most crucial screening parameter for other hormone deficiencies. Even while this incidence of GH deficiency in PES patients is consistent with previous studies[1,4,10,24,25], it should be noted that up to 57 % of all patients have been documented to have GH deficiency. Given the young ages and short follow-up duration of our patients, it would be prudent to consider the likelihood that more deficiencies may appear during the follow-up in our cohort. This prediction may be especially plausible for patients such as #10 and #11, who were considered to have constitutionally delayed growth and puberty, with delayed bone age, normal growth velocity, and predicted adult height consistent with familial patterns. Since they had not yet attained adult heights, it is questionable whether they could have benefited from GH replacement as well.

Menstrual irregularities, hirsutism, and gynecomastia are also associated with PES.[1] One patient had menstrual irregularity in our study. Obesity is also a component of the clinical spectrum, and it is involved in the pathophysiological mechanism.[1] However, none of our patients were obese, indicating that obesity could be an incidental association.

In PES, increased intracranial pressure and changes in cerebrospinal fluid dynamics, which have been reported in 60 - 77% of patients may necessitate surgical treatment.[5] Only one of our patients experienced increased intracranial pressure, which resolved with pharmacological treatment. As for the follow-up of patients, De Marinis et al.[4] suggested that patients with no abnormalities at baseline are unlikely to develop any symptoms in the future. Therefore, careful re-evaluation of symptom progression was advised for those with baseline defects. In line with this suggestion, in our study, only the patients with primary endocrine pathologies actually needed hormone replacement treatments, while those with neurological complaints had normal endocrinological assessments. This suggests that the initial clinical findings are more predictive than neuroimaging in empty sella.

Previous studies have shown conflicting results regarding the correlation between the degree of herniation, the size of the pituitary, and related dysfunctions.[25,27] Similar to Gallardo et al.[6], we also found no correlation between the pituitary size and the severity of the clinical course.

A limitation of our study is that it was conducted retrospectively on a small number of patients. Methods of radiological evaluation, laboratory tests and techniques could have changed in the past years. However, the pituitary axis was systematically assessed according to the current guidelines for each patient, and long-term follow-up examinations were presented accordingly. However, since the patients presented during childhood and the final adult assessments were unfortunately not available, various other future deficiencies may emerge over time. For this reason, it is important to monitor potential deficiencies over a longer period of time in pediatric patients.

Furthermore, among all cranial MRIs of pediatric patients ordered in our hospital in the past 10 years, only seventeen children were diagnosed with PES, which is fewer than in previous studies. This finding supports the fact that empty sella is difficult to recognize and can be easily missed without specific requests to the sellar region.

PES may be associated with different neurological and endocrinological conditions (mostly, short stature and transient delayed puberty), which may require specific treatments. The size of the pituitary gland does not relate to the severity of the symptoms. The high incidence of pituitary dysfunctions ascertains that this entity should not be considered as a normal variant, but should instead be carefully evaluated using appropriate basal and dynamic hormonal testing to identify the distinct hormonal dysfunctions.

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Ethical approval

Approval was granted by the Ethics Committee of Dokuz Eylül University Faculty of Medicine (Ethics approval number: 2021/14-2906052021).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: AA, ÖB; data collection: ÖB, EY; analysis and interpretation of results: ÖB, İME, KYA; draft manuscript preparation: AA, ÖB, KD, EB. 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

Intrathyroidal ectopic thymus: an important entity in the differential diagnosis of thyroid nodules

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ABSTRACT

Background. Intrathyroidal ectopic thymus (IET), a benign lesion due to aberrant thymic migration during embryogenesis, is often discovered incidentally. We aimed to present the ultrasound (US) features, diagnostic methods, and follow-up of IET in children and adolescents.

Methods. We searched our database of patients with a nodular thyroid lesion detected by US, between January 2007 and December 2019. In 30/255 (11.7%), IET was diagnosed.

Results. The study included 30 patients (20 males/10 females), mean age 5 years (0.1-12.2, median 5.6) with 34 lesions diagnosed by US as 'incidentalomas.' None of the patients had palpable nodules. On US, IET appeared as a hypoechoic lesion, with multiple punctuate internal echoes. 29/34 of lesions had well-defined margins. The most common location of IET was in the middle part (27/34) of the left lobe (19/34). The mean longest diameter at diagnosis was 6.4 mm (2.5–21, median 4.5). Sonographic follow-up was available in 25 patients with 27 lesions. The mean time of observation was 2.7 years (0.3-7.5, median 2.1). While 13/27 cases showed decreased size or regression during follow-up, the other 13 increased in size, and there was no change in size in one. Pubertal progression was associated with both increment and decrease in size of IET. Fine needle aspiration (FNA) was performed in 5 patients and surgery in one.

Conclusions. IET should be considered in the differential diagnosis of pediatric thyroid nodules as a cause of FNA and/or surgery. Regular US monitoring can be used safely in the follow-up of this lesion. We present one of the largest series in the literature with long-term follow-up and description of patients' pubertal status. IET prevalence was 11.7% among children and adolescents with a nodular thyroid lesion, higher than that stated in the literature.

Key words: children, ectopic thymus, thyroid, ultrasonography, nodule.

The relatively increasing use of ultrasound (US) examination in children and advances in imaging technologies may increase the detection of more thyroid lesions than there used to be. Intrathyroidal ectopic thymus (IET), which is due to aberrant thymic migration during embryogenesis, is one of such lesions. The thymus derives from the third and fourth branchial pouches and descends to the upper mediastinum, reaching its final position. Aberrant thymic migration may result in ectopic thymic rests along the normal pathway of descent, which may persist in the soft tissues of the neck.[1] Because of the close relationship between the two organs' descents, thymic tissue can get sequestered within the thyroid, the most common site for ectopic cervical thymus.[2]

While unnecessary investigations may cause anxiety for patients and families with additional costs for the health care system, thyroid lesions found in children need to be thoroughly

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examined. Although thyroid nodules are less frequent in children than in adults, a higher malignancy rate is highlighted in this group.[3] In most cases, the diagnosis of IET can be made safely with US. The most significant characteristic of this lesion is its echogenicity, which is similar to the normal thymus.[4] However, IET may also be mistaken for a thyroid nodule, especially a malignant one, due to similar sonographic characteristics. This similarity may lead to fine needle aspiration (FNA) and/or unnecessary surgeries in children.[5]

Although IET is thought to be a rare entity discovered incidentally, its prevalence is not well known. Most of the reports describe individual cases or small series. Research presenting follow-up is even more scarce.[6,7] Herein, we report a series of 30 children and adolescents with IET detected by US—and present follow-up results.

Material and Methods

We searched our database of patients under 18 years old with a nodular thyroid lesion detected by US, between January 2007 and December 2019. In 30/255 (11.7%) IET was diagnosed. We retrospectively studied the clinical and US findings of these patients. The analysis included the reason for referral, age at IET diagnosis, duration of follow-up, pubertal status at diagnosis (i.e. Tanner stages 1 to 5) and at last follow-up, levels of thyroid stimulating hormone (TSH), free T4 and thyroid peroxidase antibody (TPOAb) as well as localization, longest diameter, and US features of IET, and interventions (FNA and surgery). Serum levels of thyroid hormones and thyroid autoantibodies were determined by commercial kits. The US examinations were performed on several US machines by several well-trained radiologists under the supervision of a faculty member. Patients underwent US examinations at 3- to 12-month periods for follow-up. FNA specimens were obtained under sonographic guidance by an experienced radiologist and evaluated by qualified cytopathologists. IBM SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, N.Y., USA) was used for statistical analysis. Continuous variables are expressed as mean (range, median).

This retrospective study did not require written informed consent.

The Institutional Review Board approved the study (Karadeniz Technical University, Faculty of Medicine, 28/01/2021, 2020/386).

Results

Out of the 255 children evaluated for a thyroid nodule between 2007 and 2019, IET was incidentally found in 30 (%11.7) (20 males/10 females). The mean age at the first examination was 5 years (0.1-12.2, median 5.6 years) and only 2 patients were pubertal (Tanner stage 2 and above). The indications that led to the initial US were as follows: cervical lymphadenopathy (n=6), congenital hypothyroidism (n=2), elevated serum TSH concentration (n=4), 'thyroid nodule' found by US performed elsewhere (n=17), and follow-up imaging after hemato-oncological cancer treatment (n=1). None of the children had a palpable thyroid nodule or clinical evidence of thyroid malignancy at presentation or during the follow-up period. We observed 34 IETs in 30 patients as four patients had IETs bilaterally. While 28 cases were diagnosed by US, five cases were diagnosed by FNA, and one by surgery. Apart from the five patients with FNA and the one who had undergone surgery, all the other patients were diagnosed at the first US. A total of 19 IETs (55.9%) were located in the left lobe, and most (79%, 27/34) in the midportion of the thyroid lobe.

On US examination, IET appeared as a round, oval, or irregular hypoechoic area, with regular linear and punctate bright internal echoes. The lesions generally had a typical echo pattern consistent with the descended thymus. The mean longest diameter of IET was 6.4 mm (2.5-21, median 4.5), and 88% (30/34) were <1 cm in the longest diameter. 85.3% (29/34) had well-defined margins.

At diagnosis, serum TSH concentrations were within normal levels in 25 patients, slightly above the reference range in 4 patients, and one patient had a significantly high TSH and a low free T4. Four patients were receiving L-thyroxine therapy for primary hypothyroidism (two congenital, two acquired). TPOab was positive in 1 of the 21 patients in which the analysis was available.

A total of 25 children with 27 lesions were followed up with a mean follow-up time of 2.7 years (0.3-7.5, median 2.1 years). No change in size was observed in one lesion after 4.1 years. 13 (48%) lesions showed an increase in the longest diameter by 0.3-4 mm initially, but then the lesions were stable. A decrease in size or complete regression was observed in 13 (48%) lesions after a mean time of 2.4 years (0.3–7.5, median 1.5 years). At the last follow-up, 7 patients were pubertal, and one was postpubertal. Pubertal progression (compared to the initial examination) was associated with both increment and decrease in size of IET. While 4/8 lesions in patients with pubertal progression showed an increase in size, the other 4/8 lesions decreased in size.

In most cases, a conservative approach was led. FNA was performed in 5/30 patients, and hemithyroidectomy in one. The patients having FNA were older (8.7 vs. 4.3 years) and had lesions with a larger longest diameter (8.2 vs. 6.1 mm) compared to the patients without FNA. In these cases, interventions were performed due to the concern that these lesions might represent a malignancy. The only patient who underwent surgery was the 'first' IET case in our series and belonged to the earliest years of the study period when surgeons preferred immediate surgery due to concern for malignancy and accuracy of FNA in children with suspicious nodules. The 'nodule' size was 10 mm, and there was suspicion of increased vascularity. Regarding the cases with FNA, two of them did not present the typical echotexture of IET, both were ≥10 mm in size and showed an increase in size on follow-up. Two others also did not have the classical appearance, one of them had increased vascularity, and the other had irregular margins. The last case showed increased vascularity and ill-defined margins. Cytologic analysis revealed benign-appearing lymphocytes in all FNAs. In one patient, hemithyroidectomy was performed after two months of observation, and the histological examination confirmed the presence of IET. Intrathyroidal thymus in a 1-year-old girl is presented in Figure 1.

Discussion

Herein, we reported our experience in the evaluation of IET over twelve years. Of the 30 patients with 34 lesions, 28 cases were diagnosed by US findings, five cases were diagnosed by FNA and one by surgery. There are not many reports on IET. A recent review of the pediatric literature revealed only 59 previously published cases of IET masquerading as thyroid nodules or neoplasia in US.[8]

As IET had been identified by imaging studies performed for other indications in most cases reported in the literature, it can be defined as an 'incidentaloma.' Incidental thyroid findings detected on US examinations in children have been documented by Avula et al.[9] In a series of 287 neck US performed for non-thyroid indications, they identified 52 patients with thyroid abnormalities (18%), of whom 9 (17.3%) were diagnosed as IET. Yildiz et al.[10] reported

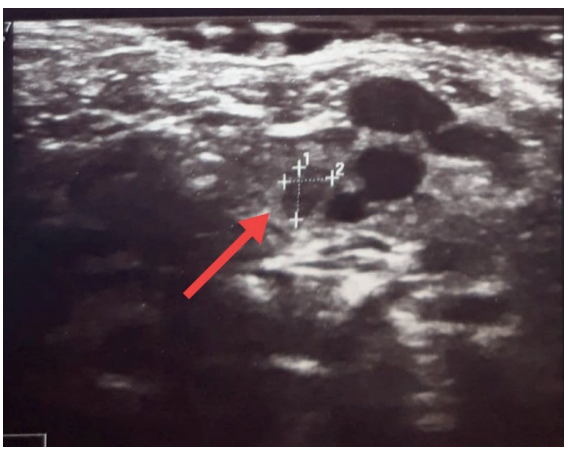

Fig. 1. Intrathyroidal thymus in a 1-year-old girl.

a prevalence of 4.2% in 216 children who had undergone a thyroid or neck US examination only in a one-year period. Kim et al.[11] found a prevalence of 0.4% during a seven year-period among 3195 children. We found this rate as 11.7% among children and adolescents with a nodular thyroid lesion between 2007 and 2019, higher than the previously published literature. The wide range of reported IET prevalence in the limited number of studies is probably due to the different patient selection methods.

Ultrasound is the recommended diagnostic modality for IET because of its unique appearance characterized by a hypoechoic pattern with multiple regular linear and punctate internal echoes.[12] These bright echogenicities in IET represent fat against lymphoid tissue or connective tissue septae and blood vessels. This echotexture is similar to the normal thymus, which is often visible in the suprasternal area in children by US.[13] Another characteristic feature of IET is its location in the middle or the lower third of the thyroid, which is explained by the embryologic origin of the thymus.[10] The predominance of IET occurrence in the middle part of the thyroid lobe was also observed in our study. Most IETs have well-defined margins, but there is inconsistency regarding the shape of IET in reports. While some authors described these lesions as not simply rounded or ovoid nodules having irregular margins, others noted diverse shapes as round, triangular, polygonal, or fusiform on different planes.[9,14,15] Our observations are similar to other authors'. Although most authors did not report vascularity, others reported isovascularity with thyroid parenchyma in some patients.[16]

While IET has typical clinical and sonographic characteristics allowing diagnosis, the US differential diagnosis between IET and malignant nodules may be challenging. Due to the hypoechoic texture of benign thymic tissue with microcalcification-like punctate echogenicities, distinguishing IET from suspicious lesions may be difficult, and it might be mistaken as a high-risk nodule that needs to be referred for FNA.[17] Further, irregular margins can additionally suggest malignancy.[3] It is essential to be aware of the sonographic pattern of IET and interpret the findings cautiously to avoid unnecessary investigations and surgery in children. To avoid unnecessary interventions, clinicians and radiologists should be familiar with the sonographic appearance of normal thymic tissue. Differentiating suspicious thyroid nodules from an IET using US requires good experience. Critical features that may help differentiate IET from thyroid nodules are a characteristic hypoechoic solid lesion with multiple linear or punctate internal echoes-an echotexture similar to the thymus, well defined margins, and mid-to low-lying location. A view of the thymus should be considered when US imaging reveals an intrathyroidal lesion with these features. Thus, if the radiologist or the clinician is well-acquainted with the typical characteristics of IET, further invasive procedures could be avoided. However, when sonography results are inconclusive, further evaluation with other techniques such as elastography or FNA, only if certainly needed, could be considered.

In most previously reported cases, the diagnosis of IET was made after FNA and/or surgery. Apart from thyroid nodules, the differential diagnoses also included hemato-oncological malignancies such as lymphoma and lymphocytic leukemia in some cases.[18,19] In addition, there are exceptionally rare cases of intrathyroidal malignancies associated with thymic tissue, e.g., spindle epithelial tumor with thymus-like differentiation.[20] Although surgery was performed in a substantial proportion of previously reported cases, the recent literature supports a conservative attitude and avoidance of surgery in most IETs.[21] Our approach is in line with this recommendation. In our center, we follow-up children with various thyroid diseases with regular long-term US monitoring. Accordingly, in our series, FNA was performed in 5/30 cases with IET and surgery in only one. We support that US and, in select cases, FNA can be used in the diagnosis and follow-up of thyroid lesions such as IET. FNA should be

avoided in cases demonstrating stability or regression at the US follow-up.

In one-half of the cases described in the current report, we observed a decrease in size or regression with time, and in one patient, there was no change in size over a time period of 4 years. This finding confirms the substantially benign course of IET, which has also been reported in the limited studies presenting follow-up.[22] It has been suggested that the regression may be reflecting the tendency toward thymic involution occurring with advancing age.[23] On the other hand, we observed an increase in size in the other half of cases. This increment may be linked to the normal growth of the thymus or rebound thymic enlargement during childhood.[1,24] However, these children are going to be followed up for any other differential diagnosis. Observations described by other authors regarding the natural history of IET have varied. While the size did not correlate with the child's age at diagnosis, an inverse relationship between the size and age was shown, suggesting that these lesions disappear or regress over time.[15,23] We suggest that the small increases in lesion diameter can be safely monitored by US, along with the rare occurrence of a tumor arising from thymus.

We showed that pubertal progression was associated with both increment and decrease in size of IET. Since eutopic thymus is expected to disappear following puberty[1], various Tanner stages of patients (ranging from 1-5) at the last follow-up might explain this finding. We couldn't recognize another study describing pubertal findings of IET patients in detail with follow-up.

Even though we have reported a relatively large series of IET with long-term follow-up, this study has several limitations. First, it was a retrospective review, and we were unable to review the US images of the lesions. Second, we were unable to use elastography, a newly proposed diagnostic modality in the evaluation of IET. Description of the patients' pubertal status over the follow-up period and its association with changes in size is the strength of the study.

In conclusion, IET may be more common than previously thought and should be considered in the differential diagnosis of incidental thyroid lesions and nodules in children and adolescents, keeping in mind that the US characteristics of IET can suggest a malignant nodule. Awareness of this entity with long-term follow-up can reduce the need for FNA or unnecessary surgery.

Ethical approval

The Institutional Review Board approved the study (Karadeniz Technical University, Faculty of Medicine, 28/01/2021, 2020/386).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: EAC, SK, İE, HD, GK; data collection: EAC, SK, İE, HD, GK; analysis and interpretation of results: EAC, İE, GK; draft manuscript preparation: EAC. 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

Cyclopentolate eye drops-induced anaphylaxis in an infant

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ABSTRACT

Background. Cyclopentolate is frequently used as a mydriatic agent during ophthalmological examinations in childhood and hypersensitivity reactions associated with this drug are rare. We aim to report an infant who experienced anaphylaxis due to cyclopentolate eye drops.

Case. A nine-month-old girl, who was being followed up with a diagnosis of retinoblastoma, presented for consultation for urticaria, cough, stridor, and dyspnea that developed after the administration of topical cyclopentolate to the eyes. The patient was diagnosed with anaphylaxis and treated with adrenaline. During the follow-up, tropicamide was used safely as an alternative drug.

Conclusions. In children, hypersensitivity reactions due to cyclopentolate are very rare. Only four pediatric patients were reported in the literature to have developed an allergic reaction after the administration of cyclopentolate eye drops. We present here the youngest patient who developed anaphylaxis with cyclopentolate eye drops. Anaphylaxis due to cyclopentolate should be kept in mind, rapidly recognized, and treated when a reaction develops.

Key words: anaphylaxis, children, cyclopentolate, eye drop, mydriatic.

Mydriatic eye drops are frequently used by ophthalmologists for both diagnosis and treatment.[1] Cyclopentolate is a widely used synthetic mydriatic agent which has an advantage of rapid onset and successful mydriasis in children. Systemic adverse reactions are rare relative to their extensive use.[2] Hypersensitivity reactions due to cyclopentolate eye drops are very rare in children.[2-4] We aim to report the case of an infant who experienced anaphylaxis due to cyclopentolate eye drops and to review the literature related to this case.

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The results are also presented as a poster at the 28th National Allergy and Clinical Immunology Congress, 13-17 October, 2021, Antalya, Türkiye.

Case Report

A nine-month-old girl who was being followed up with a diagnosis of retinoblastoma presented for consultation because of a reaction that had developed after drops were used during the eye examination. It was learned that ophthalmologists had administered five drops of Siklopleji n® (1% cyclopentolate hydrochloride, sodium chloride, disodium EDTA, benzalkonium chloride) that were administered to both eyes at an interval of five minutes. It was stated that widespread urticarial rash, cough, stridor, dyspnea, and restlessness developed within minutes after the administration. The infant's vital signs were as follows: oxygen saturation 93%, 56 breaths/min, heart rate 200 beats/min, blood pressure 105/60 mmHg. The patient was diagnosed with anaphylaxis and administered 0.01 mg/

kg adrenaline intramuscularly, and 1 mg/kg methylprednisolone and diphenhydramine intravenously. It was seen that the respiratory distress regressed rapidly and that the rash disappeared within a few hours. Since serum tryptase levels could not be studied at our hospital, this measurement could not be performed during the reaction. The patient did not take any other concomitant medication. It was learned that the patient did not have any problems with cyclopentolate administration during previous eye examinations. There was no known atopic disease or drug allergy in the patient's personal and family history. The patient was evaluated as experiencing cyclopentolate-induced anaphylaxis. Skin tests with cyclopentolate, benzalkonium chloride and latex were planned in the follow-up, but her family did not consent to this. During the follow-up, Tropamid® (0.5% tropicamide, sodium chloride, disodium EDTA, benzalkonium chloride) was used as a mydriatic for eye examination safely. A drug allergy information card was given to the family. The written informed consent was obtained from the parents of the patient for publication.

Discussion

In this case report, we present the youngest patient who has been seen to develop anaphylaxis due to cyclopentolate eye drops. It is well recognized that systemic adverse reactions may follow topical administration of any drug. Cycloplegic agents can enter the systemic circulation by absorption through the cornea, conjunctiva, nasolacrimal mucosa, and gastrointestinal tract. Most of adverse reactions due to central nervous system (CNS) disturbances include confusion, cerebellar dysfunction, and seizures.[5] Topical cyclopentolate can be measured in the systemic circulation within 5 minutes; the drug reaches peak level at 15 minutes.[6]

Anticholinergic eye drops in children are usually known to be safe and severe adverse reactions are rare.[7] In a prospective cohort study, however, Minderhout et al.[8] reported a rate of 10.3% for adverse reactions following the administration of two drops of cyclopentolate; this rate was 4.8% in children following one drop of cyclopentolate. Low body mass index and young age have been reported as risk factors in the development of adverse reactions with eye drops containing cyclopentolate. Many adverse effects were included in the cited study, but anaphylaxis was not mentioned.

In children, hypersensitivity reactions due to cyclopentolate are very rare. Only four pediatric patients were reported to have developed an allergic reaction after the administration of cyclopentolate eye drops in the literature. Two of these were patients, 3 and 12 years old, and only urticaria had developed.[2] Two other case reports presented cases who had developed severe anaphylaxis[9] after the administration of cyclopentolate eye drops.[3,4] The clinical features and diagnostic tests of these four pediatric cases and our case are summarized in Table I.[2-4] In the literature, apart from pediatric cases, the cases of two adults who developed hypersensitivity with cyclopentolate eye drops have been reported. One of these cases developed contact urticaria while the other developed generalized urticaria. However, no case of adults with anaphylaxis due to cyclopentolate has been found.[10,11] Unlike the previously reported severe anaphylaxis in two pediatric cases, our patient developed moderate anaphylaxis due to the cyclopentolate eye drops.[9] Since the skin test could not be performed due to the concerns of the family, it is not possible to distinguish whether the anaphylaxis was caused by cyclopentolate or the benzalcholium chloride in the eye drops. On the other hand, the patient's subsequent use of another eye drop containing benzalcholium chloride without any problems suggests that the causative agent of anaphylaxis is cyclopentolate.

There is no clear discussion in the literature about the mechanism of the reaction that developed in the four pediatric cases mentioned.[2-4] However, prick test positivity in two of the patients who developed anaphylaxis

Table I. Characteristics of pediatric cases who developed hypersensitivity after administration of cyclopentolate eye drops.

	Jones et al.[2]		Diesner et al.[4]	Tayman et al.[3]	The present case
Age (months)	36	144	60	48	9
Clinical symptoms of the reaction	Urticarial rash	Facial rash and redness	Dizziness Hypotension Angioedema Fatigue	Angioedema Urticaria Cyanosis Wheezing	Urticarial rash Cough Stridor Dyspnea Restlessness Tachycardia Tachypnea
Reaction time*	20 minutes	6 hours	Several minutes	10 minutes	Several minutes
Treatments	NA	Topical corticosteroid p.o.antihistamine	i.v. corticosteroid i.v. antihistamine i.v. fluid	i.m adrenaline i.v. antihistamine i.v. corticosteroid i.v. fluid nebulized albuterol Oxygen	i.m adrenaline i.v. antihistamine i.v. corticosteroid
Diagnostic tests	NA	NA	(+) Prick to prick test with Cyclopentolate %1 eye drops	(+) conjunctival challenge test with Cyclopentolate eye drops (1/10 dilution) (+) Prick test with Cyclopentolate eye drops (1/10 dilution)	NA

*Reaction time: the period between the administered dose and the appearance of clinical reaction
NA: not applicable, i.m.: intramuscular, i.v.: intravenous

suggests that these reactions developed through an IgE-mediated mechanism.[3,4] In the two cases reported in 1990 with urticaria, no diagnostic test was performed.[2] Although we could not perform skin tests in our case, we know that the resulting reaction developed after repeated exposures. This is why we think that the reaction may have developed through an IgE-mediated mechanism.

Another issue that needs to be discussed in the case seems to be an atropinergic reaction. Anticholinergic reactions may be seen due to eye drops and may present with tachycardia, tachypnea, increase in body temperature, CNS stimulation manifested by restlessness, confusion, psychiatric reactions, delirium, and seizures. A serious intoxication can cause death with CNS depression, coma, circulatory and respiratory failure.[12] Tachycardia and tachypnea can also be seen in anticholinergic reactions but urticaria and stridor are not expected in this event. Although we did not consider the presence of an anticholinergic reaction in our patient based on our clinical findings, it would be useful to study the tryptase level to differentiate both reactions. Elevated levels could support a diagnosis of anaphylaxis; on the other hand, normal levels do not exclude the previous occurrence of anaphylaxis.[13] For this reason, anaphylaxis should be recognized rapidly by considering clinical findings. Intramuscular adrenaline administration is the first-line life-saving treatment in anaphylaxis and should be

administered as soon as the diagnosis is made. Antihistamines and corticosteroids may be used as second-line therapy, but they have no place in the emergency treatment of anaphylaxis and are not a substitute for adrenaline.[14]

In conclusion, anaphylaxis can develop not only via the intravenous or oral route but also with the topical administration of drugs such as eye drops. Anaphylaxis due to cyclopentolate should be kept in mind, rapidly recognized, and treated when a reaction develops.

Ethical approval

The written informed consent was obtained from the parents of the patient for publication.

Author contribution

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

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Case Report

Severe acute kidney injury induced by crescentic glomerulonephritis in a child with infective endocarditis

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ABSTRACT

Background. Kidney involvement related to infective endocarditis (IE) may present with different clinical findings. The most common histopathological finding of renal involvement is a combination of proliferative and exudative glomerulonephritis. However, severe acute kidney injury (AKI) induced by crescentic glomerulonephritis (CGN) is extremely rare in children with IE. To date, only 4 pediatric cases with IE-induced CGN had been reported. We present a 14-year old girl with IE-induced CGN.

Case. A 14-year old girl with fever, macroscopic hematuria, oliguria, and acute kidney injury (AKI) was admitted to our clinic. The medical history revealed that the patient had undergone several cardiac interventions due to truncus arteriosus type 1, and she recovered from IE-induced glomerulonephritis following antibiotherapy six months ago. During admission, the patient was diagnosed with IE according to one major (positive imaging finding) and three minor (fever, predisposing cardiac disease, and immunological criterion) criteria. Immediate antibiotic treatment was initiated. A kidney biopsy was performed, which showed crescentic glomerulonephritis (CGN with crescents, >50%). Daily pulse steroid (3 days), monthly pulse cyclophosphamide (6 doses), and oral steroid (2 mg/kg/day) therapy were initiated with gradual dose tapering. The patient underwent 12 hemodialysis sessions until the 38th day of the treatment. She was discharged on the 45th day of treatment with normal kidney function tests and negative acute phase reactants. Treatment was maintained with mycophenolate mofetil (MMF) after a 6-month course of cyclophosphamide. MMF was discontinued in the 12th month. At the 18th-month follow-up visit the patient had mild proteinuria, and was on ramipril therapy.

Conclusions. The occurrence of CGN should be considered in children with predisposing cardiac disease, who develop hematuria, proteinuria, and severe AKI. Although antibiotic therapy alone is often sufficient in this immune complex GN induced by infection, early initiation of additional immunosuppressive therapy in the presence of CGN may be beneficial for long term preservation of kidney functions.

Key words: infective endocarditis, crescentic glomerulonephritis, children, vegetation.

Infective endocarditis (IE) and related kidney involvement may present different clinical findings. Hematuria and/or proteinuria constitute the initial clinical findings in these patients. The most common histopathological finding is proliferative and exudative glomerulonephritis.[1-8] Severe acute kidney injury (AKI) caused by crescentic glomerulonephritis (CGN) is extremely rare in children with IE.[1-3] To the best of our knowledge to date, only four cases with IE-induced CGN have been reported in PubMed/MEDLINE, Scopus, and Google Scholar databases. In this case report, CGN, related severe AKI, and the difficulties of the clinical course and treatment in a girl who had undergone several cardiac interventions due to the truncus arteriosus type 1 are discussed along with similar cases in the literature.

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Case Report

A 14-year old girl, who was referred from another health center due to macroscopic hematuria and gradual impairment in kidney functions was admitted to our clinic. Her medical history revealed that she had undergone five surgeries starting at two months of age due to the truncus arteriosus type 1 and underwent three cardiac homograft implants.

Six months ago while she was under treatment in the city in which she lived, the patient had convulsions due to a diagnosis of immune thrombocytopenic purpura (ITP) and urinary system infection presenting with macroscopic hematuria and thrombocytopenia. Cranial imaging displayed hemorrhage involving left parasagittal area. During this period, the patient was hospitalized in our clinic because of a high fever and impaired kidney function. She was diagnosed with bacterial endocarditis according to the Duke's criteria consisting of one major (*Staphylococcus aureus* growing in the blood culture) and three minor (predisposing cardiac disease, glomerulonephritis, and high fever) findings.[9] She recovered after 6-weeks of treatment with teicoplanin and cefotaxime antibiotherapy. She was discharged with normal kidney function tests and microscopic hematuria. After six months, the patient was re-hospitalized due to macroscopic hematuria and impaired kidney functions. Her physical examination revealed body weight 29 kg (<3p), height 130 cm (<3p), temperature 36.7°C, blood pressure 110/70 mmHg, oxygen saturation 99%, 4/6 pansystolic murmur in all cardiac auscultation points, and splenomegaly. Laboratory examination showed anemia, thrombocytopenia, hypocomplementemia, elevated acute phase reactants, negative antinuclear antibody (ANA) and direct Coombs tests, elevated urea and creatinine levels, proteinuria, hematuria, and elevated rheumatoid factor (RF) titration (Table I). However, the blood culture was negative. The cardiac imaging showed vegetation in the aortic valve (Fig. 1).

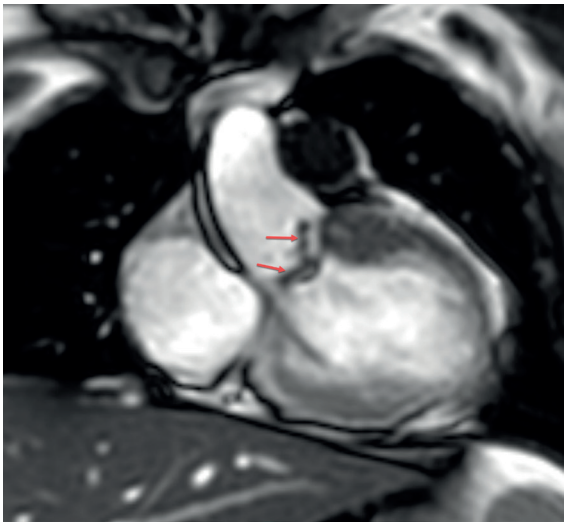

Fig. 1. Magnetic resonance imaging of left ventricular outflow tract (LVOT) showing vegetations in the aortic valve (arrows).

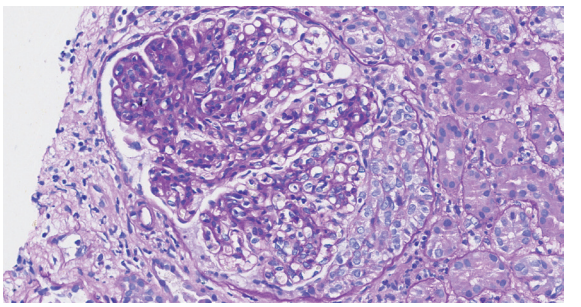

Fig. 2. Cellular crescent and mesangial proliferation (Periodic acid – Schiff statining, x400).

At this admission to our clinic, although blood culture was negative, considering other major (positive imaging finding) criterion of Duke's criteria[9] and three minor (fever, predisposing cardiac disease, and immunological) criteria, IE, and immune complex-mediated glomerulonephritis secondary to IE were diagnosed; teicoplanin and cefotaxime were initiated. However, progressive impairment in kidney function was observed. The creatinine level increased to 6.3 mg/dl, and oliguria developed. The kidney biopsy revealed diffuse endocapillary proliferation along with 56% cellular crescents (Fig. 2). The immunofluorescence images showed granular C3 and IgM deposition on the basement membrane zone. The patient was diagnosed

Table I. Laboratory findings of the patient at first admission.

Complete blood count		Serological tests	
WBC (4-12 x10^3/mm^3)	39.4	Anti-HBs Ab	positive
Hemoglobin (12-16 g/dl)	7.2	HBsAg	negative
MCV (80-100 fl)	70	Anti HCV IgM	negative
Hematocrit (35-49 %)	22	Anti HAV IgM	negative
Platelet (100-400 x10^3/mm^3)	89	Anti HIV	negative
Biochemical parameters		CMV IgM	negative
Urea (16-40 mg/dl)	98	EBV IgM	negative
Creatinine (0.57-0.87 mg/dl)	2.7	Parvovirus-B19	negative
eGFR (ml/min./1.73 m^2)	26	Anti-nuclear antibody (ANA)	negative
Total protein (6.6-8.7 g/dl)	6.4	Anti-ds DNA	negative
Albumin (3.2-4.5 g/dl)	3.2	p- ANCA	negative
Sodium (135-145 mEq/L)	138	c- ANCA	negative
Potassium (3.5-5.1 mEq/L)	3.3	Anti-phospholipid antibodies	negative
Chloride (98-107 mEq/L)	106	Direct Coombs	negative
Phosphorus (2.9-5.1 mg/dl)	5.8	Urine analysis	
Magnesium (1.7-2.2 mg/dl)	1.3	Proteinuria-24 hours (0.140 gr/day)	2.04
Uric acid (2.4-5.7 mg/dl)	7.7	Spot urine protein/creatinine ratio (<0.2 mg/mg)	3
ALP (50-117 IU/L)	66	Proteinuria-24 hours (<4 mg/m^2/hour)	73
LDH (135-214 U/L)	301	Microscopy	>50 RBC/hpf
AST (<32 IU/L)	12	Acute phase reactants	
ALT (<33 IU/L)	4	Erythrocyte sedimentation rate (<20 mm/hour)	23
Calcium (8.4-10.2 mg/dl)	7.8	C-reactive protein (<5 mg/L)	70
ASO (<200 IU/ml)	34	Fibrinogen (200-393 mg/dl)	292
Triglycerides (<200 mg/dl)	226	Serum complements	
Cholesterol (<200 mg/dl)	188	Complement 3 (0.88-1.55 g/L)	0.09
Rhematoid factor (<14 IU/ml)	146	Complement 4 (0.12-0.32 g/L)	0.04

Values in parenthesis represent normal range
eGFR: estimated glomerular filtration rate, hpf: high power field, RBC: red blood cell, WBC: white blood cell.

with IE-induced and immune complex-mediated crescentic glomerulonephritis.

The previous incident of bacterial endocarditis (*S. aureus*) and secondary glomerulonephritis were treated only with antibiotics. However, this time, due to remarkable increase in serum creatinine during antibiotic treatment and the presence of CGN, pulse steroid therapy (3 days) and monthly pulse cyclophosphamide (6 months) were initiated. Oral steroid (2 mg/kg/day) were tapered gradually. Twelve sessions of hemodialysis were performed. On the 38th day of treatment, dialysis was stopped. On the 45th day of treatment, urea and creatinine levels dropped to 58 mg/dl and 0.5 mg/dl, respectively; and the patient was discharged with negative acute phase reactants on the same day. The treatment was maintained with mycophenolate mofetil (MMF, 1200 mg/m^2) after six months of cyclophosphamide treatment. In the seventh month, a repeat kidney biopsy revealed global sclerosis in 2 of 14 glomeruli and fibrous crescents and 30% fibrosis in three glomeruli. MMF treatment was discontinued in the 12th month. At the 18-month follow-up control, proteinuria was mild (12 mg/m^2/hour) and urea and creatinine levels were 33 and 0.6 mg/dl, respectively. The levels of complement and rheumatoid factor (RF) were within normal limits. The patient is still receiving 4 mg/m^2 ramipril.

Informed parental written consent was taken for case publication.

Discussion

Severe AKI is extremely rare in patients with IE-induced immune complex-mediated glomerulonephritis. Only four case reports on severe AKI in children with IE-induced immune complex-mediated glomerulonephritis (Table II) have been published in the literature.[1-3]

Immune complex processes are responsible for the pathogenesis of IE-induced acute glomerulonephritis.[4] The infections are responsible for the development of renal disorders and they require antibiotherapy which makes the decision difficult with respect to the immunosuppressive treatment needed for the treatment of the immune complex-mediated GN. Monotherapy of IE with antibiotics may occasionally contribute to the recovery of immune complex-mediated kidney lesions. However, as CGN can potentially lead to chronic renal failure, it requires immunosuppressive therapy, therefore, it should be administered in combination with antibiotherapy.[5-7]

During the investigation of the cases reported in the literature, it was noticed that the cases with IE-induced CGN seem to occur between the ages of 6 and 14. In all cases, fever was the most important first finding and the general findings of cardiac disease were usually observed.[1-3]

The first case in the literature was reported by Sadikoglu et al.[1] A 6-year old male patient, who had undergone cardiac surgery due to pulmonary atresia and ventricular septal defect, underwent a kidney biopsy because of persistent fever and impaired kidney function. The histopathological findings were interpreted in favor of crescentic immune complex-mediated glomerulonephritis. The patient recovered by antibiotherapy, intravenous methylprednisolone (followed by tapering doses of prednisone), and intravenous cyclophosphamide.

Table II. Clinical profile of the patients reported in literature with subacute bacterial endocarditis and crescentic glomerulonephritis.

Author	Age/Sex	Urinalysis	C3/C4	ANA/ ANCA	Blood culture	Treatment	Percentage of glomeruli with crescents	Light microscopy	Outcomes
Sadikoglu (2006)	6/M	Hematuria,	Low/N	-/-	-	Ab, Cs, CYC	80%	Crescentic immune complex glomerulonephritis	Full recovery
Mantan (2013)	11/M	Proteinuria	Low/N	-/-	-	Ab, Cs, CYC	21%	Immune complex-mediated proliferative glomerulonephritis	Full recovery
Krishnamurthy (2017, Case 1)	8/F	Hematuria, Proteinuria	Low/N	-/-	-	Ab, Cs	1%	Diffuse endocapillary exudative proliferation;	Full recovery
Krishnamurthy (2017, Case 2)	9/F	Hematuria, Proteinuria	Low/N	-/-	-	Ab, Cs, HD, CYC	70%	Crescentic glomerulonephritis	Predialysis CKD, Proteinuria, Hematuria (microscopic)
Our patient	14/F	Hematuria, Proteinuria	Low/ Low	-/-	-	Ab, Cs, HD, CYC, MMF	56%	Crescentic immune complex glomerulonephritis.	Proteinuria, Hematuria (microscopic) Normal creatinine

ANA: antinuclear antibodies, Ab: antibiotic therapy, ANCA: anti-neutrophil cytoplasmic antibody, Cs: corticosteroids, CKD: chronic kidney disease, CYC: cyclophosphamide, HD: hemodialysis, MMF: mycophenolate mofetil.
–: negative, +: positive, N: normal

Mantan et al.[3] reported an 11-year old male patient with acyanotic heart disease. The patient was treated with antibiotics and oral prednisolone for four weeks due to a highly impaired kidney function and fever that persisted for the last 15 days. As proteinuria and edema persisted, a kidney biopsy was performed in the sixth week, and the patient was diagnosed with crescentic glomerulonephritis. The steroid treatment was continued combined with oral cyclophosphamide (8 weeks).

Krishnamurthy et al.[2] reported two cases. The first case was an 8-year old girl with mitral insufficiency (MI) secondary to rheumatic heart disease and was on antibiotic treatment because of fever; she underwent a kidney biopsy as the serum creatinine increased rapidly on the 5th day of treatment. The biopsy displayed diffuse endocapillary exudative proliferation and segmental crescents. The patient was treated with intravenous methylprednisolone and oral prednisolone; without dialysis.

The second case, a 9-year old girl, was under follow-up with a diagnosis of perimembranous ventricular septal defects (VSD), and had fever, hematuria, oliguria, and edema. The kidney biopsy revealed severe CGN with 70% crescent formation. The patient was treated with pulse methylprednisolone, monthly pulse cyclophosphamide and oral prednisolone. Residual renal injury developed despite aggressive immunosuppressive treatment. In the fourth month of the follow-up, her serum creatinine level was still elevated along with proteinuria and microscopic hematuria, and the need for antihypertensive treatment continued.

In our case, endocarditis was diagnosed twice, and at the first admission of bacterial endocarditis (*S. aureus*) and secondary glomerulonephritis were treated only with antibiotics. However, during the second admission, she had to take antibiotics and aggressive immunosuppression together with hemodialysis.

IE and secondary immune complex glomerulonephritis may be treated with antibiotics and/or surgical restoration of the predisposing cardiac disorder. Thereby, the kidney lesions resolved as a result of the decrease in or disappearance of the circulating immune complexes. However, it has been previously reported that aggressive immunosuppressive treatment was necessary for IE-related CGN in adults.[5] Although plasma exchange combined with the immunosuppressive treatment has been widely discussed, the benefits of this approach were demonstrated only in a few adult patients.[8,10] In our case and other pediatric cases reported in the literature, plasma exchange was not necessary. Although there is no evidence on immunosuppressive agent treatment to be initiated (high dose steroid monotherapy or in combination with cyclophosphamide), it seems that the decision should be made according to clinical and laboratory findings. In our case, considering the ongoing immune complex formation, we implemented maintenance therapy with high-dose steroids and then gradually tapered the oral steroids while we added an intensive induction treatment with monthly pulse cyclophosphamide. In addition, we maintained the immunosuppression with MMF until the 12th month.

In conclusion, CGN or severe diffuse proliferative glomerulonephritis should be considered in children who have predisposing heart disease concomitant with hematuria, proteinuria, and severe AKI. In patients with severe AKI, antibiotic treatment and early initiated aggressive immunosuppressive treatment following the kidney biopsy may be useful for long-term preservation of kidney functions.

Ethical approval

Informed parental written consent was taken for case publication.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: SY, NY; data collection: NY, SY, DG, İG, TB, MY, FU, GG; analysis and interpretation of results: SY, NY; draft manuscript preparation: SY, NY. 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

3q29 microdeletion syndrome associated with developmental delay and pulmonary stenosis: a case report

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ABSTRACT

Background. 3q29 microdeletion syndrome (OMIM 609425), first described in 2005, is a rare copy number variation (CNV), accompanied by various neurodevelopmental and psychiatric problems. Phenotypic features of the syndrome have not been fully characterized due to the new definition and rarity. Facial dysmorphology, musculoskeletal anomalies, cardiovascular abnormalities, gastrointestinal abnormalities, and dental abnormalities can be seen.

Case. A 28-month-old male patient was brought to the child and adolescent psychiatry clinic with a complaint of speech delay. He had mild dysmorphic symptoms. He was also sensitive to voice and often covered his ears. Balloon valvuloplasty was performed on the postnatal 28th day due to severe pulmonary stenosis. While karyotype was found to be normal, in array-Comparative genomic hybridization (aCGH), copy loss was detected in the long arm of chromosome 3 (arr[hg19] 3q29[196,209,689-197,601,344]x1), which contains approximately 1.4 Mb harboring 30 genes. Genetic counseling was given to the family of the patient who was diagnosed with 3q29 microdeletion syndrome.

Conclusions. In conclusion, we present 3q29 microdeletion syndrome with global developmental delay (GDD), dysmorphic face, hyperacusis, scoliosis, and severe pulmonary stenosis. Performing genetic analysis in patients with developmental delay and congenital heart disease (CHD) for which the cause cannot be explained will prevent these rare diseases from being missed, and the characteristics of the diseases will be better characterized with the reported cases.

Key words: 3q29 microdeletion syndrome, aCGH, developmental delay, child, cardiac defects.

3q29 microdeletion syndrome (OMIM 609425), first described in 2005, is a rare copy number variation (CNV), causing various neurodevelopmental and psychiatric problems.[1] The deletion is usually caused by *de novo* mutations and is rarely inherited.[2] Phenotypic features of the syndrome have not been fully characterized due to its recent definition and rarity. The neuropsychiatric aspects of 3q29 microdeletion syndrome have been emphasized in the literature. Facial dysmorphology, musculoskeletal anomalies, recurrent ear infections, ocular abnormalities, cardiovascular abnormalities, gastrointestinal abnormalities, and dental abnormalities can be detected in 3q29 microdeletion syndrome.

In this article, a 28-month-old male patient who presented with speech delay and was diagnosed with 3q29 microdeletion syndrome is presented, and the importance of genetic evaluation in cases of unexplained global developmental delay (GDD) and congenital heart disease (CHD) is emphasized. This may also contribute to the genotype–phenotype relationship of 3q29 microdeletion syndrome.

Case Report

A 28-month-old male patient was brought to the child and adolescent psychiatry clinic with a complaint of speech delay. The case had

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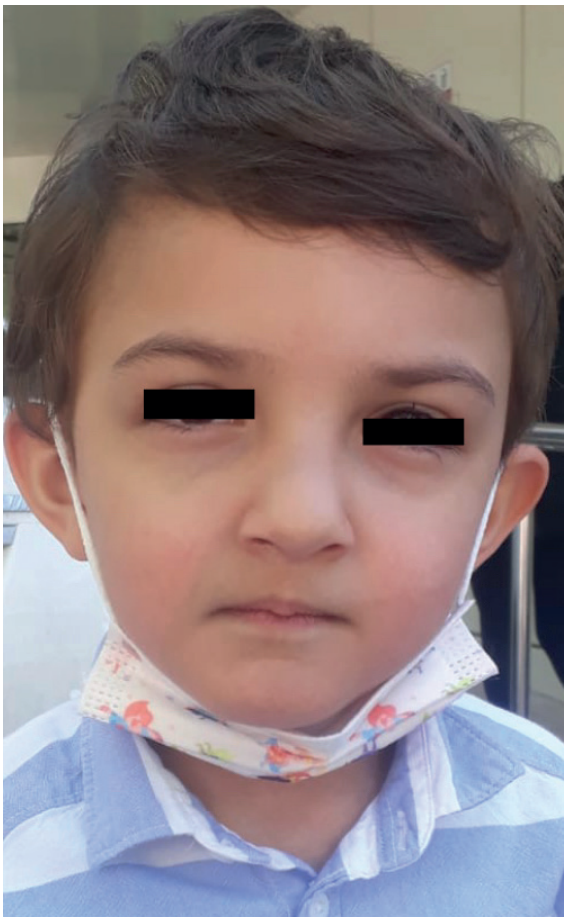
5-6 words and could not make sentences. He was also sensitive to voice, often covering his ears. When he was evaluated in the playroom with his parents, it was observed that he made eye contact, looked when his name was called, performed commands, established joint attention, and played imaginary games. The patient had no seizure history, and his neurological examination was normal.

On physical examination, he had mild dysmorphic symptoms such as posteriorly rotated ears, broad nasal tip, wide forehead, and widely spaced teeth (Fig. 1, 2). There was a *café au lait* spot on his right leg. His height was 100 cm (90th percentile), weight was 15 kg (86th percentile), and head circumference was 49.5 cm (57th percentile).

Since the high trisomy 21 risk was detected in the triple screening test, noninvasive prenatal testing (NIPT) was performed in the prenatal period and the NIPT results showed low trisomy 21 risk. He was born full-term (40th gestational week) with a birth weight of 3,350 kg (50th centile), head circumference of 36 cm (89th centile), and height 50 cm (52nd centile) via cesarean delivery. Balloon valvuloplasty was performed on the postnatal 28th day due to severe pulmonary stenosis. While there was no delay in head and neck control and walking, he spoke his first words at the age of two. He did not have toilet training. There was no abnormality in his newborn hearing screening and visual examination.

The case had a healthy 32-year-old mother and father who were not consanguineous. This case



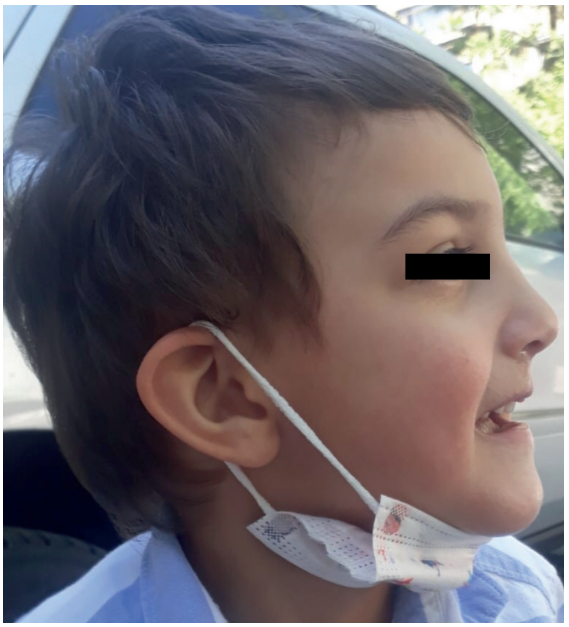


Fig. 1, 2. Patient face and profile at the age of 36 months.

was the first child in the family. A maternal uncle had died on second postnatal day for an unknown reason.

According to the Denver II Development Screening Test, while gross motor skills were comparable to his peers, personal–social (18-19 month), lingual (16-17 month), and fine motor (14-15 month) skills were behind the peers. The Childhood Autism Rating Scale (CARS) score was 21and the Autism Behavior Checklist (ABC) score was 20, both indicating absence of autism. The patient had bilateral Type A tympanograms and presented a normal auditory brainstem response (ABR).

Array-Comparative Genomic Hybridization (aCGH) using the CytoScan® Optima Assay platform and conventional karyotype analysis from peripheral blood were performed on the patient, who was referred to the Medical Genetics department because of his dysmorphic characteristics. Karyotype was found to be normal. In aCGH analysis, copy loss was detected on the long arm of chromosome 3 (arr [hg19] 3q29 [196209689-197601344] x1), which contains approximately 1.4 Mb covering 30 genes (Fig. 3). Genetic counseling was given to the family of the patient on diagnosis of 3q29 microdeletion syndrome. During examination for possible additional problems, mild thoracolumbar scoliosis was detected on direct radiography. He was referred to therapy for his developmental delay and was followed up for possible risks. The aCGH test was recommended to the family before future pregnancies to determine whether the detected anomaly is *de novo* or not, and to determine the risk of repetition of the disease. Written informed consent was obtained from the family.

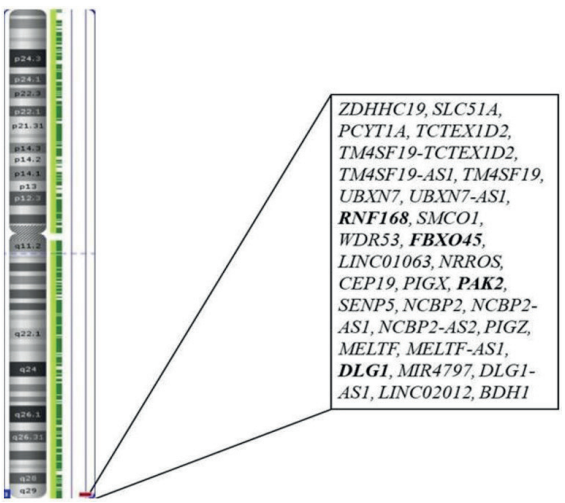


Fig. 3. Copy loss region and affected genes on the long arm of chromosome 3 in presented.

Discussion

Herein a rare case of 3q29 microdeletion syndrome with hyperacusis and pulmonary valve stenosis was presented. The deletion region was as long as 1.4 Mb. The deletion of our patient matched with the typical 3q29 microdeletion syndrome region, but it excluded the *TRFC* gene, which is one of the five disease causing genes in the region.

In addition to high rate neuropsychiatric and neurodevelopmental problems, cardiac malformations like atrial septal defect (ASD), ventricular septal defect (VSD), patent ductus arteriosus (PDA) are seen with 3q29 microdeletion syndrome at a rate of 27%-28%.[3] The cardiac defect is one of the most serious manifestations of this syndrome[4], and it may be the first clinical reflection as in this case. Pulmonary valve stenosis is rarely identified (about 5%) in 3q29 microdeletion syndrome which was also seen in this case.[5] Moreover, hyperacusis has never been reported with this syndrome previously in the literature. The findings in the present case and the frequently reported clinical features are given in Table I.[3,6]

Thirty percent of patients with CHD have genetic defects and they may emerge as various genetic syndromes. For that reason, verification of the existence or absence of genetic defects in newborns with CHD is crucial.[7] By doing this, neurological problems and non-cardiac malformations can be predicted and irreversible damage can be prevented with early intervention.[8] Related to this, research was performed with 200 fetuses with CHD. In that study, whole-exome sequencing (WES)

Table I. Comparison of the clinical features of the patient and reported cases.[4,5]

Phenotypic feature	Previous reported N (%)	Present case
Developmental/Psychiatric		
Speech delay	55/89 (61.8 %)	+
Delayed walking	53/133 (39.8 %)	-
ID*	93/136 (68.3 %)	**
Autism	38/127 (29.9 %)	-
Anxiety	28/96 (75.6 %)	-
Cranio-Facial Dysmorphism		
Broad/High nasal bridge	28/39 (30.7 %)	-
Broad nasal tip	11/28 (39.3 %)	+
Microcephaly	20/38 (52.6 %)	-
Long narrow face	12/36 (33.3 %)	-
Short philtrum	18/36 (50.0 %)	-
Dental abnormalities	36/78 (46.1 %)	+
Musculoskeletal		
Long tapered fingers	12/33 (36.4 %)	-
Scoliosis	6/19 (31.6 %)	+
Clinodactylous toes	9/25 (36.0 %)	-
Chest cavity deformity	12/40 (30.0 %)	-
Others		
Low birth weight (<3rd percentile)	31/80 (38.7 %)	-
Gastrointestinal abnormalities	37/58 (63.8 %)	-
Cardiovascular abnormalities	35/126 (27.8 %)	+
Recurrent ear infections	19/75 (25.3 %)	-
Ocular abnormalities	14/23 (60.9 %)	-
Abnormal skin pigmentation	3/22 (13.6 %)	+

* Intellectual Disability
** IQ test could not be done because of his young age.

was applied to those whose tests were negative. In prenatal chromosome microarray analysis, various chromosomal anomalies including 3q29 microdeletion syndrome were identified in half of the cases.[9] Besides, in a recently published case report, a fetus with VSD was diagnosed with 3q29 deletion prenatally and after genetic counseling, the family decided to terminate the pregnancy.[10]

3q29 microdeletion syndrome provides an important opportunity to investigate genes related to complex neuropsychiatric diseases. 3q29 microdeletion syndrome was first described in six patients with GDD or intellectual disability (ID) ranging from mild to moderate in cases.[1] In later studies, it was reported that the risk of autism increases 34 times in girls and 16 times in boys.[11] Moreover, the 3q29 deletion syndrome, which showed a relationship between cerebellar cortex volumes and psychosis tendency[12], was found to be the highest genetic risk factor (40-fold increased risk) for schizophrenia.[13] Pollak et al.[11] emphasized that these children should be screened starting from an early age, especially with neuropsychiatric and cognitive screening, and followed-up throughout their development.

Despite the strong relationship of 3q29 deletion syndrome with neurodevelopmental disorders, it is unclear how and which genes

affect the phenotype and which cellular mechanisms are impaired. In recent studies, especially *DLG1*, *PAK2*, *FBXO45* genes have come forward with their proven central roles in synaptic communication, and these genes have been associated with ID/GDD, autism, and schizophrenia pathogenesis.[14-17] DLG1 produces a synaptic scaffold protein that interacts with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors and takes a role in dendritic spine formation.[18] *PAK2* is from the family of serine/threonine kinases and it is a crucial regulator of cytoskeletal remodeling and dynamics.[19] FBXO45, on the other hand, codes for ubiquitin ligase and regulates glutamate release by mediating Munc13-1 degradation that is a necessary protein for the preparation of presynaptic vesicles.[20] Psychiatric symptoms are considered resulting from synergistic interactions of these gene products rather than individual impacts of genes in deletion area.[21,22] These genes, which have important roles in nervous system development and neurosynaptic maturation are located in the deletion area of this case and this supports the hypothesis in which synaptic dysfunction is involved in GDD/ID pathogenesis. However, despite the similar molecular interactions, the cause behind the fact that autism may arise in 3q29 microdeletion syndromes is still unknown. This may be related to additive variants in the genome or environmental factors.[22]

Furthermore, *RNF168*, which is another gene located in the deletion area is associated with DNA damage repair and is involved in the etiology of immunodeficiency.[23] Its pathological variants cause autosomal recessive RIDDLE syndrome, characterized by immunodeficiency and ID. However, in this case with a heterozygous deletion, no findings suggested immunodeficiency. Likewise, biallelic mutations in TFRC (OMIM 190010) gene is related to autosomal recessively inherited immunodeficiency 46 (OMIM 616740), and this gene is not encompassed in our patient's deletion.[10]

The aCGH method is used as a first-line clinical diagnostic genetic test for further investigation in unexplained GDD/ID cases despite detailed evaluations of history, hearing and vision, and EEG recordings (in suspicious cases). Furthermore, in cases where cardiac defects are seen in addition to dysmorphic symptoms, neuropsychiatric or neurodevelopmental problems, gastrointestinal or musculoskeletal abnormalities, it is important to test for CNVs.[24] Besides, through aCGH, it has become easier to identify many new microdeletion/microduplication syndromes in individuals with idiopathic ID/GDD or congenital anomalies. For the same microdeletion/microduplication syndrome, the size of the associated region and accordingly gene contents differ in cases. To establish phenotype-genotype relationships much clearly, detailed molecular analysis should be performed on more patients.

In conclusion, the determination of a CNV in this patient, urged us to consider other possible risks and follow the patient closer. These findings suggest to clinicians that children diagnosed with 3q29 microdeletion syndrome should be evaluated with cardiac and developmental scans. Furthermore, routine psychiatric follow-ups are recommended for these children due to developmental delays or other possible psychiatric problems.

Ethical approval

Written informed consent was obtained from the family.

Author contribution

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

The anomalous drainage of the inferior vena cava into the left atrium

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ABSTRACT

Background. Anomalies in systemic venous return most commonly involve a persistent left supe-rior vena cava draining into the left atrium. Anomalous drainage of the inferior vena cava (IVC) into the left atrium is a rare congenital vascular disorder. The diagnosis was confirmed as anoma-lous drainage of the right superior pulmonary vein and large atrial septal defect following echo-cardiography. Anomalous drainage of the inferior vena cave was confirmed with computed tomog-raphy (CT). We report a rare combination of drainage of the inferior vena cava associated with atrial septal defect (ASD) and partial anomalous pulmonary venous return.

Case. A 14-year-old girl was referred to our hospital for the evaluation of palpitations, hypoxia, exertional dyspnea, and cyanosis. Transthoracic echocardiography (TTE) revealed a large sinus venosus ASD and anomalous right superior pulmonary venous return. A cardiac CT demonstrated IVC drainage to the left atrium and an anomalous right superior pulmonary vein draining into the right atrium.

Conclusions. In older patients with cyanosis, further imaging methods together with TTE will be useful in detecting additional cardiac anomalies. Patients with inferior vena cava opening to the left atrium are different from caval type ASD's and should be surgically repaired using a patch. Corrective surgery involves repositioning of the interatrial septum via a patch.

Key words: inferior vena cava, left atrium, anomalous pulmonary venous drainage.

Anomalies in systemic venous return most commonly involve a persistent left superior vena cava draining into the left atrium (LA). Anomalous drainage of the inferior vena cava (IVC) into the LA is a rare congenital vascular disorder. It has been reported in isolation and in association with other cardiac defects.[1] It can occur with atrial septal defect (ASD), anomalous pulmonary venous drainage, and pulmonary arteriovenous fistula.[2] In this case report, we described clinical signs and symptoms which include hypoxia, exertional dyspnea and cyanosis. Diagnosis confirmed anomalous drainage of the right superior pulmonary vein and a large ASD following echocardiography. Anomalous drainage of the IVC was confirmed with computed tomography (CT). We report a rare combination of drainage of the IVC associated with ASD and partial anomalous pulmonary venous return.

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Case Report

A 14-year-old girl was referred to our hospital for the evaluation of palpitations, hypoxia, exertional dyspnea, and cyanosis. Her blood pressure was 100/60 mmHg, and her heart rate was 85 beats/min. Oxygen saturation was 94% at rest in room air. An electrocardiogram (ECG) showed normal sinus rhythm and right ventricular hypertrophy. Transthoracic echocardiography revealed a large sinus venosus ASD and anomalous right superior pulmonary venous return. A cardiac computed tomography demonstrated IVC drainage to the LA and an anomalous right superior pulmonary vein draining into the right atrium (Fig. 1). There was no evidence of other cardiac

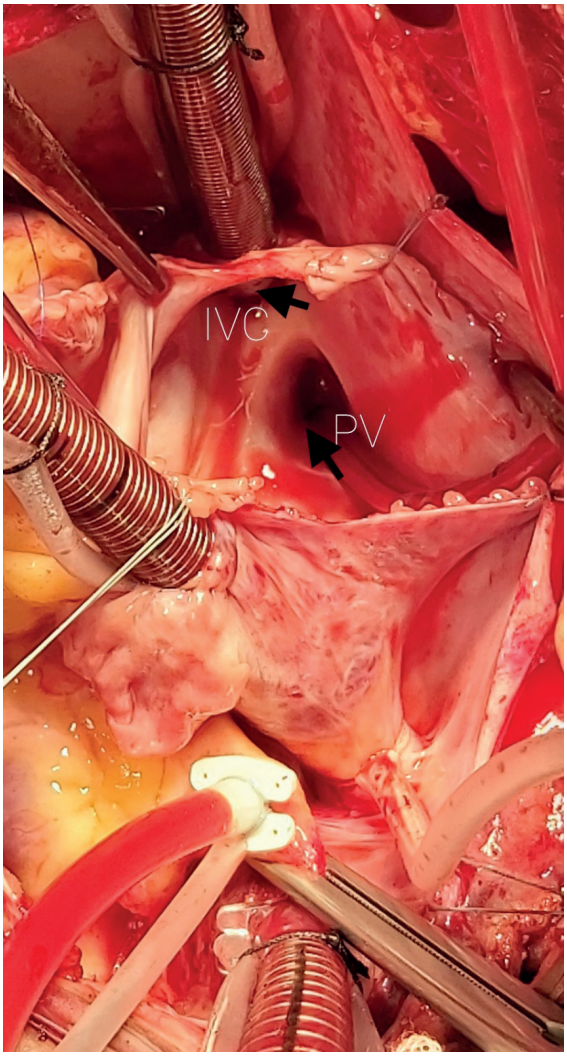

Fig. 1. Abnormal right superior pulmonary vein flowing into the left atrium and right atrium is shown.

anomalies. Informed parental written consent was taken for case publication.

The operation was performed through a median sternotomy incision. The ascending aorta and both vena cavae were cannulated directly. IVC cannulation was made as low and as close to the diaphragm, as possible. After initiated cardiopulmonary bypass under moderate hypothermia, the heart was arrested by antegrade del-nido cardioplegia. The right atrium was opened, which revealed a large ASD, 4x3 cm in size, and the inferior left-ward shift of IVC and anomalous drainage

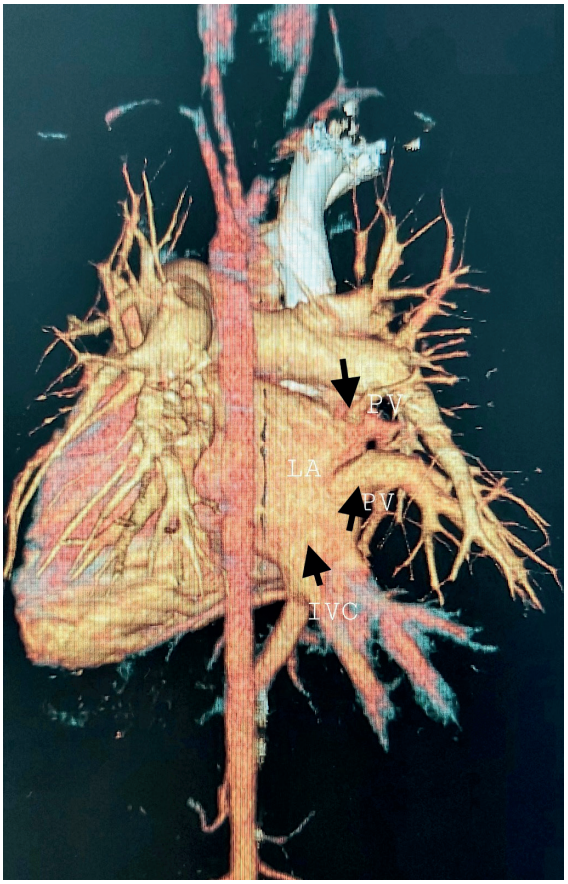

Fig. 2. Intraoperative pulmonary vein appears to open into the inferior vena cava.

of the right superior pulmonary vein (Fig. 2). The defect was closed with a patch of the fresh autologous pericardium. The IVC was redirected to the right atrium, and the right superior pulmonary vein was redirected to the LA. The postoperative course was uneventful, with the patient followed up fully saturated in room air. She was discharged on the fifth postoperative day.

Discussion

Anomalies in systemic venous return have been reported extensively, but drainage of the IVC directly into the LA is a rare condition, resulting in significant right-to-left shunts. However, anomalous drainage of the IVC into the LA is a rare congenital vascular disorder and is less commonly associated with an ASD

which Gardner first described in 1955.[1] An ASD occurring with the condition is uncommon in the reported cases. Anomalous pulmonary venous drainage and pulmonary arteriovenous fistula may also be associated.[2] This entity is different from a low or IVC secundum ASD shunting of blood from the IVC to the LA. Most patients with IVC drainage to the LA are diagnosed either congenitally or after incorrect ASD repair. If the surgeon is not careful, this can be mistaken for the inferior ASD rim, and they may iatrogenically divert IVC blood to the LA upon ASD closure.[3,4]

Patients clinically present most commonly at a young age with symptoms of right-to-left shunt. The main clinical features are shortness of breath, cyanosis, and palpitations. However, these congenital findings have been reported in asymptomatic patients referred for the evaluation of incidentally noted hypoxemia or cyanosis.[5]

Only a few cases with anomalous drainage of the IVC into the LA have been reported in the literature. Diagnosis can be difficult, as can be understood from the fact that most of the cases reported in the literature are diagnosed in adulthood.[5] More advanced imaging methods are often required to make a diagnosis. Here we present a case of a 14-year-old girl with ASD combined with anomalous drainage of the left superior pulmonary vein and drainage of the IVC into the LA. In our case, the TTE failed to demonstrate the anomalous IVC drainage into the LA. However, the presence of cyanosis made us suspect an associated disorder then the diagnosis was established by CT contrast angiography. On imaging, the IVC is positioned normally in the lower chest but then curves towards and joins the LA. In older patients with cyanosis, further imaging methods together with TTE will be useful in detecting additional cardiac anomalies. Patients with IVC opening to the LA are different from caval type ASD's and should be surgically repaired using a patch. Corrective surgery involves repositioning of the interatrial septum via a patch.

Ethical approval

Informed parental written consent was taken for case publication.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: AA, ANE; data collection: ANE; manuscript preparation: AA, ANE. All authors reviewed the results and ap-proved 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 unique case of a newborn with a hemangioma on the omphalocele sac

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ABSTRACT

Background. Mass lesions of the umbilical cord are rare anomalies. There have been rare reports of hemangiomas of the umbilical cord, but the co-occurrence of omphalocele and hemangioma of the umbilical cord has not been previously reported. Nonetheless, the condition is clinically significant as it may cause the disturbance of intrauterine fetal circulation, retardation of fetal growth and development, non-immune hydrops fetalis, morbidity and mortality.

Case. Here we aim to report a case that was prenatally diagnosed with an omphalocele and that presented after birth with a hemangioma on the omphalocele sac.

Conclusions. When dealing with umbilical mass lesions in the prenatal and postnatal periods, a hemangioma on the omphalocele sac should be considered in the differential diagnosis of patients when an omphalocele is suspected.

Key words: newborn, omphalocele, hemangioma.

An omphalocele can be separated but is more as often as possible related with other innate inconsistencies and disorders, such as Beckwith-Wiedemann disorder and trisomies 13, 18 and 21.[1] The administration of neonates born with an omphalocele comprises of the starting steps of airway stabilization, sterile wrapping of the bowel to conserve warmth in order to diminish insensate fluid privation, inclusion of an orogastric tube for bowel decompression, and the foundation of fringe intravenous access. Hemangiomas are congenital lesions originating from errors in embryonic development and are characterized by the proliferation of the vessels' endothelial cells.[2] In most instances, hemangiomas are associated with an increased risk of fetal anomalies, polyhydramnios, fetal hydrops, and perinatal morbidity and mortality.[3] We aim to report an intriguing case of a newborn with a hemangioma on the omphalocele sac who was referred with a prenatal diagnosis of omphalocele. Although many cases of umbilical cord hemangioma have been reported in the literature, to the best of our knowledge no case of hemangioma on the omphalocele sac has been reported previously.

Case Report

A 3180-gram newborn baby who was the firstborn of the first gestation of a 31-year-old mother was referred to us for a consultation. He was delivered by cesarean at 39 weeks of gestation after being diagnosed with an omphalocele at the 23rd week of gestation. During the pregnancy period, the mother had not undergone an alpha-fetoprotein test. The

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patient APGAR scores at 1min and 5min were 6 and 8 respectively in the neonatal intensive care unit. The omphalocele sac was 7x8 cm in diameter and had a pale, dark blue/yellow appearance with the umbilical cord on top. A dark red mass lesion, which was about 2x3 cm in diameter and resembled a hemangioma, was noticed over the sac (Fig. 1). Sterile wet gauze was placed on the omphalocele sac with hemangioma. The umbilical cord contained 2 arteries and 1 vein. After a short period of patient stabilization, the patient was taken to the operating room. When the sac was opened, it was found that the sac was completely occupied by the liver (Fig. 2) and that the hemangioma was originating from the sac without any obvious connection with the intraperitoneal space. After the liver was introduced into the abdomen, the defect size measured 6 cm. The intra-abdominal pressure was measured with a bladder catheter preoperatively. It was 15 cm H_2O. Upon this the omphalocele was treated by primary repair. An artificial patch was not used. On postoperative day-5, the patient required endotracheal intubation and mechanical pulmonary support due to respiratory distress and then remained intubated for 10 days. After the surgery, the patient had thrombocytosis that gradually decreased to normal values over a period of days. The patient's cranial and abdominal ultrasonography were normal, but a small ventricular septal defect (VSD) and a small secundum atrial septal defect (ASD) were identified on echocardiography.

The patient was discharged home on postoperative day-30. A histological examination of the lesion revealed large cystic dilated vascular structures with thin walls that contained scarce areas of intravascular thrombosis. On immunohistological examination, the vascular endothelium was found positive for D2-40 and CD31 expression. The final morphological and immunohistochemical diagnosis was determined to be a cavernous hemangioma (Fig. 3). A chromosomal analysis was performed, and the karyotype analysis of the patient was 46XY. Written informed consent for publication of this case was obtained from the parents.

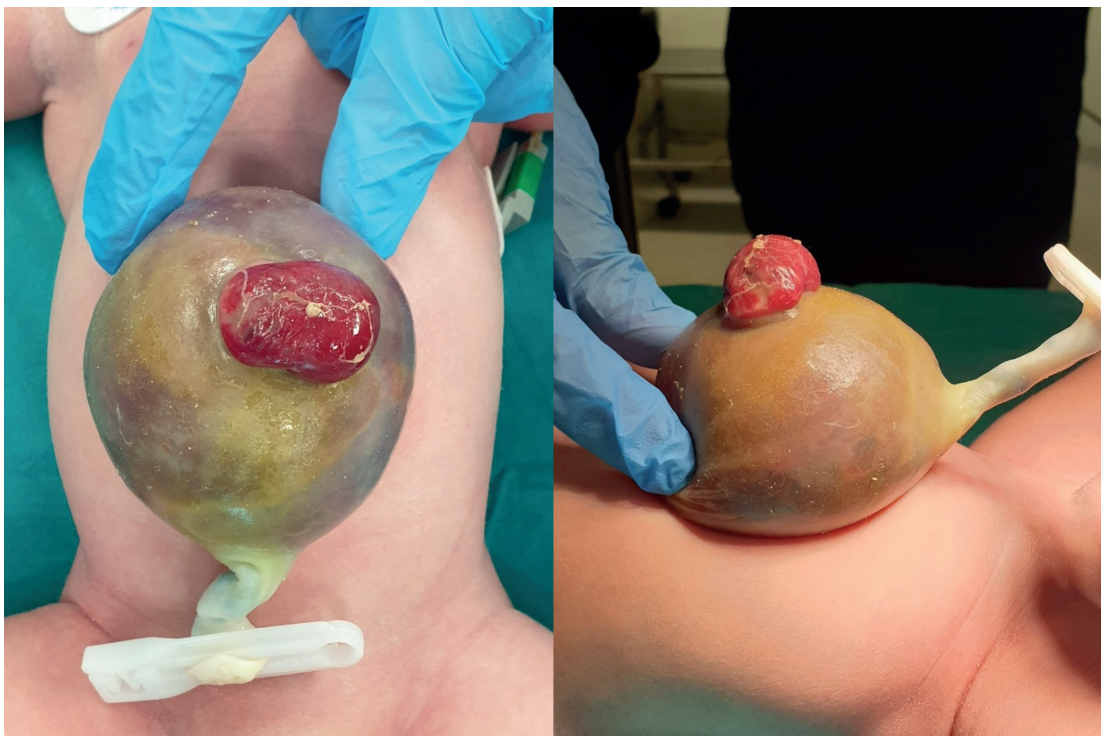

Fig. 1. Omphalocele appearance with the hemangioma and umbilical cord on it.

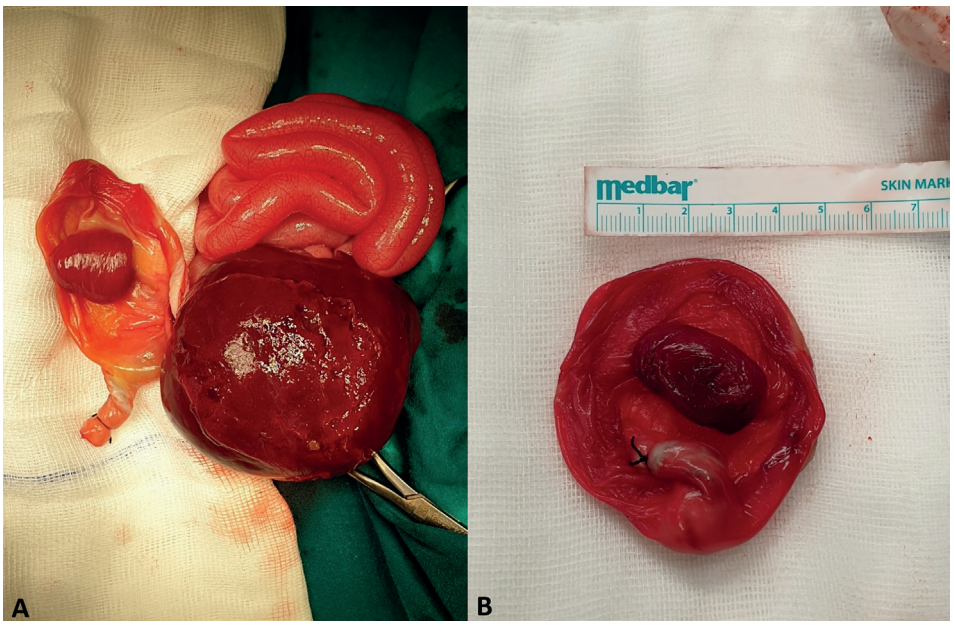

Fig. 2. A. The view of the entire liver after the omphalocele sac wass excised **B.** Macroscopic view of the sac.

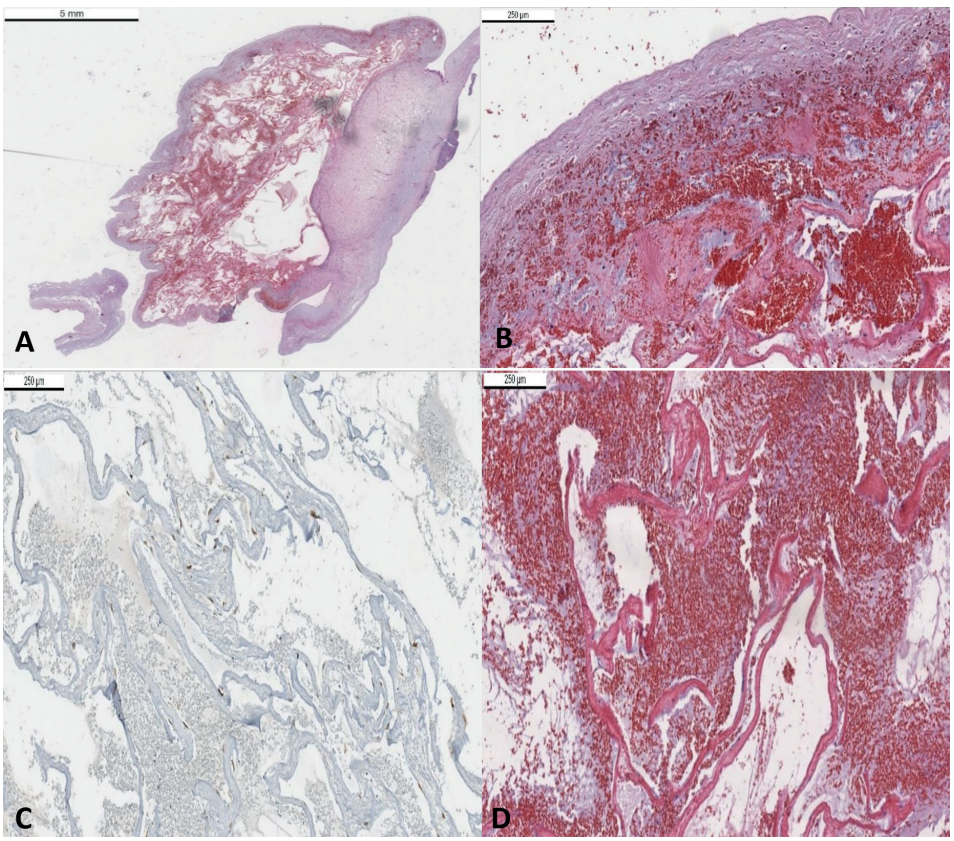

Fig. 3. A. Cystic hemorrhagic lesion with polypoid appearance on the omphalocele sac. **B.** Multiple cavernous cavities filled with blood. **C.** CD34 positivity in the vascular endothelium. **D.** Cystic cavernous structures filled with erythrocytes

Discussion

The omphalocele is a transparent sac connected to the umbilical cord that may contain intestinal structures and/or liver within it. The outer surface of the omphalocele is covered by an amniotic membrane while the inner surface is covered by the peritoneum. Between these layers, umbilical vessels and the embryological remnants of allantois and yolk sac are placed, all of which are surrounded by Wharton's jelly.[1] Associated chromosomal anomalies, such as Beckwith-Wiedemann and pentalogy of Cantrell syndromes, are encountered in about 50 to 70% of the cases.[3] The administration of neonates born with omphalocele comprises of the introductory steps of air duct stabilization, sterile wrapping of the bowel to protect warm to decrease unaware liquid misfortune, inclusion of an orogastric tube for bowel decompression, and the foundation of fringe intravenous entry.

Tumors associated with the umbilical cord are usually reported as isolated malformations without co-existing fetal anomalies.[4] To the best of our knowledge, 12 cases of umbilical cord pseudocyst have been reported to date.[5] Chromosomal anomalies are frequently reported in these cases (53.8%). Trisomy 18 was identified in 6 patients, while only one patient was reported to have trisomy 13.

Increased levels of AFP can be seen in either umbilical cord hemangioma cases or in patients with omphalocele.[6] Mass lesions of the umbilical cord may cause circulatory collapse due to stenosis, thrombosis and torsion of the umbilical vessels via compression of the mass. This process may cause intrauterine growth retardation, non-immune hydrops fetalis and even intrauterine fetal death.[7,8] Loss of the fetus has been reported due to intrauterine bleeding caused by hemangioma of the umbilical cord.[9] Although edema of the umbilical cord is a relatively frequent finding in patients with hemangioma of the umbilical cord, our patient with a hemangioma on the omphalocele sac did not have a such finding.

The presence of a deformity in the abdominal area is a precise complication for pediatric surgeons. Sustaining deficient intra-abdominal force is vital to ensure effective ventilatory procedures. Because modification of this fragile stability is authoritative for the ethics of one-fifth of patients with large omphalocele[10], multistage terminating approaches involving the exertion of patches have been suggested to permit time for the abdominal wall to adjust.[11] Within our, a primary repair was preferred because the intra-abdominal pressure measured preoperatively was not high.

Most cases with an omphalocele can be recognized during prenatal screening and should be differentiated from umbilical cord tumors, omphalomesenteric channel remnants, hemangioma, pseudocyst of the umbilical cord and exstrophy of the bladder. During the differential diagnosis of the omphalocele, it should be kept in mind that these conditions may co-exist with each other.

In our patient, a hemangioma on the omphalocele sac was protruding into the amniotic space and was located remote to the umbilical vessels in the omphalocele sac. Although it was prone to bleeding into the amniotic space due to its location, we did not experience such an event in our patient. The dressing of the patient was done with wet sponges. No additional intervention was applied, as no bleeding was observed. The hemangioma on the omphalocele sac didn't affect the manipulation of the sac and surgical management.

About 45 cases of umbilical cord hemangioma have been detailed within the literature, but to the leading of our information, our case is the primary case of co-existing hemangioma on the omphalocele sac.

When dealing with umbilical mass lesions in the prenatal and postnatal periods, a hemangioma on the omphalocele sac should be considered in the differential diagnosis of patients when an omphalocele is suspected.

Ethical approval

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

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: EEE, CİÖ, EŞ; data collection: CİÖ, TÖD, MEÖ; analysis and interpretation of results: SAB, AE, SD; draft manuscript preparation: DG, MNA, EEE, EŞ. 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

Giant cell tumor arising from the anterior arc of the rib: an extremely rare site in an adolescent girl

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ABSTRACT

Background. Giant cell tumor is a rare and locally aggressive neoplasm of the long bones in children. Rib is the least frequently affected site, seen in less than 1% of all cases and most of them occur at the posterior arc.

Case. A 12-year-old girl presented with swelling and slight pain on the left inferior-anterior chest wall for two years. Physical examination revealed a giant, hard and fixed mass on the left chest wall. Hematological and biochemical test results were in normal limits but slight elevation of alkaline phosphatase level. Computed tomography of the chest showed a large expansive mass and lytic lesion with internal calcification arising from the anterior part of the 7th rib. *En-bloc* resection was performed including the 6th-8th ribs and a small part of the diaphragm. The pathological evaluation revealed giant cell tumor of bone.

Conclusions. Herein, we aim to emphasize that giant cell tumor should be considered in the differential diagnosis of chest wall tumors in childhood whereby *en-bloc* resection and close follow up would be paramount.

Key words: giant cell tumor, costa, chest wall tumor, child, treatment.

Giant cell tumors (GCT) are rare, benign but locally aggressive tumors of bones. They account about 5% of all primary bone tumors and approximately 20% of benign bone tumors. They usually occur between the 2nd and 4th decades of life.[1,2]

GCTs mainly occur in the meta-epiphyseal regions of long bones, and the most common site is the distal end of the femur. Ribs are the least frequently affected site, seen in less than 1% of all cases of GCT.[3,4] Most of them arise from the posterior arch of the rib, and the anterior arch of origin is also extremely rare in adults. The typical histologic feature of GCTs is proliferation of multinucleated giant cells resembling osteoclasts with a stroma of spindle-shaped mononuclear cells.[5] The tumor has to be differentiated from other lesions containing benign giant cells, such as aneurysmal bone cyst (ABC) or brown tumors of hyperparathyroidism.[2] Detailed search of old and recent series and cases of GCT or rib lesions revealed that there are a few pediatric cases with GCT in the rib and no children had the involvement of the anterior arc (Table I).[5-12] Therefore, we aimed to present our case with unusual GCT location to include GCT in the list of differential diagnoses of chest wall tumors and to emphasize the necessity for *en-bloc* resection and follow up in children.

Case Report

An otherwise healthy 12-year-old girl presented with swelling and slight pain on the left inferior-anterior chest wall for two years. She had no respiratory symptoms. Past medical history was unremarkable.

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Table I. Giant cell tumor of the rib in children.

Case no	Author, year	Age (year) & Sex	Sign & Symptoms	Side and location of GCT	Size (cm)	Initial approach	Surgery	Pathology	Outcome
1	Locher GW, et al. (3), 1975	14, M	Pain	R, 6th rib, lateral arc	NA	Resection	*En-bloc* resection & bone graft	GCT - ABC	NED, 3 years
2	Locher GW, et al. (3), 1975	13.5, M	Pain, mass	L, 8th rib, posterior arc	NA	Resection	*En-bloc* resection	GCT - ABC	NED, 5 years, scoliosis
3	Locher GW, et al. (3), 1975	4.5, F	No	R, 4th rib, posterior arc	NA	Resection	*En-bloc* resection*	GCT - ABC	NED, 1 year
4	Schütte HE, et al. (4), 1993	NA	NA	NA	NA	NA	NA	GCT	NA
5	Athanassiadou F, et al. (7), 2003	12, F	Mass	R, lower rib, posterior arc	8x4x4	Needle biopsy	*En-bloc* resection	GCT	NED, 1 year
6	Özyüksel G, et al. (present case), 2020	12, F	Pain, mass	L, 7th rib, anterior arc	7x10x10	Tru-cut biopsy	*En-bloc* resection & chest wall reconstruction	GCT	NED, 14 months

M: male, F: female, NA: not available, R: right; L: left; **En-bloc* resection for recurrent tumor previously underwent incomplete resection another center, GCT: giant cell tumor, ABC: aneurysmal bone cyst, NED: no evidence of disease

Physical examination revealed a giant, hard and fixed mass on the left chest wall. The overlying skin was normal. Hematological and biochemical test results were in normal limits except slight elevation of alkaline phosphatase (ALP) level (359 U/L, normal range; 51-332 U/L). Parathyroid hormone (PTH) level was also normal (46.8 pg/ml, normal range; 12-88 pg/ml). Plain chest X-ray revealed a radiopaque area in the left lower hemithorax (Fig. 1). Computed tomography (CT) of the chest showed a large expansive mass and lytic lesion with internal calcification arising from the anterior part of the 7th rib and atelectasis of the left lower lobe (Fig. 2a, b). Dimensions of the mass were 7×10×10 cm. The differential diagnosis included ABC, primitive neuro-ectodermal tumor (PNET), and less likely chondroid matrix neoplasms; but was not specified on the CT. Tru-cut biopsy was performed but it was not possible to differentiate GCT and ABC.

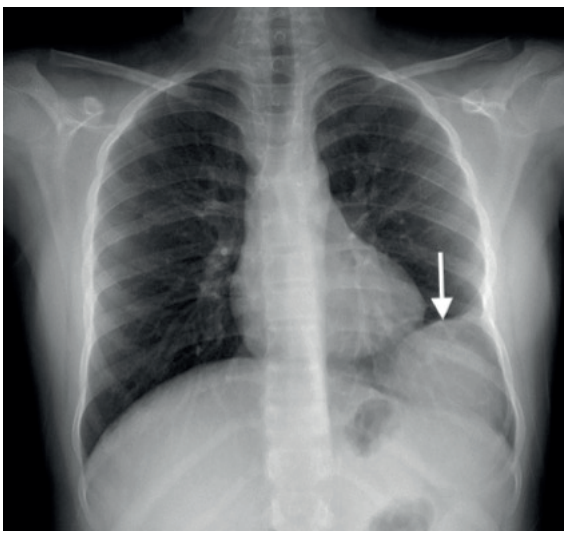

Fig. 1. Chest X-ray demonstrates destructive rib lesion with soft tissue mass (arrow).

The patient was operated via a left thoracotomy incision at the 6th intercostal space. *En-bloc* resection was performed including the 6th-8th ribs and a small part of the diaphragm, which was attached to the tumor. Chest wall reconstruction was performed with expanded-polytetrafluoroethylene (ePTFE) dual-surface (Dual Mesh, W.L. Gore & Associates, Flagstaff, AZ).

The diameter of the tumor (as measured from the gross surgical specimen) was11×10×6.5 cm and three ribs were 9 cm in length. Final pathological evaluation revealed GCT of bone and the surgical margin was clear (Fig. 3). Serum ALP levels decreased to 230 U/L and 176 U/L at 2nd and 11th months respectively following

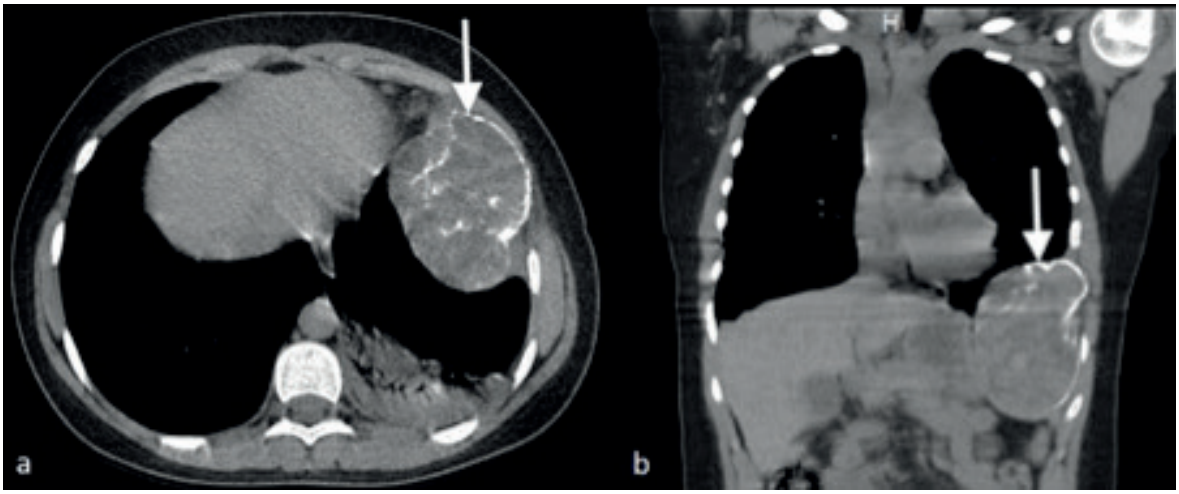

Fig. 2. (a) Axial and **(b)** coronal reformatted computed tomography scans showing an irregularly shaped expansive mass of the anterior part of left 7th rib (arrows). Note that the calcification is shown here by stippled ring and arc type pattern. Atelectasis of the left lower lobe is also evident.

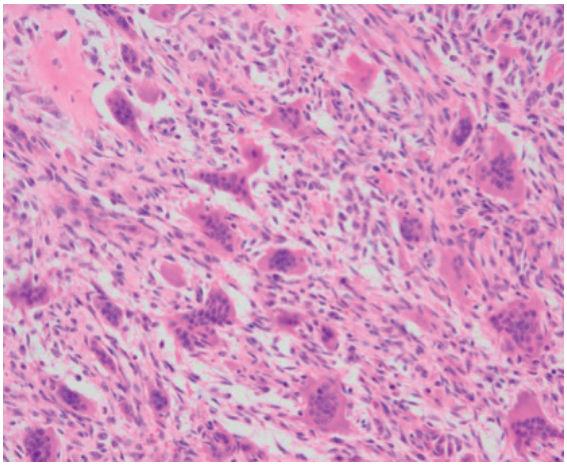

Fig. 3. Tumor composed of diffusely dispersed osteoclast type giant cells and stromal cell-like mononuclear cells (HE, ×230).

surgery, respectively. She had no symptoms of recurrence or metastasis at the one-year follow-up. Written informed consent was obtained from the patient for publication of this case report and accompanying images.

Discussion

We presented an extremely rare site of GCT in childhood. GCT is a rare tumor that usually affects the bones around the knee joint. GCT originating from the ribs is an extremely rare condition and usually arise from posterior ribs.[3,4] In this sense, the presented case is unique for its location in anterior arc. Giant cell tumor of bone represents around 4-5% of all primary bone tumors and approximately 20% of benign primary bone tumors. It is seldom seen in skeletally immature individuals. In the pediatric population, GCT of bone is seen in 2-5% of all reported cases.[1,13] The most common area of the tumor is proximal tibia, followed by vertebrae and pelvis.[2,14] A detailed review of case reports[15-17], clinical and surgical series[8-12] revealed that GCT of the rib has been encountered in only six pediatric cases including ours and there is no pediatric case with the GCT arising from the anterior arc of the rib.[2,5,6]

It is known that GCT of bone has a slightly higher female-to-male incidence in all age groups.[2,14] According to novel pediatric reports, female predominance may be seen in the GCT of bone.[10-12,15] Of note, our patient was female as well.

Serum ALP may be elevated at the time of diagnosis and normalize after surgery.[18] ALP is not a specific marker for GCT of bone, as some stromal cells express ALP.[19] However, normal level of ALP rules out brown tumor of hyperparathyroidism.[16] Our patient had slightly

elevated serum ALP level in the preoperative period, and decreased to normal in the postoperative period. Additionally, PTH level was in normal limits in the current case. Serum acid phosphatase (AcP) level has also been suggested as a useful marker for GCT of bone, because a correlation was revealed previously between the tumor volume and serum AcP level.[18] The serum AcP was not examined in the present case.

Radiologically, the tumors generally appear as expansive osteolytic defects on X-rays, eventually leading to significant local bone destruction.[14] A cross-sectional imaging provides a better evaluation of cortical destruction, calcification, penetration, and metastases. While CT is the best choice to describe the grade of cortical destruction, magnetic resonance imaging (MRI) may be useful to evaluate the invasion of soft tissues.[12,15] Furthermore, MRI can detect hemosiderin deposition seen in GCT of bone.

In a child with a chest wall mass, histopathological diagnosis should be performed, besides physical and radiological examination findings, to exclude a more likely malignancy. Therefore, tru-cut biopsy is useful in preoperative diagnosis.[10,12] Similarly, in the current case, malignancy was ruled out by the tru-cut biopsy, but the differential diagnosis between GCT of bone and ABC was not possible due to the limited amount of tissue.

The first choice of treatment is surgery in GCT of the bone. There are two options: *en-bloc* excision with reconstruction or extended intralesional curettage followed by filling the defect with bone cement or graft.[1] Wide excision to ensure clear margins is recommended because of 25-50% of local recurrence rates following intralesional curettage.[11,13] Although no statistical significance between local recurrence rates following wide resection or intralesional curettage has been reported for GCT in pediatric case series[10], it is remarkable that patients who undergo wide resection may have a greater chance in avoiding local recurrence.[11,12] *En-bloc* resection of GCT should be preferred in every patient unless surgery causes impairment of joint function, mobilization or esthetics. We preferred *en-bloc* excision to eradicate all neoplastic tissue with clear margins in our patient.

Histologically, GCT of bone is characterized by a large number of multinucleated giant cells as well as macrophage-like and stromal cell-like mononuclear cells.[19] It may contain areas of cystic degeneration, hemorrhage, hemosiderin deposition, occasional mitotic figures or increased spindle cell stroma. It is important to make the differential diagnosis of other lesions containing giant cells, such as ABC, fibrous metaphyseal defects, chondroblastoma, brown tumor in hyperparathyroidism and giant cell-rich variants of osteosarcoma.[14] In addition, GCT of bone and ABC may be seen together in the same case since GCT of bone is the most common precursor lesion of ABC.[15,17]

Malignant transformation occurs in less than 1% of GCT. Furthermore, radiotherapy should be avoided in these patients which may lead to malignant transformation. Metastases on GCT occur to the lungs with an incidence of 2-6% of patients, in 3-4 years after the primary diagnosis. They have usually a benign character and some of them regress spontaneously.[13] A small group of these metastases is progressive and causes mortality. According to a wide study on 2315 patients with GCT of bone, 5-year survival rate for malignant GCT of bone was 87%, with a total mortality rate of 16%.[20]

In conclusion, we present a unique case of GCT arising from the anterior arc of the rib in a 12-year-old adolescent girl. As such, we propose that differential diagnoses of chest wall tumors in childhood should comprise GCT as well. Due to its recurrence and malignant potential, complete wide resection with chest wall reconstruction must be the first choice of treatment for this tumor. Lastly, it is crucial to follow up these patients postoperatively for recurrent or metastatic disease.

Ethical approval

Written informed consent was obtained from the patient for publication of this case report and accompanying images.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: İK, GÖ; data collection: GÖ, BA; analysis and interpretation of results: GÖ, BA; draft manuscript preparation: İK, GÖ, BA, HNÖ, GG, AV. 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

Persistent moderate methylmalonic aciduria in a patient with methylmalonyl CoA epimerase deficiency

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ABSTRACT

Background. Methylmalonyl CoA epimerase (MCE) deficiency was first reported in 2006 and only a few cases have been reported so far. The clinical spectrum of MCE deficiency ranges from asymptomatic to life-threatening metabolic decompensation attacks.

Case. Herein we report a patient diagnosed with MCE deficiency with recurrent acute metabolic ketoacidosis attacks and moderate MMA-uria that persisted in periods without decompensation. At presentation, organic acid profiles were dominated by increased 3 hydroxybutyrate.

Conclusions. 3-Oxothiolase deficiency as a main ketolysis defects disorder was initially suspected. However, the subsequently repeated organic acid analyses demonstrated mild and persistent elevation of methylmalonic acid. This report provides a new phenotype of the clinical and biochemical characterization of MCE deficiency.

Key words: methylmalonyl-CoA epimerase, methylmalonic aciduria, ketosis.

Methylmalonyl CoA epimerase (MCE, another name being methylmalonyl-coA racemase) catalyzes the transformation of (S)-methylmalonyl-CoA to (R)-methylmalonyl-CoA in the propionyl-CoA to succinyl-CoA pathway. The pathway is responsible for the degradation of branched chain amino acids, odd chain-length fatty acids, and other metabolites. MCE is encoded in humans by the MCEE gene located on chromosome 2p13.3. MCEE is a small gene containing 4 exons.

The first MCE deficiency (OMIM 251120) case published in 2006 was followed by a small number of publications.[1-7] The clinical spectrum of MCE deficiency ranges from asymptomatic to life-threatening metabolic decompensation attacks. Two cases with neurological findings from the cases described in the literature were also affected by a second inherited disorder, sepiapterin reductase (SR) deficiency.[2,6]

Herein we report a patient diagnosed with MCE deficiency with recurrent acute metabolic ketoacidosis attacks and moderate methylmalonic aciduria that persisted in periods without decompensation, aiming to provide a new phenotype of the clinical and biochemical characterization of MCE deficiency.

Case Report

A male patient was born to healthy consanguineous parents following an uneventful pregnancy and delivery. He had an older brother and a younger sister (Fig. 1). The patient (V-2) presented acutely with vomiting and gastroenteritis at the age of 3 and half years, following a previously unremarkable medical history. Physical examination showed dehydration and severe signs of tachycardia, tachypnea, and confusion. There was severe metabolic acidosis with increased anion gap (pH = 7.11, bicarbonate 3.9 mmol/L and

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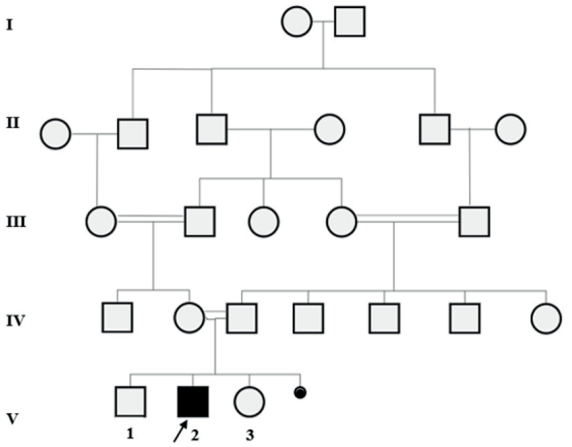

Fig. 1. Pedigree of the family.

pCO2 17 mm Hg, anion gap 20 mmol/L), accompanied by strong ketonemia in fresh capillary whole blood (5.2 mg/dl) and ketonuria (100 mg/dl). Glycemia was normal. There was no hyperlactatemia, hyperammonemia. High glucose content fluid (10 - 12 mg/kg per minute) was given to the patient in metabolic decompensation to meet his energy needs. When blood glucose was greater than 150 mg/dL, IV insulin infusion (began at 0.01 units/kg per hour and titrated according to blood sugar levels) was instituted to promote anabolism. Blood glucose was maintained between 100 and 150 mg/dL. Metabolic investigation results were consistent with a ketolysis defect with a large increase in urine 3-hydroxy-butyrate (25200 mmol/mol Creatine; N < 11) and acylcarnitine profiles were normal. Increased urinary 3-hydroxy-butyrate addressed the diagnosis of acetyl-coa acetyltransferase 1 (ACAT1) deficiency. Unexpectedly, no mutation was detected in ACAT1 gene analysis. Three months after his first decompensation attack, he had the second one with ketoacidosis. Blood β-Ketone (Beta-Hydroxybutyrate) in fresh capillary whole blood from the fingertip was elevated (6.4 mg/dl). MMA was still not detected in his urine organic acid analysis. But unlike the first attack acylcarnitine profile showed an elevation of C3 (propionyl) carnitine (7.7 μmol/L; N < 6). His plasma homocysteine level was normal (5.2 μmol/L, N: 5 – 15). No mutation was defined in the MUT gene analysis that was studied to exclude methylmalonic aciduria. During his follow-up, twelve assessments were performed, with results ranging from undetectable (twice during metabolic decompensation) to 1234 mmol/mol creatine, with a mean of 258 and a median of 145. The brain MRI and echocardiography were normal.

With these findings, as methylmalonic aciduria is present in several inborn errors of metabolism affecting different steps of cobalamin pathways a clinical whole-genome sequencing was ensued. In the MCEE gene data analysis performed due to persistent methylmalonic acid excretion in between attack periods, a previously described homozygous nonsense mutation (c.139C>T) was identified and validated by the Sanger sequencing. Following the initial acute decompensation, he was treated with carnitine supplementation (50-100mg/kg/day). Protein restriction in the diet was recommended only during metabolic decompensation periods. Now at age seven and half years, growth and development are completely normal. The patient has successful academic results in school.

Urine organic acids analysis was planned for parents and both siblings. His eleven year old brother (V-1) had a meningomyelocele which had been operated on. He has a neurogenic bladder and is treated with clean intermittent catheterization three or four times a day. He has never had a decompensation attack despite surgery for the meningomyelocele. He has normal growth parameters and is successful in school. His sister (V-3) had normal growth and development and had no decompensation attack. Urine organic acids indicated that the older brother with neurogenic bladder and younger sister also have methylmalonic aciduria, 888 mmol/mol creatinine, and 3 mmol/mol creatinine, respectively. The family did not allow genetic studies from the two siblings.

Informed consent was obtained from the parents of the patient for publication of this case.

Discussion

After the first case was described in 2006, a small number of cases have been reported so far, and the clinical presentations are very diverse.[1-7] Previously reported three patients presented with an acute metabolic decompensation with acidosis but severe ketonemia was not described in these patients (Table I). Although severe metabolic ketoacidosis was detected in both metabolic decompensation episodes of the case, ammonia and glucose levels were found to be normal. So this report provides a new phenotype of the clinical and biochemical characterization of MCE deficiency via mimicking ketolysis defects. As in the case reported by Abily-Donval et al.[5], in our case also, no MMA was detected in two decompensation attacks. This finding reveals the importance of organic acid analysis in the periods between metabolic decompensation attacks on follow-up.

An increase in creatine kinase was reported during an attack in a case presented previously.[4] No increase in muscle enzymes were detected in our patient's decompensation attacks and on follow-up.

Neurologic involvement was described in five cases.[2,3,5-7] Two of them were diagnosed with a second inherited metabolism disorder, SR deficiency.[2,6] SR deficiency is an autosomal recessive inherited defect in the biosynthesis of tetrahydrobiopterin. The SPR gene is also located on chromosome 2, like the MCEE gene. SR deficiency is characterized by dystonia, axial hypotonia, oculogyric crises, and delays in motor and cognitive development. Considering the coexistence of these two diseases in two cases in the literature, contiguous gene deletion syndrome was excluded. Because the SPR gene and MCEE gene mutations in both families were missense mutations. The evaluation of SR deficiency was not mentioned in the other two patients with neurological findings to confirm the exclusive association with MCE deficiency.[3,5]

In the MCEE gene analyses of 9 previously reported patients listed in Table I, c.139C> T was shown in 14 alleles. c.139C>T variation is a stop codon mutation.[1-7] Our case was also homozygous for the missense mutation c.139C> T. We did not make any evaluation in terms of vitamin B12 responsiveness because previous cases were unresponsive in the literature.[2,5,7] There is no consensus on dietary protein restriction or a normal protein diet yet. Examples of both applications have been reported in the cases in the literature.[1,2,5] To achieve this unity of decision, there is a need to increase the experiences and cases about MCE deficiency. Our case was not given a protein-restricted diet on follow-up, except during metabolic decompensation attacks.

In summary, we have reported a new case of the rare disease MCE deficiency, presenting with strong ketosis. So we described a new biochemical phenotype for MCE deficiency. We emphasize the importance of the metabolic sample during the attack, as well as the sample in between the attack period on follow-up.

Ethical approval

Informed consent was obtained from the parents of the patient for publication of this case.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: HY and SKU; data collection: EC,SH and HO; analysis and interpretation of results: HY, MÇ; draft manuscript preparation: HY. 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.

Table I. Clinical, biochemical and molecular findings.

Patients	P1	P2 (P1S)	P3	P4 (P3S)	P5	P6	P7	P8	P9	Case
References	1	1	2	3	3	5	5	6	7	
Sepiapterine Reductase Deficiency			Yes		Non communicated			Yes	No	No
MCE Deficiency Diagnosis age	13 months	14 years (Asymptomatic)	16 years	Non communicated (Asymptomatic)	3 years	5 years	5 years	2 years	78 years	3.5 years
Neurological Impairment	Only before ventriculo-peritoneal shunt due to hydrocephalus	No	Axial hypotonia, anormal eye movements, wheeled chair	No	Dysarthria, deteriorated motor function, spastic paraparesis, ataxia	No	Attentional difficulties, language development delay	Psychomotor development retardation, Spasticity	Parkinson disease, demans	No
Acute Metabolic Acidosis	Yes	No	No	No	No	Yes	Yes	No	No	Yes
Ketonuria		No	No	No	No	Yes	Yes	No	Non communucated	Yes
Urinary MMA (Decompansasion) (µmol/mmol Creatinine)	180-1456	No attack	No attack	No attack	No attack	53	Not detected	No attack	No attack	Not detected
Urinary MMA (µmol/mmol Creatinine)	180-1456	95-1400	60	1400	621	47-121	18-212	1175	5.5-60	0-1234
C3 (µmol/L) (Decompansasion)						21.6(<0.58)			No attack	7.7
C3 (µmol/L)						1.53-5.82			13	N-3.7
CK (IU/L)						498-3062			Unmeasured	74
HCY (µmol/L) (Decompansasion)						Unmeasured			No attack	5.2
HCY (µmol/L)						Normal			17-32	8.3
Diet	Normoprotein	Protein-restricted diet				Protein restriction in attack	Protein-restricted diet		Non communucated	Protein restriction in attack

CK: creatin kinase, HCY: homocysteine, MCE: methylmalonyl CoA epimerase, MMA: methylmalonic acid

Table I. Continued.

Patients	P1	P2 (P1S)	P3	P4 (P3S)	P5	P6	P7	P8	P9	Case
B12	Non-responder		Non-responder				Non-responder		Non-responder	
Carnitine supplement			4gr/day			100mg/kg/day				50-100 mg/kg/day
MCEE Variants	c.139C>T	c.139C>T	c.139C>T	c.139C>T	c.178A>G	c.139C>T	c.139C>T	c.139C>T	c.139C>T	c.139C>T
	c.139C>T	c.139C>T	c.139C>T	c.139C>T	c.178A>G	c.379-644A>G	c.139C>T	c.139C>T	c.419delA	c.139C>T
SPR Variants			c.751A>T		Non communicated				No mutation	No mutation
			c.751A>T							

CK: creatin kinase, HCY: homocysteine, MCE: methylmalonyl CoA epimerase, MMA: methylmalonic acid

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Case Report

Pyruvate kinase deficiency mimicking congenital dyserythropoietic anemia type I

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ABSTRACT

Background. Pyruvate kinase (PK) deficiency is the most common enzyme abnormality in the glycolytic pathway. Here, we describe two siblings with PK deficiency that mimicked congenital dyserythropoietic anemia (CDA) type I.

Case. The siblings were referred to our hospital for evaluation of anemia when they were newborns. Their PK enzyme activities were normal. Their bone marrow aspirations and electron microscopies showed CDA-like findings. A CDA panel with next-generation sequencing showed no mutation. Though their PK enzyme levels were normal, a molecular study of the *PKLR* gene showed a homozygous variant c.1623G>C (p.Lys541Asn) in exon 12 of our patients.

Conclusions. Although the diagnosis of pyruvate kinase deficiency is difficult, it can be confused with many other diagnoses. Bone marrow findings of these cases are similar to congenital dyserythropoietic anemia. In patients with normal pyruvate kinase enzyme levels, the diagnosis cannot be excluded and genetic analysis is required.

Key words: pyruvate kinase deficiency, congenital dyserythropoietic anemia, PKLR gene.

Pyruvate kinase (PK) deficiency is the most common enzyme abnormality in the glycolytic pathway, which leads to an anemia secondary to decreased ATP synthesis.[1] The disease exhibits autosomal recessive inheritance and is caused by mutations in the *PKLR* gene.[2] The protein encoded by this gene is a pyruvate kinase that catalyzes the phosphorylation of phosphoenolpyruvate into pyruvate and ATP, therefore a mutation in the PKLR gene leads to ATP deficiency in erythrocytes. This ATP deficiency presumably results in a reduced capacity to maintain the red cell membrane and diminished erythrocyte deformability, resulting in a shortened lifespan and destruction in the spleen. Defects in this enzyme, due to gene mutations or genetic variations, are the common cause of chronic hereditary nonspherocytic hemolytic anemia.[3]

The diagnosis of PK deficiency is based on the presence of clinical signs and symptoms of hemolytic anemia, evidence of extravascular hemolysis in laboratory findings, measurement of PK activity or antigen levels and detection of mutations in the *PKLR* gene.[4] The clinical severity of PK deficiency varies widely, ranging from mild anemia and jaundice to severe transfusion dependent hemolytic anemia. Even within the same family, individuals can have different symptoms and severity.[5,6]

In some patients with PK deficiency, surprisingly, PK levels are normal. PK levels depend on the age of red blood cells and younger cells have higher PK levels. Reticulocytosis and

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large quantities of nucleated red blood cells in the peripheral blood due to hemolysis produce higher levels of PK.[7] Multiple and frequent transfusions can obscure enzymatic defects and finally, the PK isozyme in white blood cells cannot be totally eliminated from the sample, which can influence detection of PK levels.[8]

Here, we describe two siblings with PK deficiency that was misdiagnosed as congenital dyserythropoietic anemia (CDA) type I.

Case Report

Patient 1 was referred to our hospital for the evaluation of anemia when she was 32 days old. She was born as a term baby of a first pregnancy and her birth weight was 3550 g. She was hospitalized in another hospital due to anemia and jaundice on the first postnatal day and received phototherapy and erythrocyte suspension. The initial family history was unremarkable except that parents were first-degree cousins. On physical examination, she had an icteric appearance, but did not display hepatosplenomegaly. Complete blood count revealed a hemoglobin of 4.8 g/dl (normal range 12-18.5 g/dl), mean corpuscular volume 92 fl (normal range 86-118 fl), mean corpuscular hemoglobin 24.9 pg/cell (normal range 29-36 pg/cell), mean corpuscular hemoglobin concentration 33 g/dl (normal range 28-38 g/dl), white blood cell count of $13.5x10^9$/L (normal range 5-$20x10^9$/L) and platelets $387x10^9$/L (normal range 150-$450x10^9$/L). The corrected reticulocyte count was 8.6% (normal range 0.5-1.5%). Indirect bilirubin level was 2.12 mg/dl and LDH 432 U/L. Her peripheral blood smear revealed mild hypochromia, polychromasia and rare schistocytes. Direct and indirect antiglobulin tests were negative. Serum folic acid, ferritin, vitamin B12 and haptoglobulin levels were normal. The patient was transfused for the second time because of anemia at 32 days of age. On follow-up, pyruvate kinase, glucose-6-phosphate dehydrogenase and 5'-nucleotidase enzyme activities, hemoglobin electrophoresis and osmotic fragility tests were normal 3 months after the first transfusion when the patient was 4 months old. In further investigations, a diagnosis of paroxysmal nocturnal hemoglobinuria was excluded since CD59 and CD55 were normally expressed on erythrocytes. Her parent's complete blood counts and erythrocyte morphologies were normal. She underwent bone marrow aspiration at 5 months of age. Erythroid hyperplasia with many bi-nucleated erythroblasts with nuclei of different maturities and internuclear chromatin bridges, which raised the concern of CDA, was seen in bone marrow aspiration. Spongy appearance (Swiss cheese appearance) of heterochromatin in all normoblasts and expansion of the perinuclear areas and the extension of the cytoplasm towards the nucleus in some, were observed with electron microscopy. These findings were assumed to be compatible with CDA type I. In her follow-up, since the hemoglobin values were between 5-6 g / dl, she was enrolled in a transfusion program.

Patient 2, the four year younger brother of patient 1, was admitted to our clinic with pallor and icterus at the age of one month. He did not have hepatosplenomegaly on physical examination. On peripheral blood smear, mild hypochromia and polychromasia were seen but red blood cell morphology showed no acanthocytes or nucleated erythrocyte. His corrected reticulocyte count was 6.5%. Serum folic acid, ferritin, vitamin B12 and haptoglobulin levels were normal similar to patient 1. Activities of pyruvate kinase, glucose-6-phosphate dehydrogenase and 5'-nucleotidase enzyme activities and osmotic fragility test were normal. Bone marrow examination showed erythroid hyperplasia with significant dyserythropoiesis similar to his sister and electron microscopy was not performed (Fig. 1). He was enrolled in an erythrocyte transfusion program due to consistent anemia (Hb < 7 g/dl).

A congenital dyserythropoietic anemia panel with next generation sequencing (NGS) including *CDAN1, C15orf41, SEC23B, KLF1* and *GATA1* genes showed no mutations in the patients. While they were being followed-up, a

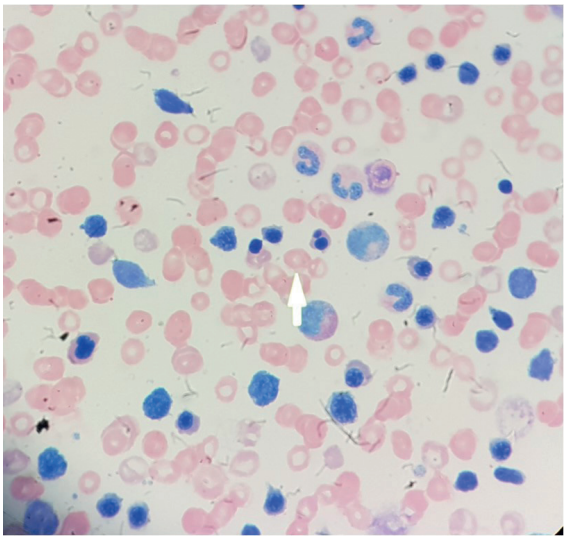

Fig. 1. Bone marrow smear showing erythroid hyperplasia with bilobulated erythroblasts and internuclear chromatin bridges.

newborn cousin with similar complaints was examined and diagnosed with PK deficiency due to a homozygous c.1623G>C (p.Lys541Asn) mutation in the *PKLR* gene in another center. Though PK enzyme levels of patients 1 and 2 were normal, a genetic test for PK deficiency was performed. In the molecular study of the *PKLR* gene, a homozygous c.1623G>C (p.Lys541Asn) in exon 12 was found in our patients. Subsequently, the heterozygous c.1623G>C mutation was identified in DNA samples of their parents by sequence analysis. Informed consent was obtained from the patients' families.

Discussion

Pyruvate kinase deficiency is a rare cause of hemolytic anemia and the differential diagnosis includes a heterogeneous group of both congenital and acquired hemolytic disorders. Though jaundice, severe indirect hyperbilirubinemia and significant anemia requiring transfusions are frequent in the neonatal period, as with our patient, some newborns have no evidence of jaundice or severe anemia.[5] In children with PK deficiency, the most frequent symptoms are those related to anemia, splenomegaly, jaundice, gallstones and secondary hemochromatosis.[1,9] Due to the increased red cell 2,3-DPG content, which is responsible for a rightward shift in the oxygen dissociation curve of hemoglobin, the anemia may also be well tolerated. However, many patients with PK deficiency have fatigue related to their anemia.[10]

Our patients had neonatal onset severe hemolytic anemia with significant dyserythropoiesis. Dyserythropoiesis is considered to be present when erythroblasts show dysplastic features such as bi/multinuclearity, nuclear karyorrhexis, ring sideroblasts, cytoplasmic vacuolation, cytoplasmic PAS positivity, maturation asynchrony, megaloblastic changes, nuclear budding, internuclear chromatin bridges or duplication of the nuclear membrane. This can be seen in several congenital and acquired disorders such as CDA, some thalassemia syndromes, iron deficiency, vitamin B12 or folate deficiency, aplastic anemia, malaria and kala azar.[11] Onset of CDA also generally occurs in childhood, even clinical signs can occasionally be observed in the neonatal period.

Congenital dyserythropoietic anemia type I is characterized by erythroid hyperplasia and binucleated erythroblasts of different size and shape and thin chromatin bridges between nuclei of erythroblasts in the bone marrow.[12] The electron microscopy reveals unusual and significant morphological aberrations selectively within the erythroid series with progressing maturation. The pores of the nuclear envelope become more numerous and wider than normal and the nucleoli lack the filamentous component and have a purely granular appearance. This Swiss cheese appearance of heterochromatin, which is characteristic for CDA was also seen in our patient.[13] Because the electron microscopy findings alone were not sufficient to diagnose CDA, we analyzed the mutation of our patients. However, no mutation was detected in the CDA type I related genes, *CDAN* and *C15orf41.* A 3-month-old child with evidence of dyserythropoiesis in bone marrow electron microscopic findings

suggesting CDA type I and coexistent unilateral multicystic dysplastic kidney and persistent PK deficiency was reported.[14] Pereira et al.[15] described another patient with clinically significant anemia who had dyserythropoiesis in the bone marrow suggestive of CDA and low PK activity. Of interest, coinheritance of *PKLR* and *GATA1* mutations were detected in that patient. In another study, Roy et al.[16] analyzed 57 patients with congenital anemia with a novel 33-Gene targeted resequencing panel. In their study, an 18-month-old transfusion dependent girl was diagnosed with CDA because her bone marrow analysis showed erythroid hyperplasia with significant dyserythropoiesis and electron microscopy showed 'Swiss cheese heterochromatin'. Molecular analysis revealed compound heterozygosity for *PKLR* mutations, low PK levels were found and the bone marrow morphology was felt to be consistent with non-specific dyserythropoiesis rather than CDA.

In our patients; the c.1623G>C (p.Lys541Asn) mutation was found in exon 12 of the *PKLR* gene, which was reported previously as a novel mutation from Turkey.[17] Unal et al.[18] also described two patients with homozygous c.1151C> T and a novel homozygous c.880G>A mutation in the *PKLR* gene with erythroid hyperplasia along with double and multi-nucleated erythroid precursors, which suggested CDA. Recently, Hamada et al.[19] performed whole-exome sequencing (WES) for 10 CDA patients who did not carry *CDAN1*, *SEC23B*, and *KLF1* mutations and reported that WES unexpectedly identified *G6PD* and *SPTA1* gene mutations known to cause congenital hemolytic anemia in two patients.

The causative mechanism for such hemolytic anemia exhibiting dyserythropoiesis similar to that exhibited in CDA remains unknown. Some authors thought dyserythropoiesis was secondary to ineffective erythropoiesis due to ATP depletion.[14] However, others hypothesized that impaired erythrocyte membrane proteins cause incomplete chromosome segregation and cytokinesis in erythroblasts, resulting in dyserythropoietic morphology.[19] Coexistence of abnormalities of transcription factors or epigenetic modifiers of erythropoiesis might have a role in the development of dyserythropoiesis.

Given the rarity and the clinical heterogeneity, the diagnosis of these diseases can be difficult, mostly in atypical forms. Patients with PK deficiency are typically diagnosed in the neonatal period or early childhood. Patients have mild-to-moderate anemia that can be misdiagnosed as hemoglobinopathies such as thalassemia, more common hemolytic anemias such as hereditary spherocytosis, or inflammatory anemia. It is important to evaluate patients in terms of thalassemia mutation and other congenital membrane defects. Complications such as hyperbilirubinemia and gallstones can often be mistaken as Gilbert syndrome or hereditary hemochromatosis.

PK deficiency should be considered in the differential diagnosis of CDA. In addition to the enzyme activity, comprehensive genetic analysis is warranted for more effective diagnosis of patients with suspected CDA and congenital hemolytic anemia.

Ethical approval

Informed consent was obtained from the patients' families.

Author contribution

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

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Conflict of interest

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Case Report

Three Afghani siblings with a novel homozygous variant and further delineation of the clinical features of *METTL5* related intellectual disability syndrome

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ABSTRACT

Background. *METTL5* gene is one of the members of methyltransferase superfamily and biallelic variants cause intellectual disability syndrome (ID) with microcephaly. This article reports three new cases with *METTL5* related ID syndrome in a consanguineous family.

Case. Afghanistan descent family was affected by a novel homozygous c.362A>G (p.Asp121Gly) *METTL5* gene variant. This variant is predicted to be "pathogenic" by multiple in-silico tools. Patients had dysmorphic and neurodevelopmental features including intellectual disability, microcephaly, poor/absent speech, delayed walking, aggressive behavior, large/posteriorly rotated ears, broad nasal base and short stature, which seem to be the cardinal findings of the designated syndrome.

Conclusions. While the data reported in these individuals indicate characteristic clinical features of *METTL5* related ID syndrome, further investigations and study of additional cases are needed to improve the understanding of disease pathogenesis, and management.

Key words: METTL5, intellectual disability, whole exome sequencing, WES.

Intellectual disability (ID) is a neurodevelopmental condition that affects approximately 1-2% of the population all over the world, characterized by impaired learning and behavioral impairment.[1,2] While genetic factors, congenital metabolism errors and brain malformations are the main factors in ID etiology, approximately 50% of affected cases remain undiagnosed.[3,4] However, in recent years, next generation sequencing (NGS) technologies have improved the rates of diagnosis of rare diseases and the identification of ID related genes. The *METTL5* gene encodes a methyl transferase and plays a key role in the methylation of 18S ribosomal RNA.[5] Recently, biallelic pathogenic *METTL5* variants have been associated with a new ID syndrome (OMIM #618665) which is characterized by moderate to severe ID, developmental delay, microcephaly, various facial dysmorphisms and behavioral abnormalities.[6] To date, only 7 *METTL5* related ID patients have been reported from 3 unrelated consanguineous families, including 3 from Pakistani, 2 from Yemeni and 2 from Iranian descents.[6-8]

Here, we report the clinical and molecular genetic findings of three new *METTL5* related ID cases from a consanguineous Afghanistan family. To the best of our knowledge, this is the first Afghanistan descent family reported in the literature.

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Case Report

Three patients from a consanguineous Afghani healthy couple were referred to our clinic (Fig. 1a). Written informed consent were obtained from parents to undertake genetic investigations and for the publication of any potentially identifiable images or data included in this article.

Patient III-1

He was 11-years-old at the time of examination. He was born by normal and uneventful labor at term. Records on neonatal physical measurements and developmental milestones (head control, sitting and standing) were insufficient. Patient III-1 started to walk at age about 2. He had normal vision and hearing but speech was slurred. Besides, he had learning impairment, temper and aggressive behaviors. On examination, his physical measurements were as follows: Weight (W) 35 kg (10th-25th centiles), height (H) 141 cm (25th-50th centiles) and head circumference (HC) 51 cm (<1st centile). Microcephaly, large and posteriorly rotated ears, broad nasal base, long philtrum and thin upper lip were the dysmorphic features of the patient III-1 (Fig. 2a). Magnetic resonance imaging (MRI) did not reveal any structural brain abnormality.

Patient III-2

He was 8-years-old and was born by normal labor. The labor was complicated with cord entanglement. He started to walk at about 7 years of age. However, the patient III-2 had an ataxic gait and could not climb up and down the stairs without support. III-2 had a febrile seizure at postnatal day 11, and then he had tonic-clonic seizures until the age of 6. III-2 had no speech and vision and hearing were normal. Besides learning impairment, temper and aggressive behavior, he also had self-mutilating behavior. His physical measurements were as follows: W: 23 kg (10th-25th centiles), H: 116 cm (3rd centile) and HC: 49.5 cm (<3rd centile). On physical examination, microcephaly, large and posteriorly rotated ears, broad nasal base, full lips and epicanthal folds were detected (Fig. 2b). MRI did not reveal any structural brain abnormality.

Patient III-3

She was 6-years-old and was born by normal and uneventful labor at term. She started to walk at about 3 years of age. III-3 had a febrile seizure history at postnatal day 14. She had no speech. Vision and hearing were normal. Her physical measurements were as follows: W: 16 kg (10th centile), H: 110 cm (3rd centile) and HC: 47 cm (<1st centile). The proband had learning impairment, temper and aggressive behavior, self-mutilating behavior and attention deficit hyperactivity disorder. Other findings included microcephaly, large and posteriorly rotated ears, broad nasal base, long philtrum, thin upper lip, and epicanthal folds (Fig. 2c).MRI did not reveal any structural brain abnormality.

Genetic Analyses

Karyotype and microarray analyses were normal for all 3 children and did not reveal any structural or numerical chromosome abnormalities. Whole exome sequencing (WES) was performed on the genomic DNA of patient III-2 (Fig. 1a). The WES analysis of the patient III-2 revealed homozygosity for a c.362A>G (NM_014168.3, p.Asp121Gly) variant of the *METTL5* gene. The variant was consistent with *METTL5* related ID phenotype and no other *METTL5* variants were identified. In addition, no other alternative homozygous gene variants were identified in other genes related to similar diseases or ID phenotype. Confirmation of the WES result and testing of the genomic DNA of the parents, patient III-1 and III-3 were performed by sanger sequencing (Fig. 1b). While patients III-1 and III-3 were homozygous for c.362A>G variant, mother and father were heterozygous carriers. The c.362A>G variant is located in third exon of the *METTL5* gene, which encodes the S-adenosyl-L-methionine-

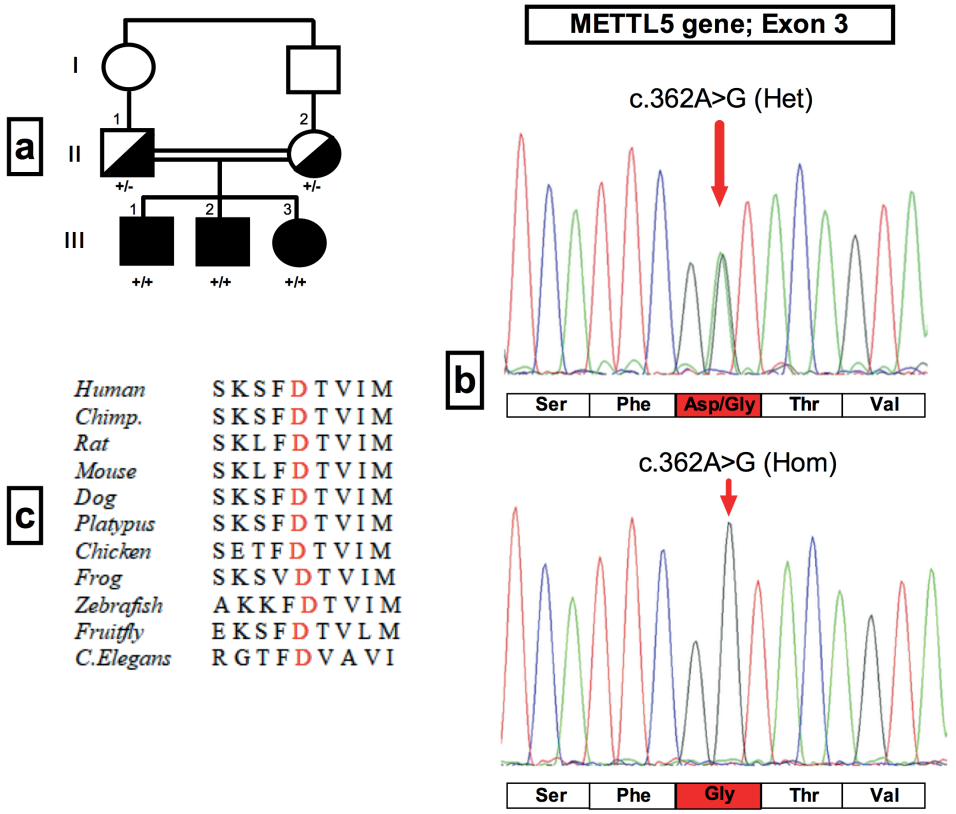

Fig. 1. (a) Pedigree of Afghanistan descent family segregating recessive intellectual disability, microcephaly, poor/absent speech and psychomotor developmental delay. The filled symbols represent affected individuals with homozygous c.362A>G variant in *METTL5* gene. The semi-filled symbols represent heterozygous carrier individuals. [(+/-): Heterozygous; (+/+): Homozygous] **(b)** Sanger sequencing chromatograms depicted *METTL5* c.362G>A homozygous mutation in the affected individuals (lower panel) and carrier status in the parents (upper panel). Amino acid sequences for each codon are also shown. (Het: Heterozygous; Hom: Homozygous) **(c)** Conservation alignment indicating that the affected amino acid of *METTL5* is conserved across different species. (Color figures can be viewed at online version of the manuscript).

dependent methyltransferase domain. Aspartate at position 121 is highly conserved (Fig. 1c). In contrast, the protein change to glycine is found only at a very low frequency in population databases [GnomAD_exome; G=0.000012 (3/246538), ExAC; G=0.000008 (1/120920)]. The p.Asp121Gly variant is predicted as "pathogenic" by multiple in silico tools, including Mutation Taster, Mutation assessor, SIFT, PROVEAN and REVEL. However, using American College of Medical Genetics (ACMG) criteria the variant is classified as a "*variant of uncertain significance (VUS)*" (PP1, PM2, PP3).[9] The variant data has been submitted to ClinVar (https://www-ncbi-nlm-nih-gov.gate2.inist.fr/clinvar/), accession number SUB8342013.

3D Modelling of METTL5 Variant

3D-dimensional structure of human *METTL5* was created on the crystal structure of the human *METTL5-TRMT112* complex, the 18S rRNA m6A1832 methyltransferase at 2.5A resolution.[5] YASARA software was used for modeling and subsequent analysis.[10] The 3D modeling analysis for *METTL5-TRMT112*

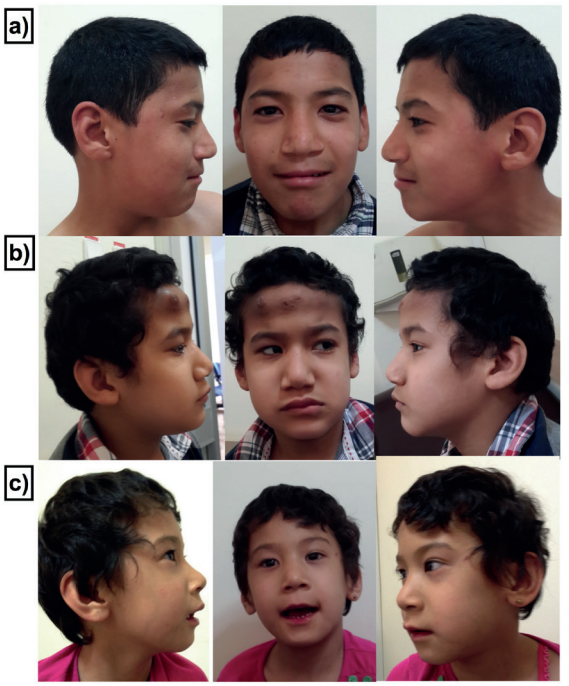

Fig. 2. Facial appearance of affected individuals, patient III-1 **(a)**, patient III-2 **(b)** and patient III-3 **(c)**. All affected individuals show characteristic facial features including microcephaly, large and posteriorly rotated ears and broad nasal base. Long philtrum and thin upper lip (III-1 and 3), full lips (III-2) and epicanthal folds (III-2 and 3) were also noted. Patient III-2 had skin lesions on his forehead caused by self-injurious behaviors. Parental written permission has been obtained to publish the patients' photos. (Color figures can be viewed at online version of the manuscript).

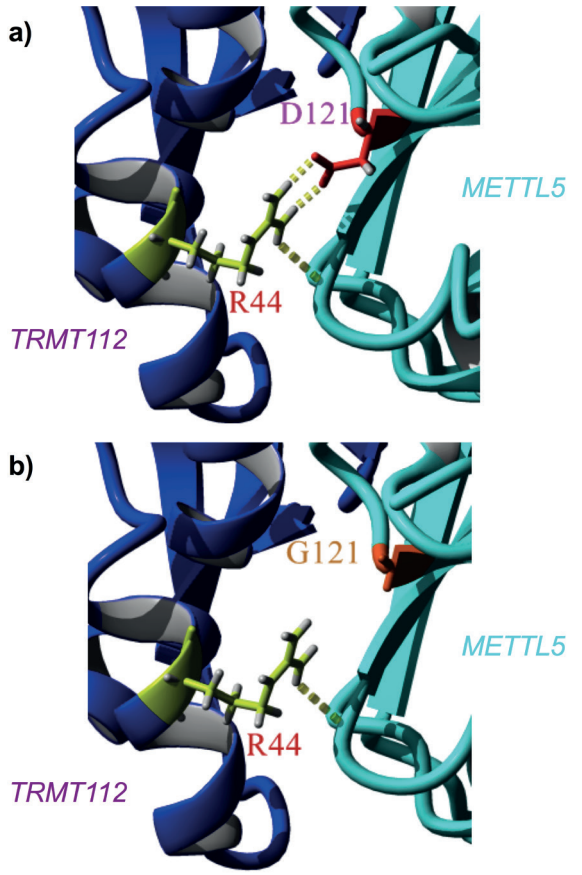

Fig. 3. 3D modeling of the *METTL5-TRMT112* complex. (a) D121 of *METTL5* (red) is able to form salt-bridges with R44 of *TRMT112* (yellow) and contribute the interactions of proteins. (b) c.362A>G variant causes D121 to be replaced by glycine (orange) and disturbs the formation of salt-bridges.

complex revealed that wild-type residue (D121) is located in S-adenosyl-L-methionine-dependent methyltransferase domain and is involved in formation of a salt-bridge with R44 of *TRMT112* (Fig. 3a) c.362A>G variant causes the D121 to be replaced by glycine. This substitution disturbs the salt bridge between *METTL5-TRMT112* complex (Fig. 3b).

Discussion

Intellectual disability related disorders show extensive clinical and/or genetic heterogeneity. To date, more than two thousand genes related to ID have been identified and the application of NGS technologies such as WES continues to reveal new genes. The *METTL5* gene has been postulated as a candidate gene for ID in two different clinical studies.[7,8] In 2019, a detailed functional and clinical study in seven patients with intellectual disability, microcephaly, poor/absent speech and aggressive behavior phenotypes identified *METLL5* as a causative gene for autosomal recessive ID.[6] Here, we report three new *METTL5* related ID syndrome cases in a consanguineous family of Afghanistan descent with a novel homozygous missense variant. The clinical and genetic findings of this case and previous cases are compared in Table I.

METTL5 is one of the members of methyltransferase superfamily and act as m^6A-

Table I. Clinical manifestations of *METTL5* gene related cases.

	Richard et al., 2019; Riazuddin et al., 2017			Richard et al., 2019		Hu et al., 2019		Present Study		
	Family PKMR43M			F47949		M8600616				
	II-1	II-2	II-3	III-2	III-3	III-3	III-5	III-1	III-2	III-3
	F	M	M	M	M	M	F	M	M	F
	c.344_345delGA (p.Arg115Asnfs*19)			c.571_572delAA (p.Lys191Valfs*10)		c.182G>A (p.Gly61Asp)		c.362A>G (p.Asp121Gly)		
	Clinical Findings									**Total**
Dysmorphic features										
Short stature	+	+	+	+	+	-	-	-	+	+	7/10 (70%)
Long philtrum	-	-	-	-	-	+	+	+	-	+	4/10 (40%)
Large ears	+	-	+	-	-	+	-	+	+	+	6/10 (60%)
Posteriorly rotated ears	-	-	-	+	+	-	-	+	+	+	5/10 (50%)
Strabismus	-	-	-	-	-	+	+	-	-	-	2/10 (20%)
Broad nasal base	-	+	-	+	+	-	-	+	+	+	6/10 (60%)
Narrow nasal base	-	-	-	-	-	+	+	-	-	-	2/10 (20%)
Overhanging nasal tip	-	+	+	-	-	-	-	-	-	-	2/10 (20%)
Thin upper lip	-	-	-	-	-	+	+	+	-	+	4/10 (40%)
Abnormal dentition	-	-	+	-	-	N/A	N/A	-	-	-	1/8 (12.5%)
Neurodevelopmental features										
Hypotonia	-	+	-	-	-	N/A	N/A	-	-	-	1/8 (12.4%)
Intellectual disability	+	+	+	+	+	+	+	+	+	+	10/10 (100%)
Microcephaly	+	+	+	+	+	+	+	+	+	+	10/10 (100%)
Seizure	-	-	-	-	-	-	+	-	+	+	3/10 (30%)
Delayed walking	+	+	+	+	+	-	-	+	+	+	8/10 (80%)
Unbalanced gait	-	-	-	N/A	N/A	-	+	-	+	-	2/8 (25%)
Poor or absent speech	+	+	+	+	+	+	N/A	+	+	+	9/9 (100%)
Spasticity	-	-	-	+	+	N/A	N/A	-	+	-	3/8 (37.5%)
Aggressive behavior	-	+	+	-	-	+	+	+	+	+	7/10 (70%)
Self-mutilating behavior	-	-	-	+	+	-	-	-	+	+	4/10 (40%)
ADHD	+	+	+	-	-	-	-	-	-	+	4/10 (40%)
Additional findings	-	-	-	Fetal tachycardia, hydrops fetalis	ASD, PS	-	-	Brachydactyly, hypoplastic nails	Full lips, epicanthus, mild SNHL	Epicanthus	

(+), the feature is present; (-), the feature is absent; F: female, M: male, N/A: not available, ADHD: attention deficit hyperactivity disorder, ASD: atrial septal defect, PS: pulmonary stenosis, SNHL: sensorineural hearing loss

methyltransferase that methylates the 18S rRNA gene.[6,11] *METTL5* is expressed in the human brain from the embryonic period to adulthood. Taken together, defects in *METTL5* functions are likely to lead to neurodevelopmental disorders due to impairment in the epigenetic processes. Recently, Ignatova et al.[11] revealed that *METTL5* knock-out (KO) mice had craniofacial and brain abnormalities. It was also postulated that pre/post synaptic effects and altered translation capacity due to defects in rRNA modification seem to be the cause of abnormal development. In addition, Richard et al.[6] defined an autosomal recessive ID and microcephaly syndrome in humans which is caused by the defects in *METTL5* gene (OMIM #618665). *In vitro* studies in COS7 cells confirmed that biallelic truncating variants (c.344_345delGA and c.571_572delAA) in *METTL5* gene decrease the expression and stability of METTL5, but the missense variant (c.182G>A) does not.

In this study, a novel biallelic c.362A>G missense variant was detected in a consanguineous Afghanistan descent family with three affected individuals. Clinical features of these cases were similar to the ID phenotypes reported by Richard et al. (Table I).[6] The homozygous c.362A>G *METTL5* variant was predicted as "pathogenic" by multiple *in silico* tools, although by ACMG criteria, it was classified as a VUS (PP1, PM2, PP3). PP1 is supporting evidence for the pathogenicity and indicates that the variant is co-segregating with disease in multiple affected family members. PM2 is considered as a moderate piece of evidence for pathogenicity and indicates that the variant is absent from the control population or is extremely low in frequency for recessive diseases. PP3 is also supporting evidence for the pathogenicity and reflects multiple lines of computational evidence supporting a deleterious effect on the gene or gene product. The lack of functional studies supporting the deleterious effects of the variant is the most important reason why the variant was identified as a VUS. Therefore, we observed the functional effect by revealing the effects of the c.362A>G variant on the 3D structure of the *METTL5* protein. *TRMT112* is a methyltransferase activator and *METTL5* must form a heterodimeric complex with *TRMT112* to gain metabolic stability in cells.[5] The interaction between the *METTL5-TRMT112* complex is provided by eight hydrogen bonds and two salt-bridges. Salt bridges are combination of two non-covalent interactions: hydrogen bonding and ionic bonding and forms bonds between oppositely charged residues of proteins that are close enough to each other.[12] Although non-covalent interactions are known to be relatively weak interactions, they contribute to protein structure and to the specificity of interaction of proteins with other biomolecules. Wild-type D121 residue of METTL5 is a negatively charged amino acid and forms a salt bridge with positively charged R44 residue of TRMT112. c.362A>G variant causes the wild-type residue (D121) to be replaced by uncharged glycine. It is most likely that this change would impair the metabolic stability of METTL5 by disrupting the interaction between METTL5-TRMT112 complex (Fig. 3b).

Although it was classified as a VUS by ACMG criteria, the variant we detected was assumed to be pathogenic for the following reasons; 1) The close resemblance of the clinical findings of patients with other cases reported to date, 2) Cosegregation of the biallelic missense variant with disease in multiple affected family members, 3) Presence of previously described cases with similar clinical findings and missense variants[7], 4) Structural changes in protein interactions revealed by 3D-modeling.

Apart from the variants identified by Richard et al.[6] the CLINVAR database includes 17 more pathogenic or likely pathogenic variants associated with the *METTL5* gene. Sixteen out of 17 are copy number gain/loss variants and gene dosage effect plays a major role in the pathogenesis, and the phenotype is a direct result of the cumulative effect of the imbalance of individual genes located on the deleted/duplicated chromosome region. Remaining 1 out of 17 is a splice site mutation, c.541+1G>C is located in a canonical splice-site and is

predicted to affect mRNA splicing resulting in a significantly altered protein (CLINVAR accession number: VCV000917596.1). These data show that different types of mutations may play a role in the pathogenesis of *METTL5* related ID.

Review of the clinical data showed that intellectual disability, microcephaly and poor/absent speech findings were present in all of the seven previously reported patients as well as in three new patients (Table I). The three new patients we reviewed also had delayed walking, aggressive behavior, large/posteriorly rotated ears and broad nasal base findings which were present in majority of the previously reported patients. Except for patient III-1, patient III-2 and III-3 from our study also had short stature which was present in 70% of the all reported cases. Taken together, intellectual disability, microcephaly, poor/absent speech, delayed walking, aggressive behavior, large/posteriorly rotated ears, broad nasal base and short stature comprise the cardinal findings of this ID syndrome. Besides, long philtrum, thin upper lip, seizure, self-mutilating behavior, attention deficit hyperactivity disorder (ADHD) and spasticity appear as the most common clinical findings after the cardinal findings. Dysmorphic features including narrow nasal base, overhanging nasal tip, strabismus and unbalanced gait as a neurodevelopmental abnormality constituted the less frequently reported clinical findings. Abnormal dentition and hypotonia findings were only reported in one case. The patients in the present study also had some features that were not reported previously. These included full lips, epicanthal folds, brachydactyly, hypoplastic nails and mild sensorineural hearing loss. Whether these findings are related to the c.362A>G *METTL5* missense mutation or to another unknown genetic lesion, remains to be substantiated in future patients with the phenotypic spectrum of this disorder. In addition, Ignatova et al.[11] reported that skull abnormalities or ossicle malformations might be the cause of hearing impairment in *METTL5* knock-out mice. This observation may explain the cause of mild sensorineural hearing loss in patient III-2.

In conclusion, clinical findings of the cases reported here are consistent with a *METTL5* related ID syndrome. To the best of our knowledge, the homozygous missense variant of *METTL5* gene that we found by WES has not been reported before and, these three new cases are the first patients from Afghanistan descent. Although, there is some conflicting data about the role of missense variants in the pathogenesis of the *METTL5* related ID syndrome, the variant found in this study was assumed to be the primary cause of clinical features in the three patients. However, understanding the functional impact of missense variants in *METTL5* functions does require further investigations and thus further examination of any new cases will help to provide a better understanding of the disease pathogenesis.

Ethical approval

Written informed consents were obtained from parents to undertake genetic investigations.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: DT, MA; data collection: DT, MA, BÇ, HA; analysis and interpretation of results: DT, BÇ, DSC; draft manuscript preparation: DT, DSC. 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 child presenting with bullous emphysema

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ABSTRACT

Background. Placental transmogrification of the lung (PTL) is a clinical spectrum varying from asymptomatic to severe pulmonary impairment; such as recurrent pneumothorax, bronchopneumonia, respiratory distress syndrome and chronic obstructive airway disease. PTL usually presents as a bullous lesion, and rarely can appear in nodule or cyst formation on chest imaging. PTL with giant bullous emphysema has a male preference, is more commonly unilateral and mostly affects one lobe, but can rarely involve more than one lobe.

Case. Here we report a 13-year-old boy presenting with bullous emphysema and coexisting with a borderline testicular tumor. He had no complaints of cough, sputum, or shortness of breath. He had a past medical history of pneumonia five years ago. In order to elucidate the underlying lung pathology, a wedge lung biopsy was performed and the patient was diagnosed with PTL. Scrotum ultrasonography was performed because of hydrocele in both testes, and bilateral epididymal cysts with papillary solid projections were reported. Pathological examination of the epididymal tumor revealed a "Mullerian type borderline epithelial neoplasm" which is an analogue of the ovarian serous borderline tumor.

Conclusions. In conclusion, we reported the youngest PTL case in the literature, a rare disease with unknown pathophysiology, presenting as bullous emphysema and coincidental Mullerian type borderline epithelial neoplasm. It is important to diagnose placental transmogrification of the lung in a child with bullous emphysema because compared to other cystic lung diseases it is a benign disease and if no additional malignity exists, lobectomy or pneumonectomy is the cure for the disease.

Key words: placental transmogrification, child, bullous emphysema, Mullerian type borderline epithelial neoplasm, hydrocele.

Placental transmogrification of the lung (PTL) which was named after the resemblance to immature placental villous structures was first described by McChesney in 1979.[1] Placental transmogrification can be asymptomatic, however severe pulmonary impairment; such as recurrent pneumothorax, bronchopneumonia, respiratory distress syndrome and chronic obstructive airway disease can be seen. Radiological differential diagnosis of PTL includes pneumonia, bronchogenic cyst, alveolar adenoma, sclerosing hemangioma, congenital pulmonary airway malformation, hamartomas.[2] PTL frequently appears as a bullous lesion. However, it can rarely appear in nodule or cyst formation on chest imaging.[3] PTL with giant bullous emphysema has a male preference, is more commonly unilateral and mostly affects one lobe, but can rarely involve more than one lobe.[2]

Here we report a 13-year-old boy presenting with giant bullous emphysema and coexisting with a borderline testicular tumor with review of the literature. The young age of our patient and the rarity of this pathological diagnosis and the coexisting testicular tumor makes this case unique.

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Case Report

A 13-year-old boy was admitted to our pediatric pulmonology outpatient clinic due to a chest deformity recognized by his parents. He had no complaints of cough, sputum, or shortness of breath. He had a past medical history of pneumonia five years ago, in which he was hospitalized in a different facility and treated for, and discharged without any morbidities. During this hospitalization a chest computed tomography (CT) was performed indicating ground glass opacities and interlobular septal thickening, no further work up was performed. In the same year, the patient had an endovenous laser ablation procedure due to Vena Saphena Magna ectatic venous formation. For the following five years he had no complaints, and was lost to follow up. On admission to our hospital in 2019, the physical examination revealed that his weight and height were within the normal percentile, transcutaneous oxygen saturation was 96% at room air, he had an asymmetrical chest wall with protrusion of the left chest wall, decreased lung sounds on the right lower lobe, levoscoliosis at the level of thoracic vertebra, and bilateral hydrocele in testis. He had no signs of hemihypertrophy. Spirometry revealed a restrictive pattern (Vital capacity (VC): 53% forced VC (FVC): 50%, forced expiratory volume in 1 Second (FEV1): 50% FEV1/FVC: 83 forced expiratory flow at 25-75% of the pulmonary volume (FEF25/75): 45%). Chest CT was performed displaying fibrotic bands, septal thickening, emphysematous regions, honeycombing and air trapping mainly localized to the right lower and middle lobe and a leftward mediastinal shift (Fig. 1). A progression was observed at the right lung parenchyma within five years. Because of the localized nature of the pulmonary disease and a history of venous pathology at the right leg, we wanted to exclude venous lymphatic drainage diseases, so we performed a lymphoscintigraphy in which no lymphatic pathology was found. Scrotum ultrasonography was performed because of hydrocele in both testes, and bilateral epididymal cysts with papillary solid projections were reported (Fig. 2). Bilateral tumor was successfully resected by pediatric surgeons.

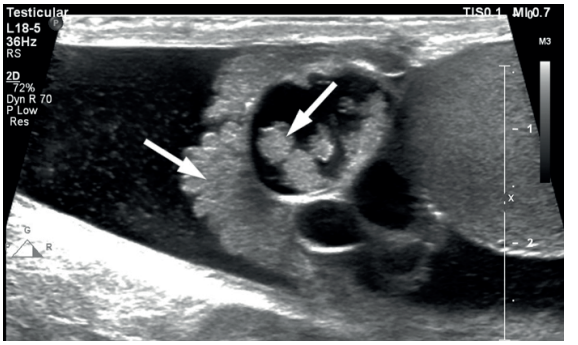

Fig. 2. Ultrasonography image demonstrates a cystic neoplasm with papillary projections of the epididymis (arrows). Note the normal testis parenchyma.

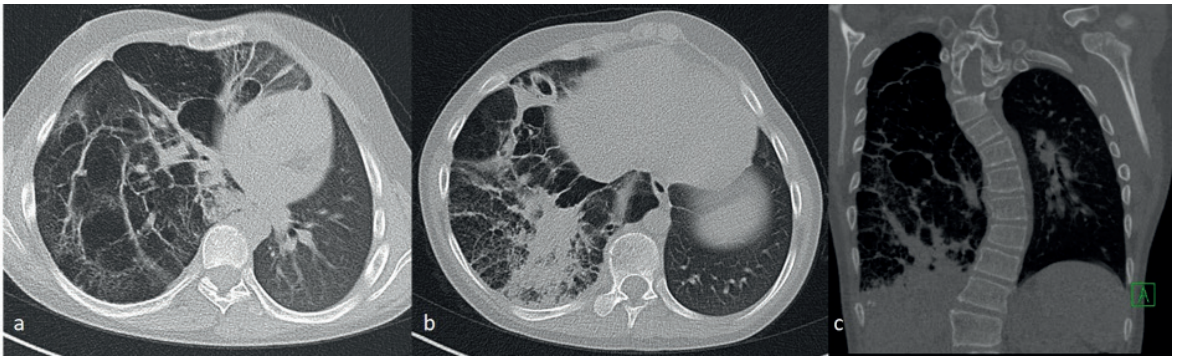

Fig. 1. A, B) Axial chest CT images show, fibrotic bands, septal thickening, bullous/emphysematous changes, air trapping and leftward mediastinal shift, honeycombing mainly localized to right lower lobe. **C)** Coronal reformatted image demonstrates levoscoliosis of the thoracic spine.

Pathological examination of the epididymal tumor revealed a "Mullerian type borderline epithelial neoplasm" which is an analogue of the ovarian serous borderline tumor (Fig. 3A). The tumor was located in the right epididymis, causing a cystic dilatation with numerous intra-cystic blunt papillae lined by ciliated stratified columnar cells with mild cytological atypia. The cyst wall had a variable amount of fibrous tissue without any invasion or necrosis detected. Immunohistochemical studies performed for ER, PR and CK7 revealed positivity in neoplastic cells, while very focal CD10 expression was detected only in the apical part of the cells. D2-40 and calretinin were negative, excluding a tumor with mesothelial origin. Ki-67 proliferation index varied from 2-3% to 10% in the lowest and highest areas (mean 5%), respectively. Considering both morphological and immunohistochemically findings, the biopsy confirmed a diagnosis of borderline serous tumor with a Mullerian origin. Because of the intact surgical margin, and negative tumor markers (Ca-125) total resection of the tumor was thought to be curative and clinical follow up was planned by pediatric oncology.

In order to elucidate the underlying lung pathology, a wedge lung biopsy was performed. The histopathologic examination showed numerous irregular cystic/emphysematous parenchymal areas accompanied by papillary structures surrounded by flattened alveolar pneumocytes, morphologically resembling placental chorion villi (Fig. 3B), especially at low power view magnifications. The patient was diagnosed with PTL. Because of the bullous/

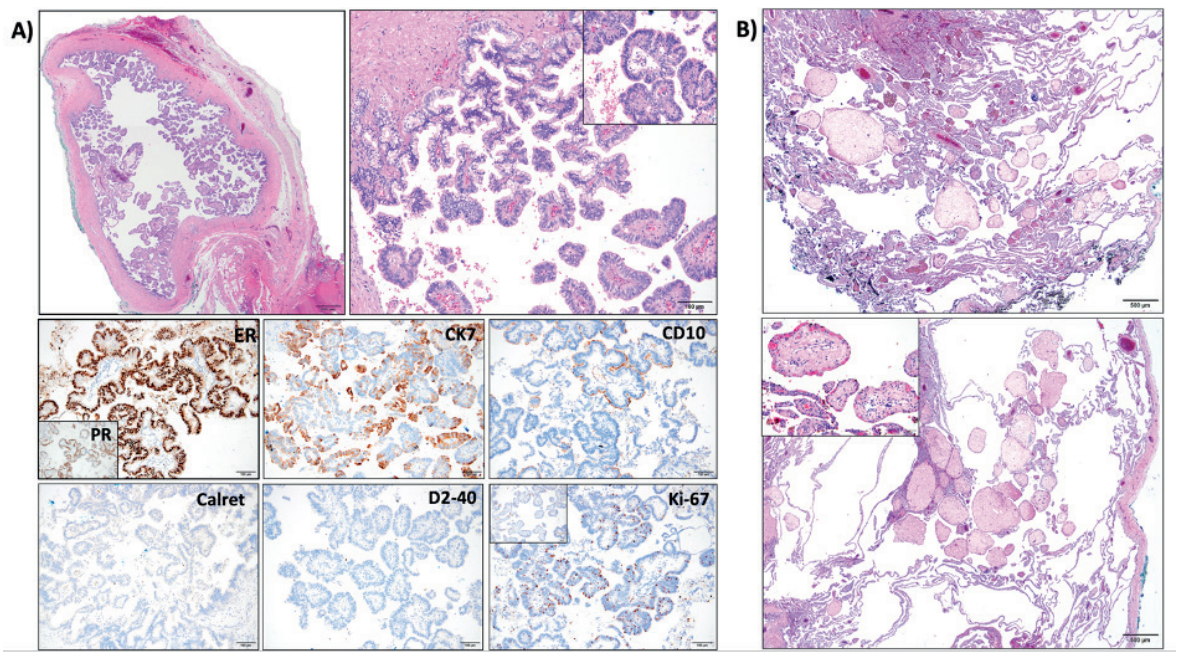

Fig. 3. A) Low power view of the epididymal cystic neoplasm having papillary projections towards the lumen (upper left: HE, x20); papillary projections showing «medusa head» micropapillary pattern (upper right: HE, x200); intra-cystic blunt papillae lined by stratified epithelial cells with mild cytologic atypia (inlet: HE, x200); summary of the immunohistochemical findings suggesting a Mullerian origin rather than a mesothelial one; ki-67 with high and low areas (lower pictures: IHC, x100 for each, respectively). **B)** Low power view of the pulmonary placental transmogrification within large cystic emphysematous areas (upper: HE, x20); pulmonary parenchyma includes cystic lesions with variable amounts of intra-cystic papillary proliferations (lower: HE, x20); papillary structures, which morphologically resemble mature chorionic villi, contain congested capillaries and are surrounded by alveolar pneumocytes (inlet: HE, x200).

emphysematous changes which severely compressed the normal lung tissue and leftward mediastinal shift, right middle and right lower lobectomy were planned. During the procedure the right upper lobe was not inflated after resection of the right middle and lower lobe, so right pneumonectomy was performed. The pathological result was compatible with placental transmogrification.

During the post-operative period, the patient had no complaints except for exertion dyspnea during heavy physical exercise. Spirometry revealed a restrictive pattern (VC: 68% FVC: 67%, FEV1: 72% FEV1/FVC: 88 FEF25/75: 74%). The patient was referred to orthopedic surgeons and no surgical intervention was planned for scoliosis.

The patient and the caregiver gave written consent for the publication of this case.

Discussion

PTL is a rare disease. As of 2019, less than 40 adult patients with PTL were defined in the literature. Until 2017, there were no children diagnosed with PTL in the literature. This is the third case of a child in the literature that has been reported so far. The young age of our patient and the rarity of this pathological diagnosis and coexisting testicular tumor makes this case unique. In this case report, we tried to emphasize the importance of differential diagnosis in a patient presenting with giant bullous emphysema who was nearly asymptomatic when he was first admitted to our unit. It is important to diagnose PTL in a child with giant bullous emphysema because compared to other cystic lung diseases it is a benign disease and if no additional malignity exists lobectomy or pneumonectomy is the cure for the disease.

Knowledge concerning PTL in children is limited. To date, there are only two case studies reporting PTL in children, and in both of the cases giant bullous emphysema cases were restricted to one side of the lung and more than one lobe was affected.[4,5] In both of the cases the patients were 14-year-old males, presenting with back pain, and no other significant physical findings. One child had chest CT findings in past years where pneumonia or bleb formation could not be differentiated, both patients were cured by lobectomy of the right middle lobe and right lobe, intervention. There was no comorbidity in both patients. In contrast, our case was diagnosed with testicular Mullerian type borderline epithelial neoplasm - the analogue of ovarian serous borderline epithelial tumor-, at the same time interval. The borderline serous tumor one of the epithelial paratesticular tumors with Müllerian characteristics was first reported by Young and Scully in 1986.[6] To date only four children with testicular ovarian epithelial tumor, serous borderline have been reported. A painless mass with hydrocele was detected in all of these patients. In our case, Ki67 proliferation index was 5% which is consistent with the literature where Ki67 proliferation index ranges between 1.3-10%.[7,8] However, none of the cases with testicular Mullerian type serous borderline epithelial tumor, had a coexisting lung pathology. Because of the papillary structures, it can be tricky to differentiate a metastatic papillary neoplasm from PTL's papillary figures in a low power view. On the other hand, cytologic details of the epithelial component of the serous tumor can be easily distinguished from single layered bland pneumocytes of PTL. Besides, another major histopathological difference between these two lesions - architectural simplicity of PTL and "medusa head" pattern seen in serous tumor- was proof that they are not linked with each other. Therefore, we considered these two diseases co-existing in our case as two different clinical entities.

PTL usually presents as a bullous lesion, and rarely can appear in nodule or cyst formation on chest imaging.[2] In our case, in addition to bullous/emphysematous changes, other radiological findings such as: air trapping, fibrotic bands, septal thickening, mediastinal shift and honeycombing localized to the

unilateral lung were also present. We believe these additional radiological findings can be explained by the delayed diagnosis because the patient was asymptomatic and did not apply to a hospital for almost five years.

Because of the extreme rarity of PTL the pathogenesis remains unknown. However, Narula et al.[9] suggested that the mechanism of disease can range from lymph vascular proliferation in the setting of an emphysematous lung to congenital malformation, which can both be the case in our patient as he had abnormal lymphovascular proliferation history of the right leg and existing lung lesion on chest CT when he was 8 years old. One of the other possible mechanisms for PTL development is increased fat tissue expression inside the villi arising from lipomatosis.[3] In one case series, it was stated that emphysematous changes can be caused by the primary inciting event: interstitial clear cell proliferation.[2]

In our case, the patient had bullous/emphysematous changes which severely compressed the normal lung tissue and leftward mediastinal shift. Multiple (right middle and lower lobe) lobectomy was planned however after the multiple lobectomies, the right upper lobe was not inflated, so right pneumonectomy was performed. Ma et al.[10] stated in their study that among 33 adult patients the most common choice for operation was lobectomy, but pneumonectomy was performed in 7 patients. The authors concluded that the size and extension of the lesions are associated with the type of surgery. They highlighted that when giant bullae were present which severely compressed the normal lung tissue multiple lobectomy or pneumonectomy had to be performed.[10]

In conclusion, we report the youngest PTL case in the literature, a rare disease with unknown pathophysiology, presenting as giant bullous emphysema and coincidental Mullerian type borderline epithelial neoplasm. PTL should be kept in mind in a child presenting with unilateral emphysema because it is a curable disease, unlike most cystic lung diseases of childhood.

Ethical approval

The patient and the caregiver gave written consent for the publication of this case.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: BO DAT, MÜ, HNÖ, EN, NE, TS; draft manuscript preparation: BO, DO, EY, DD, UO, NK. All authors approved the final version of the manuscript.

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The authors declare the study received no funding.

Conflict of interest

The authors declare that there is no conflict of interest.

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