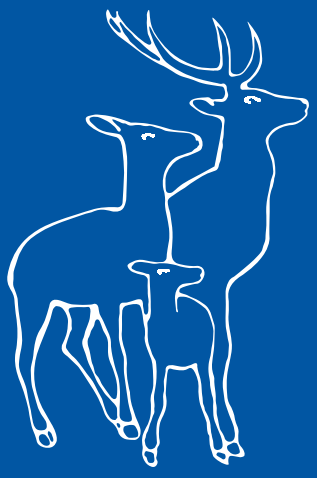

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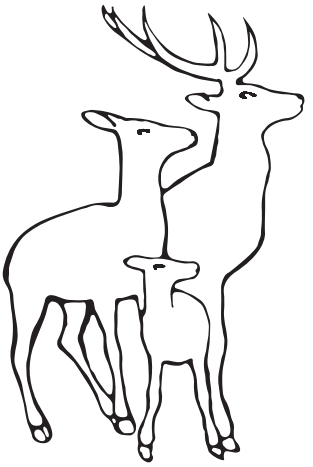

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Recommendations on phenylketonuria in Turkey

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ABSTRACT

Background. Phenylketonuria (PKU), is an autosomal recessive disease leading to the conversion defect of phenylalanine (Phe) into tyrosine. Severe neurocognitive and behavioral outcomes are observed in untreated cases.

The present paper aims to review clinical experiences and expert recommendations in diagnosis, treatment, and follow-up of pediatric PKU patients in Turkey.

Methods. Two advisory board meetings were held in the year 2016 and 2017 with contributions of four leading experts in this field, and an online update meeting was held for final decisions about statements, and conclusions in January 2021. Considering management gaps in diagnosis, treatment, and follow-up of PKU, discussion points are defined. The Committee members then reviewed the Turkish and general literature and the final statements were formulated.

Results. The diagnostic cut-off for dried blood spots should remain at 2 mg/dl. Treatment cut-off value is acceptable at 6 mg/dl. Compliance with an ideal follow-up list is strongly recommended. Total protein intake should not be limited. Age-related safe levels of protein intake should be encouraged with an additional 40% from L-amino acids supplements, a 20% compensatory factor to account for the digestibility and utilization of amino acids from the supplement, and a further 20% compensation to optimize Phe control. Cognitive impairment and intelligence quotient evaluations should be performed at least twice before 3 years of age. In pregnant women, the target Phe level should be < 5 mg/dl, and they should be followed-up weekly in the first trimester, then every 2 weeks after organogenesis. Novel pharmacological treatments are promising, but some of them have limitations for our country.

Conclusions. Early diagnosis and treatment initiation; determination and standardization of diagnostic and treatment thresholds; treatment modalities and follow-up parameters are significant steps in treating PKU in the long term. PKU follow-up is a dynamic process with uncertainties and differences in clinical practice.

Key words: phenylketonuria, hyperphenylalaninemia, phenylalanine hydroxylase deficiency, management, diet, tetrahydrobiopterin, sapropterin.

Phenylketonuria (PKU; OMIM 261600) is an inborn error of metabolism caused by mutations in the phenylalanine hydroxylase *(PAH)* gene. The mutations lead to a decrease in enzymatic activity of PAH, which is predominantly a hepatic enzyme. PAH requires the cofactor tetrahydrobiopterin (BH4) to convert phenylalanine (Phe) to tyrosine (Tyr).[1] Therefore, if there is a deficiency either in PAH or its cofactor BH4, then the result is the accumulation of excess phe in the blood and brain.[1]

Recently neonatal screening programs for PKU enable physicians to identify most of the cases, and for early interventions to prevent

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severe consequences in later years of life. In cases of inadequately controlled blood Phe, neurocognitive findings, such as low intelligence quotient (IQ), decreased attention span, poor executive functions (such as planning, working memory, inhibition, flexibility, and behavioral issues), and psychosocial effects are observed in later childhood, and they extend into the adulthood.[2]

The prevalence of PKU varies worldwide. In Europe, the mean prevalence is approximately 1:10,000 newborns, and it is most commonly encountered in Ireland and Turkey whereas less commonly in Finland.[3] It is reported that the disease prevalence in Turkey is one in every 4000 births that is believed to be caused partly by the high rate of consanguinity within the population.[4] According to the statistics of the Turkish Ministry of Health, PKU prevalence is one in 6228 births, which is still one of the highest prevalence rates in the world.[5]

A group of pediatric metabolism specialists who are well-known for their clinical experience in the management of PKU patients were invited to discuss major issues and offer a standard for diagnosis, treatment, and follow up for PKU as well as to determine and inquire into specific national problems in hindering treatment success. Since the management of PKU shows wide variations across Europe[3], and in the world[6,7], the committee members decided to prepare a recommendation paper for clinicians of interest for PKU management for Turkish patients of all age groups. For this purpose, face-to-face advisory board meetings were held twice in December 2016 and December 2017 on twelve selected topics in PKU management. They discussed unmet needs in diagnosis, treatment, and follow-up of PKU patients in Turkey. Recommendations about discussion points were collected and advisory board reports were prepared for each meeting. After the participants reviewed the reports, an online update meeting was held for the final decisions about the statements, and conclusions in January 2021. The authors of this paper have suggested the key clinical recommendations that should be prioritized for implementation for PKU in Turkey.

Discussion Topics

Diagnostic cut-off levels for PKU

Similar to published recommendations and guidelines in PKU management, neonatal dried blood spot (DBS) screening, performed within the first 48-72 hours of life, is the crucial step in the diagnosis, and early initiation of treatment. Although it is recommended in the literature that blood samples for PKU screening should be obtained after 72 hours of birth, the samples are sometimes obtained within 24 hours in Turkey, because the newborns are discharged early due to an overload of maternity hospitals. If the blood is sampled before the second day of life or without sufficient feeding of the baby, a second sample should be taken on the 5th-7th days of life. Hyperphenylalaninemia (HPA) is defined as any blood Phe ≥2 mg/dl (≥120 μmol/L).[3] Therefore, it is obligatory to have DBS from each newborn, and the procedures are defined by the legislation of the Ministry of Health of the Turkish Republic. Filter paper for newborn screening for PKU is present at all healthcare units and hospitals. After samples are obtained on the filter paper, they are sent to two centers affiliated with the Ministry of Health of the Turkish Republic. If the results of Phe determination are suspicious, then newborns are immediately referred to expert metabolic centers for the determination of blood Phe levels, and confirmation of the diagnosis of HPA. The current algorithm is given in Figure 1.[5]

The committee members discussed that the cut-off level for blood Phe was 3 mg/dl (180 μmol/L) previously in Turkey and had then been decreased to 2 mg/dl (120 μmol/L). It is also important to emphasize that even blood value below 2 mg/dl alone may be misleading in some cases (i.e. 1.98 mg/dl) because complete and correct management of PKU depends on laboratory findings integrated with clinical symptoms and signs.

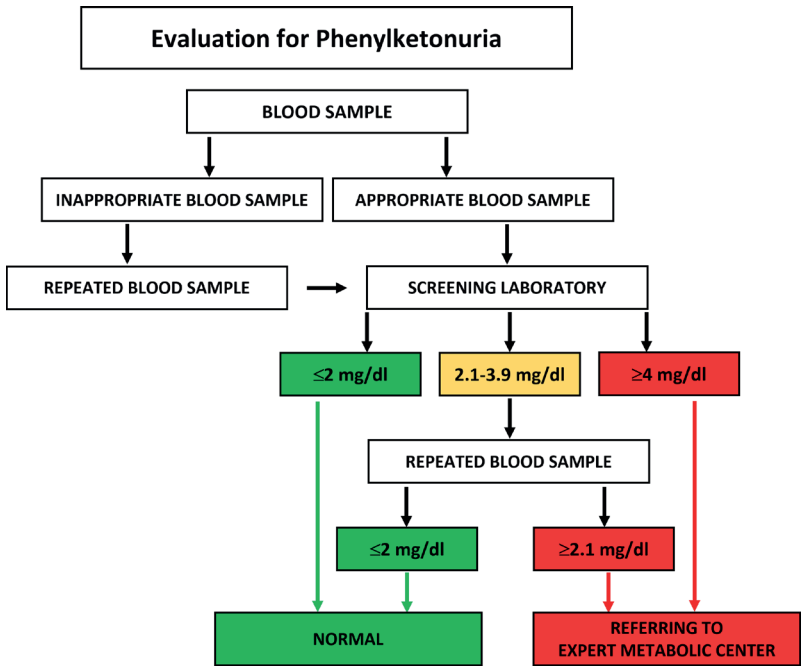

Fig. 1. PKU screening algorithm in Turkey.

Statement #1: Cut-off level for DBS should remain at 2 mg/dl (120 μmol/L).

Classification criteria for PKU

There is no consensus in the literature about patients with blood Phe concentrations >10 mg/dl (>600 μmol/L) should be treated who do not receive any treatment. However, Phe level between 2 and 6 mg/dl is generally accepted as hyperphenylalaninemia (HPA), 6-10 mg/dl as mild; 10-20 mg/dl as moderate; and >20 mg/dl as severe PKU.

Since blood Phe levels may increase with age, patients with Phe levels <360 μmol/L should be monitored (at a lower frequency) during the first year of life at minimum.[3] The evidence regarding initiation of treatment with blood Phe levels between 6 and 10 mg/dl (360 and 600 μmol/L) is incoherent. Waisbren et al. reported a decrasing trend toward of intelligence quotient (IQ) in those with higher Phe levels after having compared 3 groups (<400, 400–500 and >500 μmol/L), and recommended that treatment should maintain Phe levels <400 μmol/L throughout childhood in all PKU forms. It was predicted that for every 1.66 mg/dl (100 μmol/L) increase in mean Phe levels, IQ would decrease by approximately 6 points.[3]

Based on evidence from small size studies, it is recommended in the European (EU) guidelines that patients without treatment and who have the Phe concentrations between 6 and 10 mg/dl (360 and 600 μmol/L) should be treated during the first 12 years of age particularly to provide a good metabolic control during childhood to prevent cognitive function impairment.[3]

In a recent single-center study in Turkey, cognitive functions and attention-related problems were compared between healthy children and untreated patients with HPA.[8] A total of 60 children were recruited to the study, 41 hyperphenylalaninemia patients aged 6-16 years with untreated blood Phe levels between 240 and 600 μmol/L and 29 healthy controls. The children with untreated Phe levels between 240-360 μmol/L compared to their healthy peers, and the children with Phe levels 360-600 μmol/L compared to the children with Phe levels between 240-360 μmol/L were found at higher risk for cognitive and attention-related

problems. The outcome indicates that the "safe" upper Phe level should be considered to be lowered.[8] Considering Turkish PKU patients, the experts have decided that <6 mg/dl level indicates mild disease and patients can be followed up closely without treatment if their clinical pictures denote no sign of the disease. However, it is agreed that a decimal system should be accepted for the classification of patients in the national PKU database such as 6-10 mg/dl mild, 10-20 mg/dl moderate, and >20 mg/dl severe. It is determined that this method will be easy to use and help better classify PKU patients.

The Phe tolerance is the amount of Phe per kg of body weight or mg/day to maintain the blood Phe concentrations within the target range. This may also be described as natural protein tolerance expressed as grams per day.[3] In PKU, the individual dietary Phe tolerance is influenced by many factors such as severity of the clinical picture, net protein catabolism-synthesis ratio, energy intake, dosage and distribution of Phe-free L-amino acid supplements, and target blood Phe concentrations.[3] Children with moderate or severe PKU usually tolerate 200–500 mg/day of phenylalanine. Phe requirements are highest in early infancy ranging from 55 mg/kg/day at 0–3 months of age to 27 mg/kg/day at 12 months.[3] After the age of 1 year, there is a slow and steady decline in tolerance per kg of body weight. It has been recognized that children with classic PKU usually only tolerate between 200 and 500 mg Phe/day. Patients with a milder form of PKU, namely those with untreated blood Phe concentrations less than 16.6-20.0 mg/dl or 1000–1200 μmol/L, usually tolerate ≥500 mg/day of dietary Phe. By comparison, the third US National Health and Nutrition Examination Survey (NHANES III) showed that mean daily dietary Phe intakes for all ages and gender groups in the population without PKU was as high as 3400 mg/day.[3] A clear relationship between Phe tolerance at 2 years and 10 years of age was found.[9] However, it is unclear how this tolerance relates in older patients aiming to achieve a target blood Phe of 120 to 600 μmol/L. All patients' Phe tolerance should be evaluated periodically, especially during at times of rapid growth, changes in body composition, or use of different treatment modalities [e.g. tetrahydrobiopterin (BH4)].[3]

Experts have discussed which method will provide a more robust classification for PKU patients, blood Phe levels at the baseline or Phe tolerance. Considering the literature data, and achieving rapid and sustained treatment success, it is recommended that the national database should be classified both according to Phe levels and tolerance.

Statement #2: PKU patients can be classified according to blood Phe levels as 6-10 mg/dl mild, 10-20 mg/dl moderate, and >20 mg/dl severe cases. Classification due to Phe tolerance should be taken into account for individualized treatment for each PKU patient.

Phe levels for treatment initiation

In UK recommendations[6], USA guidelines[7] and EU guidelines[3], it is reported that PKU treatment should be started as early as possible, preferably within the first week of life to have blood Phe in the treatment range within the first 2 weeks of life. Generally accepted blood Phe level for initiation of diet is 6 mg/dl (360 μmo/L).[8] Still there is no current consensus on whether to initiate treatment between 6-10 mg/dl (360-600 μmol/L) because evidence regarding the clinical outcome in untreated patients within these limits is not definite.[10-12] However, the cut-off value to initiate treatment is accepted as 6 mg/dl (360 μmol/L) both in USA and EU guidelines.[3,7]

We decided to determine a definite cut-off level for treatment in Turkey because there are cases with no treatment up to 10 mg/dl in some centers. It is considered that the most important argument point is the definition of levels according to the patients' age because it is easier to manage blood levels when the diet is completely managed by parents. However, patients are expected to take responsibility for

their diseases during adolescence which may be quite a difficult period of their lives.

Blau et al.[13] reported that deciding the target blood Phe concentrations during the follow-up had no standard in patients with PKU. According to treatment policy data from 17 countries, the lower target Phe levels for PKU patients were in the range of 0.7-2.[14] mg/dl (40 to 130 μmol/L). During the lifetime period, the upper target blood Phe concentrations were recommended in two to five stages by different countries. It is defined in three stages in Turkey; <240 μmol/L for 0-2 years of age, <900 μmol/L for 3-15 years of age and <1,200 μmol/L for ≥16 years of age.[14]

Statement #3: National cut-off value for treatment can be accepted as 6 mg/dl (360 μmol/L).*

* Considering increased risk in executive and neurocognitive functions, experts at one center prefer to initiate treatment at 4 mg/dl (240 μmol/L). Follow-up is the preferred approach only in selected cases with mild PKU *(personal communication)*.[8]

Age limit for treatment initiation

Similar to current guidelines[3,6] and recommendations[5] the committee members have agreed on the initiation of treatment as soon as possible ideally before 10 days of life for prompt control of neurocognitive functions. Examples have been given concerning late-diagnosed PKU patients, who have improved outcomes after initiation of nutritional therapy. It is also emphasized that the number of late-diagnosed PKU cases is increasing now in Turkey due to Syrian refugees on the Southeast border, although there is not enough published data *(personal communication)*.

It is concluded that high Phe levels should always be treated no matter what the clinical picture indicates.

Statement #4: Treatment should be initiated as soon as PKU is diagnosed without considering age.

Phe limits according to age groups

The evidence for patients <12 years of age is strongly indicating that a Phe concentration of 6 mg/dl (360 μmol/L) should be considered as the upper target Phe concentration. There are also some articles indicating that the upper target Phe levels need to be lower in this age group, but at present, the evidence to lower the upper target for Phe is not robust enough. If possible, a meta-analysis of the available data should be performed to investigate the relationship between neurocognitive and neuropsychological outcome and blood Phe concentrations. Also, it should be examined whether higher Phe levels other than 360 μmol/L would result in better results. This requires an international collaborative work.[3]

According to EU guidelines, the evidence for patients >12 years of age is mainly indirect, because there are no studies investigated the effect of Phe levels during adolescence in patients with good metabolic control during childhood. Taking into account the lower grade of evidence, an upper target Phe level at 600 μmol/L between ages 12 and 18 years is recommended.[3]

During adulthood, it is reported that the main troubling issue is the determination of PKU outcomes associated with the increased Phe levels. There are no large-size controlled longitudinal studies to determine the optimal upper target blood Phe levels for adults. Therefore, further data collection is required for definite recommendations.[3]

The committee members have explained that Phe levels are determined generally be high after the age of 1-2 years until adulthood. The underlying causes are described as low treatment compliance due to low socioeconomic

levels, and cultural factors. It is decided that it will be more practical for clinical practice if target Phe levels are defined according to patient age groups.

Statement #5: Three-stage upper target Phe concentration is most convenient for our country. PKU patients should be divided into three main age groups: newborn to adolescence (<240 μmol/L), adolescence to adulthood (10-16 years/<360 μmol/L), and adulthood (<600 μmol/L). Although it may be difficult to achieve these thresholds due to the rapid growth and active daily life of patients especially during the school-age, treating physicians should try to achieve normal Phe levels as soon as possible.

Monitoring frequency of Phe levels according to ages

Close monitoring (at least weekly) is recommended in newborns up to age 1 with increased surveillance during periods of rapid growth and transitions of diet, such as with the introduction of solid foods.[3,6,7] However, recommended monitoring frequency differs after the first age: biweekly to monthly up to 12 years of age in USA guidelines[7], twice weekly in EU guidelines[3], and biweekly until school entry in UK recommendations.[6]

Experts have discussed problems of blood sample collection, outpatient clinics, and laboratory load in different centers. Since Turkey has one of the highest disease prevalences, inadequate infrastructure capacity may present obstacles to efficient healthcare service both for PKU and other patients. It is further discussed that equipment capacity, trained healthcare personnel, and materials for testing are commonly insufficient in such cases.

Another noteworthy issue is the demographic characteristics of parents of children with PKU. They are mostly undereducated and have low socioeconomic status, so it may be quite difficult to help them understand the disease and the necessity of a specific and lifelong diet therapy even when interviewed face-to-face.[15] Therefore, although home monitoring is a practical method and is being performed in some countries with a low number of PKU patients and a high socioeconomic state, it may be misleading for some Turkish patients. Physicians may not be sure if the Phe level is increased due to the wrong sample collection method or requirement for new diet adjustments. Moreover, there are important drawbacks to PKU patient follow-up in Turkey. The main ones are the absence of a registry system for dieticians, an increased number of patients, and the absence of a call center follow-up system. The committee members have decided that PKU patients should be monitored in well-equipped metabolic centers by trained medical personnel. However, this is a major insufficiency in the monitoring of Turkish PKU patients. Under these circumstances, it is admitted that even weekly follow-up of infants cannot be carried out in some reference centers.

In EU guideline[3], the minimum frequency of blood Phe measurements and outpatient clinic visits for each age group are listed (Table I and II).

Table I. Minimum frequency of blood Phe measurements for metabolic control according to age (the European guidelines on PKU, 2017).[3]

Age	Frequency
0-1 year	Weekly
1-12 years	Fortnightly
>12 years	Monthly
Pregnancy preconception	Weekly
During pregnancy	Twice weekly

Table II. Minimum frequency of outpatient clinic visits according to age (the European guidelines on PKU, 2017).[3]

Age	Frequency
0-1 year	Every 2 months
1-18 years	Twice per year
>18 years	Once per year
Pregnancy	Once per trimester

Since each age group has its specificities, such as starting school, changing school, puberty problems, and each country has its facilities and resources for healthcare, it is resolved that individualization of monitoring frequency may be accepted. The follow-up frequency of blood Phe measurements for Turkish patients is presented in Table III.[16] These are the recommended frequencies for an office visit with a pediatric metabolic diseases specialist because the growth and vaccination program of all children are managed by family physicians and general pediatricians in Turkey. Also as the daily workload of pediatric metabolic diseases specialists is quite high in our country, the effective activation of the reference system is the best way for the prioritization of PKU patients.

Statement #6: Individualized frequency for blood Phe levels may be endorsed for some PKU patients. However, compliance with an ideal follow-up frequency list is strongly recommended.

PKU treatment requires interdisciplinary teamwork

In 2017, it was recommended in the "The complete European guidelines on phenylketonuria: diagnosis and treatment" paper that all patients with PKU should be treated at specialized metabolic centers having specialized laboratory facilities.[3] It is stated that the minimum number of health professionals within an interdisciplinary team for patients of all ages should be a metabolic physician and a dietician with experience in inherited metabolic disorders (IMD), and it is strongly advised to have a (neuro)psychologist and social worker on the team. Along with the core team, support from other medical professionals such as pediatricians, biochemists, nurses specialized in IMD, and genetic specialists and support groups may be included in the management of PKU patients.

Table III. Follow-up frequency of blood Phe in the majority of Turkish centers (the recommendations from the Turkish group of pediatric metabolism and endocrinology specialists).[16]

Age	Frequency
<1 year	Once a month (once a week in breastfeeding infants)
1-6 years	Every 2 months
7-10 years	Every 3 months
11-16 years	Twice in a year
>16 years	Once a year
Pregnancy	Once a week

The process of transferring children to adult care is another important issue in patient management. The transfer should be a carefully structured 'transitional' process, beginning from around the age of 12 years.[3] The patient should take over the lead of disease management from parents/caregivers. The patient-controlled process must occur even if the patient is staying at the same pediatric service. It is recommended that patients and families should have an individualized care plan and timetable for transition, together with detailed information about the adult center. This should be jointly agreed upon and written down with teenagers, caregivers, and health professionals. This plan should include treatment goals, a timetable for transfer, and ensure there is a consistent approach between all health professionals. It should also provide a mutual understanding of the transition process. It has been demonstrated in PKU, that most patients can make a successful transition to adult care with careful planning, close liaison between pediatric and adult teams, and patient and caregiver involvement. There is no right time or age for the subsequent transfer of patient care to the adult treatment center to occur but is commonly between 16 to 18 years of age, although some flexibility may be required depending on the maturity and circumstances of the patient.[1] Unfortunately, there is no established transition system in Turkey, so patients who are diagnosed at pediatric age continue being followed by their first physicians. The committee members would like to emphasize that a system is crucial for

uninterrupted treatment during the transition period.

Method for Phe determination

In the 1960s, Guthrie developed a simple semi-quantitative test to detect hyperphenylalaninemia (HPA) in large populations. However, newborn screening (NBS) for PAH deficiency in the United States is now primarily performed by tandem mass spectrometry (MS/MS). It is accepted in the USA that MS/MS-based NBS is far more accurate in determining blood Phe concentration than older screening methods.[7] According to EU guidelines, fluorometric enzyme analysis is more reliable than the Guthrie test, but more recently amino acid concentrations are usually measured by high-performance liquid chromatography (HPLC) and tandem mass spectrometry which are more precise.[3] Studies indicate that differences between these two methods are relatively small.[17-19]

Committee members discussed that there is more deviation in tandem mass spectrometry, and a consensus should be reached about the method. One of the critically important issues is that cut-off levels of NBS laboratories are not standardized nationwide, because laboratories are not under the control of a national quality control system. It is underlined that different laboratory results may be misleading for daily clinical practice.

Statement #7: HPLC is the most reliable method for the determination of blood Phe levels.

Crucial nutritional issues: how much natural and how many protein substitutes should be used?

A low Phe diet should be initiated at the time of diagnosis for PKU patients. The PKU diet mainly consists of fruits and vegetables low in Phe, variable amounts of low-Phe special foods, and Phe-free protein substitutes providing essential amino acids (especially tyrosine), vitamins, and minerals. There are many difficulties in the nutritional management of PKU because firstly it should be lifelong. Secondly, it is complex and time-consuming. Thirdly, it requires knowledge of foods and recipes, as well as cooking skills, and continuous food measurement.[14] The constraint of a diet that is ultimately focused at the threshold of a calculated Phe intake, and an astringent diet bears the risk of micronutrient deficiencies, such as iron, zinc, selenium, and vitamin B12.[2]

For infants with PKU, human breast milk should be given greater consideration in PKU than is currently granted, because it has greater biological and developmental advantages. Therefore, it is recommended by the World Health Organization (WHO) that breastfeeding combined with the prescribed amount of an infant Phe-free protein substitute should be promoted.[14] Protein substitutes are available as amino acid powders, gels, ready-to-drink liquids, and tablets/capsules. Ready-to-drink liquids are suitable for children from 3 years of age. In the case of adult PKU patients, the long-term use of ready-to-drink liquid protein substitutes is associated with better compliance by lowering blood Phe and improving nutritional biochemical markers.[14,20]

Preparation of low Phe diet from natural foods and protein substitutes should be diligently performed. In most patients, precursor-free L-amino acids will likely supply 52 to 80% of the total protein intake. But, the optimal amount of L-amino acids is still undetermined. The recent Cochrane review concludes there was insufficient data to define a conclusion regarding the dosage of Phe-free L-amino acid supplements for PKU treatment.[3]

It is reported in the EU guidelines that many centers in Europe and beyond prescribe L-amino acids/total protein between 2 and 3 g/kg/day in infants aged 0–1 y; 1.5–2 g/kg/day in children aged 1–10 y; and 1 g/kg/day in patients >10 years. This data was confirmed by a survey of 63 PKU centers from 18 countries, demonstrating that prescription patterns of total protein intake were influenced by country and location in Europe. In general, no more than 20% of energy

should be supplied as protein.[3] Ahring et al.[2] reported their opinion paper about dietary management of PKU in European countries that most centers preferred to use protein substitutes with protein equivalent content >30% by weight, with or without added carbohydrate or fat, and all preferred substitutes enriched with vitamins and minerals (Table IV). MacDonald et al.[21] pointed out that both Phe tolerance and total protein requirement (i.e. natural protein+Phe-free amino acid= total protein intake) play an important role in the determination of dosage of Phe-free L-amino acids or protein substitution. It is a common practice that recommendations for the optimal amount of Phe-free amino acids are based on protein recommendations for healthy individuals. However, additional factors may influence protein utilization in PKU.[21]

According to the literature, although no data supports a higher dose of Phe-free amino acid, an incremental compensation factor of 1.2 is being used in Dutch guidelines, which is parallel to the compensatory ratio of 20% in USA guidelines. This compensation is for losses due to indigestibility and protein quality for mainly vegetarian diets.[21] The recommended calculation of L-amino acid requirement by EU guidelines is total protein intake (56 g/day) - natural protein intake (6 g/day) = 50 g/day. This is corrected with an additional 40% of L- amino acids from the protein substitute = 50 g/day × 1.4 = 70 g/day.[3]

The Committee members have also emphasized that they frequently encountered inadequate protein intake especially during childhood, so clinicians should be cautious about protein restriction in a growing child (Table IV). A consensus has been reached that the amount of protein substitute according to age and severity of the disease should be determined first, and then the amount of dietary Phe intake should be decided. Studies have indicated that even when the total protein amount is increased they do not cause problems, unlike intact proteins, as there are L-amino acids in the substitutes and they are easily eliminated. Thus, a common decision has been reached that total protein amount should not be limited, and it should be calculated as 40% more than a healthy child at the same age, whereas at least 80% of the total amount should be provided by protein substitutes, which should be administered in divided doses (at least 3-4 doses in a day).

Statement #8:

- The total protein intake should not be limited and should supply age-related safe levels of protein intake (FAO/WHO/UNU 2007) with an additional 40% from L-amino acids supplements and a 20% compensatory factor to account for the digestibility and utilization of amino acids from the supplement, and a further 20% compensation to optimize Phe control.
- Phe-free amino acids should supply >75% of the total protein intake.
- Protein substitutes should be administered in divided doses (at least 3-4 doses in a day).

Follow-up parameters and frequency

Experts have discussed that PKU patients are followed-up under two main titles in Turkey;

Table IV. Dosage of protein equivalent from protein substitutes (g/kg per day) reported by individual centers (dietary recommendations on PKU across European centers, 2009).[2]

		B	D	DK	E	I	N	NL	PL	TR	UK
Age	0-1 y	≤2	2.0-2.3	2-3	3	Dependent on patient Phe tolerance	2.5	2.5-2.0	2.4	2	3[a]
	1-3 y	1.2	1.7	2	2.5		2.5-2.0	2.0-1.8	1.6	1.5	3[a]
	4-10 y	1.2	1.4-1.6	2	2		2.0-1.5	1.5	1.6	1.5	2[a]
	>10 y	1.0	0.8-1.1	10-14 y: 1.5 >14 y: 1	1.5		1.5-1.0	1.2-1.0	1.2	1.2	1-1.5[a]

[a]Total protein including protein exchanges.
Centers: B: Belgium, D: Germany, DK: Denmark, E: Spain, I: Italy, N: Norway, NL: the Netherlands, PL: Poland, TR: Turkey, UK: United Kingdom.

the first is dealing with problems related to biochemical pathways of PKU, and the second is the follow-up of blood Phe levels, other amino acid levels, and nutritional parameters. According to the literature, deficiencies of some micronutrients are more common in PKU patients. These are mainly vitamins A, C, and E, selenium, coenzyme Q10, vitamins B2, B6, and B12, and folates (which can increase homocysteine levels in the blood), iron, zinc, calcium, carnitine, long-chain polyunsaturated fatty acid (LCPUFAs) and vitamin D.

The most comprehensive PKU patient follow-up schedule was published in the EU guidelines in 2017 (Table V).[3] Minimum requirements for the management and follow-up of PKU patients are given according to age groups in a wide range from an outpatient visit to age group-specific investigations.

In PKU, the main factors influencing bone density are calcium and vitamin D status, the quality of bone proteins, endocrine status (alkaline phosphatase and PTH associated with an increase of calciuria and C-terminal telopeptide)[3], and genetic and environmental factors. Therefore, adequate calcium and vitamin D intake, regular physical activity, and optimization of natural protein intake are recommended in the EU guideline. Moreover, experts suggest that although there is no sound research data about follow-up by dual-energy X-ray absorptiometry (DXA), bone mineral density (BMD) of PKU patients should be followed up during late adolescence.[3]

In Turkey, despite physical constraints, and the absence of adequate infrastructure for PKU patients, practically experts follow up pediatric patients every three to six months until puberty, and once or twice a year from then onwards except for unexpected conditions. However, the follow-up schedule of blood Phe levels is close to the "ideal follow-up frequency of blood Phe" mentioned in Table III.[16]

Neurocognitive follow-up in PKU

Children with PKU, whose treatment is initiated during the early days of life, encounter generally better developmental milestones, and they attend normal schools.[14] Although much literature on early and continuously-treated PAH deficiency reports IQ scores in the average range, pediatric data suggest that even under these circumstances, children with PKU have IQ scores that are six to nine points lower than their siblings and parents. It is recommended that considering different literature data on normal IQ scores but failing functional outcomes in some of the children with PKU, deficits in executive functions in these patients warrant special attention during the follow-up.[7] When adult PKU patients are considered, both EU and USA guidelines recommend annual routine neurological examinations.[3,7] Committee members discussed achievements obtained by the Trust for Children Phenylketonuria and Other Metabolic Diseases (METVAK), such as performing different tests for neurocognitive examination under the supervision of Child and Adolescent Psychiatrists. While only IQ has been tested previously, cognitive functions are being evaluated currently. These improvements have clarified that even patients with normal IQ scores may have problems in executive functions. Therefore, IQ scores alone are not a reliable indicator for healthy upper cortical functions. On the other hand, it is underlined that the insufficiency of trained manpower, infrastructure, and tools for evaluation, such as validated tests are primary drawbacks in detailed data collection. Members have agreed that the first evaluation of neurocognitive functions should be performed at least 2 times before 3 years of age, and once before 6 years of age by using the Denver Developmental Screening Test. It is proposed that Wechsler Intelligence Scale for Children–IV (WISC-IV) should also be performed at the preschool age and during adolescence. They have recommended neurocognitive evaluation in

Table V. Minimum requirements for the management and follow-up of PKU patients (the European guidelines on PKU, 2017).[3]

	Childhood (<12 y)	Adolescence (12-18 y)	Adulthood (≥18 y) excluding maternal PKU	Maternal PKU
Outpatient visit	Given good clinical and metabolic control: Age 0–1 year: every 2 months Age 1–12 years: twice per year Extra clinic visits as indicated	Given good clinical and metabolic control: twice per year Extra clinic visits as indicated	Given good clinical and metabolic control: once per year Extra clinic visits as indicated	Given good clinical and metabolic control: once per trimester Extra clinic visits as indicated
Clinical nutritional assessment	Every outpatient visit: dietary assessment (3-day food record/24 h recall), anthropometric parameters (weight, height, BMI) and clinical features of micronutrient and Phe deficiency (especially anorexia, listlessness, alopecia, perineal rash)	Every outpatient visit: dietary assessment (3-day food record/24 h recall), anthropometric parameters (weight, height, BMI) and clinical features of micronutrient and Phe deficiency	Every 12–24 months: dietary assessment (3-day food record/24 h recall), anthropometric parameters (weight, height, BMI), and clinical features of micronutrient and Phe deficiency	Every outpatient visit: dietary assessment (3-day food record/24 h recall) and weight
Metabolic control	Age 0–1year weekly Phe Age 1–12 years fortnightly Phe Increased frequency as indicated Annually: plasma amino acids	Monthly Phe Increased frequency as indicated Annually: plasma amino acids	Monthly Phe Increased frequency as indicated Annually: plasma amino acids	Pre-conceptionally: weekly Pregnancy: weekly Increased frequency as indicated Pre-conceptionally: plasma amino acids
Biochemical nutritional assessment	Annual measurement of plasma homocysteine and/or methylmalonic acid, hemoglobin, MCV, and ferritin. All other micronutrients (vitamins and minerals including calcium, zinc, selenium) or hormones (parathyroid hormone) if clinically indicated			Pre-conception and at the start of pregnancy: folic acid, vitamin B12, plasma homocysteine and/or methylmalonic acid, ferritin, full blood count Pregnancy: when indicated

Table V. Continued.

	Childhood (<12 y)	Adolescence (12-18 y)	Adulthood (≥18 y) excluding maternal PKU	Maternal PKU
Bone density	BMD measurement only indicated when there are specific clinical reasons or when patients are known to be at a particular risk of metabolic bone disease	The first measurement of BMD should be undertaken during late adolescence - When BMD is abnormal, DXA (with or without change of treatment) should be repeated after 1 year. If osteoporosis (BMD < -2.5 SD) persists despite optimization of diet and physical activity, other possible causes of osteoporosis should be investigated. Treatment (including consideration of bisphosphonates) should be determined by osteoporosis severity. - If BMD results are still low but stable, yearly measurement is unnecessary. - When BMD is normal, no-repeat measurement is necessary. Further study needs only be considered when there are clinical reasons to do so.	BMD measurement is only indicated when there are specific clinical reasons or when patients are known to be at particular risk of metabolic bone disease	Not indicated
Neurocognitive functions	Only neurocognitive tests when indicated.	Testing at age 12 years Proposed domains of neurocognitive testing: IQ, perception/visuospatial functioning, EF (divided into inhibitory control, working memory, and cognitive flexibility), and motor control. Extra neurocognitive tests as indicated.	Testing at age 18 years Proposed domains of neurocognitive testing: IQ, perception/visuospatial functioning, EF (divided into inhibitory control, working memory, and cognitive flexibility), and motor control. Extra neurocognitive tests as indicated.	Not indicated

Table V. Continued.

	Childhood (<12 y)	Adolescence (12-18 y)	Adulthood (≥18 y) excluding maternal PKU	Maternal PKU
Adaptive issues (e.g. Clinical relevant behavioral problems)	Annually: clinical assessment/ discussion	Annually: clinical assessment/ discussion Screening at age 12 years	Annually: clinical assessment/ discussion Screening at age 18 years	Not indicated
Neurological complications	If neurodegeneration occurs	If neurodegeneration occurs	Annually: clinical examination	Not indicated
Psychosocial functioning and wellbeing and QoL	Annually: Clinical assessment/ discussion Once during childhood: (PKU-) QOL questionnaire	Annually: Clinical assessment/ discussion Once during adolescence: (PKU-) QOL questionnaire	Annually: Clinical assessment/ discussion Once during adulthood: (PKU-) QOL questionnaire	Especially in case of not becoming pregnant, the patient may need support
Psychiatric examination	At the onset of symptoms of psychiatric disturbances	At the onset of symptoms of psychiatric	At the onset of symptoms of psychiatric	Not indicated
White matter abnormalities (MRI)	When there is an unexpected clinical course and/or unexpected neurological deficits	When there is an unexpected clinical course	When there is an unexpected clinical course	Not indicated
Age group-specific investigations	/	/	/	Ultrasound at 18–22 weeks of pregnancy with screening for organ development (especially if there is a lack of optimal metabolic control) Echocardiogram in all infants who are conceived by women with either high blood Phe levels or poor maternal blood Phe control during pregnancy

the following years during adulthood. Treating physicians should consider more sophisticated tests such as Bayley Scales of Infant and Toddler Developmental during the assessment, if available. For further assessments, patients with PKU should be referred to a certified clinical psychologist, if possible. In centers without a certified clinical psychologist, treating pediatric metabolic diseases specialists should perform the Denver Developmental Screening Test every 6 months as a part of the follow-up plan.

Brain MRI

It is reported in the literature that MR neuroimaging is almost always abnormal in adults with blood Phe levels consistently above 800 μmol/L showing evidence of white matter changes. The correlation of such abnormalities to clinical manifestations has not been demonstrated in the majority of studies and adverse long-term consequences have yet to be proven.[3,14] White matter (WM) variations in MRI are reported reversible in PKU patients. Studies are showing an improvement of WM abnormalities (3 to 6 months) after lowering of blood Phe levels. However, the blood Phe values determined at outpatient control visits may not always reflect the absolute values. Clinically it is experienced in some patients that they have high blood Phe values between control visits, and they perform a depletion diet sometimes before they attend the visits. Recent advances in neuroimaging techniques such as diffusion kurtosis imaging (DKI), diffusion-weighted imaging (DWI), and diffusion tensor imaging (DTI) have enabled clinicians to assess the WM integrity and subtle changes in brain parenchyma.[22,23] It has been reported that these novel neuroimaging techniques are sensitive to cognitive impairment as well as decreased diet compliance of PKU patients, thus may provide a significant tool for close follow-up of the central nervous system.[3,22,23]

Under the light of literature, committee members have agreed that MR imaging should be performed to evaluate myelinization once in childhood, adolescence, and adulthood if there is no additional problem. However, the limited number of experienced radiologists and MRI centers are noted as the main drawbacks of this issue.

Statement #9:

- Blood Phe levels should be measured as stated in Statement 7. Blood samples of PKU patients who cannot attend control visits may be obtained in the nearest healthcare unit and can be sent to a reference unit. PKU treatment should be individualized in the required conditions.
- BMD should be performed during early adolescence. If there is a problem, it should be repeated once a year.
- Neurocognitive functions should be first evaluated at the preschool age (at least 2 times before 3 years of age, and once before 6 years of age). They should be evaluated during adolescence, and if possible, during adulthood.
- IQ testing should be first performed at the preschool age, and then during adolescence. Counseling centers may be used for this purpose.
- During preschool years, Denver Developmental Screening Test is a practical tool for follow-up and should be performed every 6 months in these patients.

Parameters for anthropometric measurements

In PKU patients, frequent dietary manipulations are required to respond to normal growth rate, and life stages, and avoid concurrent diseases and comorbidities. Several studies in the literature indicate that transient growth retardation is common in children treated for PKU, especially from birth to the age of 3–5 years.[24-26] On the other hand, Acosta et al.[27] showed that PKU children undergoing nutrition management, aged from 2 to 13 years old, presented a normal linear growth. Moreover, they determined that many children were overweight according to mean body mass index Z-scores. Studies show that children with PKU might weigh

more than normal children.[28,29] Scaglioni et al.[30] reported that the rate of overweight at the age of 8 years was around 25%. Even if PKU children are routinely long-term monitored for dietary intake, there are data showing evidence of overweight in this population. Therefore, ongoing nutritional assessment of energy intake and quality of carbohydrates in PKU diet, physical activity, and body weight in PKU patients should be carefully monitored over the life cycle (Table V and VI).[3,31] If a patient is not consuming an adequate amount of protein substitute distributed throughout the day, not only will plasma Phe concentration be elevated due to protein catabolism, but growth failure may occur because protein substitutes provide the majority of vitamins and minerals (Table VI).[31]

The committee members discussed whether growth charts defined for Turkish children were still useful in the determination of obesity and malnutrition in children with PKU. Experts stated that these charts were being used to follow-up growth rate, whereas National Institutes of Health (NIH) standards were being in some other centers. The main discussion points were switching to WHO criteria, which were more valuable for malnutrition, and enabled earlier diagnosis, Center for Disease Control and Prevention (CDC) curves used for obesity evaluation, and the absence of specific growth charts for children with PKU. It was decided that weight, height, body mass index, and height for age along with head circumference would be the most useful anthropometric criteria for the follow-up.

Statement #10:

- Height and weight for age should be the anthropometric criteria used for the follow-up.
- Head circumference measurements should be continued up to 3 years.
- Since the circumference of the upper arm, and skin thickness can vary according to individual measuring, they should not be accepted as criteria for growth follow-up.

Maternal PKU (MPKU)

The teratogenic effects of Phe on the developing fetus, termed MPKU syndrome, refers to the

Table VI. Guidelines for daily protein, energy, and daily Phe intake for PKU.[31]

Age	Protein requirement[1] g/kg	Minimum Phe requirement mg/kg	Range of Phe intake mg/day [26]	Energy [26]	
				kcal/kg/day	kcal/day
0-6 months	3-3.5	20-70 [26]	-	95-145	
7-12 months	2.5-3 (1.31) [26]	10-35 [26]	-	80-135	
1-3 years	2-3 (1.02)	NA	200-400		900-1800
4-6 years	2 (0.87)	13-20 [12]	210-450		1300-2300
7-10 years	2 (0.92)	13-20 [12]	220-500		1650-3300
Males					
11-14 years	2 (0.90)	NA	225-900		2000-3750
15-18 years	2 (0.87)	NA	295-1100		2100-3900
≥18 years	NA (0.84)	4.6-13.6 [11]	290-1200		2000-3300
Females					
11-14 years	2 (0.89)	NA	250-750		1500-3000
15-18 years	2 (0.84)	NA	230-700		1200-3000
≥18 years	NA (0.84)	4.6-13.6 [11]	220-700		1400-2500

NA= Sufficient evidence is not available for this age group.
[1]Protein requirements for PKU are based on increased need with consumption of protein substitute[32,33], and values in parentheses reflect WHO safe level recommendations for the typical population.[34]

physical and cognitive effects on the fetus of *in utero* exposure to elevated Phe levels including microcephaly, and poor fetal growth, congenital heart defects (CHD), non-familial facial features, and intellectual disability.[7] Since the identification of MPKU syndrome, concerns have been raised regarding the degree to which inadequate maternal treatment could negate the positive societal and economic effects of early identification through NBS.[7] According to the literature, approximately 65% of mothers with PKU have poorly controlled Phe before 8 weeks gestation.[35] In the first published series of 71 pregnancies and 45 live births of 32 women with PKU from Turkey, it was reported that microcephaly, intellectual disability, and dysmorphic faces were more prevalent in the offspring of untreated than treated pregnancies with classical PKU (100% vs. 0%, 91% vs. 0%, and 73% vs. 23%).[36] It was also noted that Phe levels were higher during weeks 6–14 than other periods of gestation in treated pregnancies, and the authors concluded that more frequent Phe measurements during the late first trimester are crucial to improve outcomes in treated pregnancies.[36]

Since the maternal to fetal concentration gradient results in Phe levels 1.5 to 2 times greater in the fetus than in the maternal plasma, it is recommended that women achieve plasma Phe concentrations of 120 to 360 μmol/L[37] before pregnancy, as critical development of the fetal central nervous system and heart occurs between 5 and 8 weeks' gestation.[31]

In line with the accumulated evidence, women with PKU are strongly recommended to start a Phe-restricted diet before conception. For MPKU, although minimal outpatient clinic visits of once during each trimester is recommended, more frequent follow-up and intense monitoring according to individualized needs and metabolic control are generally preferred in the daily practice (Table V).[3] Metabolic control is based on weekly Phe blood spots pre-conception and during pregnancy. Women with PKU should receive routine obstetric care and should return to standard dietary or pharmacological treatment after delivery.[3]

The committee members pointed out that the main issue in MPKU was increasing awareness of obstetricians. It was discussed that joint meetings might be held about the association of metabolic disease with pregnancy. It was emphasized that if PKU is diagnosed during pregnancy or a female with uncontrolled PKU gets pregnant, abortion should be considered. Therefore, activities in Turkey should be planned and performed under the leadership of the Child Nutrition and Metabolism Association. It was agreed that a national database should be developed shortly to collect health data on all critical issues such as the prevalence of pregnant women with PKU.

Statement #11:

- Pregnant women with PKU should be followed up once a week in the first trimester.
- They should be followed up fortnightly when the organogenesis takes place.
- The target Phe level is < 5 mg/dl.

Alternative therapies

Glycomacropeptide (GMP)

GMP is a protein derived from cheese whey that is naturally low in Phe and is rich in valine, isoleucine, and threonine.[1] Short-term data from a small controlled study in older patients indicated that GMP caused lower fasting Phe concentrations and blood urea nitrogen compared with Phe-free L-amino acid supplements.[3] There is also the suggestion that GMP lowers post-prandial concentrations of the appetite-stimulating hormone ghrelin and may help promote satiety.[3]

As there is no experience with GMP in Turkey, there is a need to test its palatability, acceptability, and its control on blood Phe levels in Turkish PKU patients.

Large Neutral Amino Acids (LNAAs)

The LNAAs, Phe, tyrosine, tryptophan, and the branched-chain amino acids share the same amino acid transport system across the blood-brain barrier. Therefore, at high concentrations, Phe in the blood will compete with other LNAAs for transport across the blood-brain barrier. LNAA supplementation has been shown to reduce cerebral Phe concentrations while an increase in plasma Phe levels was observed.[1] LNAA treatment appears to have a beneficial effect on executive functioning, however this treatment is presumed to be suitable for adults who are not adhering to a low Phe diet.[1]

Besides, these supplements are not suitable for children under the age of 8 years as well as during pregnancy. There has been little evidence to support their routine use in PKU. Moreover, ideal dose determination and safety still require further studies.[3] Therefore, it should be the treating physician's discretion to decide on when and to whom to initiate LNAAs supplementations. The LNAA supplementations may be recommended starting from adolescence.

The committee members discussed that although there were very limited data on LNAA use in Turkey, the results were satisfactory so far, mainly showing improved cognitive functions. It was emphasized that patient selection was critical, and all of the clinical experience was collected from old patients with PKU who had low adherence to treatment. The most commonly faced problem was the patient's expectation of an abrupt decrease in blood Phe levels. It was decided that the most objective criteria to determine the efficacy of LNAA treatment were cognitive tests.

Polyethylene glycol phenylalanine ammonia-lyase (PegPAL)

Until recently, there have been no pharmacological treatment options for patients who are non-responsive to sapropterin other than a low Phe diet.[38] It is a known fact that control of Phe levels is difficult especially in adolescents and young adults due to incompliance with diet therapy, decreased quality of life due to difficulty in coping with daily life, and nonattendance to follow-up visits among all patients with hyperphenylalaninemia.[39] A recently introduced enzyme substitution therapy Pegvaliase (rAvPAL-PEG) has obtained approval for PKU patients older than 18 years of age by the FDA in May 2018, and for patients older than 16 years of age by the European Medicines Agency (EMA) in May 2019.[40,41] Therefore, treatment with PAL enzyme, which metabolizes Phe independently from PAH enzyme, has in use for PKU treatment.[42] It not only is effective in decreasing blood Phe levels but also has manageable side effects.

During treatment, statistically significant improvements in blood Phe concentrations have been reported.[42] Moreover, it is determined that patients have sustained reductions in blood Phe concentrations that reached guideline-recommended levels.[42] Clinical study results have indicated that Phe levels are markedly reduced in patients who have responded to pegvaliase treatment, even reduced below target levels.[39,42]

However, due to the long duration of dose adjustment and titration, injectable route of administration, potential side effects extending to the risk of anaphylaxis, and the higher cost than conventional diet therapy it is not commonly used.[38,42] In many centers, the decision for pegvaliase treatment depends on the patient-based selection of health authorities, disease burden, and degree of compliance with the diet.[38,39,42,43]

In Turkey, Pegvaliase (rAvPAL-PEG) is approved by the scientific council for usage but is not reimbursed by social security institutions, yet. Since it is not available in clinical practice, clinical experience is limited in efficacy, safety, and side effect profile. The committee members have concluded that they will re-evaluate this issue when there is enough data.

Tetrahydrobiopterin (BH4)

BH4, also known as sapropterin dihydrochloride is used to treat a subset of PKU patients. Patients with high residual activity of the PAH enzyme have a greater probability of BH4 response, but a minority of patients with classical PKU also may benefit from BH4 treatment. As efficacy and safety of BH4 have been shown in children <4 years of age BH4 got European approval for in this age category.[3]

In a retrospective Turkish study conducted on 44 children with PKU who were younger than 4 years of age (median age=3.8 years), it was reported that the median BH4 treatment period of 26.7 months (12-45 months) was safe and effective (Phe tolerance was significantly increased by a median of 2.26 folds from 47.5 mg/kg/day to 114 mg/kg/day).[44] Authors from European centers report that BH4-responsiveness should be determined on an individual case basis. The degree of responsiveness will be characterized by the extent of improvement in biochemical control and an increase in natural protein intake. It is defined as 'establishing an increase in natural protein tolerance of ≥100% with blood Phe concentrations remaining consistently within the target range'. It can also be defined by 'improved' metabolic control >75% of blood Phe levels and remaining within the target range without any decrease in natural protein intake associated with BH4 treatment'. Moreover, it is recommended that BH4 should only be prescribed in cases of proven BH4-responsiveness.[3]

If a newborn is diagnosed with PKU during neonatal screening, and the blood Phe level is <20 mg/dl, then the neonate is tested with a single oral dose of BH4. Continuation of the treatment is decided according to the test result. In older children and/or patients with low blood Phe levels, the diet liberalization or Phe loading is performed to increase the Phe levels over 10 mg/dl as reported in the literature. After 72 hours, patients have hourly oral BH4 tests. Also in patients with mutations, if they are known to have a responsive mutation listed in the database, BH4 is initiated and the diet is liberalized according to blood Phe levels. Therefore, it may be noted that there are three protocols:

- Newborns with blood Phe levels<20 mg/dl: 20 mg/kg BH4 over 24 hours.
- Older children, previously untested and under treatment: Phe overload by diet or Phe supplementation followed by 20 mg/kg/day BH4 challenge for 72 hours.
- Patients with mutations included in the database and known to be responsive: Direct initiation of treatment and gradual liberalization of the diet (if the sibling is known to have a responsive mutation, treatment may be initiated directly, even though the mutation is not defined).

The committee members discussed the limited data in BH4 treatment in Turkey, and outcomes were controversial because the patient populations were small, and there was no national standardization for eligibility and criteria of monitoring for BH4 treatment. For the time being, individualization of BH4 according to the patient with PKU was the most commonly preferred clinical practice.

Gene therapy

Recently gene therapy for PKU has been reported as a promising approach for both protection from neurocognitive problems, and providing a normal diet without Phe restriction for PKU patients. Preclinical studies using adeno-associated virus (AAV)-mediated gene addition therapy in the *Pah*$^{enu2/enu2}$ mouse model in classical PKU[45,46], genetically engineered probiotics for PKU treatment (phenylalanine lyase gene from *Anabaena variabilis (AvPAL)* in the *Pah*$^{enu2/enu2}$ mouse model)[47], genetically engineered live bacteria (*Escherichia coli* Nissle)[48,49], gene correction therapy using CRISPR-Cas-9 associated base editors[50] have reported these investigational therapeutic approaches might be novel interventions for PKU. Gene correction therapy using CRISPR-

Cas-9 associated base editors have been shown to provide >20% of the normal activity of PAH activity in *Pah*^{enu2} mice. It has been shown that the correction of PAH enzymatic activity has been improved with time and is not associated with unwanted DNA changes in genomic regions with homology to the guide RNA utilized.[50] In (AAV)-mediated gene addition, the corrected genes are demonstrated to pass to new liver cells and maintain enzymatic activity with hepatocyte proliferation.[50]

An alternative way to provide PAH gene is to supply PAH mRNA enclosed within lipid nanoparticles and the liver takes it up leading the hepatocytes to produce the enzyme without initiation of the immune response.[50] There are new Phase 1 and Phase 2 studies initiated to provide more effective therapeutic approaches in PKU by using live biotherapeutic products (LBPs) such as SYNB1618 in addition to *Escherichia coli* Nissle 1917 (EcN).[49]

Statement #12:

- GMP is unavailable in Turkey, so there is no experience with this product.
- Selection criteria for LNAA may be advanced age, refusal of protein substitute intake, and hearing about the product from other patients with PKU.
- Current information about PegPAL indicates that it is too early to be used in pediatric patients, and there are many issues to be resolved before it is accepted as a safe and reliable treatment option.
- BH4 should be used in every suitable patient. Criteria for selecting eligible patients for BH4 treatment should be reviewed for Turkey, too.

Similar to many congenital metabolic diseases requiring a diet, PKU is not only a metabolic emergency but also a social one.

Early diagnosis and treatment initiation; determination and standardization of diagnostic and treatment thresholds to minimize errors, treatment modalities, and follow-up parameters according to the country's conditions will be the utmost significant step to obtaining the best outcomes in PKU in the long term.

This Recommendations Paper is prepared as a training document on main issues in PKU follow-up. It may function as a recommendation for clinicians in troubling pitfalls during daily practice.

European and USA consensus data are combined with accumulated knowledge from experienced clinics in PKU in Turkey and discussed with the literature.

It is pointed out that PKU follow-up is a dynamic process with uncertainties and differences in clinical practice, all of which have been carefully considered. Nevertheless, frequent revisions will be necessary.

The authors believe that when the national patient database is completed, the present review will have significant contributions to the current literature.

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Author contribution

The authors confirm contribution to the paper as follows: Study concept and design, drafting the manuscript, critical revision of the manuscript for important intellectual content, administrative support, data input and interpretation: TC; drafting the manuscript, critical revision of the manuscript for important intellectual content, administrative support, data input and interpretation: HGÖ; data input and interpretation, drafting the manuscript: MÇ, NÖM, SS. All authors reviewed the results and approved the final version of the manuscript.

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Original Article

Evaluation of ketogenic diet therapy in children diagnosed with drug-resistant epilepsy: a single-center experience

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ABSTRACT

Background. We evaluate here the effect of the ketogenic diet (KD) on children with drug-resistant epilepsy (DRE) in terms of clinical effectiveness, anthropometric measurements, and some electroencephalogram (EEG) and biochemical findings.

Methods. Included in the study were 18 children (median age 70 months, 61.1% female) who received the classical KD and modified Atkins diet (MAD) for at least one year due to DRE. The patients' demographic and laboratory data; weight, height and body mass index values; EEG and electrocardiographic findings; abdominal ultrasonography findings; and biochemical parameters were recorded at baseline and at 12 months after the initiation of the diet. A reduction of ≥50% in the number of seizures was accepted as a response to KD.

Results. Classic KD was chosen for 14 patients (77.8%), and MAD for four patients (22.2%). The response to KD therapy (≥50% reduction) was 55.5% (n = 10) (p = 0.008), and one patient even became seizure-free. By the 12th month of treatment, 10 patients had experienced a reduction of more than 50% in epileptiform discharges, as indicated by EEG findings. There was no difference in seizure reduction between the patients who received classical KD and MAD. A total of 11.1% of the children lost weight during KD treatment. The most common side effect was constipation (n = 10, 55.6%). At the end of one year of treatment, total cholesterol and low density lipoprotein cholesterol (LDL-C) LDL-C levels had increased dramatically, while fasting blood glucose levels had decreased significantly.

Conclusions. Our study suggests that KD treatment provides good clinical efficacy in the treatment of pediatric DRE, and can significantly reduce the frequency of epileptic discharges. Also, total cholesterol and LDL-C levels increased significantly, and fasting blood glucose levels decreased significantly compared to the baseline levels.

Key words: child, classical ketogenic diet, drug-resistant epilepsy, efficacy, modified Atkins diet.

Epilepsy incidence rates in children range from 0.5 to 8 per 1,000 according to population-based research.[1] Nearly, 20–40% of children with seizures fail to respond to medical treatment due to uncontrolled epilepsy or drug resistance.[2-4] Drug-resistant epilepsy (DRE) refers to epilepsy that fails to respond to the trials of two or three antiepileptic drugs (AEDs)[5], in which epilepsy surgery, vagal nerve stimulation, deep brain stimulation and ketogenic diet (KD) therapy are the most prominent treatment options.[6,7] Epilepsy surgery is the primary treatment option when there is an epileptic focus that can be removed in drug-resistant epilepsies. Ketogenic diet is a good treatment option for children who cannot undergo surgery, as in most studies, more than half of the patients treated with KD report a reduction in seizure frequency of at least 50%.[3,8,9]

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Ketogenic diets are rich in fat, low in carbohydrates, and contain sufficient amounts of protein, and mimic the effect of fasting on the body.[10] The use of fasting in epilepsy treatment dates back to the time of Hippocrates, being a nonpharmacological treatment approach to DRE in all age groups, from infancy to adulthood. Recent studies have shown that KD can be applied in children older than six weeks[11], and it has been widely used in adolescents and adults, as these patients can more easily follow the diet application and management.[12,13]

International consensus has been reached on the use of KD in DRE, and its positive effects on seizure control, through consensus studies by the International League against Epilepsy (ILAE).[14]

The KD type, application, follow up and management of side effects are the key factors in the use of KD for DRE. In the present study, we evaluate the effect of KD on seizure frequency, anthropometric measurements, EEG results and biochemical findings in children with DRE, whose seizures could not be reduced with two or more AEDs.

Material and Methods

The files of the patients followed up at the Pediatric Metabolism and Pediatric Neurology departments of our hospital with the diagnoses of DRE between January 2015 and January 2020 were reviewed retrospectively. Included in the study were 18 children – 11 female (61.1%) and 7 male (38.9%) – who had used at least two AEDs and had been receiving KD treatment for at least one year. Demographic data, AEDs used, seizure frequency, type of administered KD, complications and diet efficiency were determined retrospectively from the patients' files.

The age, gender and ideal weight of the patients were taken into consideration for calculation of energy requirements. For the patients advised to start the KD, a specific diet was prepared for each child on the basis of the food consumed over the previous three days. In accordance with the general principle, the caloric needs of the children were set to 75% of the recommended energy according to their age and body mass. The children were hospitalized and followed up, and a gradually increasing protocol was applied to these patients without fasting.

The participants' histories of allergy/food intolerance, dysphagia, reflux and nutritional habits were considered in the selection of the type of diet. Conventional KD is calculated in grams as fat/protein + carbohydrate, with the most commonly used ratio being 4:1 – meaning for every 4 grams of fat, 1 gram of carbohydrate and protein are consumed. Since the protein requirement of infants is higher than that of older children, the classical KD ratio was set as 3:1 (87% of energy provided by fat). In the older children who were fed enterally, the established diet ratio was 4:1, although each patient's diet ratio was adjusted according to their ketone level, fasting blood sugar level, seizure frequency and dietary tolerance. The modified Atkins diet was preferred in three adolescent children because it is less restrictive (release of energy and protein intake, etc.) and easier to apply than classical KD and does not need hospitalization, as well as a 68-month boy per the family. The diet ratios for MAD were determined as 1:1 and 1.5:1, and the fluid needs of the children were determined as 1.25–1.5 ml/kcal. The formula KetoCal® (Nutricia) 4:1 was used for all patients, and the fat-to-nonfat (protein plus carbohydrate) ratio in their diet was increased gradually from 0.5:1.0 to 4.0:1.0. Blood ketone levels were measured daily in the first week, twice a week for the next three weeks, and once a week thereafter. The patients' blood ketone levels were maintained at between 4 and 5 mmol/L, and the target levels were reached in five to seven days. Ketogenic diet was administered for at least three months for the evaluation of its efficacy. All drugs taken by the patients were reviewed for carbohydrate content, and all suspensions were replaced by tablets.

In the study, the cessation of seizures or a ≥50% decrease in the number of seizures was considered a response to the KD. During the diet application, all patients were supported with a low-carbohydrate supplement containing multivitamins and minerals, omega 3 and probiotics.

Weight for age, height for age, weight for height, body mass index (BMI) and fasting blood sugar, total cholesterol, high density lipoprotein cholesterol (HDL-C), LDL-C, triglyceride (TG), calcium, phosphorus, alanine aminotransferase (ALT), aspartate aminotransferase (AST), hemoglobin, blood urea nitrogen (BUN), creatinine, blood gases, thyroid function, electroencephalogram (EEG) findings, echocardiography (ECHO) results, and urinary ultrasonography (USG) values at baseline and at the 12th month of the diet were recorded from the patient files. Dyslipidemia was determined as total cholesterol >200 mg/dl, LDL-C >130 mg/dl, and TG >130 mg/dl. Serum zinc and selenium levels are not measured at our hospital, and are not included in the study.

Ethical approval for the research was obtained from the Ethics Committee of Eskisehir Osmangazi University, Medical Faculty (Dated 12.05.2020, number: 25403353-050.99-E.51610, decision number: 18), and the study was conducted in accordance with the Declaration of Helsinki.

Statistical analysis

The statistical evaluation of the data was conducted using IBM SPSS Statistics (Version 21.0. Armonk, NY: IBM Corp.). Quantitative variables were expressed as mean±standard deviation or median (Q1–Q3), while qualitative variables were expressed as frequency and percentage. The compatibility with the normal distribution of the differences between the quantitative variables measured at the beginning and at the 12th month of treatment was evaluated with Shapiro–Wilk and Kolmogorov–Smirnov tests. The changes in the variables that followed (or did not follow) a normal distribution between the beginning and the 12th month of treatment were investigated with a Paired samples t-test (Wilcoxon test). The relationship between qualitative variables was analyzed with a Chi-square analysis, where $p < 0.05$ was deemed to indicate statistical significance.

Result

Included in the study were 18 children (11 female [61.1%]; 7 male [38.9%]) who received classical KD and MAD for at least one year due to DRE. The median age of the children was 70 months (5–192 months). The demographic data of the patients are summarized in Table I. The primary diagnoses of the patients were West syndrome (n = 2), pachygyria (n = 2), Lennox-Gastaut syndrome (n = 1), encephalitis sequela (n = 1), malignant migrating epilepsy (n = 1), Miller-Dieker syndrome (n = 1), Rett syndrome (n = 1), Ohtahara syndrome (n = 1), schizencephaly (n = 1), lissencephaly (n = 1) and hydrocephalus (n = 1). No additional diagnosis was found in the remaining five patients.

Table I. Demographic data of patients.

	Number (n)	Percentage (%)
Gender		
Female	11	61.1
Male	7	38.9
Feeding		
Orally	11	61.1
PEG	7	38.9
Ketogenic diets type		
Classical KD	14	77.8
MAD	4	22.2
Number of drugs per day		
2	1	5.6
3	7	38.9
4	3	16.7
5	5	27.8
6	2	11.1

PEG: percutaneous endoscopic gastrostomy, KD: ketogenic diet, MAD: modified atkins diet

Of the children participating in the study, 14 (77.8%) used levetiracetam, 12 (66.7%) valproic acid, 10 (55.6%) clobazam and eight (44.4%) vigabatrin. The other AEDs used were topiramate, carbamazepine, clonazepam, zonisamide, phenytoin, phenobarbital and oxcarbazepine. The average number of daily AEDs used by the children was 4±1.18 (min: 2, max: 6) (Table I), and seven patients (38.9%) used three AEDs.

Of the total, 11 patients (61.1%) were fed orally, while seven others (38.9%) were fed through percutaneous endoscopic gastrostomy (PEG). Before starting KD, all patients were evaluated with a tandem mass spectrometry analysis for carnitine metabolism disorders and fatty acid beta-oxidation defects, revealing no contraindications. Considering the needs of the children according to their ages, classical KD was preferred in 14 patients (77.8%), whereas MAD was preferred in four (22.2%) (Table I). In the patients who were on classical KD, 4:1 (n = 5) and 3:1 (n = 5) ratios were the most common. All KD rates are summarized in Table II.

The mean body weight of the children at the beginning of the diet was 18.30±7.45 kg, and 20.85±7.12 kg at the 12th month, indicating a statistically significant increase was noted in body weight after one year of KD treatment (p = 0.004), while two patients (11.1%) experienced weight loss. The average height at the beginning of the diet was 104.96±21.05 cm, and that in the 12th month was 111.65 ± 21.30 cm, indicating a significant increase in height measurements (p = 0.001). When the yearly growth rates of all patients were analyzed according to their age groups, we found that the annual growth rate of our patients, especially those aged 5-72 months, who had a higher protein requirement, fell behind compared to their age [mean 4.1 cm (2-6 cm)]. The growth in height in four MAD patients was found to be appropriate for their age groups. Table III presents the changes in body weight (kg, SDS), height (cm, SDS), body weight for height (BGVA, %) and BMI (SDS) at baseline and after starting the KD.

Table II. Classical ketogenic diet ratios of patients.

Classical KD rate	Number (n)	Percentage (%)
4:1	5	35.7
3:1	5	35.7
2.5:1	2	14.3
2:1	1	7.1
3.5:1	1	7.1
Total	14	100

KD: ketogenic diet

The number of seizures in the first year of the diet was compared with the figure from before the diet, and a decrease of more than 50% was noted in 10 patients (55.6%), indicating a statistically significant difference (p = 0.008). The seizures ceased completely in one patient (5.55%) who had hydrocephalus secondary to prematurity, while in another patient, the seizures decreased by more than 50% in the first six months, but increased afterwards. While the initial EEG finding of this patient showed epileptic discharges originating from both hemispheres, completely normal EEG findings were reported after sixth month of the diet. The initial EEG findings of three patients were normal; two patients had electrical status epilepticus findings; and the EEG findings of 10 patients showed a decrease of more than 50% in epileptiform discharges at the 12th month of treatment (p = 0.219). Myoclonic seizures were initially present every day in two patients on MAD, and the number of seizures reduced to once a week within the first six months of therapy, rising again to three days a week. In the two other patients who received MAD, the length of the seizures reduced, and the patients' social skills improved. There was no difference in seizure responsiveness of those on the classical KD and MAD. A comparison of the classical KD patients on different diet ratios revealed no significant difference in the number of seizures (p = 0.380). More than 50% reduction in the number of seizures was 62.3% in patients using three or less AEDs, compared to 50% in those using four or more AEDs (p = 0.664). The EEG findings of five patients using three or less AEDs (%62.5) and seven patients using four or

Table III. The difference between anthropometric measurements of patients between baseline and 12th month of treatment.

	Mean ± S.D.	Median	(Min)-(Max)	P
Weight for age (kg)				
0.month	18.3 ± 7.45	16.85	(7.2)-(34)	
12.month	20.85 ± 7.12	21	(8.8)-(31.5)	0.004[P]*
Weight for age (SDS)				
0.month	-1.34 ± 3.19	-0.75	(-11)-(2.97)	
12.month	-1.07 ± 2.32	-0.84	(-6.61)-(3.53)	0.816[W]
Height for age (cm)				
0.month	104.96 ± 21.05	110.5	(62)-(136)	
12.month	111.65 ± 21.30	114.25	(76)-(145)	0.001[P]*
Height for age (SDS)				
0.month	-0.94 ± 2.3	-0.83	(-5.85)-(2.75)	
12.month	-1.12 ± 1.66	-1.25	(-4.38)-(2.11)	0.522[P]
Weight for height (%)				
0.month	97.59 ± 14.39	96.93	(70.53)-(125.71)	
12.month	98.19 ± 13.95	99.31	(77.55)-(119.05)	0.629[P]
Body mass index				
0.month	15.44 ± 2.45	15.36	(10.88)-(20.14)	
12.month	15.01 ± 1.78	16.02	(12.5)-(18.26)	0.589[P]
Body mass index (SDS)				
0. month	-0.99 ± 2.23	-0.45	(-6.49)-(1.53)	
12. month	-0.74 ± 1.83	0.14	(-3.79)-(1.48)	0.638[W]

[P] Paired samples t-test/ [W]Wilcoxon test
*p<0.05 is significant, kg: kilogram, cm: centimeter, SDS: Standard deviation score, SD: Standard deviation, (Min)-(Max): minimum-maximum

Table IV. Seizure outcome and EEG findings in relation to antiepileptic drugs in patients receiving ketogenic diet.

		≤ 3 AED (n: 8 patients)	≥ 4 AED (n: 10 patients)	p[F]
Seizure control*	a decrease of more than 50%	5 (62.5%)	5 (50%)	0.664
	a decrease of less than 50%	3 (37.5%)	5 (50%)	
EEG findings[&]	a decrease of more than 50%	5 (62.5%)	7 (70%)	1.00
	a decrease of less than 50%	3 (37.5%)	3 (30%)	

EEG: electroencephalogram
AED: antiepileptic drug
* A reduction of ≥50% in the number of seizures was accepted as a response to ketogenic diet.
[&] A reduction of more than 50% in epileptiform discharges in EEG was accepted as a response to ketogenic diet.
[F] Fischer exact test, p<0.05 is significant

more AEDs (%70) showed a decrease of more than 50% in epileptiform discharges (p = 1.00) (Table IV).

The most common dietary side effect in our study was constipation (n = 10, 55.6%), and no nausea, vomiting, infection or hepatic side effects were observed. The distribution of side effects experienced during the diet is presented in Table V. Echocardiogram evaluations of the patients at the beginning and at the 12th month of treatment were normal. Of the 14 patients with urinary USG reports, nephrolithiasis was detected in one patient at the beginning of the

Table V. Side effects observed in patients during ketogenic diets and their distribution.

Side effects	Number (n)	Percentage (%)
Constipation	10	55.6
Halitosis	5	27.8
Hypoglycemia	4	22.2
Nephrolithiasis	4	28.5*
Weight loss	2	11.1
Metabolic acidosis	1	5.6
Diarrhea	1	5.6

*Data from 14 patients

KD treatment; and nephrolithiasis was detected in the follow-up USG of four patients at the 12th month of the diet, though these values were not statistically significant (p = 0.308). Only one patient received topiramate therapy, according to our findings. In none of our patients did we keep a record of the urine calcium/creatinine ratio. There was no increase in the stone size of the patient who had nephrolithiasis at baseline. In the remaining two individuals, we attributed the kidney stone finding to KD.

At the end of the year-long treatment, total cholesterol (p = 0.014) and LDL-C (p = 0.019) levels were found to have increased significantly, while fasting blood glucose levels had decreased significantly when compared with the values at the beginning of the diet (p = 0.031). As for the other biochemical parameters, no statistically significant difference was noted between the findings before and after treatment. The biochemical parameter values of the patients at the beginning of the diet and in the 12th month are presented in Table VI.

Discussion

The KD is a safe and effective approach to the treatment of DRE in children.[15] Ketogenic diet directs the body's metabolism to source energy from essential oils rather than glucose, although the mechanisms of action of KD related to seizure control remain unclear. Normally, the use of ketones in the brain is minimal. In patients receiving KD, ketone bodies cross the blood-brain barrier and are used as the energy source of the brain for the replacement of glucose. The main effects of the production of ketone bodies appear to be neurotransmitter modulation and antioxidant effects on the brain.[6,16] The

Table VI. Biochemical findings of the patients before the diet and at the 12th month of the diet.

Biochemical parameters	Before the diet (0.month)		After the diet (12. month)		p^P
	Mean ± S.D.	Min.-Max.	Mean ± S.D.	Min.-Max.	
Fasting blood glucose (mg/dL)	87.05 ± 18.78	69-151	74.37 ± 6.59	62-89	0.031*
Total cholesterol (mg/dL)	161.5 ± 6.59	62-89	190.75 ± 38.46	134-262	0.014*
LDL cholesterol (mg/dL)	91.90 ± 6.41	82.6-97	142.45 ± 31.37	100.8-176	0.019*
HDL cholesterol (mg/dL)	44.44 ± 12.39	22-62	41.12 ± 8.53	25-54	0.133
Triglyceride (mg/dL)	123.33 ± 61.48	40-261	166.50 ± 119.92	65-454	0.205
BUN (mg/dL)	9.93 ± 4.59	1.7-22	8.50 ± 3.93	3-16	0.221
Creatinine (mg/dL)	0.29 ± 0.091	0.13-0.49	0.26 ± 0.081	0.10-0.45	0.115
ALT (U/L)	6 ± 2.94	3-10	8.5 ± 5.80	3-14	0.071
AST (U/L)	23.35 ± 7.63	16-34	16.75 ± 3.5	13-21	0.11
Albumin (g/dL)	4.12 ± 0.095	4-4.2	4.26 ± 0.47	3.64-4.80	0.996
Hemoglobin (gr/dL)	11.93 ± 0.90	11.4-13.5	12.77 ± 1.61	10.8-17.10	0.71
TSH (uIU/mL)	4.53 ± 3.52	1.86-9.4	3.83 ± 1.65	1.9-5.78	0.911
fT4 (ng/dL)	1.47 ± 0.37	1.15-2.01	1.65 ± 0.25	1.42-1.91	0.490

*p<0.05 is significant

[P]Paired samples t-test, SD: standard deviation, (Min)-(Max): minimum-maximum, mg/dL: milligram/deciliter, U/L: units per liter, gr/dL: gram/deciliter, uIU/mL: micro-international units/milliliter, ng/dL: nanogram/deciliter, LDL: low-density lipoprotein, HDL: high-density lipoprotein, BUN: blood urea nitrogen, ALT: alanine transaminase, AST: aspartate transaminase, TSH: thyroid stimulating hormone, fT4: free thyroxine

levels of inhibitory neurotransmitters gamma-aminobutyric acid (GABA) and agmatine were found to be increased, whereas glutamate, the excitatory neurotransmitter, remained unchanged in rat hippocampus following exposure to ketogenic diet.[16] The anticonvulsant effect of KD is also based on the inhibition of the rapamycin pathway.[17] Ketogenic diet is the first treatment option for a number of metabolic diseases, such as glucose transporter type 1 deficiency syndrome (GLUT-1) and pyruvate dehydrogenase deficiency (PDHD), and it has also been shown to be effective in some epilepsy and genetic syndromes, such as myoclonic epilepsy, severe myoclonic epilepsy in infancy (Dravet syndrome), myoclonic-astatic epilepsy (Doose syndrome), West syndromes resistant to corticosteroids and other drugs, Lennox–Gastaut syndrome, Lafora disease, Rett syndrome and Landau-Kleffner syndrome.[18-20] Qiong et al.[7] reported KD to be the most effective treatment for Doose syndrome (100%), and an impact rate of 57.9% was shown on West syndrome. In the present study, similar to the literature, four patients with West, Lennox-Gastaut and Rett syndromes, showed more than a 50% reduction in the rate of seizures after classical KD treatment.

There are four basic forms of KD therapy: classic KD (long-chain triglyceride), MAD, medium-chain triglyceride (MCT) diet and low-glycemic-index (LGI) diet.[21]

In the present study, eight (57.1%) of the 14 patients given the classical KD and two (50%) of four patients who received MAD experienced a 50% decrease in the number of seizures. In the other patients, the duration of seizures reduced. In a Cochrane review of studies conducted between 1966 and 2003, at least 38% of the patients experienced a 50% reduction in seizures when compared to the controls in the third month of KD.[22] Martin-McGill et al.[21] reported seizure-free rates in classical KD patients to reach 55%, and the decrease in seizure rates to reach 85% after three months. Studies evaluating the effectiveness of MAD report seizure-free rates of up to 25% and seizure reduction rates of up to 60% in children. Caraballo et al.[23] reporting the results of the KD treatment of 11 refractory myoclonic-astatic epilepsy patients, identified a seizure reduction of 75–99% in two, 50–74% in two, and 100% in two patients. Güzel et al.[24] administered olive oil-based KD treatment to 389 patients, and in the third month of treatment, a 39.8% seizure-free rate and a 50% reduction in seizures (rate of 34.9%) was recorded. In the same study, 43.1% of 160 patients whose treatment was completed in the 12th month were completely seizure-free, and 40% had experienced a 50% reduction in seizures. In the first year of the diet, a decrease of more than 50% in seizures was found in 10 patients (55.6%), and this difference was statistically significant, while the seizure-free rate was 5.55% (n = 1). A meta-analysis showed no difference in the efficacy of classical KD and MAD in children and adolescents with intractable epilepsy.[2] Poorshiri et al.[25] found the efficacies of classical KD and MAD in the treatment of refractory epilepsy to not be significantly different, although MAD was reported to be more favorable in terms of tolerability and the fewer associated side effects.

Ketogenic diet can also significantly improve the EEG findings of children with epilepsy. In the present study, the initial EEG findings of three of the 18 DRE patients were normal, and two patients had electrical status epilepticus findings. The EEG findings of 10 patients showed a decrease of more than 50% in epileptiform discharges at the 12th month of treatment. In one patient, the seizures decreased by more than 50% in the first six months, but increased again afterwards. The initial EEG findings revealed that epileptic discharges originated from both hemispheres, while completely normal EEG findings were found after the sixth month. Hallbök et al.[26] found that the frequency of interictal epileptic discharges decreased significantly after three months of KD treatment. Dressler et al.[27] reported a significant improvement in the frequency of interictal epileptic discharge and background activity after six months of KD treatment. In another

study, the EEG results of 34 children with DRE were examined, and significant decreases were noted in the epileptic discharge index during sleep and clinical seizures after six months of KD treatment.[28] In the same study, prolonging the duration of KD treatment significantly improved the patients' background activity irregularities, and it was reported that some children may show improvement in motor function, language ability and cognitive function.[29] When we evaluated according to the number of AED, the EEG findings of five patients using three or less AEDs (%62.5) and seven patients using four or more AEDs (%70) showed a decrease of more than 50% in epileptiform discharge. This result is not statistically significant, and further studies are needed in terms of baseline AED number and seizure responsiveness to KD treatment.

Growth may be negatively affected in children receiving KD due to energy and protein restriction. In Peterson et al.[30] the height-for-age Z-scores significantly decreased in children who received KD for 12 months. In a study from Turkey, a decrease of approximately 7% was found in the body weight of children in the third month of KD treatment.[31] Contrary to the literature, a statistically significant increase in body weight was found after one year of KD treatment in children in the present study. Weight loss was observed in only two patients (11.1%). One of the most serious adverse effects of the ketogenic diet is stagnation in height development, which cannot be prevented in long-term use due to its low protein content. Contrary to the literature, a statistically significant increase in body weight was found after one year of KD treatment in children in the present study. However, the yearly growth rates of all patients were analyzed according to their age groups. We found that the annual growth rate of our patients, especially those aged 5-72 months, who had a higher protein requirement, fell behind compared to their age [mean 4.1 cm (2-6 cm). The growth in height in four MAD patients was found to be appropriate for their age groups. Evidence suggests that alterations in the lipid profile, particularly an increase in TC and LDL-C values, may be evident in individuals receiving KD therapy.[3] In the present study, total cholesterol and LDL-C levels increased significantly at the end of the one-year treatment when compared with those at baseline, and fasting blood glucose levels decreased significantly, although remaining within normal limits. There are many doubts about the negative impact of KD on TC, TGs, and lipoprotein concentrations. However, some studies suggest that extreme carbohydrate restriction, which is typical of KD, results in favorable changes in plasma lipid concentrations, with a reduction in TC, LDL-C, and TGs and a rise in HDL-C. Chronic elevations in blood LDL-C and TC concentrations may raise the risk of cardiovascular disease, particularly in adult patients on long-term KD treatment.[32] Before the diagnosis of KD, persons with a higher cardiovascular risk should be screened to ensure that the patient understands the risks and advantages of dietary therapy.

Ketogenic diet causes thyroid dysfunction, and L-thyroxine treatment may be required, meaning that thyroid function should be monitored regularly in epileptic patients treated with KD.[33] Finally, in the present study, KD had no significant effect on thyroid function, hematological parameters, liver function or kidney function. In the study by Güzel et al.[34], the mean cholesterol, LDL-C and TG levels were significantly higher than at pretreatment levels in the first, third, sixth and 12th months of an olive-oil-based KD treatment. The mean BMI SDS and HDL-C levels did not differ significantly between baseline and follow up.

After starting KD, patients should be followed up regularly for side effects. In our study, the most common side effect related to the diet was constipation, while other side effects were halitosis, hypoglycemia, weight loss, metabolic acidosis, diarrhea and nephrolithiasis. The most common side effects of the KD treatment for DRE are vomiting and constipation[21], while other side effects include diarrhea, dysphagia, drowsiness, lower respiratory tract infection,

hyperammonemic encephalopathy, weight loss, nausea, infection, acute pancreatitis, decreased bone matrix density, gallstones, fatty liver, nephrocalcinosis, hypercholesterolemia, status epilepticus, acidosis, dehydration, hypoglycemia, abdominal pain, hypercalcemia and kidney stones.[22] We could not study serum selenium levels in our patients. Since there is an irreversible risk of cardiomyopathy due to selenium deficiency in children receiving ketogenic diet therapy, we evaluated our patients with baseline and 1st-year ECHO examinations, and we did not detect cardiomyopathy in any of the patients.

Ketogenic diet therapy is a reliable treatment approach to the treatment of DRE in children, and can also play a favorable role in cognitive and motor functions in children. Our study findings suggest that KD treatment provides good clinical efficacy against DRE, and can significantly reduce the frequency of epileptic discharges on EEG. The small number of patients can be considered a major limitation of our study, and further research is needed to evaluate the long-term effects of KD. Finally, KD therapy should not be considered as the final option for patients whose seizures cannot be controlled with medication, but it should be started early, especially in patients with epileptic syndromes.

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Ethical approval

Ethical approval for the research was obtained from the Ethics Committee of Eskisehir Osmangazi University, Medical Faculty (Dated 12.05.2020, number: 25403353-050.99-E.51610, decision number: 18), and the study was conducted in accordance with the Declaration of Helsinki.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: GKY, MY; data collection: GKY, MY, AÇU; analysis and interpretation of results: HÖ; draft manuscript preparation: GKY, CY, KBÇ. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Original Article

Evaluation of children diagnosed with a lower respiratory tract infection due to Human metapneumovirus

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ABSTRACT

Background. Human metapneumovirus (hMPV) is one of the leading causes of acute respiratory infections and bronchiolitis in infants. A history of prematurity and chronic diseases such as congenital heart disease or asthma/reactive airway disease (RAD) increases the risk of severe lower respiratory tract infection (LRTI) due to hMPV. In this cross-sectional study, we aimed to analyze the clinical outcome and risk factors for severe disease in children with LRTI due to hMPV.

Methods. The current cross-sectional study included children between 28 days and 18 years of age with the diagnosis of hMPV-associated LRTI hospitalizations, over two years from January 2016 to September 2018 in Health Science University Dr. Behçet Uz Child Disease and Pediatric Surgery Training and Research Hospital. hMPV virus was detected by the multiplex polymerase chain test (PCR) (Commercial Multiplex Real-Time PCR: FTD Respiratory 21 plus, Fast Track Diagnostics, Luxembourg) from a nasopharyngeal swab. Patients who had positive results in multiplex PCR tests with other viral agents simultaneously were not included in the study. Data were retrospectively collected from the computerized hospital system.

Results. In this cross-sectional study, 62 patients who were hospitalized with the diagnosis of LRTI due to hMPV infection were included. Thirty-five (55.7%) of the patients were male. The median age was one year (2 months-15 years). Fifty-one (82.2%) patients were younger than two years. The median hospital length of stay was found to be 10 days (2-33 days) in patients with an underlying disease and 7,5 days (ranging from 2 to 20 days) in the patients without an underlying disease, this difference was significant (p=0.031).

Conclusions. Clinicians should consider hMPV as an important pathogen of LRTI even in healthy children, although we expect a poor course of disease in children with an underlying disease.

Key words: human metapneumovirus, lower respiratory tract infection, hospitalized, length of stay.

Human metapneumovirus (hMPV) is an enveloped, single-stranded, negative-sense RNA virus that was first discovered in 2001 and categorized in the genus Metapneumovirus of the family Paramyxoviridae.[1,2] The hMPV season occurs during the late winter and early spring months in temperate locations and overlaps with those of Respiratory Syncytial Virus (RSV) and influenza viruses.

hMPV causes acute respiratory tract illnesses, such as pneumonia, asthma exacerbations or croup in people of all ages and also it is one of the leading causes of bronchiolitis in infants.[2] The proportion of hMPV detected in nasopharyngeal samples from children with unexplained respiratory infections has varied from 1.5 to 25%.[1-3]

The clinical presentations in children with hMPV infection range from mild upper

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respiratory tract disease to severe bronchiolitis and pneumonia, similar to RSV infection, Moreover, very young hMPV-infected children may require hospitalization and mechanical ventilation.[1,3] A history of prematurity, chronic diseases such as congenital heart disease or neuromuscular disorders, and pulmonary diseases including asthma/reactive airway disease (RAD) increase the risk of severe lower respiratory tract infection (LRTI) due to hMPV.[4-8]

This study was conducted for evaluating the severity, length of hospital stay, and outcome of hMPV infections. We specifically aimed to analyze the clinical outcome and risk factors for severe disease in children with LRTI due to hMPV.

Material and Methods

The current cross-sectional study included children between 28 days and 18 years of age with the diagnosis of hMPV-associated LRTI hospitalizations, over two years from January 2016 to September 2018 in Health Science University Dr. Behçet Uz Child Disease and Pediatric Surgery Training and Research Hospital. This hospital is a referral center for pediatric patients in the Aegean Region of Turkey, with an annual 600.000 outpatients and approximately 24.000 hospitalizations in 2018. We evaluated a total of 102 hMPV cases during the study period.

Multiplex PCR for hMPV

Aspirate samples from each nostril and one nasopharyngeal swab were obtained in the supine position with the head positioned at the midline. Within a maximum period of 4 hours after collection, the samples were mixed and added to a Ringer lactate solution to a total of 4 mL. After homogenization, approximately 1 mL were separated into aliquots in cryotubes and stored at -80 °C. The DNA/ RNA was extracted from samples using an extraction kit (RTP DNA/RNA Virus Mini Kit, Stratec Molecular, Germany). The sensitivity and specificity were monitored by standard quality control for molecular diagnostics. The qualitative detection of 20 respiratory viruses including influenza [IF A, H1N1, and B], coronavirus [CoV 43, 63, 229, and HKU], parainfluenza [PI1, PI2, PI3, and PI4], human rhinovirus [HRV], respiratory syncytial virus [RSV A/B], human metapneumovirus [hMPV A/B], adenovirus [AdRV], enterovirus [EV], parechovirus, and human bocavirus [hBoV] were done by real-time multiplex PCR (FTD, Fast Track Diagnostics, Belgium).

A total of 102 patients were included in the initial analysis. Nine patients with missing data and 31 patients co-infected with other viral agents were excluded from the study. We included cases if they were hospitalized and had laboratory-confirmed h-MPV by PCR at presentation or during their hospital stay. All participants had an acute LRTI with symptoms of cough, shortness of breath, wheezing, or tachypnea plus consolidation recorded on a chest X-ray. Patient files were recorded from the computerized hospital system.

Statistical Analysis

Statistical analysis was performed using SPSS statistical software (version 22; SPSS, Chicago, IL, USA). Student's t-test was used to compare continuous parametric variables, the Mann-Whitney U test was used to compare continuous nonparametric variables, and $\chi 2$ or Fisher's exact tests were used for categorical variables when appropriate. A two-tailed p-value of < 0.05 was considered to be statistically significant.

Ethical approval was received for this research from Dr. Behçet Uz Child Disease and Pediatric Surgery Training and Research Hospital Ethics Committee. The patients or legal guardians of the participants provided signed informed consent forms for inclusion in the study.

Results

In this cross-sectional study, 62 patients who were hospitalized with the diagnosis of LRTI due to hMPV infection were included. Thirty-

five (55.7%) of the patients were male, and 27 (44.3%) were female. The median age was one year (ranging from 2 months to 15 years). Forty-one patients (66.1%) were younger than one year, and 51 (82.2%) patients were younger than two years.

Thirty patients (48.4%) had an associated underlying disease, and the most common one was allergic diseases (n=9, 30%) followed by neurological diseases including cerebral palsy (n=7, 23.3%), prematurity (n=5, 16.6%), congenital heart diseases (n=3, 10.0%), immunodeficiency (n=3, 10.0%), malignancies (n=2, 6.6%) and a genetic syndrome (Table I).

The median length of stay (LOS) of the patients was eight days (ranging from 2 to 33 days). The median length of stay was found to be ten days (ranging from 2 to 33 days) in patients with an underlying disease and 7.5 days (ranging from 2 to 20 days) in the patients without, this difference was statistically significant (p=0.031). The relationship between the LOS (measured by days) and age (measured by months) was investigated using the Spearman correlation coefficient. There was no significant correlation between the two variables (r = -.138, n = 62, p=0.283). The median length of stay was not significantly different between male and female patients (8 days, 3 to 33 days, vs 8 days, 2 to 17 days. p>0.05). A total of 12 (9.2%) patients required respiratory support, including 2 mechanical ventilation and intubation, 9 high-flow nasal cannulas (HFNC) and 1 continuous positive airway pressure (CPAP). Among these twelve patients who were transferred into the intensive care unit because of severe respiratory failure, nine patients (75%) had an underlying disease. The rate of patients requiring mechanical support (invasive and non-invasive support) within the patients with an underlying disease was significantly higher compared to the rate of mechanical support in the previously healthy group (p=0.04).

Table I. General features of patients.

Gender	n (%)
Female	27 (44.3)
Male	35 (55.7)
Years	n (%)
<1 year	41 (66.1)
<2 years	51 (82.2)
> 2 years	11 (17.7)
Underlying diseases	n (%)
Allergic diseases	9 (30)
Congenital heart disease	3 (10)
Median length of stay in the hospital	Day
Underlying diseases	10
No underlying diseases	8

Discussion

In our study, the absence of an underlying disease in approximately half of the patients showed that hMPV was a cause of severe pneumonia that required hospitalization even in healthy children. The other remarkable finding is that the duration of hospitalization was longer in patients with an underlying disease. In reviewing the literature, hMPV is a relatively newly recognized pathogen, but there are many studies on this subject. Since the initial description of hMPV in 2001, hMPV has been reported in many studies as a low respiratory tract infection pathogen in different countries around the world and the rate varies between 2.2 and 9%.[2,9-13]

Although hMPV can be seen at any age, including the adult age group, it is more common, especially before 5 years of age and has a more severe clinical course in this age group. According to the results of this study, the mean age of the patients was 1 year (2 months-15 years) and, a total of 51 (82.2%) patients were <2 years of age. In previous studies, the median age of the children ranged from 6 months to 3 years.[7-9,13-16] Heikkinen et al.[14] found that the incidence rates of hMPV decreased gradually with age.[14] Approximately 80% of cases were reported to be under 5 years of age.[14,17] However, the more intensive accumulation in age is present under 2 years of age which is supported by our findings.

The duration of hospitalization of patients varies, and having certain risk factors has an impact on this duration. Previous studies have shown that the median duration of LOS ranged from 3 days to 7 days.[7,8,11-13] Trenholme et al.[12] detected hMPV as a causative agent in 7% of the patients who were admitted to the hospital with a LRTI and hMPV was associated with increased LOS.[12] In this study, the duration of hospitalization was compared between the patients with and without an underlying disease. The LOS was longer in patients with an underlying disease compared to the patients without an underlying disease (median 10 vs 8 days, p = 0.046). This finding supports the information in the literature. Similarly, Han et al.[7] reported that the LOS of previously healthy patients was significantly shorter than those with risk factors.[7]

There are well-defined risk factors for hMPV, and these include asthma, prematurity, congenital heart disease. In these cases, the course of the disease is more severe. In the study by Pancham et al.[18], children with a history of prematurity had more severe hMPV disease regardless of age, ethnicity or asthma history.[18] Prematurity was identified as a risk factor for hospitalization due to hMPV infection in previous studies.[5-7,13,15,19] In our study, 30 patients (48.4%) had an associated underlying disease, and the most common one was allergic diseases (n=9, 30%) followed by cerebral palsy (n=7, 23.3%), prematurity (n=5 16.6%) and congenital heart diseases (n=3, 10.0%). The median LOS was found significantly higher in patients with an underlying disease (p=0.031). The other remarkable point, the rate of patients requiring mechanical support (invasive and non-invasive support) was significantly higher among the patients with an underlying disease, compared to the rate of mechanical support in the previously healthy group (p=0.04).

This study has limitations due to its retrospective design. First of all, data, including patients with hMPV and possible underlying disease, were collected retrospectively from the medical files, and microbiology laboratory. Secondly, our sample size, especially for the patients requiring respiratory support and follow up in the intensive care unit, was small. However, to the best of our knowledge, this is one of the few studies focusing on hMPV infections in children. Additionally, our data is largely consistent with the literature. Most of the patients in this study were under two years of age, and the duration of hospitalization increased in patients with an underlying disease.

In this study, we found that approximately half of the hospitalized patients with LTRI due to hMPV did not have an underlying disease. This finding indicates that hMPV is an important pathogen of LRTI also in healthy children. On the other hand, the duration of hospitalization was longer in patients with an underlying disease. Therefore, clinicians should consider hMPV as an important pathogen of LRTI even in healthy children, while we should expect a poorer course of the disease in children with an underlying disease.

Ethical approval

This study was approved by the Ethical Committee of Dr. Behçet Uz Child Disease and Pediatric Surgery Training and Research (approval number: 462; 2020/14-05, approval date: 08.10.2020).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: NB, İD; data collection: EK, EB, İÇ, YS, FG, ÇÖE, ÖBS, HA, TÇ; analysis and interpretation of results: NB, İD, EK; draft manuscript preparation: EK. 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

Identification of candidate genes in a family with cancer overload by whole-exome sequencing

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ABSTRACT

Background. Approximately 120 out of every 1 million children in the world develop cancer each year. In Turkey, 2500-3000 children are diagnosed with new cancer each year. The causes of childhood cancer have been studied for many years. It is known that many cancers in children, as in adults, cause uncontrolled cell growth, and develop as a result of mutations in genes that cause cancer.

Methods. The investigation of family history within this context in the study, a total of 13 individuals consisting of all children and adults in the family were examined using the whole-exome sequencing (WES) with the individuals who were diagnosed with cancer in the family, who were detected to have different cancer profiles, and defined as high risk and to determine the gene or genes through which the disease has developed.

Results. At the end of the study, a total of 30 variants with a pathogenic record in the family were identified. A total of 10 pathogenic variants belonging to 8 different genes from these variants have been associated with various cancer risks.

Conclusions. A significant scientific contribution has been made to the mechanism of disease formation by studying a family with a high cancer burden and by finding the genes associated with the disease. In addition, by the results obtained, family members with cancer predisposition were selected after a risk analysis conducted in this family, and the necessary examinations and scans were recommended to provide an early diagnostic advantage.

Key words: cancer, gene mutation, candidate genes, whole exome sequencing.

Cancer is a disease known to be caused by the accumulation of different genetic changes in a cell over the years. These changes lead to abnormal cell proliferation and clonal expansion, which can eventually invade other tissues. In most cases, genetic changes that promote tumor formation occur in somatic cells and do not involve germline mutations.[1]

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A large number of genes that cause tumor development have been identified and classified into 3 different categories: tumor suppressor genes, proto-oncogenes, and genes involved in genome stability. Tumor-suppressor genes control cell proliferation, inhibit the progression of the cell cycle, or induce apoptosis. Generally, a single functional copy of the gene is sufficient for the development of cancer. Inactivation of both alleles allows uncontrolled proliferation and thus contributes to the development of tumors. On the contrary, proto-oncogenes promote cell proliferation and contribute to tumor progression when they are permanently activated as a result of mutations. In such a

case, mutations in a single allele are sufficient for uncontrolled reproduction. Genes involved in DNA stability do not play a direct role in the regulation of cell proliferation, however, the dysfunction in these genes contributes to an increased number of mutations and consequently, to an increased likelihood of tumor development.[2]

Childhood cancers were detected in 11.5 per 100,000 children in 1975, and at a rate of 14.8 in 2004.[3] This means that about 150 out of every 1 million children worldwide will develop cancer before the age of 20 years.[4] The incidence rates of childhood cancers are highest in children aged 0-4 years.[5] The causes of childhood cancer have been studied for many years. Although the genetic basis of childhood cancer is currently not fully elucidated, the immune system and exposure to environmental factors are suggested to have a significant contribution. The hereditary syndromes caused by the high-penetration germline DNA mutations[6,7], chromosomal aneuploidy[8] or epigenetic disorders[9] are known to account for 5-10% of childhood cancers.[10] It is believed that many cancers that occur in children, as in adults, are suggested to develop as a result of uncontrolled cell growth and ultimately due to the mutations in the genes that lead to cancer. In adults, these gene mutations reflect the cumulative effects of aging and long-term exposure to cancer-causing substances. However, it is difficult to identify the potential environmental causes of childhood cancer.

Overall, a hereditary predisposition to cancer is suspected in cases such as families with 2 or higher number of relatives with the same cancer type on the same side of the family, with multiple primary cancer individuals, the emergence of different types, presence of the genetically associated cancers (such as breast and ovarian cancer or colon, and uterine cancer), in increased bilateral or multifocal prevalence in contrast to unilateral involvement, and with the emergence of malignant changes in the same individual or the family.[11]

The application of new gene sequencing techniques in children with cancer allows us to expand our view of the molecular basis of childhood tumors. Unlike conventional molecular techniques, high-productive techniques such as large sequencing or next-generation sequencing (NGS) can sequence millions of DNA fragments, at ever-decreasing costs and in less time. They can also detect different types of genomic changes with a single test.[12]

In a recent study, 1120 cancer patients aged 0-19 years were screened with next-generation sequencing, and germline mutations that cause a predisposition to cancer, which were detected as 1% in the control group, were observed in 8.5% of pediatric patients.[13] Pediatric cancer patients having germline mutations at a higher rate than the general population which raises some questions: Is developing routine genetic screening of newborns to identify patients at risk ethical and cost-effective? Is it possible to track all these patients? Can they benefit from early diagnosis? The answer to all these questions can be found by studying the genes that affect the formation of diseases in families with a high cancer burden at the entire genome level and clinically evaluating the obtained results.

Genetic features are known to be the most important cause of cancer formation, especially in childhood tumors. The transmission of the disease, which is genetically inherited, to the next generation is also inevitable. In addition, the risk of developing a secondary tumor is also significantly increased. Therefore, the early diagnosis of childhood cancers which are known to have high genetic inheritance, and demonstrating whether they showed genetic structure are highly important for the patient's own life, the quality of life, and for the next generation.

When family history is examined, it is very difficult to determine which gene or genes cause the disease in individuals diagnosed with cancer in families which were identified as having many and different types of cancer,

and defined as high-risk. Therefore, panel gene testing applications are needed that allow the investigation of multiple genes in the study of genes that may be the cause of disease in these families. Our goal in the project is to identify candidate genes that may affect the incidence of cancer in a family with individuals that were diagnosed with different cancers and are considered as high-risk. For this purpose, a total of 13 individuals diagnosed with cancer in the family, and who were considered at high risk were examined using the whole-exome sequencing, and high-risk genes were detected in this family.

Material and Methods

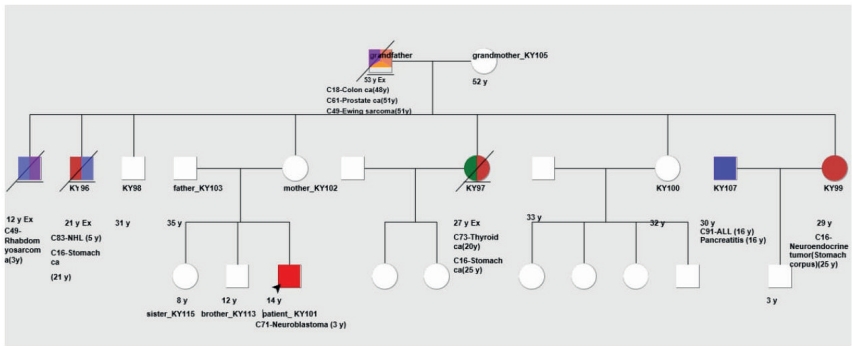
The whole-exome sequencing (WES) analyses were performed from the peripheral blood samples of 13 individuals who were selected after genetic counseling from patients with childhood cancer diagnoses with high-risk cancer profiles, and from the family members who presented to Istanbul University Institute of Oncology, Department of Clinical Oncology, Pediatric Hematology-Oncology Unit. Patient consent was obtained from all the patients included in the study that they accepted the test, and this study was approved by the Ethics Committee of İstanbul University.The data of the pediatric patient and the family members is shown in Table I. The disease codes and descriptions of the family was shown in Figure 1.

After receiving the approvals for the project, about 10 ml of peripheral blood was taken into the EDTA tube, and lymphocyte cells were isolated using the Fikol (Sigma-Aldrich, Germany) method.[14] DNA was isolated from lymphocytes obtained from blood samples using the QIAamp DNA mini kit (Qiagen, Germany) following the manufacturer's instructions.[15] First, the genomic DNA was measured with a Qubit fluorimeter (Life Technologies), and then the genomic DNA concentration was adjusted to be 10 ng/μL using a 10 mM pH of 8.5 Tris-HCl. Fluorometric measurement was repeated, and the same buffer solution was readjusted to obtain the concentration as 5 ng/μL, and 50 ng was made ready for use in sequencing. The obtained genomic DNA samples were stored in the -80 device or nitrogen tanks until the whole-exome sequencing process was performed.

Table I. The clinical features of the proband, and other family members.

Family ID	Patient no.	Gender	Cases	Last Status	Consanguinity
F1	KY96	Male	NHL (5y) + Stomach ca (21y)	Ex (21y)	Maternal-Uncle
F1	KY97	Female	Tyroid ca (20y) +Stomach ca (25y)	Ex (27y)	Maternal-Aunt
F1	KY98	Male	Healthy	Alive	Maternal-Uncle
F1	KY99	Female	Neuroendocrine tumor (Stomach corpus) (25y)	Alive	Maternal-Aunt
F1	KY100	Female	Healthy	Alive	Maternal-Aunt
F1	KY101	Male	Neuroblastoma (3y)	Alive	Proband
F1	KY102	Female	Healthy	Alive	Mother
F1	KY103		Healthy	Alive	Father
F1	KY105	Female	Healthy	Alive	Maternal-Grandmother
F1	KY107	Male	ALL (16y) + Pancreatitis (16y)	Alive	Brother in law (Husband of KY99)
F1	KY108	Male	Healthy	Alive	Maternal-Cousin (Child of KY99)
F1	KY113	Male	Healthy	Alive	Brother
F1	KY115	Female	Healthy	Alive	Sister

NHL: non-hodgkin lymhoma, ALL: acute lymphoblastic leukemia.



Disease Codes and Descriptions

C16- Stomach malignant neoplasm* Stomach ca/Neuroendocrine tumor (Stomach Corpus)

C18- Colon malignant neoplasm* Colon ca

C49- Other malignant neoplasm of connective tissue and soft tissue* Rhabdomyosarcoma/Ewing sarcoma

C61- Prostate malignant neoplasm* Prostate ca

C71- Brain malignant neoplasm* Neuroblastoma

C73- Malignant neoplasm of thyroid gland* Thyroid ca

C83- Non-Hodgkin lymphoma(NHL)

C91- Acute Lymphoblastic Leukemia(ALL)

Fig. 1. The pedigree of the F1 family.

Whole exome sequencing is a DNA sequencing strategy that allows the investigation of base matches in genomic coding regions and other interested regions. Since the encoded part of the genome covers only 1-2% of the entire genome, this approach is preferred because it is a more cost-effective strategy for detecting DNA changes that can change protein function compared to the whole genome sequence. Although the research community reveals and identifies the functional effects of sequence changes in non-coding regions of the genome, WES is a test that provides valuable information for both exploratory research and precision medicine applications.[16] The whole-exome sequencing process was performed on the GenoXome_MGISeq G-400 Platform. According to the panel protocol, DNA was fragmented after DNA quality determination was made. Later, adapters were connected to this fragment with the ligation process to the DNA. For the sequencing quality to be at the optimum level, non-specific DNA was removed by purification. Then, to obtain the amplified DNA, the marked library was replicated by a 10-cycle PCR process. The enriched library was loaded into the "Flow Cell" before being put on the device, and the "Flow Cell" loaded with the sample was placed in the HiSeq device for the sequencing process.

The raw data in the BCL format obtained from the device was primarily converted to the VCF file format. The resulting raw data were examined using the computer programs such as VarAFT (https://varaft.eu/) and VariED (http://varied.cgm.ntu.edu.tw/) in accordance with the filter and quality control scores, and then genomic changes that are seen to exist in gene regions were evaluated. The Variant Effect Predictor (VEP) is a central source for the annotation of transcript results. VEP also uses the databases such as NCBI Reference Sequence Database (RefSeq) and algorithms such as Polymorphism Phenotyping (PolyPhen) and SIFT. Information

about the known disease relationship was obtained from the Catalog of Somatic Mutations in Cancer (COSMIC), from the ClinVar database and Online Human Mendelian Inheritance (OMIM) catalog. The resources such as dbSNP, Ensembl 1000 Genome Project and the Exome Variant Server provide information about the occurrence and frequencies of variants within a population. As a result, the descriptions of the variants indicated in all relevant databases and algorithms were obtained. Various filtering options were also used to determine the relationships of the annotation-treated variants with the phenotype. In particular, variants with a pathogenic record of the Clinvar were examined in detail. Variants that have not been previously reported in the literature or databases were defined as candidate (novel) variants. The variables obtained in the study were evaluated considering those with a Q30 quality score above 80%. According to the emerging pathogenic variants, a detailed clinical report was prepared for each person and was given accompanied by genetic counseling.

Furthermore, Copy number variations (CNVs) were evaluated with the CNVkit tool using WES data. We used WES data of 13 individuals, who have high-risk cancer load in the family. The Likely pathogenic/Pathogenic deletion/duplication were included in the study whereas Uncertain significance/Benign deletion/duplication were excluded. The reference Genome HG38 were used to analyze the CNV data.

Results and Discussion

At the end of the study, a total of 30 variants with a pathogenic record in the family were identified. A total of 10 pathogenic variants belonging to 8 different genes from these variants have been associated with various cancer risks. These pathogenic gene mutations, and individuals detected with these gene mutations, and the records of the clinical database of these mutations are shown in Table II.

Table II. The pathogenic gene mutations detected in F1 family and ClinVar records.

Family ID	Cases	Mutation	ClinVar_Associated Diseases
F1	KY96, KY97, KY98, KY99	FCN3 (NM_003665), Exon5, HET, c.349delC, p. (Leu117Serfs*65)	Immunodeficiency_due_to_ficolin_3_deficiency
F1	KY96, KY97, KY98, KY99, KY100, KY102, KY107, KY108	KLKB1 (NM_000892), Exon5, HET, c.428G>A, p. (Ser143Asn)	Prekallikrein_deficiency
F1	KY96, KY97, KY98, KY100, KY101, KY102, KY105, KY113, KY115	C7: NM_000587: exon12:c.C1561A: p.R521S	Complement_component_7_deficiency|C7_and_C6_deficiency, combined_subtotal
F1	KY96, KY97, KY98, KY100, KY101, KY102, KY103, KY113	IRGM (NM_001145805), Exon2, HET, c.313C>T, p. (Leu105Leu)	Inflammatory_bowel_disease_19
F1	KY96, KY97, KY98, KY99, KY100, KY102, KY105, KY107, KY113, KY115	PRSS1 (NM_002769), Exon2, HET, c.86A>T, p. (Asn29Ile)	Hereditary_pancreatitis|not_provided
F1	KY99, KY101, KY102, KY103, KY107, KY108, KY115	PRSS1 (NM_002769), Exon2, HET, c.161A>G, p. (Asn54Ser)	Hereditary_pancreatitis
F1	KY96, KY102, KY107, KY108, KY115	MBL2 (NM_000242), Exon1, HET, c.161G>A, p. (Gly54Asp)	Mannose-binding_protein_deficiency
F1	KY98, KY99, KY105	MBL2 (NM_000242), Exon1, HET, c.154C>T, p. (Arg52Cys)	Mannose-binding_protein_deficiency
F1	KY96, KY97, KY99, KY100, KY105, KY108	PTPRJ (NM_001098503), Exon5, HET, c.827A>C, p. (Gln276Pro)	Carcinoma_of_colon
F1	KY101, KY103, KY107, KY108	FGFR4 (NM_001354984), Exon9, HET, c.1162G>A, p. (Gly388Arg)	Cancer_progression_and_tumor_cell_motility

In our study, **FCN3 (NM_003665), Exon5, HET, c. 349delC, p. (Leu117Serfs*65)** variant were detected in individuals with the codes KY96, KY97, KY98, and KY99. This variant is registered in the ClinVar database as: RCV000005603.6 (Uncertain significance*- Immunodeficiency due to ficolin 3 deficiency). In the dbSNP database, it is registered as rs532781899 (MAF/MinorAlleleCount:-=0.019/2- Clinical significance: CLIN_pathogenic). This variation creates a frameshift starting from the Leu106 codon. The new reading frame creates a STOP Codon at position 65. Ficolin-3 (FCN3), is a circulating model recognition molecule of the lectin pathway that participates in host immune responses against cancer.[17] Ficolin-3 is mainly synthesized in the liver and lung and is known to participate in both systemic and local natural immune responses.[18] Recent studies suggested that the FCN3 gene may be an indicator of ovarian and prostate cancer.[19,20] Also, FCN3 was reported to have a distinctive potential biological marker role in leukemia aggressiveness.[21] H-ficolin(FCN3) serum concentration in pediatric cancer patients is shown to be effective in susceptibility to fever and neutropenia.[22] FCN3 has an important role in the innate immune response to infections, and there is convincing evidence that its deficiency is associated with a predisposition to infections and autoimmunity.[23] Considering family members with a high burden of cancer, detection of **FCN3 (NM_003665), Exon5, HET, c.349delC, p.(Leu117Serfs*65)** variant in FCN3 gene in four siblings, and three of which have been diagnosed with many different types of cancer were striking. In addition, according to the segregation assessment, this variant was not found in the mother. Their father was diagnosed with colon ca+prostate ca+ewing's sarcoma and died at the age of 55 years. Therefore, genetic testing could not be performed on the father, however, considering the intensive clinical condition of the father, it is assumed that this variant was most likely transferred from the father. The reason why this variant has not been detected in a patient with the code KY101 diagnosed with cancer in the family is that his mother and father do not carry this variant. The evaluation of the results found in the light of clinical data showed that the **HET, c. 349delC, p. (Leu117Serfs*65)** variant detected in the FCN3 gene was suggested to have been associated with cancer aggressiveness.

As a result of the study, individuals with the codes KY96, KY97, KY98, KY99, KY100, KY102, KY107, and KY108 were found to have the **KLKB1 (NM_000892), Exon5, HET, c. 428G>A, p. (Ser143Asn)** variant. In the Clinvar database was indicated as RCV000012817.26 (conflicting interpretations of pathogenicity- Prekallikrein deficiency), and in the dbSNP database as rs3733402 (MAF/MinorAlleleCount: G=0.395/783- Clinical significance: CLIN_ pathogenic). KLKB1 secretes bradykinin by cutting the plasma kallikrein enzyme, the Lys-Arg and Arg-Ser bonds in the kininogen and has functions related to blood clotting, fibrinolysis, hemostasis, and inflammatory response.[24] In various databanks, **KLKB1 (NM_000892), Exon5, HET, c.428G>A, p. (Ser143Asn)** variant was reported to be associated with Prekallikrein deficiency.[25] In the investigation of the association of the KLKB1 gene with cancer, it was reported that its expression increases when treated with the demethylating agent 5-azacytidine in lung cancer cells, and therefore it can act as a tumor suppressor gene.[26] PSA, which is used as a biomarker to detect prostate cancer and reduce cancer deaths, is a protein product copied from the KLKB1 gene and is secreted by the epithelial cells of the prostate.[27] Considering the cancer history of the family, although four of the eight individuals with whom the variant was detected were diagnosed with cancer, none of them had lung or prostate cancers. Additional studies are required in the wider patient and control groups for clearly understanding the clinical effect of **KLKB1 (NM_000892), Exon5, HET, c.428G>A, p. (Ser143Asn)** variant.

Within the scope of the study, people with the codes KY96, KY97, KY98, KY100, KY101, KY102, KY105, KY113, and KY115 were detected to have the variant **C7 (NM_000587),**

Exon12, HET, c.1561C>A, p. (Arg521Ser). In the ClinVar database it was registered as RCV000788233. 1 (Pathogenic-Complement component 7 deficiency) and in the dbSNP database it is registered as rs121964920 (MAF/MinorAlleleCount: A=0.001/0- Clinical significance: CLIN_pathogenic). It is associated with complement C7 deficiency in various databases.[28,29] The complement system connects innate immunity to adaptive immunity. The complement system is an important component of the inflammatory response and is involved in various stages of inflammation, tumorigenesis, and cancer progression.[30] Activation of the complement regulates the adaptive immune response and is involved in the regulation of the T cell response in tumors.[31,32] Complement deficiency disrupts both B and T cell responses.[31] The inflammation that stimulates the tumor has an important role in carcinogenesis and cancer progression.[33] Various studies reveal that complement system activation is an important component of tumor-stimulating inflammation.[32,34] When the distribution of variants in the family was evaluated, this variant was found in five of the six siblings. Two of these siblings were diagnosed with cancer. This variant was detected to be transferred from the healthy mother. Also, it has been described in a patient diagnosed with cancer from the third generation. This variant was detected to be transmitted from the mother to both the sick child and to his two healthy siblings. When the variant was evaluated considering the clinical data, the variant could not be directly associated with the pathogenesis of the disease. There is a need for additional studies for understanding the association of **C7 (NM_000587), Exon12, HET, c.1561C>A, p. (Arg521Ser)** variant, and clinical findings.

Within the scope of the study, individuals with the codes KY96, KY97, KY98, KY100, KY101, KY102, KY103, and KY113 were detected to have the **IRGM (NM_001145805), Exon2, HET, c. 313C>T, p. (Leu105Leu)** variant. In the Clinvar database it is regşstered as RCV000023694.2 (Pathogenic- 19 inflammatory bowel disease (Crohn's disease (CD)), in the dbSNP database it is registered as rs10065172 (MAF/MinorAlleleCount: T=0.304/462- Clinical significance: CLIN_pathogenic). Crohn's disease (CD) is a common form of chronic inflammatory bowel disease. The genetic evidence associated with the **IRGM (NM_001145805), Exon2, HET, c. 313C>T, p. (Leu105Leu)** indicates that there are defects in the early immune response in the pathogenesis of CD, in particular in the innate immune pathways and the processing of intracellular bacteria. With the conducted IRGM expression analysis, it was noted that it shows transcription in many tissues, including the colon, small intestine, and peripheral blood leukocytes.[35] Only the immunity-related guanosine triphosphatase family M (IRGM) gene out of three IRG genes located in the human genome (IRGC, IRGQ, and IRGM) encodes a functional IRG.[36] Immunity-associated guanosine triphosphatase has critical importance in defense against pathogens by regulating the progression of autophagy.[37] Autophagy is an "autodigestive" process that plays an important role in the enabling of intracellular components for terminal degradation and recycling.[38] This process has been associated with various aspects of innate and adaptive immunity and has a role in many autoimmune diseases such as the abnormalities in the autophagy pathway, rheumatoid arthritis, systemic lupus erythematosus (SLE).[39] IRGM plays an important role in autophagy. IRGM genetic polymorphisms were confirmed to have been associated with many types of inflammatory, and autoimmune diseases. Also, **IRGM (NM_001145805), Exon2, HET, c. 313C>T, p. (Leu105Leu)** variant is known to be associated with autoimmune thyroid disease (AITD).[40] Recent research has suggested that autophagy may play a critical role in tumorigenesis. The GTPase family M (IRGM) associated with immunity, is a human protein that has been highlighted for its contribution to autophagy on inflammation, and infections. Studies have shown that IRGM plays a role in the development of several cancers. One study reported that the genetic polymorphisms in

IRGM were associated with a predisposition to stomach cancer.[41] In another study, a positive correlation was reported between IRGM upregulation, and IRGM levels in stomach cancer tissues and cancer stages, and the IRGM gene might have a role in the pathogenesis of stomach cancer and may reflect the progression of the disease.[42] The evaluation of the clinical data of the family including all this information, the detection of (KY96, KY97) IRGM gene mutation in 2 siblings who have been diagnosed with stomach cancer out of 3 siblings was noteworthy. However, the fact that this gene mutation, which was also detected in a patient with the code KY101, was seen in both his healthy mother and his healthy father raises doubts about the pathogenicity of this variant. Additional studies should be conducted to understand the level of contribution of **IRGM (NM_001145805), Exon2, HET, c. 313C>T, p. (Leu105Leu)** variant to the process of cancer formation.

PRSS1 (NM_002769), Exon2, HET, c. 86A>T, p. (Asn29Ile) variant was detected in individuals with the codes KY96, KY97, KY98, KY99, KY100, KY102, KY105, KY107, KY113, and KY115; and **PRSS1 (NM_002769), Exon2, HET, c. 161A>G, p. (Asn54Ser)** variant was detected in individuals with the codes KY99, KY101, KY102, KY103, KY107, KY108, and KY115. In the ClinVar database **PRSS1 (NM_002769), Exon2, HET, c.86A>T, p.(Asn29Ile)** variant is registered as RCV000763166.1 (Pathogenic* - hereditary pancreatitis), in dbSNP database it is registered as rs111033566 (dbSNP entry validated Clinical significance: CLIN_pathogenic). Hereditary pancreatitis (HP) is a genetic disease in which the risk of pancreatitis and pancreatic cancer can be passed from generation to generation in a family. The most commonly associated gene with HP is the PRSS1 gene. The mutations in the PRSS1 gene create an increased risk of pancreatitis and pancreatic cancer in the individual.[43] The assessment of the family history showed that it is worth noting that **PRSS1 (NM_002769), Exon2, HET, c. 86A>T, p. (Asn29Ile)** variant was found in all 6 siblings. Three of these individuals had various cancer diagnoses, however, there were no pancreatic cancer diagnoses. Perhaps because two of the three siblings diagnosed with cancer had died at an early age, pancreatic cancer had not yet been detected by that date. The fact that this variant, which is also found in other siblings, was transferred from their 53-year-old healthy mother has raised doubts about the pathogenicity of this variant. Further studies are required on this topic. **PRSS1 (NM_002769), Exon2, HET, c.161A>G, p. (Asn54Ser)** variant was detected only in patients with the codes KY99 and KY101 who were diagnosed with cancer. It is noteworthy that this variant was detected both in the mother and father of the patient with the code KY101. Therefore, it raises doubts about its pathogenicity property. Further studies are needed on this issue.

MBL2 (NM_000242), Exon1, HET, c.161G>A, p. (Gly54Asp) variant was detected in individuals with the codes KY96, KY98, KY99, KY102, KY107, KY108, and KY115, however **MBL2 (NM_000242), Exon1, HET, c.154C>T, p. (Arg52Cys)** variant has been detected in the individuals with the code KY105. **MBL2 (NM_000242), Exon1, HET, c.161G>A, p.(Gly54Asp)** variant in Clinvar database is registered as RCV000015424.24 (Pathogenic - Mannose-binding protein deficiency), and as rs1800450 (MAF/MinorAlleleCount: T=0.122/75 - Clinical significance: CLIN_pathogenic) in dbSNP database. **MBL2 (NM_000242), Exon1, HET, c.154C>T, p. (Arg52Cys)** variant in Clinvar database is registered as RCV000015426.28 (Pathogenic*-, mannose-binding protein deficiency), and in the dbSNP database as rs5030737 (MAF/MinorAlleleCount: A=0.027/4- Clinical significance: CLIN_pathogenic). Mannose-binding lectin deficiency (MBL) is a condition that affects the immune system. People with this condition have a deficiency in the levels of an immune system protein called mannose-binding lectin in their blood. It is not clear whether this deficiency predisposes the affected individuals to recurrent infections. Individuals with the deficiency of mannose-

binding lectin can develop infections of the upper respiratory tract and other body systems. People with this condition can also get more serious infections, such as pneumonia and meningitis. Depending on the type of infection, the frequency and severity of the symptoms caused by infections vary. Infants and young children with mannose-binding lectin deficiency appear to be more susceptible to infections than the affected adults, however, adults may also develop recurrent infections.[44] In addition, affected people who have undergone chemotherapy or are taking drugs that suppress the immune system are especially prone to infections. Increased susceptibility to sepsis and chemotherapy-related infections has been shown in people with MBL deficiency.[45,46] Mannose-binding lectin is a gene that is a key activator in the lectin complement pathway. The complement pathway has recently been found to play a role in oncogenesis.[47] Genetic polymorphisms in the MBL gene have been associated with risk for several cancers including breast cancer[48], stomach cancer[49,50], colon cancer[51] and cervical cancer.[52] In these studies, MBL2 polymorphisms were reported to have been resulted in lower serum levels and were associated with a high risk of cancer. On the contrary, in a study on lung cancer, it has been reported that low serum MBL levels due to MBL2 gene polymorphisms created positive effects on survival.[53] No association was reported on MBL2 gene and colon cancer survival in another study.[54] The evaluation of the distribution of **MBL2 (NM_000242), Exon1, HET, c.161G>A, p. (Gly54Asp)** variant among the family members showed that this variant was found in 2 out of 6 siblings. However, only one of these 2 siblings was diagnosed with cancer. Therefore, additional studies are required for the pathogenic effect of the **MBL2 (NM_000242), Exon1, HET, c.161G>A, p. (Gly54Asp)** variant. This variant has not been found in the mothers of individuals (KY105). Their father, on the other hand, was diagnosed with three different cancers (colon ca, prostate ca and Ewing's sarcoma and died at the age of 55 years. Therefore, genetic testing could not be performed on the father. However, considering the intensive clinical condition of the father, it is assumed that this variant was most likely transferred from the father. **MBL2 (NM_000242), Exon1, HET, c.154C>T, p. (Arg52Cys)** variant was found in the mothers of the siblings with the codes KY98 and KY99, and KY105. The assessment of the cancer history in the family showed that the detection of this variant in the healthy mother raises doubts about its pathogenic effect. In the HGMD data bank **MBL2 (NM_000242), Exon1, HET, c.154C>T, p. (Arg52Cys)** variant is registered as DFP (Disease-associated polymorphism with supporting functional evidence). Further studies are needed to understand the relationship between the **MBL2 (NM_000242), Exon1, HET, c.154C>T, p. (Arg52Cys)** variant and cancer risk. However, given the effects of the MBL gene on the oncogenesis process, clinical follow-up especially against some cancers such as breast cancer, stomach cancer, and colon cancer of people carrying variants is recommended.

PTPRJ (NM_001098503), Exon5, HET, c.827A>C, p. (Gln276Pro) variant has been found in individuals with the codes KY96, KY97, KY99, KY100, KY105, and KY108. In The Clinvar database it is registered as RCV000009227.4 (Pathogenic- Carcinoma of the colon) and as rs1566734 (MAF/MinorAlleleCount: C=0.190/181- Clinical significance: CLIN_ pathogenic) in dbSNP database. In the HGMD database, it has been associated with the risk of thyroid cancer. PTPRJ is a good candidate for the cancer predisposition gene. It has functional roles in tumor suppression, including inhibition of cell growth, migration, and angiogenesis.[55] PTPRJ also seems to play a role in human CRC as well.[56] PTPRJ is also a candidate tumor suppressor gene for other types of cancer. Loss of 11p11, the locus hosting the PTPRJ, is observed in 50% of lung cancers, 78% of breast cancers, and 38% of thyroid anaplastic carcinomas.[55,57,58] Considering the distribution of variants in the family in the light of this information, it is noteworthy that 4 out of 6 siblings have this

variant and three of them have been diagnosed with cancer regarding the pathogenic effect of the variant. In addition, the fact that one of the sick siblings had thyroid cancer increases the doubts. However, the fact that this variant has also been found in their healthy mother has led to the question of its pathogenic effect. Further studies are needed to clearly understand the effect of the **PTPRJ (NM_001098503), Exon5, HET, c.827A>C, p. (Gln276Pro)** variant on the risk of developing cancer.

GLUD2 (NM_012084), Exon1, HET, c.1492T>G, p.(SER4986) variant has been found in individuals with the codes KY96, KY98, KY99, KY100, KY105, KY107, and KY108. In the The Clinvar database is registered as: RCV000022827.24 (Pathogenic- as in Parkinson's disease), and as rs9697983 (MAF/MinorAlleleCount: G=0.033/4- Clinical significance: CLIN_pathogenic) in the dbSNP database. The age of the onset of Parkinson's disease (Gomes, #234), a common neurodegenerative disorder characterized by progressive loss of dopaminergic neurons and their termination in the basal ganglia is suggested to be associated with the **GLUD2 (NM_012084), Exon1, HET, c.1492T>G, p.(SER498Ala)** variant.[59] When evaluated from this point of view, it is recommended that people carrying this variant must undergo detailed examinations for Parkinson's disease.

FGFR4 (NM_001354984), Exon9, HET, c.1162G>A, p. (Gly388Arg) variant has been found in individuals with the codes KY101, KY103, KY107, and KY108. In the Clinvar database it is registered as RCV000017723.29 (Pathogenic Cancer progression and tumor cell motility), and in the dbSNP database as rs351855 (MAF/MinorAlleleCount: A=0.300/449- Clinical significance: CLIN_pathogenic). FGFR4 gene is a member of the fibroblast growth factor (FGF) family. Acts as a cell surface receptor for fibroblast growth factors, and plays a role in many mechanisms such as cell proliferation, differentiation, tissue repair, invasion, regulation of fat metabolism, bile acid biosynthesis, glucose uptake, vitamin D metabolism, and phosphate balance.[60] According to a research, cancer progression and tumor cell motility were associated with the 1162G>A (p.Gly388Arg) variant in the FGFR4 gene, and the increase in FGFR4 expression was associated with the development of breast and colon cancer. They also noted that it is statistically associated with lymph node metastasis and increased TNM stage, thereby indicating that it triggers the progression of cancer.[61] In another study it was emphasized that FGFR4 expression was associated with pancreatic cancers.[62] According to another research, this variant in the FGFR4 gene was effective in the onset and progression of prostate cancer.[63] Moreover, this variant in the FGFR4 gene was suggested to be used as a marker in Cushing's disease.[64] The c.G1162A:p.G388R variant in the FGFR4 gene which is also known for its oncogenic transformation activity was reported as a candidate gene for predicting the clinical development and assessing the stage of the disease in patients with advanced-stage retinoblastoma.[60,65] The evaluation in the light of this information shows that the detection of **FGFR4 (NM_001354984), Exon9, HET, c.1162G>A, p.(Gly388Arg)** variant only in the spouses of the siblings (KY107, and KY103), and in their children (KY108, and KY101) but not in the family members with high cancer burden suggest that additional studies are required for its pathogenic effect.

Individuals with the code KY107 and KY108 were detected to have **CST3 (NM_000099), Exon1, HET, c.73G>A, p. (Ala25Thr)** variant. In the Clinvar database it was defined as RCV000005989.4 (conflicting interpretations of pathogenicity- age-related macular degeneration 11), as rs1064039 in dbsnp database (MAF/MinorAlleleCount: T=0.212/226- Clinical significance: CLIN_pathogenic). Age-related macular degeneration (AMD) and Alzheimer's disease (AD) are degenerative, multifactorial diseases that involve the age-related accumulation of extracellular deposits due to the irregularity of protein homeostasis. There is evidence that the **CST3 (NM_000099), Exon1, HET, c.73G>A, p. (Ala25Thr)** variant in CST3 (cysteine proteinase inhibitor cystatin C) gene

which was confirmed to have been associated with AD with meta-analysis, is associated with AMD.[66] Within the scope of the study, it is recommended that these people (KY107 and KY108) should be followed up clinically, as the **CST3 (NM_000099), Exon1, HET, c.73G>A, p. (Ala25Thr)** variant detected in KY107 and KY108 (The son of KY107) increases the risk of age-related macular degeneration (AMD) and Alzheimer's disease (AD

SAA1 (NM_000331), Exon3, HET, c.209C>T, p. (Ala70Val) variant has been found in individuals with the code KY96, KY97, KY98, KY99, KY100, KY101, KY102, KY103, KY105, and KY115. In the Clinvar database it is registered as RCV000019736.27 (Pathogenic- a variant of serum amyloid) and as rs1136743 in dbSNP database (dbSNP entry validated Clinical significance: CLIN_pathogenic). In the HGMD database, the SAA1 (NM_000331), Exon3, HET, c.209C>T, p. (Ala70Val) variant has been associated with familial Mediterranean fever (FMF).[67] When the family profile was examined, this variant was found in all 6 siblings and their mothers. Detailed clinical screening of these family members (KY96, KY97, KY98, KY99, KY100, KY101, KY102, KY103, KY105, and KY115) for FMF is recommended.

During the examination, people with the codes KY96, KY97, KY98, KY99, KY101, KY103, KY105, KY107, and KY115 were found to have the **TP53 (NM_000546), Exon4, HET, c.215C>G, p. (Pro72Arg)** variant. It is registered in the Clinvar database (December 2020) as RCV000211212.1 (drug response***- cyclophosphamide response-Efficacy), and in the dbsnp database as rs1042522 (MAF/MinorAlleleCount: G=0.457/1046 Clinical significance: CLIN_uncertain_significance, CLIN_benign, CLIN_drug_response). This change has been investigated by different researchers in different groups of diseases and has been associated with drug response and apoptosis. In a study conducted in gastric cancers, it was reported that people with advanced-stage gastric cancer treated with paclitaxel and cisplatin who carry this polymorphism were advantageous in terms of their response to chemotherapy and the longer time until progression.[68] A study conducted on patients with ovarian cancer reported that there was no effect of the corresponding polymorphism.[69] In a study conducted in breast cancer patients receiving neoadjuvant chemotherapy, it was emphasized that it is effective in responding to 5-FU and cyclofosfamide-based neoadjuvant chemotherapy.[70] As can be understood, **TP53 (NM_000546), Exon4, HET, c.215C>G, p. (Pro72Arg)** variant has advantages in response to apoptosis and chemotherapy. Therefore, drug selection should be made according to the identification of the **TP53 (NM_000546), Exon4, HET, c.215C>G, p. (Pro72Arg)** variant in these family members (KY96, KY97, KY98, KY99, KY101, KY103, KY105, KY107, KY115).

In addition to detection of the mutations, we also evaluated large deletions and duplications. After annotation by using the CNVkit tool, CNV results of 13 individuals were obtained. Although high number of deletions and duplications were detected in all individuals, only Likely pathogenic/Pathogenic variations were shown in Table III.

Both children and family members were informed in detail about the risks they may encounter later on. In addition, considering the candidate variants identified, various clinical follow-up recommendations are presented to minimize the risks. Their physicians were also

Table III. Evaluation of the Likely pathogenic/Pathogenic CNV results using WES data.

Patient no.	Chromosome	Type of variations	ACMG Classification of variations	Dosage-sensitive genes	Protein coding genes
KY96	chr15	Deletion	Likely pathogenic	*SPRED1*	*SPRED1*
KY98	chr11	Deletion	Likely pathogenic	*KMT2A*	*ATP5MG, KMT2A*
KY115	chrX	Deletion	Likely pathogenic	*DMD*	*DMD*

informed in detail about other tumors that may occur during the pediatric patient's long life process. Approaches such as radiation therapy for cancer treatment has additional risk of causing secondary tumors, especially in individuals known to have a genetic predisposition; therefore, it must be avoided as much as possible. In addition, since there is a risk of transmission to future generations in individuals with a genetic mutation, preimplantation genetic options for preventing a decrease or increase in the incidence of the disease were recommended to both patients and parents. In conclusion, the risk of developing cancer in people with the candidate genes was identified in the family with a high risk of cancer, and all follow-up plans have been formed in this direction. However, research with wider patient group and healthy controls will clarify in what direction pathogenic effects would the candidate variants create in the disease formation process.

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Ethical approval

This study was approved by the Ethics Committee of Istanbul University (25.02.2020, 341).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: DAO, HY, RK; data collection: DAO, HY, RK; analysis and interpretation of results: DAO, RK, SBB, OSE, SBT, MH, SK, BC and HY; draft manuscript preparation: DAO, HY, RK. 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

Incidental pulmonary nodules in children: characteristic features and clinical course

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ABSTRACT

Background. There exists insufficient information about the natural course of incidental pulmonary nodules (IPN) determined on tomography in children. The aim was to determine the characteristic features and factors affecting the course of IPN.

Methods. This retrospective study included patients who presented at the Pediatric Pulmonology, Allergy & Immunology Section of Akdeniz University Hospital between January 2014-2020, and were determined with pulmonary nodules on high-resolution computed tomography (HRCT). The patients were separated into two groups as those with a nodule decreased in size or which had disappeared on the follow-up HRCT (Group 1) and those with a nodule which had remained at the same size (Group 2). These two groups were compared in respect to demographic data, nodule size and characteristics, and accompanying findings on HRCT.

Results. A total of 177 nodules were determined in the 66 patients included in the study. A follow-up HRCT was taken within mean 16.29±11.38 months in 27 patients. In these patients, 78 nodules were determined on the initial HRCT. On the follow-up, twelve of the nodules were seen to have shrunk or disappeared compared to the initial images, 66 had remained the same size, and none had grown. The mean age of the patients in Group 1 was statistically significantly lower than that of patients in Group 2 ($p<0.001$). The rates of an accompanying mosaic attenuation pattern ($p<0.001$) on HRCT and subsolid density ($p=0.011$) of the nodules in Group 1 were statistically significantly higher compared to Group 2 and the rate of calcification content was statistically significantly lower ($p=0.002$). No suspicious or confirmed malignancy was observed in any case throughout the mean follow-up period of 38.33±16.5 months after the initial HRCT.

Conclusions. The young age of patients, subsolid structure of nodules, calcification content and the presence of an accompanying mosaic attenuation pattern on HRCT, could be useful factors in the estimation of size in the follow-up of nodules.

Key words: pulmonary nodule, incidental nodule, HRCT.

Pulmonary nodule is defined as a rounded or irregular opacity, well or poorly defined, which measures up to 3 cm in diameter on radiological imaging.[1] The causes of pulmonary nodules in pediatric patients include infections (e.g. tuberculosis, histoplasmosis), sarcoidosis, vasculitis (e.g granulomatosis with polyangiitis, eosinophilic granulomatosis with polyangiitis), hypersensitivity pneumonitis, lymphomatoid granulomatosis, primary lung malignancies, and cancers with pulmonary metastases (wilms tumour, osteosarcoma and ewing sarcoma).[2] There is no clear relation between malignancy and nodule size.[3]

The widespread use of high-resolution computed tomography (HRCT) for pediatric patients has increased the frequency of

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incidental determination of pulmonary nodules. This creates a challenging decision-making situation for clinicians. When it is considered that there may be an accompanying infection, immune deficiency disorder, malignancy or congenital airway malformations in pediatric cases determined with pulmonary nodules, it must be determined whether or not the nodule is incidental.[4] Pulmonary nodules may be an important indicator of metastasis in children with known malignancy, and incidental pulmonary nodules determined in cases with no underlying malignancy are generally considered benign.[5,6]

As a result of concerns regarding radiation exposure, there exists insufficient information about the frequency, characteristics and natural course of incidental pulmonary nodules (IPN) determined on tomography in children, and there are no general rules that can be formulated in the management of these nodules.[4,7,8] Therefore, adult guidelines are usually used for children with pulmonary nodules. To be able to determine the applicability of using these guideline recommendations in pediatric cases, it is extremely important to evaluate the course of IPN determined in children.[4,7]

The aim of this study was to examine the demographic data of pediatric cases determined with IPN on HRCT and to determine the characteristic features and factors affecting the course of the nodules.

Material and Methods

Study design and setting

A retrospective screening was made of the results of patients aged <18 years who presented at the Pediatric Pulmonology, Allergy & Immunology Section of Akdeniz University Hospital between January 2014 and January 2020, and were determined to have pulmonary nodules on HRCT, which was performed for various reasons. Patients were excluded from the study if they had any known malignancy, congenital lung disease, immune deficiency disorder, cystic fibrosis, rheumatological disease, fungal infection, latent tuberculosis infection/tuberculosis disease, or any findings of lower respiratory tract infection clinically or radiologically and also diagnosed with these diseases after HRCT with microbiological examinations (bronchoalveolar lavage or sputum culture), immunological (tuberculin skin test and/or interferon gamma release assay) and other laboratory tests.

Approval for this retrospective study was granted by the Ethics Committee of Akdeniz University (decision no:421, dated: 06/12/2020).

Patient data

Data related to patient age, gender, indications for HRCT and smoking/exposure to cigarette smoke, were retrieved from the hospital records system (MIA MED, 1.0.1.3295). Some patients had control HRCT: the decision was made on a case-by-case basis by the responsible physician using clinical judgment. The patients were separated into two groups, one with patients with a nodule which decreased in size or had disappeared on the follow-up HRCT (Group 1) and another comprised of patients with a nodule which had remained at the same size (Group 2). These two groups were compared in respect to demographic data, indications for HRCT, exposure to smoking, nodule size and characteristics, accompanying findings on HRCT, as well as interval between the two HRCT examinations. The clinical follow-ups of the patients in respect to findings or suspicion of malignancy after HRCT, were evaluated from the hospital records system.

CT Technique

The CT examinations were performed with 128-detector multidetector CT devices (Somatom Definition Edge, GERMANY). All chest CT studies were performed with the following parameters: 1) 0.625 mm collimation; 2) weight-based kilovoltage and tube current; 3) high-speed mode; 4) pitch equivalent of 1.0–1.5. A slice thickness of 0.5 mm was used

to reconstruct the data set for review. All CT images were evaluated using a picture archiving and communication system (PACS; Sectra Workstation IDS7; Sectra AB, Linköping, SWEDEN) and standard soft tissue (level, 40–50 Hounsfield units [HU]; width, 400-450 HU) and lung (level, -450 to -550 HU; width, 1,600 – 1,800 HU). PACS allowed multiplanar (e.g., coronal and sagittal reformation) imaging, which was used routinely for the evaluation.

Image review

Two radiologists with experience evaluating pulmonary nodules on CT studies, (A.K. 15 years of experience in radiology; A.E., 13 years of experience in radiology) independently reviewed the lung parenchyma on the CT images using a PACS Workstation, and the final decision was made in consensus.

Maximum intensity projection (MIP) images were used to identify pulmonary nodules. All visualized pulmonary nodules were counted. First, perifissural nodules and parenchymal pulmonary nodules were identified and evaluated separately. The size, density, shape, and contour characteristics of the pulmonary nodules were evaluated and recorded. On axial CT images, the long and short axis diameters of the pulmonary nodule were totalled and averaged as in the Fleischner guidelines and recorded. Pulmonary nodule shape was characterized as round, ovoid, rectangular or triangular. Pulmonary nodular margins were described as regular or irregular. Attenuation was assessed as solid or subsolid. The lobe in which the pulmonary nodule was located was recorded, and whether the pulmonary nodule contained cavitation or calcification. The pulmonary nodules were subsequently compared on follow-up CTs and recorded.

Statistical Analysis

Data obtained in the study was analysed statistically using the SPSS v. 23.0 software. Descriptive statistics were stated as number and percentage or mean, standard deviation, median, 25th and 75th percentile, minimum and maximum values. In the analysis of categorical data, the Fisher's Exact test was applied if the expected value was <5 in >20% of cells and the Pearson Chi-square test if <20%. Conformity of the data to normal distribution was assessed with the Shapiro Wilk test. In the analysis of the difference between two groups, the Mann Whitney U-test was used when the data did not show normal distribution. A value of $p<0.05$ was accepted as statistically significant.

Results

A total of 394 HRCT images of 286 patients were evaluated and pulmonary nodules were determined in 90 (31.46%) patients. Of these, 24 were excluded from the study because of known malignancy (n=4), immune deficiency disorder (n=6), a diagnosis of cystic fibrosis (n=6), and clinical-radiological findings of lower respiratory tract infection (n=8). Thus, analysis was made of the data of 66 patients, comprising 42 (63.63%) males and 24 (36.36%) females. The most common indication for HRCT was treatment-resistant asthma (n=22, 33.33%), followed by chronic cough (n=21, 31.81%), determination of abnormality on plain radiographs (n=16, 24.24%) and recurrent pneumonia (n=10, 15.15%) (Table I). None of the patients smoked, whereas 12 (18.18%) were exposed to cigarette smoke.

A total of 177 nodules were determined in the 66 patients included in the study, at mean 2.68±2.79

Table I. Indications for HRCT.

Indication	Number of patients (%)
Treatment-resistant asthma	22 (33.33%)
Chronic cough	21 (31.81%)
Determination of abnormality on direct radiography	16 (24.24%)
Recurrent pneumonia	10 (15.15%)
Unexplained shortness of breath	1 (1.51%)
Hemoptysis	1 (1.51%)
Finger clubbing	1 (1.51%)
Nasal polyp	1 (1.51%)

(range, 1-13) nodules per patient. Nodule localisation was 89 (50.28%) parenchymal and 88 (49.71%) perifissural, some 173 (97.74%) nodules were solid and four (2.25%) of them had subsolid density. Calcification was present in 26 (14.68%) nodules and cavitation in one (0.56%) nodule. The most common indication for HRCT in the cases with calcified nodules was treatment-resistant asthma (51.6%), followed by chronic cough (41.9%) and in the cases with subsolid density nodules, it was treatment-resistant asthma (50%) and recurrent pneumonia (50%). The nodule margins were regular in 165 (93.22%) and irregular in twelve (6.77%). The nodules were most often located in the right lung upper lobe (49 nodules, 28%), followed by the left lung lower lobe and the right lung lower lobe (Fig. 1).

In twenty (30.30%) patients, there were additional findings accompanying the nodule on HRCT; linear atelectasis in thirteen patients, mosaic attenuation pattern in eleven, and air cyst in one. In patients with mosaic attenuation, the most common indication for HRCT was treatment-resistant asthma (45.4%), followed by recurrent pneumonia (27.3%) and chronic cough (18.2%), respectively.The mean nodule diameter was 3.11±1.31 mm (range, 1.30-7.10 mm), and a weak positive correlation was determined between mean nodule size and age (r=0.219, p=0.003). The mean nodule diameter was determined as 3.83 ± 1.34 mm in the right lower lobe, 2.99 ± 1.16 mm in the right upper lobe, 2.93 ± 1.15 mm in the right middle lobe, 2.90 ± 1.63 mm in the left upper lobe, and 2.79 ± 1.12 mm in the left lower lobe. The mean diameter of nodules localised in the right lower lobe was determined to be statistically significantly higher than that of the other lobes (p<0.0001). No statistically significant difference was determined between mean nodule diameter according to gender, perifissural location, and parenchymal location (p=0.21, p=0.12, p=0.12, respectively).

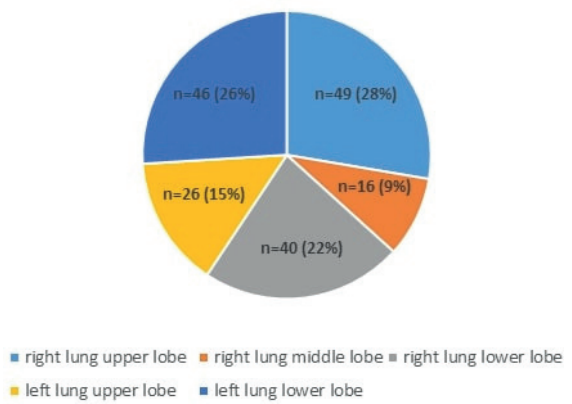

Fig. 1. Localisation of the nodules.

A single nodule was present in 34 (51.51%) patients and multiple nodules were present in 32 (48.48%). No statistically significant difference was determined between the presence of single or multiple nodules according to age, gender, exposure to cigarette smoke, indication for tomography and accompanying findings on tomography.

A follow-up HRCT was present in the system for 27 (40.90%) patients, taken at mean 16.29±11.38 months (range, 6-50 months) after the first imaging. On the first HRCT of these 27 patients, there were 78 nodules present with a mean diameter of 3.67±1.53 mm (1.50-7.10 mm). On the follow-up HRCT examination, twelve (15.38%) of the nodules were seen to have shrunk (n=2) or disappeared (n=10) compared to the initial images, 66 (84.61%) had remained at the same size, and none had grown. No suspicious or confirmed malignancy was observed in any case throughout the mean follow-up period of 38.33±16.5 months after the initial HRCT.

Comparisons between Group 1 and Group 2

The mean age of the 27 patients with a follow-up tomography was 10.24±4.26 years (1-17 years). The mean age of the patients in Group 1, where nodules had shrunk or disappeared (4.50 ± 2.65 years), was statistically significantly lower than that of patients in Group 2, where nodules had remained the same (11.29 ± 3.63 years) (p<0.001) (Table II).

The rate of subsolid density of the nodules and mosaic attenuation pattern accompanying the

Table II. Factors affecting nodule size during follow-up.

	Group 1	Group 2	p
Age (years), mean ± SD	4.50 ± 2.65	11.29 ± 3.63	<0.001
Gender, male/female	11/1	50/16	0.446
The time between the two CT examinations (months)*	15.50 ± 12.82	13.89 ± 8.48	0.786
Indication for first HRCT; abnormality on chest x ray, n (%)	2 (16.66)	29 (43.93)	0.110
Indication for first HRCT; treatment-resistant asthma, n (%)	4 (33.33)	14 (21.21)	0.457
Indication for first HRCT; chronic cough, n (%)	2 (16.66)	15 (22.72)	0.99
Indication for first HRCT; hemoptysis, n (%)	3 (25)	4 (6.06)	0.069
Indication for first HRCT; recurrent pneumonia, n (%)	3 (25)	4 (6.06)	0.069

*Values presented as mean±SD

nodule on HRCT in Group 1 was statistically significantly higher compared to Group 2 (25% vs 1.51%, p=0.011, and 50% vs 1.51%, p<0.001, respectively) and the rate of calcification and solid density of the nodules was statistically significantly lower (0% vs. 18.8%, p=0.002 and 75% vs. 98.48%, p=0.011, respectively) (Table III). No statistically significant difference was determined between Group 1 and Group 2 in respect of mean diameter, lobe localisation, gender, perifissural/parenchymal localisation, shape, margin features, exposure to cigarette smoke, single or multiple nodes, indication for HRCT and time interval to follow-up HRCT.

Discussion

The aim of this study was to investigate the characteristic features of IPN determined in children and to ascertain the factors affecting

Table III. Characteristics of pulmonary nodules on the first HRCT in cases with follow-up HRCT.

	Nodules shrank or disappeared on the follow-up HRCT (n=12)	Nodules remained at the same size on the follow-up HRCT (n=66)	p
Mean diameter (mm)*	2.95 ± 1.37	3.80 ± 1.54	0.068
Location in right upper lobe, n (%)	6 (50.00)	15 (22.72)	0.059
Location in right middle lobe, n (%)	0 (0.00)	4 (6.06)	0.050
Location in right lower lobe, n (%)	1 (8.33)	21 (31.81)	0.088
Location in left upper lobe, n (%)	1 (8.33)	12 (18.18)	0.361
Location in left lower lobe, n (%)	4 (33.33)	14 (21.21)	0.282
On HRCT, accompanying linear atelectasis, n (%)	5 (41.66)	18 (27.27)	0.322
On HRCT, accompanying mosaic attenuation pattern, n (%)	6 (50)	1 (1.51)	<0.001
Round shape, n (%)	6 (50)	34 (51,51)	0.99
Oval shape, n (%)	6 (50)	32 (48.48)	0.99
Regular margins, n (%)	10 (83.33)	56 (84.84)	0.99
Irregular margins, n (%)	2 (16.66)	10 (15.15)	0.99
Containing cavitation (n)	0 (0)	1 (1.51)	0.99
Containing calcification, n (%)	0 (0)	12 (18.18)	0.002
Perifissural nodule, n (%)	6 (50)	40 (60.60)	0.536
Parenchymal nodule, n (%)	6 (50)	26 (39.39)	0.536
Solid density, n (%)	9 (75)	65 (98.48)	0.011
Subsolid density, n (%)	3 (25)	1 (1.51)	0.011

the course of the nodules. The results revealed that the size of pulmonary nodules determined at a young age shrank at a higher rate during follow-up. A subsolid density of the nodules and mosaic attenuation pattern accompanying the nodule on HRCT, were determined to be important factors in regards to shrinkage or the disappearance of the nodule during follow-up, whereas calcification content was significant in regards to remaining at the same size.

The mean age of the patients where nodules were seen to have shrunk or disappeared on the follow-up HRCT, was statistically significantly lower than the mean age of those where nodules had remained the same. This is thought to be associated with younger children contracting viral infections more often and that no clinically infectious conditions were determined. As there is a high probability of the disappearance of nodules determined at a young age and radiation exposure increases the risk of the development of cancer[9,10], it can be considered that follow-up tomography should not be applied at a very young age.

According to the guidelines recommended by Westra et al.[8] for the follow-up of incidental nodules determined in children, the probability of malignancy is very low when IPN determined in asymptomatic children have a solid structure with the classic benign features (fat/popcorn calcification, peripheral location, elongated, pleural tag, uniformly calcified). In another study by Westra et al.[4], ground glass opacities were reported to be secondary to infection or inflammation and there was a very low possibility of being an indicator of malignancy in asymptomatic pediatric cases with no known malignancy. In the current study, malignant characteristics were not observed in any of the solid or subsolid nodules during the follow-up period of mean 38.33±16.5 months. The majority of the nodules determined in this study were of solid structure (97.74%). The nodules that had shrunk or disappeared on the follow-up HRCT were determined to be of subsolid character at a statistically significantly higher rate, than the nodules that stayed at the same size. This suggested that in asymptomatic pediatric cases, there was a much higher probability of regression during follow-up of IPN of subsolid character determined in clinical practice, and there was no need for follow-up HRCT.

The frequency of calcification of IPN determined in children has been reported in previous studies to be 10.7-19%.[7,11] Although the nodule calcification of IPN determined in children generally shows a benign character, it can be an indicator of malignant character in cases with malignancy.[12,13] In the current study, calcification was determined in 14.68% of the IPN and none of these nodules were seen to have shrunk or disappeared on the follow-up HRCT, which was consistent with the literature. This finding shows that in the clinical follow-up of calcified nodules, it must be kept in mind that they can remain stable without shrinkage.

There are few studies that have reported the prevalence of pulmonary nodules in children with no malignancy and the frequencies are in a wide range of 33 to 75%.[6,7,11] In the current study, the pulmonary nodule prevalence was determined to be 31.46%, and the mean diameter of the nodules determined was 3.11±1.31mm (range, 1.30-7.10mm), similar to findings in the literature.[6,7,11] In a study by Renne et al.[7] which examined 131 pulmonary nodules determined on tomography and taken because of trauma, nodules <5mm were reported to be determined more often in children with no malignancy. Alves et al.[11] evaluated a total of 225 pulmonary nodules in 99 pediatric patients applied with HRCT because of pectus excavatum and pectus carinatum, and reported at the end of a 2-year clinical follow-up period that there was a very low possibility of nodules ≤6mm being pathological. That no malignant course was seen in our study during follow-up was thought to be due to the mean nodule diameter being below these limits. In a recent review of the literature, Liang et al.[14] stated that a conservative recommendation would be that children with unexpected solid pulmonary nodules smaller than 5 mm in the absence of a malignancy, should not require dedicated follow-up CT

scans unless there is an underlying clinical concern requiring further follow-up.

Previous studies have reported that the majority of incidental nodules determined in children are located in the lower lobes[7,11] and no relationship has been determined between nodule diameter and the lobe, as per where it is located.[11] In contrast to the literature, the most common site of localisation in the current study was the right lung upper lobe and the diameter of nodules located in the right lung lower lobe, was determined to be statistically significantly higher than that of nodules in other lobes. This difference in data shows that localisation and nodule diameter cannot be used in the prediction of whether or not the nodule is incidental.

In the current study, there were found to be mean 2.68±2.79 (range, 1-13) nodules per patient, and there were multiple nodules in 48.48% of the patients. The frequency of multiple nodules in IPN in children has been reported to be between 5% and 19%.[6,7] It has been reported that multiple nodules, large nodules and nodules not completely benign in character (non-calcified, non-perifissural) can be seen in children with no malignancy.[6] The relatively higher rate of multiple nodules in the current study compared to literature was thought to be associated with the difference in definitions. While two or more nodules were defined as multiple nodules in the current study, other studies have defined multiple nodules as the presence of four or more nodules. Therefore, there is a need for consensus on the definition of multiple nodules to be able to attain standardisation of the data.

There are very few studies in literature related to the course of the size of IPN determined in children. In a study of 36 pediatric patients, Assefa et al.[15] evaluated the data of 22 patients with nodules >4mm on follow-up tomography taken at 3-12 months; they reported that 54% of the pulmonary nodules remained at the same size, 19% shrank, and 27% disappeared. In the current study, 12 (15.38%) of the 78 nodules were seen to have shrunk (n=2) or disappeared (n=10) compared to the initial images, 66 (84.61%) had remained at the same size, and none had grown. Control HRCT ranged from 6 to 50 months that showed wide variation, so the possibility of disappearance of some pulmonary nodules in the late course is the limitation of our study. To the best of our knowledge, our study included the highest number of IPN determined in children and the longest clinical and radiological follow-up period.

In conclusion, the results of this study demonstrated that IPN of varying dimensions and character are frequently seen in childhood and throughout a 3-year clinical follow-up period, no malignancy developed. Although there is scant data in literature, these findings suggest that the likelihood of IPN determined in healthy children being primary lung cancer or findings of extrathoracic malignancy, is so low as to be negligible[16] and as routine tomography follow-ups can lead to the harmful effects of radiation exposure, the follow-up recommendations for adults are not valid for children. Nodules determined on HRCT, especially at a young age, with a subsolid density, calcification content and the presence of an accompanying mosaic attenuation pattern, could be significantly useful factors in the estimation of size in the follow-up of nodules.

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Ethical approval

The study was approved by the ethics committee of Akdeniz University (decision no:421, dated: 06/12/2020).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: AB, AEB, AB, AK; data collection: AB, AK,AE, SD, TK, DFU; analysis and interpretation of

results: AB, AEB; draft manuscript preparation: AB, AEB,AK, AB. 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 association of angiotensin-converting enzyme gene polymorphism with respiratory distress syndrome in premature neonates

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ABSTRACT

Background. There was a contradiction in the previous literature on whether the D/D genotype of angiotensin-converting enzyme (ACE) is a protective or risk factor for respiratory distress syndrome (RDS) in premature neonates. To solve this debate, we intended to examine the association between ACE gene polymorphism and RDS in premature neonates.

Methods. We enrolled a total of 100 premature neonates with gestational age below 37 weeks. They were divided into 2 groups, the case group included 50 premature neonates diagnosed with RDS. While the control group included 50 premature neonates with no signs of RDS. We assessed ACE gene polymorphism using polymerase chain reaction. All neonates underwent chest x-ray, echocardiography, and routine laboratory investigations.

Results. D/D and D/I genotypes were higher in the control group (48% and 50%) than in the case group (26% and 40%). Whereas, I/I genotype was lower in the control group (2%) than in the case group (34%) ($p<0.001$). By counting D alleles among members of both groups, D-alleles were significantly higher in the control group (73%) than in the case group (46%) ($p <0.001$).

Conclusions. In premature neonates, D/D and D/I genotypes and D-alleles are protective factors for RDS. Whereas, I/I genotype and I-alleles are associated with the incidence of RDS with complications.

Key words: angiotensin-converting enzyme, gene polymorphism, neonates, premature, respiratory distress syndrome.

Respiratory distress syndrome (RDS) is the most prevalent cause of comorbidity and mortality in preterm neonates. It is mainly triggered by the deficiency of alveolar surfactants in the lung. It commonly presents in premature neonates below 37 weeks of gestation. The severity of RDS increases over the first two days of life, which ends with death due to progressive hypoxia and respiratory failure if was not appropriately managed.[1-3]

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The Renin-angiotensin system (RAS) is a crucial modulator of blood pressure, liquid, and electrolyte homeostasis.[4] Angiotensin-converting enzyme (ACE) is a membrane-bound exopeptidase enzyme. It has a crucial role in converting angiotensin-1 to angiotensin-2, which is a growth factor and vasoconstrictor.[5] ACE degrades bradykinin and kallidin, vasodilator peptides. Consequently, ACE increases growth and vasoconstriction and decreases vasodilation.[6]

ACE is highly presented in the vascular endothelial cells of the neonatal lung.[7,8] The human ACE gene is located on chromosome 17. It consists of 26 exons and 25 introns with a

total length of 21 kbp.[1] ACE has many types of gene polymorphism, where the most common form is the existence or absence of 287bp to the intron 16, known as the insertion/deletion (I/D).[1,9,10] The ACE insertion/deletion gene polymorphism results in three genotypes: deletion/deletion (D/D) and insertion/insertion (I/I) homozygotes, and deletion/insertion (D/I) heterozygote.[10]

It has been suggested that the D/D genotype and D-alleles genotype is associated with RDS patients with sepsis, increased risk of bronchopulmonary dysplasia, myocardial infarction and ischemic heart disease, and diabetic kidney disease.[4,11,12] Furthermore, the D/D genotype is associated with the high activity of ACE in serum and tissues of the preterm neonates with RDS.[13]

Sivasli et al.[14] revealed that the D/D genotype is a protective factor from RDS in preterm neonates. Whereas, Yimenicioglu et al.[1] and Hussein et al.[4] demonstrated that it is a risk factor for RDS in preterm neonates. Satar et al.[15] reported no link between D/D genotype and RDS. Consequently, to solve this debate, we intended to assess the association between ACE gene polymorphism and RDS in premature neonates.

Material and Methods

We performed this case-control study between January 2019 and June 2021 at the Menoufia University hospital, Egypt. The study protocol was approved by the Menoufia Faculty of Medicine Committee for Medical Research Ethics (IRB: 4-2018PEDI32). We followed the Helsinki Declaration of 1964, as revised in 2013. Written informed consent was obtained from the neonates' parents included in the study.

Eligibility criteria

We included preterm neonates with gestational age below 37 weeks, examined for RDS within the first 48 hours after delivery, and both sex. We excluded neonates with genetic and/or metabolic disorders, minor or major anomalies, pulmonary hypertension, congenital pneumonia, wet lung, and meconium aspiration.

Study Process and Evaluations

The study was performed on 100 preterm neonates at gestation age below 37 weeks admitted to the neonatal intensive care units at Menoufia University Hospital. Of them, the case group consisted of 50 neonates with RDS and 50 premature neonates served as the control group with no signs of RDS. All neonates were subjected to a detailed history, clinical examination, chest x-ray, echocardiography, as well as laboratory investigations.

RDS was diagnosed according to the following standards: (1) respiratory rate of more than 60 per minute, (2) dyspnea, (3) grunting, nasal flare, cyanosis, (4) respiratory acidosis with pH < 7.25, pCO2 > 60 mmHg, and PaO2 < 50mmHg in blood gases test, (5) radiological and clinical signs of RDS.[1,4]

Genotyping analysis

Peripheral venous blood was aseptically sampled in EDTA tubes. Total genomic DNA was extracted using Gene JET Whole Blood Genomic DNA Purification Mini Kit (Thermofisher) according to the manufacturers' instructions. We performed polymerase chain reaction using the primers, sense 5`-CTG GAG ACC ACT CCC ATC CTT TCT-3` and antisense 5`-GAT GTG GCC ATC ACA TTC GTC AGA T-3`.[9] Depending on the D/I flanking sequence, two DNA fragments were amplified, D-alleles (190 bp) and I-alleles (490bp). The reaction (total volume 25µl) was conducted in 2720 thermal cycler Singapore Applied Biosystem. Following the 5 min initial denaturation step at 95°C, 35 cycles were conducted as the following: 30s for denaturation at 95°C, 45s for annealing at 58°C, and 90s for extension at 72°C followed by 5 min for the final extension. DNA products were separated by gel electrophoresis, then stained by ethidium bromide (Sigma), Fig. 1.

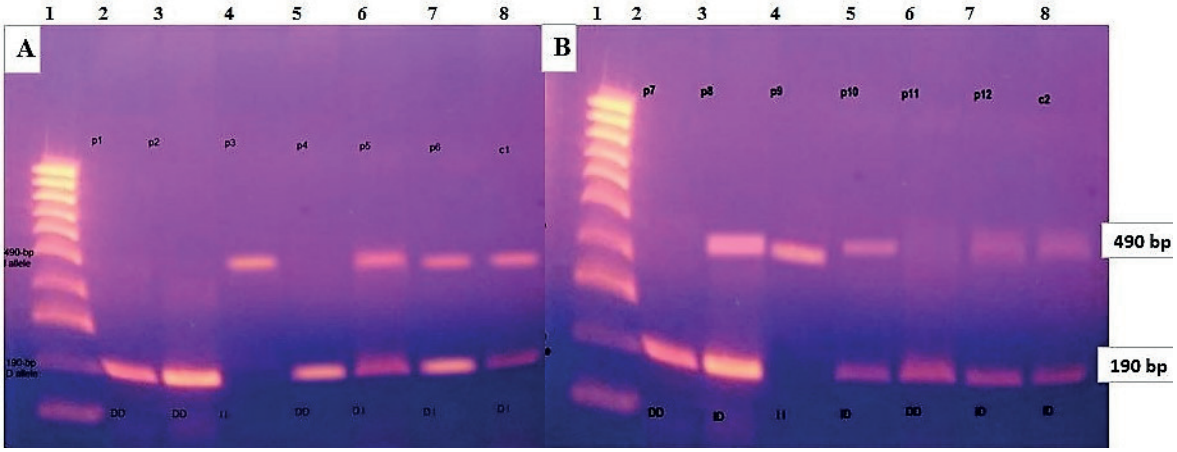

Fig. 1. Agarose gel visualization of the angiotensin-converting enzyme genotypes (DD, ID and II) fragments results from the polymerase chain reaction..(A) Lane 1 is the ladder; lane 8 is a control; lanes 2, 3, and 5 are the D/D genotype; lanes 6, 7 are D/I genotype; and lane 4 is the I/I genotype. (B) Lane 1 is the ladder; lanes 2 and 6 are the D/D; lanes 5, 7, and 8 are the D/I genotype; and lane 4 is the I/I genotype.

Statistical Methods

We analyzed the data using IBM SPSS advanced statistics version 25 (IBM Corp, NY, US). We presented the qualitative data in frequencies and percentages. Chi-squared test was used to measure the association between two or more qualitative variables. We performed the Shapiro-Wilk test to determine the type of data distribution.[16,17] Parametric data were stated as mean ± SD. The Student's t-test was performed for parametric data to compare quantitative variables between two groups. P-value <0.05 was considered significant.

Results

General characteristics of the included population

The study included 100 preterm neonates distributed into two groups. The case group included 50 premature neonates with RDS, with a mean gestational age of 32.16 weeks (±1.95), while the control group included 50 premature neonates with no signs of RDS, with a mean gestational age of 34.74 weeks (±0.94). Of the case and control group 62% and 56% were males, respectively. The control group participants had a significantly higher mean gestational age, birth weight, birth length, and head circumference than neonates with RDS (p<0.05). Premature neonates with RDS had significantly lower mean Apgar scores at 1 minute (2.74 vs. 5.86, p<0.001) and 5 minutes (5.36 vs. 7.26, p<0.001). Among the case group, three neonates (6%) developed intra-ventricular hemorrhage, twelve neonates (24%) had sepsis, and during follow-up, ten neonates (20%) died, Table I.

Regarding premature neonates with RDS, respiratory distress grade two was the most frequent in the case group (70%). Further, weak muscle tone was encountered in 4 neonates (8%) and absent moro-reflex was detected in 19 neonates (38%) of the case group. In contrast, none of them were detected in the premature neonates without RDS (p=0.041 and p<0.001, respectively). Weak suckling was more prevalent in the case group than in the control group (58% vs. 30%, p=0.005). Convulsions were detected only in one neonate, which is in the case group, Table I.

ACE gene genotyping

ACE gene D/D and D/I genotypes were significantly lower in the premature neonates with RDS than in the premature neonates without RDS (26% and 40% vs. 48% and 50%). On the other hand, the I/I genotype was

Table I. General and clinical characteristics of the study groups.

Neonates Data	Case group (n=50)	Control group (n=50)	P-value
Sex (male/female)	31/19 (62%/38%)	28/22 (56%/44%)	0.542
Gestational age (weeks)	32.16 ± 1.95	34.74 ± 0.94	<0.001[a]
Birth weight (g)	1872.8 ± 316.9	2035.2 ± 386	0.024[a]
Birth length (cm)	42.42 ± 1.85	43.28 ± 1.62	0.015[a]
Head Circumference (cm)	31.36 ± 1.27	31.94 ± 1.20	0.021[a]
APGAR Score (at 1 min)	2.74 ± 0.78	5.86 ± 0.35	<0.001[a]
APGAR Score (at 5 min)	5.36 ± 0.48	7.26 ± 0.44	<0.001[a]
IntraVentricular Hemorrhage	3 (6%)	-	-
Sepsis	12 (24%)	-	-
Mortality	10 (20%)	-	-
Respiratory distress (RD) grades			
RD I	5 (10%)	-	-
RD II	35 (70%)	-	-
RD III	7 (14%)	-	-
RD IV	3 (6%)	-	-
Muscle tone			
Normal	46 (92%)	50 (100%)	0.041[a]
Weak	4 (8%)	0 (0%)	
Moro-reflex			
Normal (Positive)	31 (62%)	50 (100%)	<0.001[a]
Abnormal (Absent)	19 (38%)	0 (0%)	
Suckling			
Normal	21 (42%)	35 (70%)	0.005[a]
Weak	29 (58%)	15 (30%)	
Convulsions			
No	49 (98%)	50 (100%)	0.315
Yes	1 (2%)	0 (0%)	

Parametric data are expressed as mean ± SD. Qualitative data are expressed as frequency (percentage). [a] p<0.05 *is significant.*

significantly higher in the case group than in the control group (34% vs. 2%), p<0.001, Table II.

By counting D alleles and I-alleles in all neonates in both groups, D-alleles were significantly lower in the case group than the control group (46% vs. 73%), Whereas the I-alleles were higher in the case group than the control group (54% vs. 27%), p<0.001, Table II.

Association of ACE with morbidity and mortality

There was no significant association between the D/D, D/I, and I/I genotypes of the ACE gene with intraventricular hemorrhage, sepsis, and mortality (0.423, 0.328, and 0.342, respectively), Table III.

Discussion

ACE gene polymorphism is correlated with the prevalence of several diseases and morbidities. However, only few studies have been conducted on premature neonates. These studies revealed a contradictory association between the ACE gene and RDS.[4,11-13] Consequently, we measured the ACE gene polymorphism in premature neonates with and without RDS.

Table II. Distribution of ACE gene polymorphism in the study groups.

Gene testing Data	Case group (n=50)	Control group (n=50)	p-value
Genotype			
• D/D	13 (26%)	24 (48%)	
• D/I	20 (40%)	25 (50%)	<0.001[a]
• I/I	17 (34%)	1 (2%)	
Allele (n=100)			
• D	46 (46%)	73 (73%)	<0.001[a]
• I	54 (54%)	27 (27 %)	

Qualitative data are expressed as frequency (percentage). [a] p<0.05 *is significant*.

Table III. Complications of RDS according to the genotypes.

Complications of RDS	D/D (n=13)	D/I (n=20)	I/I (n=17)	p-value
Intra Ventricular Hemorrhage				
Yes	1 (7.69%)	2 (10%)	0 (0%)	0.423
No	12 (92.31%)	18 (90%)	17 (100%)	
Sepsis				
Yes	2 (15.39%)	7 (35%)	3 (17.65%)	0.328
No	11 (84.61%)	13 (65%)	14 (82.35%)	
Mortality				
Yes	2 (15.39%)	6 (30%)	2 (11.75%)	0.342
No	11 (84.61%)	14 (70%)	15 (88.25%)	

Qualitative data are expressed as frequency (percentage). RDS: Respiratory distress syndrome.

Our study showed that D/D and D/I genotypes and D-alleles were significantly higher in premature neonates without RDS than those with RDS. In comparison, I/I genotype and I-alleles were significantly higher in the RDS premature neonates than in those without RDS. ACE gene polymorphism was not associated with intraventricular hemorrhage, sepsis, and mortality in premature neonates.

RAS activation may influence lung injury by modifying vascular permeability by changing the intracellular calcium levels in the endothelial cells and vascular tone by changing the calcium inflow and vascular smooth muscles activity. Moreover, RAS has been detected with high levels in the bronchoalveolar lavage of acute respiratory distress syndrome patients.[14,18]

D/ D genotype and D-alleles may increase ACE activity, which increases the risk of RDS through its increased levels of ACE and its product angiotensin-2, increasing lung inflammation and modifying the pulmonary vascular status.[19] Angiotensin-2 increases pulmonary inflammation by stimulating interleukin-6, tumor necrosis-alpha, and chemoattractant protein-1 production in the pulmonary endothelial cells and vascular smooth muscles, which may cause edema in the lungs through increasing pulmonary endothelial permeability.[19] Furthermore, the D/D genotype has a significant role in developing a cardiorespiratory disease in premature neonates.[20] D/D genotype may upsurge the risk of neonatal bronchopulmonary dysplasia.[19]

Our findings are in line with those of Sivasli et al.[14], who reported that D/D and D-alleles were significantly higher in premature neonates without RDS than in neonates with RDS (43.5% and 68.5% vs. 26.8% and 47.6%, p<0.05), which suggested that D/D and D-alleles are protective factors from RDS in premature neonates. In contrast, Yimenicioglu et al.[1] and Hussein et al.[4]

demonstrated that D/D genotype and D-alleles were significantly higher in the RDS neonates than in the control ($p<0.05$), revealing that they are risk factors for RDS. In addition, Satar et al.[15] demonstrated no link between D/D genotype and RDS.

Rigat et al.[21] studied the frequency of the genotypes and alleles of the ACE gene polymorphism in healthy subjects. They observed that D/D genotype and D-alleles were higher than I/I genotype and I-alleles (36% and 59.4% vs. 18% and 40.6%). These results are matching with the findings of the present study. Taking all this together, this disparity may be attributed to the different genetic factors between the different populations and the limited number of studies.[4,19,22]

Cardinal-Fernanandez et al.[11] demonstrated that D-alleles were associated with sepsis. However, Hou et al.[23] observed that D/D was a protective factor for sepsis in both the adult and pediatric populations in their meta-analysis study, and ACE inhibitor drugs may be harmful to patients with sepsis. These findings matched with our results that there is no relationship between ACE polymorphism and sepsis. Also, Hou et al.[23] elucidated no association between the I/D genotype and infant mortality, which enhances our results. However, during sepsis, ACE activity is decreased with the blood pressure.[24,25] This decrease may have resulted from the damage of endothelial cells or ACE inhibition by lipopolysaccharide of the bacterial infection.[25]

In the current study, multivariate analysis showed birth weight, birth length, and head circumference did not interfere with the difference in the ACE gene polymorphism between the case and control group except for the gestational age, which was proposed by other studies to be associated with ACE gene polymorphism.[26,27]

We concluded that in premature neonates, D/D genotype and D-alleles are protective factors from RDS. I/I genotype and I-alleles are associated with more severity and complications of RDS. In addition, there is no link between the ACE gene polymorphism and intraventricular hemorrhage, sepsis, and mortality. Further studies are required to examine the correlation of ACE gene polymorphism and activity with racial and genetic distribution.

Ethical approval

The study protocol was approved by the Ethics Committee of Menoufia Faculty of Medicine (IRB 4/2021PEDI2).

Author contribution

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

Urinary C-peptide creatinine ratio is a significant indicator of non-alcoholic fatty liver disease in children with obesity

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ABSTRACT

Background. Nonalcoholic fatty liver disease (NAFLD) is the commonest etiology of chronic hepatic problems in children with obesity. This study aimed to assess whether urinary C-peptide creatinine ratio (UCPCR) might be a potential indicator of NAFLD in obese children.

Methods. The study included 240 children with simple obesity. Hepatic ultrasonic examination, anthropometric and laboratory measurements including fasting plasma glucose, fasting insulin, fasting C peptide, liver, renal profile, lipid profile, and UCPCR were obtained in all cases. According to the results of the hepatic ultrasonography, cases were classified into two categories, those with NAFLD (n=98) and without NAFLD (n= 142).

Results. In cases with NAFLD, UCPCR was significantly higher than those without NAFLD ($P < 0.001$). A significant positive correlation between UCPCR and waist circumference (WC SDS), triglyceride, fasting C-peptide, HOMA-IR and alanine aminotransferase (ALT) was found ($P < 0.001$ for each). Adjusting for other variables, UCPCR was the most significant predictor of NAFLD in children with obesity with higher odds ratio (OR = 3.26) than fasting C peptide (OR = 2.87), triglyceride (OR = 1.89), ALT (OR = 2.20), WC SDS (OR = 1.32) and age (OR=1.27) . UCPCR cut-off value of 0.755 nmol/mmol was able to discriminate cases with NAFLD from those without NAFLD with a sensitivity of 95%, a specificity of 87%.

Conclusions. We concluded that UCPCR is a useful, practical and non-invasive predictor of NAFLD in children with obesity with high sensitivity and specificity.

Key words: non-alcoholic fatty liver disease, urinary C-peptide creatinine ratio, obesity, fasting C- peptide, insulin resistance.

The commonest etiology of chronic hepatic disorders in obese children is nonalcoholic fatty liver disease (NAFLD) (36.1%).[1] It includes a range of hepatic diseases from NAFLD to nonalcoholic steatohepatitis (NASH), which may proceed to cirrhosis, hepatocellular carcinoma, as well as hepatic failure.[2] Insulin resistance is one of the characteristic features of NAFLD and is crucial in the occurrence of the disorder as associated with obese.[3] NAFLD is difficult to diagnose without diagnostic procedures because people with NAFLD are generally asymptomatic or have nonspecific symptoms.[4] Early detection of NAFLD is required, although the optimum timing, frequency, and mode of screening remain undetermined.[5] The gold standard test for the detection of NAFLD is a liver biopsy. However, it is hard to perform a liver biopsy in younger children because it is invasive and has potentially life-threatening complications.[4] Moreover, liver biopsy is subjected to, sampling errors, micro-inhomogeneity, presence of un-fragmented cores, and inter-observer variability.[6] So, in both adults and children, imaging procedures as abdominal ultrasonography are frequently used to assess the presence of fatty liver

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disease and its severity.[4] However, abdominal ultrasonography is useful in moderate to severe cases, and cannot distinguish simple steatosis from NASH.[7] A new, non-invasive, and early marker is needed for the diagnosis and prognosis of NAFLD in obese children.

C-peptide is a short-chain polypeptide that is produced with insulin in identical proportions. Because C-peptide is eliminated unchanged in the urine, it is possible to measure it in the urine.[8] Previous studies reported that 24-hour urinary C-peptide correlates well with serum C-peptide. 24-hour urine collection can be difficult and deficient urine collection is another constraint.[9,10] Evaluation of the possibility of use of C-peptide/creatinine ratio allows the use of a single-spot urine sample in a similar way as protein creatinine ratio. Up to our knowledge, no research has been done to evaluate the role of UCPCR in children with obesity and its relation to NAFLD. In this work the aim was to evaluate UCPCR in children with obesity and its relationship with NAFLD and clinical and laboratory profiles.

Material and Methods

A total of 240 obese children took part in this hospital-based cross-sectional case-control study. Obesity in children was defined as a BMI of 95th percentile or higher.[11] According to hepatic ultrasonography, obese subjects were split into two categories, obese with NAFLD (n=98) and obese without NAFLD (n=142). During the months from June 2018 to May 2019, cases were randomly selected from pediatric outpatients clinics at Assiut University Children's Hospital. Children with glucose intolerance or type 2 diabetes mellitus (HbA1C 5.7% or higher) were excluded from the study. Obese children with underlying chronic medical disorders such as genetic syndromes, secondary obesity due to endocrinopathies, familial or primary hypercholesterolemia, hereditary inborn metabolic error, or consuming medicines known to produce fatty liver were also excluded from the study. The Institutional Review Board gave their approval to the research. The parents' informed consent was obtained before the patients were enrolled.

A comprehensive medical history and examination were performed on all individuals. Anthropometric measurements included determining weight (Wt) and height (Ht) as well as body mass index (BMI). A digital weighing scale was used to measure weight in kilograms to one decimal point, and a standard stadiometer was used to measure height to one decimal place. The Egyptian Growth Reference Data were used to compute BMI using the formula: weight (kg)/height^2 (meters), as well as BMI SDS using the reference ranges.[12] Waist circumference (WC) was measured halfway between the lowest rib margin and the iliac crest and WC SDS was computed using American percentiles.[13] Tanner criteria were used to determine the pubertal stage.[14]

Laboratory investigations

The serum levels of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), fasting glucose, fasting insulin, and fasting C-peptide were measured at 8:00–10:00 a.m. following an overnight fast of at least 12 hours. Standard enzymatic procedures and reagents (Boehringer Mannheim GmbH, Penzberg, Germany) were used to measure the levels of TC, TG, HDL-C, and LDL-C using a fully automated analyzer. The YSI 2300 STAT Plus TM Glucose & Lactate Analyzer (Ohio, USA) was used to assess fasting glucose. Insulin ELISA Kit (LDN, Nordhorn, Germany) was used to assess fasting insulin. The homeostasis model of insulin resistance (HOMA-IR) was used to determine IR using the following formula: fasting glucose (mg/dl) x fasting insulin (lU/ml)/405.[15] A cut-off level for diagnosing insulin resistance was > 2.7.[16] Fasting C-peptide was measured by human C-peptide enzyme-linked immunosorbent assay (ELISA) kit (DRG Instruments GmbH, Germany). An auto-analyzer (Abbott AXSYM system-UK) was used to determine the liver profile which

included total serum bilirubin, direct bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and albumin. The serum creatinine (Cr, mg/dl) was measured using Dimension Xp and plus chemistry analyzer using its kits; which were supplied by Siemens Technology (Illinois). A human C-peptide ELISA kit (DRG Instruments GmbH, Germany) was used to determine the level of C-peptide in the urine. Urine samples were collected in boric acid preservative 2 hours after lunch meal, following a premeal void and sent to the laboratory on the same day. On the Roche Cobas e311 platform, urinary creatinine was measured using the creatinine Jaffé reagent, and the data were utilised to calculate UCPCR (nmol/mmol).

Ultrasonography

Two radiologists who were uninformed of the study's goals and blinded to laboratory results used ultrasound to diagnose fatty liver. All ultrasonic instruments were high-end models from General Electric Company, Philips, and Mindray. Diffuse fatty liver was diagnosed in patients who met two of the following three criteria in acoustic performances[17]: (a) the liver's near-field echo is diffusely enhanced, and its echo is stronger than the kidney's; (b) the structure of the intrahepatic duct is unclear; (c) the liver's far-field echo is gradually attenuated.

Statistical analysis

The SPSS package (version 22.0), SPSS Inc., Chicago, IL, United States, was used for statistical analysis. The data were presented as a mean ± standard deviation. Pearson's analysis was performed to compute the correlation coefficients for the parameters with normal distribution, and the Student's t-test was employed to calculate the differences in means between the two groups. The correlation coefficients for the parameters with non-normal distributions were calculated using Spearman's rank correlation analysis, and the Mann-Whitney U test was used to evaluate the differences in means between the two groups. The logistic model is a suitable model for assessing if predictors are significantly linked with the response variable because the response variable is a dichotomous variable (with NAFLD or without NAFLD). As a result, logistic regression was employed to see if NAFLD is linked to any single predictor at the univariate level. The relevant factors from the univariate analysis were then used as predictors in step-wise logistic regression to find significant predictors at the multivariate level. The optimum UCPCR cutoff value for detecting NAFLD in obese children was determined using a receiver operating characteristic (ROC) curve. In all studies, a p-value of less than 0.05 was considered significant.

Results

Demographic, anthropometric, and clinical data of the study groups (Table I) shows that children with obesity and NAFLD had significantly higher BMI SDS, WC SDS, TC, TG, LDL-C, fasting insulin, fasting C-peptide, HOMA-IR, ALT and URPCR compared to children with obesity without NAFLD. The difference was statistically significant ($p < 0.05$).

Table II shows the correlation between UCPCR and clinical and laboratory variables in children with obesity and NAFLD. UCPCR was positively and significantly correlated with fasting C-peptide ($r=0.31$, $p < 0.01$), HOMA-IR ($r=0.32$, $p < 0.01$), TG ($r=0.18$, $p < 0.05$), serum ALT ($r=0.58$, $P<.001$), WC SDS ($r=0.25$, $p < 0.01$), and BMI SDS ($r=0.25$, $p < 0.01$), and inversely correlated with HDL-C ($r= - 0.32$, $p < 0.01$).

Table III shows the predictors of NAFLD and adjusted odds ratio estimated by multivariate logistic regression. Adjusting for other variables, we found that UCPCR was the most significant predictor of NAFLD in children with obesity with higher odds ratio (OR) (OR = 3.26; 95% CI: 1.467 -5.818; p = 0.000) than fasting C-peptide (OR = 2.87; 95% CI: 1.457 -3.870; p = 0.001), TG (OR = 1.89; 95% CI: 1.164- 1.251; p = 0.037), ALT

Table I. Baseline characteristics of obese children with NAFLD and without NAFLD.

Variable	NAFLD (n=98)	without NAFLD (n =142)	P value
Age (year)	11.41 ± 2.349	10.52 ± 2.52	0.001
Male/female	56/42	98/44	0.072
Obesity duration (year)	5.9 ± 3.36	5.4 ± 3.22	0.059
BMI-SDS	3.12 ± 0.60	2.23 ± 0.86	0.001
WC-SDS	3.18 ± 1.6	2. 44 ± 1.7	0.001
Puberty, n (%)			
Prepubertal	71 (72.45%)	97 (68.31%)	0.11
Pubertal	27 (27.55%)	45 (31.69%)	
Albumin (g/L)	42.35 ± 9.01	42.56 ± 7.88	0.825
Total bilirubin (mg/dl)	0.78 ± 0.29	0.64 ± 0.37	0.18
Direct bilirubin (mg/dl)	0.22 ± 0.14	0.18 ± 0.17	0.65
ALT (U/L)	39.5 ± 5.9	18 .8 ± 6.8	0.001
AST (U/L)	28.1 ± 5.7	22.5 ± 3.7	0.001
ALP (U/L)	294.27 ± 105.83	273.11 ± 87.02	0.058
GGT (U/L)	27.4 ± 8.5	18.7 ± 4.9	0.001
Serum creatinine (mg/dl)	0.66 ± 0.14	0.62 ± 0.20	0.765
TC, mg/dl	188.2 ± 45.5	156.3 ± 22.1	0.01
TG, mg/dl	153.56 ± 43.12	121.10 ± 13.56	0.01
LDL-C, mg/dl	115.5 ± 34.2	99.6 ± 11.2	0,01
HDL-C, mg/dl	44.1 ± 6.8	50.2 ± 5.8	0.02
Fasting blood glucose, mg/dl	104.3 ± 8.8	106.7 ± 9.7	0.862
Fasting insulin (mU/L)	27.2 ± 3.7	18.8 ± 4.1	0.001
Fasting C-peptide(ng/ml)	1.52 ± 0.7	1.01 ± 0.4	0.001
HOMA-IR	7.3 ± 2.7	5.0 ± 1.7	0.001
UCPCR (nmol/mmol)	0.88 ± 0.32	0.65 ± 0.23	0.001

Data are expressed as mean ± SD; ALP: alkaline phosphatase, BMI SDS: body mass index, standard deviation score, ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: gamma glutamyl transferase, TG: triglycerides, TC: total cholesterol, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, NAFLD: nonalcoholic fatty liver disease, HOMA-IR: insulin resistance by homoeostasis model, UCPCR: Urinary C-peptide to creatinine ratio

(OR = 2.20; 95% CI: 1.32 -2.154 ; p = 0.001), WC SDS (OR = 1.32; 95% CI 1.74- 1.581; p = 0.018) and age (OR = 1.27; 95% CI 1.21-1.54; p = 0.032) .

Receiver-operating characteristic (ROC) curves were created to determine the accuracy of UCPCR to predict NAFLD in children with obesity. UCPCR cut-off value of 0.755 nmol/mmol was able to differentiate subjects with NAFLD from those without NAFLD in children with obesity with 95% sensitivity and 87% specificity (area under the curve [AUC] 0.98; confidence interval (CI) 1.02–1.31; (p < 0.001).

Discussion

This study focused to determine the utility of UCPCR testing to identify NAFLD in a cohort of children with obesity. We observed that children with obesity with NAFLD had significantly higher UCPCR compared with those without NAFLD. Another important finding was that UCPCR is the strongest significant predictor of NAFLD in children with obesity as compared with the other factors with excellent sensitivity and specificity. To our knowledge, this is the first study to suggest that UCPCR can be utilized as a novel predictor of NAFLD in

Table II. Correlation between UCPCR and other variables in obese children with NAFLD.

Variable	NAFLD (n = 98)	
	r	P
Age (year)	0.388	0.001
Obesity duration (year)	0.067	0.566
BMI SDS	0.456	0.001
WC SDS	0.587	0.001
ALT (U/L)	0.404	0.001
AST (U/L)	0.098	0.133
GGT(U/L)	0.102	0.98
TC, mg/dl	0.112	0.021
TG, mg/dl	0.16	0.010
LDL-C, mg/dl	0.188	0.019
HDL-C, mg/dl	- 0.109	0.099
Fasting glucose, mg/dl	0.037	0.667
Fasting insulin, μU/mL	0.821	0.001
Fasting C-peptide, ng/ml	0.765	0.001
HOMA-IR	0.641	0.001

WC-SDS: waist circumference standered deviation score, BMI SDS: body mass index standered deviation score, ALT: alanine aminotransferase, AST: aspartate aminotransferase, TG: triglycerides, TC: total cholesterol, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, HOMA-IR: insulin resistance by homoeostasis model, UCPCR: urinary C-peptide to creatinine ratio

obese children with 95% sensitivity and 87% specificity. In accordance with the present results, another study reported that UCPCR had also high sensitivity of 97% in identifying T2DM from T1DM in children.[8] Also, another study concluded that, in non-diabetic obese children and adolescents, UCP and UCPCR are simple, fast, and reliable indicators of IR. The sensitivity of UCP and UCPCR to diagnose IR in children with obesity was 71.4% and 87.6%, respectively and the specificity was 70% and 84%, respectively.[18]

Our results were expected as the measurement of UCPCR reflects the endogenous insulin secretion which has already been established to be higher in children with obesity and NAFLD and this can discriminate it from those without NAFLD.[11,12] UCPCR is a simple, practical, non-invasive, easy test that is stable at room temperature for 3 days in a preservative which allows outpatient and home samples to be collected and sent for analysis later on.[9]

In our study, we reported that fasting C-peptide is a good predictor of detection of NAFLD in children with obesity but it is not superior to UCPCR. This was in agreement with Han et al.[19] who reported a similar result in a cross-sectional study of children with simple obesity. In line with our data, Besser et al.[10] reported that UCPCR obtained 2 hours after a home evening meal is highly correlated with 90-min stimulated C-peptide during a mixed meal tolerance test and can distinguish between maturity-onset diabetes of the young (MODY) and type 2 diabetes mellitus with a 96% sensitivity and a 97% specificity. C-peptide is a well-known indicator of B-cell secretion. Jones et al.[20] on the other hand, found that 24-hour urine C-peptide can be utilized instead of stimulated serum C-peptide measurement in the assessment of

Table III. Risk factors of NAFLD by stepwise multiple logistic regression analysis.

Variable	Beta	Standard error	Odds ratio	95% CI	p-Value
Age	0.134	0.28	1.27	1.21-1.54	0.032
WC-SDS	0.108	0.23	1.32	1.74- 1.581	0.018
TG , mg/dL	0.118	0.54	1.89	1.164- 1.251	0.037
Fasting C-peptide (ng/ml)	0.612	0.61	2.87	1.457 -3.870	0.001
HOMA-IR	0.537	0.23	1.92	1.567-1.975	0.001
ALT (U/L)	0.548	0.66	2.20	1.020 -2.154	0.001
UCPCR (nmol/mmol)	0.861	0. 13	3.26	1.467 -5.818	0.001

WC-SDS: waist circumference standard deviation score, NAFLD: non-alcoholic fatty liver disease, ALT: alanine aminotransferase, TG: triglycerides, TC: total cholesterol, HOMA-IR: insulin resistance by homoeostasis model, UCPCR: urinary C-peptide to creatinine ratio

late-onset insulin-treated diabetes. C-peptide moves through the liver without being extracted in any significant amount. As a result, the level of C-peptide is a well-known indicator of B-cell secretion. The function of fasting C-peptide in distinguishing people who have or don't have fatty liver is well recognized.[10] The assessment of serum C-peptide needs access to centrifugation followed by freezing by special laboratory techniques which are restricted only to the hospital laboratory.[19]

Our study showed a significant positive correlation between UCPCR and HOMA-IR that remains significant after regression analysis, which may indicate that UCPCR can also be a potentially useful method for the assessment of the degree of insulin resistance in NAFLD in children with obesity.[19] This is in accordance with Hassan et al.[18], who reported that in obese children, UCPCR was a better predictor of IR than UCP in a multivariate logistic regression research. This showed that UCPCR is more reliable marker for predicting IR in obese children than UCP; nonetheless, both markers have good sensitivity for predicting IR.[18]

Insulin resistance is a common feature of obesity and the link between the two has long been known, with substantial scientific and therapeutic implications.[5]

Insulin resistance may have a role in the occurrence of NAFLD in both adults and children. It is significantly linked to compensatory hyperinsulinemia in children, which inhibits free fatty acid oxidation and lipid peroxidation in mitochondria. The synthesis and buildup of harmful lipid metabolites as a result of this cascade of reactions promotes oxidative stress and hepatocellular damage.[19,21]

Up to our knowledge we reported the first work that specified a cutoff value for UCPCR and the value of 0.755 nmol/mmol at which the existence of NAFLD in obese children with obesity may be predicted with high specificity and sensitivity, at the time of diagnosis. This could lead to better management and, as a result, a better prognosis for obese children. This threshold will need to be validated in prospective trials.

It is crucial to keep in mind several limitations of this study: 1-This study was cross sectional and cannot explain the causality of various factors. Further longitudinal studies are necessary. 2- The study was conducted in a single location with a relatively small sample size. More research is needed to confirm the validity of our findings. 3- NAFLD diagnosis was based on the results of hepatic ultrasound which may have resulted in a missed diagnosis in some patients. 4- Other aspects, including lifestyle-related characteristics and nutrient consumption in the diet, should be investigated further in future studies. 5- If there is renal impairment, UCPCR test cannot be used.

This study concluded that UCPCR is a useful, practical and non-invasive predictor of NAFLD in obese children with high sensitivity and specificity.

Ethical approval

The study protocol was approved by the Ethics Committee of Faculty of Medicine, Assiut Children's University Hospital, Assiut, Egypt (33/5, may.2018). Written informed consents were obtained from the parents of all participants.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: HSF, KAM, YG; data collection: HSF, KAM; analysis and interpretation of results: YG, GMS, YF; draft manuscript preparation: KAM, YG, YF. 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

Difficulties in the diagnosis and management of eight infants with secondary pseudohypoaldosteronism

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ABSTRACT

Background. Type 1 pseudohypoaldosteronism (PHA1) is a rare condition characterized by the resistance of the kidney to the effect of aldosterone. Secondary PHA1 is a syndrome that is most often related to urinary tract anomalies (UTAs) and/or urinary tract infections (UTIs). A similar pattern of electrolyte impairment is seen in congenital adrenal hyperplasia (CAH) and secondary PHA1, and CAH is a condition that requires urgent treatment. In our study, eight patients aged between 15 days and 8 months (seven males and one female) were included in the evaluation. It was aimed to evaluate cases of secondary PHA1 in our clinic and to identify the problems encountered in diagnosis and follow-up.

Methods. The records of the patients who presented to our hospital between February 2010 and 2021 were retrieved and retrospectively scanned.

Results. In all cases, hyponatremia, hyperkalemia, hyperaldosteronism, and hyperreninemia were detected. Other biochemical and hormonal tests were normal. Leukocytosis was detected in urine analysis, and urine cultures were productive. UTA was detected in five cases. Nine episodes of PHA1 occurred in eight patients and fungal infections were responsible for causing two episodes. Four episodes of PHA1 needed mineralocorticoid treatment. On the third day, serum electrolytes normalized. Fludrocortisone treatment was continued for 1 week. In one case, UTIs were repeated with PHA1, but in the follow-up, there were no additional problems.

Conclusions. Secondary PHA1 should be kept in mind when hyponatremia and hyperkalemia are seen, especially in infants aged under 3 months or older, up to 8 months, who present with non-specific symptoms. Fungal infections should not be forgotten in UTI etiology because PHA1 episodes can be initiated. If CAH is suspected, mineralocorticoid treatment should be rapidly initiated.

Key words: fungal urinary infection, secondary pseudohypoaldosteronism, urinary tract anomaly, urinary tract infection.

Aldosterone plays a major role in the control of blood pressure through the control of sodium balance, fluid homeostasis, and transepithelial sodium transport.[1] The main effect of aldosterone in epithelial target tissues is to regulate sodium reabsorption, and potassium and hydrogen secretion. Aldosterone regulates this pathway through intracellular mineralocorticoid receptors (MR) and amiloride-sensitive epithelial sodium channels. Interruption of the intracellular MR signal pathway leads to the appearance of clinical signs of pseudohypoaldosteronism (PHA). Type 1 PHA (PHA1) is a rare condition characterized by the resistance of the kidney to the effect of aldosterone. In patients, metabolic acidosis associated with elevated plasma renin and aldosterone levels manifests with hyperkalemia and salt loss.[2] This rare syndrome starts in the neonatal period and early infancy and has two forms; (a) Genetic forms: There are two types, the renal form (autosomal dominant) and systemic form (autosomal recessive), (b) The secondary form of PHA1 which is limited to the kidney, most often related to urinary tract anomalies (UTAs) and/or urinary tract

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infections (UTIs) and is identified in newborns and infants.[3] Secondary PHA1 is a problem that causes difficulties in diagnosis and treatment, which can be overlooked in early childhood and confronted with different causes.

A similar pattern of electrolyte impairment is seen in congenital adrenal hyperplasia (CAH) and PHA1 in infancy. CAH remains the most common cause and is a condition that requires urgent treatment.[4] The incidence of the classic form of CAH is reported to range from 1:5000 to 1:15,000 and varies among ethnic/racial backgrounds.[5] CAH is a group of autosomal recessive disorders encompassing enzyme deficiencies in the adrenal steroidogenesis pathway. Hyperpigmentation of skin creases and genitalia may be early signs of adrenal insufficiency. Depending on excessive androgen exposure, virilization findings occur in the external female genitalia; the external genitalia in males are usually unaffected, except for subtle penile enlargement. In CAH, low levels of aldosterone and cortisol, and elevated levels of renin, 17 hydroxyprogesterone (17 OHP), and adrenocorticotropic hormone (ACTH) are detected.[6] The management of secondary PHA1 involves sodium supplementation and water replacement, ion exchange resins in cases of high potassium values, in some cases bicarbonate, antibiotics for UTIs, and surgical intervention for UTA when necessary.[7] Stress-dose corticosteroids may be given to patients until the end of the hormone tests taken for differential diagnosis.[4]

In this study, it was aimed to evaluate cases of secondary PHA1 in our clinic, and to identify the problems encountered in diagnosis and follow-up.

Material and Methods

The records of the patients who presented to our hospital between February 2010 and 2021 were retrieved and retrospectively scanned. The study protocol was approved by the Institutional Ethics Committee of Ankara University (approval number: 14-675-16). Informed consent was received from the parents of all patients included in the study.

Patients

Eight patients were included who were evaluated for hyperkalemia and hyponatremia. Hyperkalemia was accepted when the potassium level was ≥5.1 milliequivalents (mEq)/Liter (L) and hyponatremia, sodium level was <136 mEq/L. The bicarbonate level for acidosis was considered as <22 mEq/L, 17 OHP, cortisol, ACTH, plasma renin activity (PRA), and aldosterone levels were measured when hyponatremia and hyperkalemia were detected. Urinary system obstructions were evaluated using urinary ultrasonography (US) and voiding cystourethrography (VCUG). UTI was considered to be above five leukocytes/high-power field (hpf) in urine analysis microscopy and urine culture with a single pathogen greater than 10^5 colonies (col)/milliliter (mL). Reproduction above 10^4 col/mL was considered significant for fungal infections. Urine samples were taken using a urine catheter. Treatment with intravenous (IV) fluid and sodium replacement, IV antibiotics and/or antifungal agents was started. It was recorded whether glucocorticoids and/or mineralocorticoids were given.

Case Presentation

The clinical features and laboratory findings of the patients are shown in Tables I and II. On initial examination, all patients had hyperkalemia (6.9±1.0 mEq/L), hyponatremia (118.5±5.5 mEq/L), hyperaldosteronism (462.5±167.4 ng/dL), and hyperreninemia (116.6±67.6 ng/mL/hr). There was no history of parental consanguinity, no signs of atypical external genitalia, or hyperpigmentation in our cases.

Patient 1 was admitted to the emergency department due to weakness and the inability to feed orally. His physical examination showed decreased skin turgor and poor peripheral

Table I. Clinical features of patients.

Patient	Sex	Age at admission	Birth weight/ week	Type of urinary malformation	Surgical treatment	Urinalysis-Urine culture on admission
1	Male	3 months	1060 g/27 week	-	-	44 leucocyte/hpf-10^5 col/mL *Klebsiella Pneumoniae*
2	Male	1.5 months	2350 g/34 week	PUV	Stenosis excision at 2.5 months, Left nephroureterectomy and right ureteronephrostomy at 9.5 months	234 leucocytes/hpf-10^5 col/mL *Candida Glabrata*
3	Male	2.5 months	2980 g/35 week	PUV	Stenosis excision at 3 months old	885 leucocytes/hpf-$8x10^4$ col/mL *Candida Albicans*
		4.5 months (2nd episode)				24 leucocytes/hpf-10^5 col/mL *Klebsiella Pneumoniae*
4	Girl	2.5 months	2990 g/40 week	Neurogenic bladder secondary to meningomyelocele	-	219 leucocytes/hpf-10^5 col/mL *Klebsiella Pneumoniae*
5	Male	6 months	3180 g/38 week	Left UP stenosis	Left pyeloplasty at 3.5 months old	213 leucocytes/hpf-10^5 col/mL *Enterobacter cloacae*
6	Male	8 months	2870 g/40 week	-	-	7 leucocytes/hpf-10^5 col/mL *Escherichia coli*
7	Male	1.5 months	2950 g/38 week	Double collecting system in the right kidney, Bilateral VUR	-	259 leucocytes/hpf-10^5 col/mL *Klebsiella Pneumoniae*
8	Male	15 days	3900 g/40 week	PUV	-	1746 leucocytes/hpf-10^5 col/mL *Enterobacter Bugadensis*

UP: ureteropelvic, PUV: posterior urethral valve, hpf: high-power field

circulation. A UTI was detected in a urine test. The patient was evaluated as having secondary PHA1 due to UTI. Hydrocortisone (single-dose) and fludrocortisone were added to antibiotic and IV sodium replacement therapy for possible surrenal insufficiency. Fludrocortisone was discontinued at the end of the first week when the 17 OHP result was normal.

Patient 2 presented with vomiting. His clinical examination showed dehydration. Antenatal US had shown bilateral hydronephrosis (HN). His laboratory findings suggested PHA1 secondary to UTI and UTA. He was treated first with IV saline. The IV fluconazole treatment was completed in 3 weeks.

Patient 3 was admitted with vomiting and irritability. Physical examination showed dehydration. Bilateral HN had been detected at the 31st week of gestation. On postnatal day 5, the patient was diagnosed as having a posterior urethral valve (PUV). At admission, he was considered to have secondary PHA1 associated with UTI and UTA. IV sodium replacement treatment for hyponatremia was started. IV fluconazole treatment was continued for 2 weeks. After the PUV resection surgery, when

Table II. Laboratory features of the patients on admission and clinical follow-up.

Patients	BUN	Cr	Na	K	Cl	pH	HCO3	PRA	Aldosterone	ACTH	Cortisol	17 OHP	Treatment
Patient 1	15	0.09	123	6	100	7.30	17	277.6	552	7.5	25.52	1.63	IV antibiotic, IV fluid, Hydrocortisone (single-dose), Fludrocortisone (1 wk)
Day 3			136	4.1									
Day 14								1.27	0.81	50.01	6.6		
Day 21								1.8	17				
Patient 2	41	0.40	112	7.7	102	7.28	12.5	121.6	551	50.80	39.52	2.65	IV antibiotic + antifungal, IV fluid
Day 3	13	0.50	137	4.3	102								
Day 14								0.9	15.1				
Patient 3 1st PHA episode	52	0.66	115	8.1	105	7.27	13.2	97.7	242	11.36	31.69	1.89	IV antifungal, IV fluid
Day 3	3	0.34	137	5									
Day 14								1.1	16	45.05	7.5		
2nd PHA episode (2 mths later)	12	0.55	123	6.8				97.7	480	5.07	30.81		IV antibiotic, IV fluid
Day 2			138	4.9									
Day 14								30.6	187.9				
Day 28								1.9	17				
Patient 4	8	0.18	128	5.2	99	7.33	18	143.8	497.8	7.7	7.71	2.33	IV antibiotic, IV fluid
Day 3			136	4.2									
Day 21								0.92	15.5				
Patient 5	23	0.42	119	6.2	101	7.28	13	105	390	22.86	6.25	2.55	IV antibiotic, IV fluid
Day 2	2	0.17	138	5.1									
Day 14								1.2	14.5				
Patient 6	6	0.24	119	7.2	104	7.36	20.6	86.3	300	7.9	60.60	1	IV antibiotic, IV fluid, Hydrocortisone (single-dose), Fludrocortisone (1 wk)
Day 3			136	4									
Day 7								0.51	5.93				

Normal Laboratory Values: BUN: 5-18 mg/dl, Creatinin (Cr): 0.32-0.6 mg/dl, Sodium (Na): 136-145 mEq/L, Potassium (K): 3.5-5.1 mEq/L, Clor (Cl): 98-108 mEq/L, Bicarbonate (HCO3): 22-26 mEq/L, Plasma Renin Activity (PRA): 0.5-1.9 ng/mL/hr, Aldosterone: 0.8-17.2 ng/dL, Adrenocorticotropic hormone (ACTH): 7.2-63.3 pg/mL, Cortisol: <10 mcg/dL, 17 hydroxyprogesterone (17 OHP): <3 ng/mL

Table II. Continued.

Patients	BUN	Cr	Na	K	Cl	pH	HCO3	PRA	Aldosterone	ACTH	Cortisol	17 OHP	Treatment
Patient 7	90	1.16	111	7.6	105	7.04	5.2	30	350	10.60	24.92	2.89	IV antibiotic, IV fluid, Hydrocortisone (single-dose), Fludrocortisone (1 wk)
Day 3	17	0.49	136	4.3									
Day 7								1.72	4.6				
Patient 8	40	0.56	117	8.1	99	7.27	12.6	90	800	7.5	25.9	2.5	IV antibiotic, IV fluid, Hydrocortisone (single-dose), Fludrocortisone (1 wk)
Day 3	16	0.40	137	4.2									
Day 28								1.8	53				

Normal Laboratory Values: BUN: 5-18 mg/dl, Creatinin (Cr): 0.32-0.6 mg/dl, Sodium (Na): 136-145 mEq/L, Potassium (K): 3.5-5.1 mEq/L, Clor (Cl): 98-108 mEq/L, Bicarbonate (HCO3): 22-26 mEq/L, Plasma Renin Activity (PRA): 0.5-1.9 ng/mL/hr, Aldosterone: 0.8-17.2 ng/dL, Adrenocorticotropic hormone (ACTH): 7.2-63.3 pg/mL, Cortisol: <10 mcg/dL, 17 hydroxyprogesterone (17 OHP): <3 ng/mL

he was aged 4.5 months, he underwent a second PHA1 episode after a UTI.

Patient 4 presented for evaluation routinely. Her history was characterized by the presence of meningomyelocele and flaccid paralysis of the lower extremities. Clean intermittent catheterization was performed since the 2nd month. Physical examination showed a meningomyelocele sac in the sacral region. She was accepted as having PHA1 due to UTI.

Patient 5 presented with vomiting. The physical examination was normal. Dilatation of the left renal collecting system had been detected during the antenatal period. Previous imaging studies revealed grade 4 ectasia and ureteropelvic (UP) stenosis on the left kidney using US. He was considered as having secondary PHA1 caused by obstructive uropathy and UTI.

Patient 6 was admitted to the emergency department due to lethargy and decreased oral acceptance. His physical examination showed dehydration and circulatory collapse. A urine microscopy examination revealed UTI. He was accepted as having PHA1 due to UTI. Corticosteroids were added to the therapy for possible surrenal insufficiency. Fludrocortisone was discontinued at the end of the first week.

Patient 7 presented with vomiting and oral intolerance. He had dehydration and pallor and did not look well. In his abdominal US, a double collecting system was detected on the right kidney and grade 2 dilatation on the left kidney. Bilateral grade 5 vesicoureteral reflux (VUR) was observed in VCUG. UTI was detected in a urine test. He was considered to have secondary PHA1 caused by UTA and UTI. Hydrocortisone (single-dose) and fludrocortisone were started. CAH was excluded when the 17 OHP level was normal.

Patient 8 presented to the emergency department with a one-week history of poor feeding, vomiting, and lethargy. Urine microscopic examination revealed UTI. Antenatal US had shown bilateral HN and PUV was detected in the postnatal period. He was accepted as having

PHA1 due to UTI and UTA. Corticosteroids were added to the therapy. Mineralocorticoid therapy was discontinued at the end of the first week.

Among our patients, males were in the majority (87.5%, 7/8) and 62.5% (5/8) were in the under 3 months age group. Although UTI was present in eight of our patients, there was no underlying UTA in two of these patients; one patient had a neurogenic bladder secondary to meningomyelocele. UTA and UTI were observed in 62.5% (5/8) of our patients. All patients recovered from their electrolyte imbalances on the third day of hospitalization with antibiotic and/or antifungal treatment for underlying infections and intravenous saline. Hydrocortisone (single-dose) and fludrocortisone were added to the therapy for possible surrenal insufficiency in *Patient 1*, *Patient 6*, *Patient 7*, and *Patient 8*.

Discussion

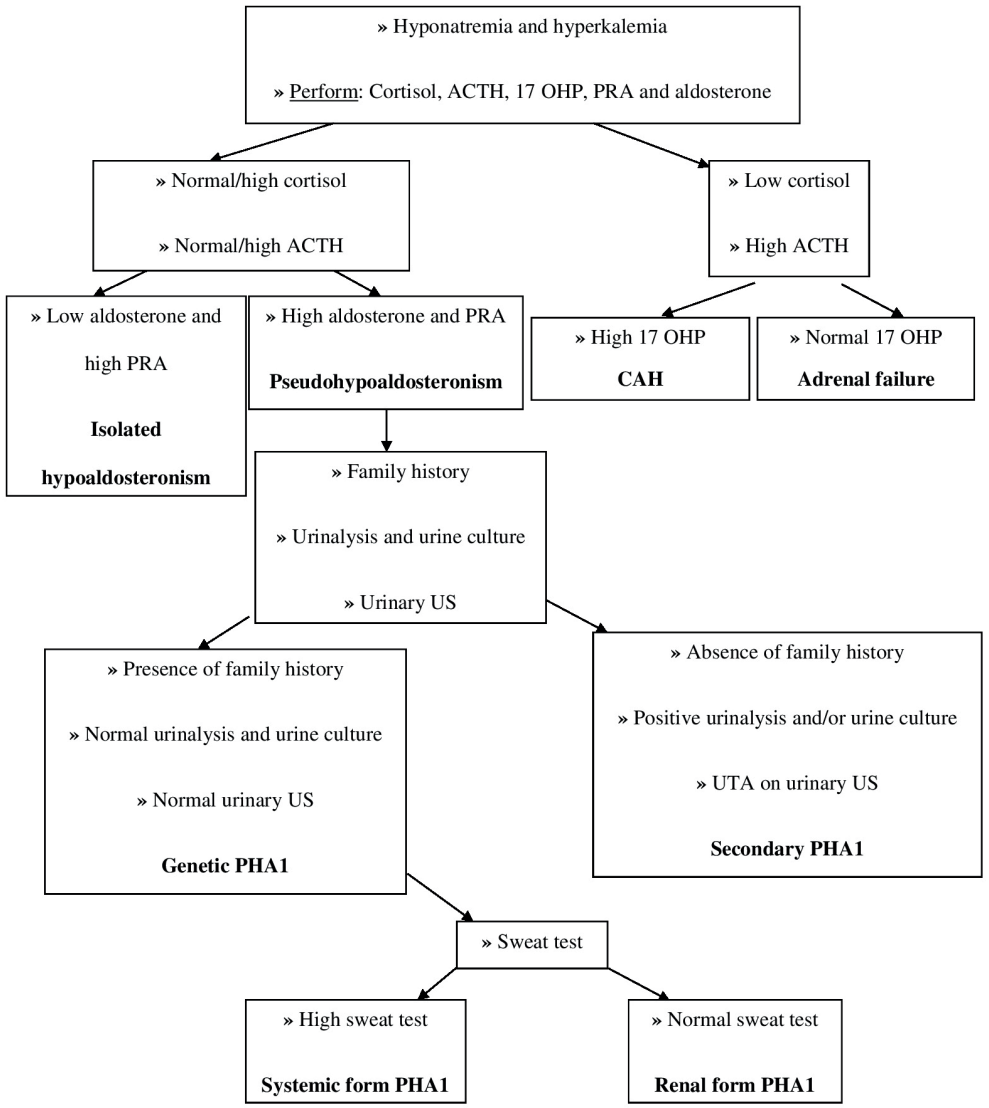
Secondary PHA1 is due to transient aldosterone resistance and has been described in infants with urinary tract malformations or UTIs or both.[8] Searching MEDLINE, we found 137 cases of transient PHA1 since 1983.[4,9-20] Most of the reported cases by sex were male 70.5% (91/129, 1:1.41), and 84.6% (116/137, 1:1.18) were in the under 3 months age group. In our study, male sex was more prevalent, on the other hand, the age group under 3 months was found to be lower than in the literature.

In the literature, UTA was present in 125 of 137 patients (91.1%), UTA and UTI were seen together in 87.2% (109/125) of cases.[4,9-20] UTA and UTI were observed in 62.5% of our cases. Uncircumcised male infants aged under 3 months have the highest baseline prevalence of UTI and the incidence of obstructive uropathy is higher in males than females.[21] The risk of severe electrolyte imbalance in children with obstructive uropathy is significantly reduced after 3 months and this age-related condition seems to be related to immaturity in kidney tubules.[22] We believe that the frequency of PHA1 is higher in boys aged under 3 months for these reasons. Although the true prevalence of PHA1 associated with UTI and/or UTA is not known, it may be higher than reflected in the published literature because ascertainment is dependent on physician awareness.

The underlying pathogenesis of secondary PHA1 is unclear. Possible mechanisms include the emergence of bacterial endotoxin damage at the aldosterone receptors secondary to cytokines such as transforming growth factor-β (TGF-β), and parenchymal scarring secondary to obstruction.[23] Urinary tract obstruction increases the intrarenal synthesis of many cytokines, such as interleukin (IL)-1, IL-6, tumor growth factor (TGF)-β1, and tumor necrosis factor alfa (TNF-α).[24] TGF-β1 is produced from infiltrated macrophages and renal tubule cells and suppresses the effect of aldosterone by reducing MR susceptibility.[25]

Severe hyponatremia, hyperkalemia, and dehydration may be seen with CAH, isolated aldosterone deficiency, and other forms of hypoadrenalism, or PHA.[9] The most common cause of hypoaldosteronism is adrenal failure, which is usually caused by CAH during early life. Treatment should begin as soon as possible for the possibility of adrenal insufficiency. If CAH is excluded, hydrocortisone is stopped.[4] In cases of hyponatremia and hyperkalemia, the key finding of elevated serum aldosterone and renin level strongly suggests the diagnosis of PHA1.[9] Two forms of PHA1 can be distinguished at the clinical and genetic level. Family history, laboratory studies, and the presence of urinary tract malformations and/or UTI may help to differentiate genetic PHA1 from secondary PHA1. The sweat test helps when considering the genetic form; if the sweat test result is normal, the renal form should be considered, and if it is high, the systemic form should be considered (Fig. 1).[3]

In our cases [*patient 2, 3* (first episode)], Candida spp. appeared as the causative agent of two PHA1 episodes. Candida infections were



Abbreviations: ACTH: Adrenocorticotropic hormone, CAH: congenital adrenal hyperplasia, PHA: Pseudohypoaldosteronism, PRA: Plasma renin activity US: Ultrasonography, UTA: Urinary tract anomaly, 17 OHP: 17 hydroxyprogesterone

Fig. 1. Approach to hyponatremia and hyperkalemia.

the most important causes of clinically proven UTI in our patients, who produced no other uropathogens. Initially, IV antibiotic therapy was started for the patients but there was no reproduction in the first urine culture and IV fluconazole treatment was added to two urine cultures in the presence of the same amount of Candida spp. In our patients, the causes of susceptibility to Candida-related UTIs appeared to be congenital and structural abnormalities of the urinary tract and urinary stasis.[26] It may also be another important risk factor for premature birth history in both patients.[27] Although obstruction and/or urinary tract stones have been identified for renal involvement, the mechanism of settlement and proliferation in the calyx has not been fully elucidated.[28] Further studies are needed on how UTIs due to Candida species lead to secondary PHA1.

These case series show the need for testing for UTIs in afebrile infants with non-specific symptoms such as vomiting and agitation

at admission, and to consider PHA1 as a differential diagnosis when co-existent with hyponatremia and hyperkalemia. Renal US should be performed, especially in male infants when secondary PHA1 is suspected. Obstructive uropathy supports the diagnosis of secondary PHA1. Our patients also demonstrated the transient nature of the aldosterone resistance; therefore, genetic analysis was not performed.

It is controversial in the literature that the most important risk factor leading to aldosterone resistance is UTA or UTI.[29] With younger age, the risk for secondary PHA1 increases, and severe electrolyte imbalance significantly increases in patients with obstructive uropathy.[22] Preterm birth history also facilitates the development of PHA1 by increasing kidney immaturity.[30]

In *patient 1*, UTI, prematurity, and age 3 months were the most important risk factors as the secondary cause of PHA1. *Patient 2* and *patient 3* were found to have UTA, UTI, prematurity, and age under 3 months. *Patient 3* underwent PUV resection surgery in the third month after the first PHA1 attack. However, this patient had PHA1 for the second time after UTI at 4.5 months. The ongoing bilateral grade 3-4 VUR appears to be the cause of the second attack. Possible risk factors for PHA1 in *patient 4* are clear intermittent catheterization, UTI, and age under 3 months. *Patient 5* underwent surgery at the age of 3.5 months due to left UP stenosis. At the age of 6 months, *patient 5* had PHA1 as a consequence of ongoing grade 3-4 HN and UTI. In *patient 6*, UTI was found to be the only secondary cause of PHA1. UTA, UTI, and age less than 3 months in *patient 7* and *patient 8* were possible risk factors.

The management of secondary PHA1 involves sodium supplementation and water replacement, ion exchange resins when there are high potassium values, bicarbonate in some cases, antibiotics for UTIs, and surgical intervention for UTA when necessary.[7] The abnormalities quickly disappear after medical or surgical therapy.[31] The normalization of biochemical tests may be seen within 24 hours.[22] In our study, all patients recovered from their electrolyte imbalances on the third day of hospitalization with antibiotic and/or antifungal treatment for underlying infections and intravenous saline. In *patient 1* and *patient 5*, PRA and aldosterone levels normalized at 2 weeks. Despite the underlying PUV in *patient 2*, PRA and aldosterone post-UTI normalized at 2 weeks. On the other hand, although *patient 8* was given antibiotic treatment for UTI, aldosterone levels did not normalize at 4 weeks. This situation may be related to the ongoing PUV. In the first PHA episode in *patient 3*, PRA and aldosterone levels were normalized at 2 weeks in combination with UTI and PUV, whereas in the second PHA episode caused by UTI alone, the improvement was observed at 4 weeks. Single-dose hydrocortisone and fludrocortisone treatment were given to *patient 1, 6, 7*, and *8* for the possibility of CAH due to poor hemodynamic circulatory findings and biochemical profiles. Glucocorticoid and mineralocorticoid treatment were started without waiting for hormone test results (cortisol, aldosterone, 17 OHP). In the literature, there are cases in which glucocorticoid and mineralocorticoid treatment were initiated and these therapies were discontinued during follow-up.[3,4] In our study, fludrocortisone was discontinued at the end of the first week when the 17 OHP result was normal.

In patients presenting with non-specific symptoms during early infancy, when hyperkalemia and hyponatremia in biochemical parameters are noticed, CAH or adrenal insufficiency and secondary PHA1 should be considered. Urine analysis, urine culture, and renal US imaging are very important in terms of rapid identification, especially in children younger than 3 months or older, up to 8 months. If a pathologic condition is detected in these studies, PHA1 should first come to mind. Fungal infections should not be forgotten in the etiology of UTI because PHA1 episodes can be initiated.

Ethical approval

Ankara University Ethics committee approval for the study was received. (approval number: 14-675-16).

Author contribution

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

Seasonal coronaviruses infections in children: do they always cause mild illness?

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ABSTRACT

Background. Human coronaviruses (HCoVs) cause a comprehensive clinic ranging from asymptomatic course to pneumonia. We aimed to describe the HCoV infections in children to determine the clinical status and coinfection effects in a five-year retrospective surveillance study. The primary outcome was admission to the intensive care unit (ICU) and the secondary outcome was the need of high oxygen support.

Methods. Between September 2015 and November 2020, all patients whose reverse transcription polymerase chain reaction (RT-PCR) tests were positive were determined and patients with HCoVs were included in the study. Demographical characteristics, underlying chronic diseases, clinical diagnosis, laboratory data, subtypes of HCoVs, radiological findings, treatments, hospitalization, and ICU admission were analyzed.

Results. Of the 2606 children, the overall respiratory tract virus detection rate was 82.4%. Among these, 98 cases were HCoVs positive and of these 80 (81.6%) were under five years of age and most of the patients were admitted to the hospital in spring and 70% were a mixed infection with other respiratory viruses. Since lower respiratory tract infections are more common in HCoV coinfections, a significant difference was found in clinical diagnosis ($p<0.001$). The presence of hypoxia ($p=0.003$) and underlying disease ($p=0.004$) were found to be significantly more common in patients admitted to the ICU. The presence of hypoxia, infiltration on chest X-ray, and elevated C-reactive protein levels were more frequently determined in patients who received high oxygen support ($p=0.001$, $p=0.036$, $p=0.004$, respectively).

Conclusions. Clinical findings may be more severe if HCoVs, which generally cause mild respiratory disease, are coinfected with another viral agent.

Key words: HCoV, children, respiratory tract infections, coinfection, lower respiratory tract infection.

Human coronaviruses (HCoVs) have been known to infect humans and animals since the late 1960s.[1] There are seven subtypes, including OC43, NL63, 229E, HKU1, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2. HCoV-OC43 is the most common subtype and is commonly observed in children <5 years of age.[2] Although the infection can be asymptomatic, it can present with upper respiratory tract infections, croup, bronchiolitis, and pneumonia. HCoVs are thought to be responsible for approximately 10% of all upper and lower respiratory tract diseases.[3] For seasonal HCoVs, several including OC43, 229E, NL63, and HKU-1 have generally been associated with mild respiratory illnesses and have been assessed mainly in epidemiological studies.[4] Still, severe diseases, accompanied by lower respiratory tract infection, might also occur, especially in the elderly, neonates, and patients with underlying

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conditions and risk factors.[5] HCoVs are often detected in coinfections with other respiratory viruses. Although the impact of infection has not been clearly demonstrated in HCoV coinfections, some studies have found that patients with coinfections experience worse morbidity.[6,7]

Although HCoV is widespread globally, the frequency of detection of its four major subtypes varies significantly both by geography and over time. Despite these epidemiology features, there is no study focusing on the frequency of HCoVs strains and their clinical manifestations from Turkey. In general, coronaviruses are thought to cause mild respiratory illnesses, but severe respiratory diseases or even intensive care admission may be seen in some patients. This study aimed to determine the frequency of HCoVs in a series of hospitalized infants and young children with symptoms of respiratory tract diseases. The present study's main focus was to detect clinical pictures and factors that affect the disease severity of the HCoVs in hospitalized children.

Material and Methods

Study Design and Definitions

This retrospective cohort study was conducted by the Division of Pediatric Infectious Diseases, Health Sciences University Izmir Tepecik Research and Training Hospital in Turkey between September 2015 and November 2020. The medical records of patients diagnosed with HCoVs infections were analyzed. Relevant information including demographical characteristics, clinical symptoms, prematurity, underlying diseases, clinical diagnosis, laboratory data, subtypes of HCoVs, radiological findings, treatments, hospitalization, and ICU admission were recorded.

Common cold and croup were regarded as upper respiratory tract infections. Symptoms of a common cold are nasal discharge, nasal obstruction, sneezing, sore throat, and cough. Croup is often seen with sudden onset of a bark like cough, hoarse voice, and nocturnal respiratory stridor.[7] The clinical diagnoses of acute bronchiolitis, asthma, and pneumonia defined the lower respiratory tract infections. Acute bronchiolitis is characterized by wheezing in children less than two years old.[8] Patients with infiltration on chest radiographs and without wheezing were classified as pneumonia.[9] In the diagnosis of asthma, the National Asthma Education and Prevention Program guidelines were followed.[10]

In this study, the term viral coinfection indicates the simultaneous detection of two or more respiratory viruses in the same sample taken from a respiratory infection patient.

The study protocol was approved by the Institutional Ethics Committee of Izmir Tepecik Training and Research Hospital (Protocol number: 2020/13-15)

Viral Assays

Nasopharyngeal swab samples were examined for the *Respiratory syncytial virus A/B, Rhinovirus, Influenza virus A and B, Parainfluenza virus 1, 2, 3 and 4, Human metapneumovirus, Adenovirus, Human coronavirus OC43, 229E* and *NL63*, *Bocavirus*, and *Enterovirus* with multiplex reverse transcription-polymerase chain reaction (RT-PCR) method (Anyplex II RV 16 Detection; Seegene, Seoul, South Korea was used until December 2018; after January Bosphore, Respiratory pathogens panel kitv4, Anatolia, Turkey was used). One nasopharyngeal swab was collected from each enrolled child. A specimen was defined as being HCoVs positive when the RT-PCR assay was positive for HCoVs 229E, NL63, and OC43.

Primary and secondary outcome

The primary outcome was considered as admission to ICU. Participants were divided into two main groups according to oxygen treatment to determine the secondary outcome. In the first group, patients did not receive any oxygen treatment or need oxygen with a mask. The second group consisted of patients who

received high flow nasal cannula (HFNC), noninvasive mechanical ventilator (MV), and invasive MV treatment.

Statistical Analysis

Statistical data were analyzed with IBM SPSS for Windows version 25.0 (Chicago, IL). Values for numerical variables were given as median (interquartile range) (IQR) or mean ± standard deviation, depending on the normality distribution. Categorical variables were presented as numbers and percentages. Continuous variables following normal distribution were compared using one-way analysis of variance or t-tests. When distribution was not expected, the Kruskal-Wallis test was used. Categorical variables were compared using the Chi-Square test. A p-value of <0.05 was considered statistically significant for all predictions.

Results

A total of 2606 pediatric cases were diagnosed with respiratory tract infections and a nasopharyngeal swab was obtained from these cases. Overall, in 82.4% (n=2147) of samples, one or more viruses were confirmed with the RT–PCR. Of whom 98 (4.6%) were infected with HCoVs. Single HCoVs infection was detected in only 29 (29.6%) cases; in 69 (70.4%) of the patients, at least one other viral agent was detected in addition to HCoVs.

Characteristics of HCoVs Infection

A total of 98 children infected with HCoVs were enrolled in this retrospective study, 80 (81.6%) were under five years, and 62 (63.3%) were male. Considering the seasonality of HCoVs, 48 cases (49%) presented in spring. With regard to the distribution of cases in proportion to months, the highest admission was determined in March with 25 cases (25.5%). Half of the patients (50%) had an underlying disease, while 19 patients (19.4%) had a history of prematurity. The most common symptoms on the first presentation were fever (56/98, 57.1%), and cough (53/98, 54.1%). Physical and clinical findings on presentation were consistent with acute pneumoniae in 46 patients (46.9%). Infiltration was detected in the chest radiography of 51 cases (52%). It was observed that 77 cases (78.6%) received antibiotic treatment. Although 90 patients (91.8%) were hospitalized during follow-up, only 22 (22.4%) of them required the ICU.

Clinical Comparison Between HCoV Single Infections and HCoV Coinfections

When patients with single and coviral infections were compared in terms of age, gender, prematurity history, underlying diseases, and laboratory findings, all variables were similar between groups. Although lower respiratory tract infections and infiltration in the chest X-ray were more common in HCoV coinfections, the differences were statistically significant ($p<0.001$ and $p=0.007$, respectively). While 26 (89.6%) patients were hospitalized with a single HCoV infection, 64 (92.7%) patients with coinfection had a hospitalization history. However; the difference was not statistically different between groups ($p=0.609$). The clinical comparisons in both groups are summarized in Table I.

Clinical Comparison Between HCoV Subtypes

In terms of subtypes of HCoVs observed in the virally positive group OC43 was found in 53 cases (54%), NL63 was found in 32 (32.7%), and 229E was found in 13 (13.3%) cases. According to seasonal distribution, 15 cases (45.4%) with NL63, 26 cases (50%) with OC43, and seven cases (53.8%) with 229E were admitted in spring. There was a significant difference between the seasonal distribution of the three subtypes of HCoVs ($p=0.001$). When the clinical findings were evaluated, it was seen that the groups were similar in terms of fever, cough, rhinorrhea, and nasal obstruction. Wheezing was detected more frequently in nine patients (69.2%) infected with 229E, and the difference was statistically significant compared to the other groups ($p=0.042$). There was no difference

Table I. Clinical comparison between single HCoV infections and HCoV coinfections.

	Single HCoV Infections (n=29)	HCoV Coinfections (n=69)	*P* Value
Gender*			0.223
Male	21 (72.4)	41 (59.4)	
Female	8 (27.6)	28 (40.6)	
Age (mo)**	14 (6-71)	11 (3-30)	0.320
Season*			0.741
Autumn	2 (6.9)	5 (7.2)	
Winter	6 (20.7)	21 (30.4)	
Spring	15 (51.7)	33 (47.8)	
Summer	6 (20.7)	10 (14.5)	
Clinical findings*			
Fever	13 (44.8)	43 (62.3)	0.267
Cough	12 (41.4)	41 (59.4)	0.249
Wheezing	9 (31)	36 (52.2)	0.151
Rhinorrhea	3 (10.3)	11 (15.9)	0.594
Nasal congestion	1 (3.4)	3 (4.3)	0.718
Hypoxia*	9 (31)	24 (34.8)	0.292
Prematurity*	8 (27.6)	11 (15.9)	0.183
Underlying disease*	17 (58.6)	32 (46.4)	0.269
Clinical condition*			**<0.001**
Upper RTI	18 (62.1)	16 (23.2)	
Lower RTI	11 (37.9)	53 (76.8)	
Laboratory findings			
Total WBC (10^3/uL)***	10.526±4.394	11.309±6.715	0.577
Platelet counts (10^3/uL)***	344.444±178.900	354.042±169.931	0.807
C-reactive protein (mg/L)**	6.5 (0.8-28.7)	11.3 (3.1-24.6)	0.845
Procalcitonin (μg/L)**	0.06 (0.03-0.27)	0.14 (0.05-1.8)	0.243
Infiltration in chest graphy*	9 (31)	42 (60.9)	**0.007**
Antibiotic treatment*	22 (75.9)	55 (79.7)	0.672
Hospitalization*	26 (89.6)	64 (92.7)	0.609
Days of hospital stay**	12 (8-31)	9 (7-16)	0.309
ICU admission*	9 (31)	13 (18.8)	0.187
Days of ICU stay**	4 (3-10)	5 (3-13)	0.545

HCoV: human coronavirus, IQR: interquartile range, RTI: respiratory tract infection, WBC: white blood cell, ICU: intensive care unit, SD: standart deviation.
*n, % **median (IQR) ***mean±SD

between the clinical diagnoses of the cases, hospitalizations, and treatments. Clinical characteristics of cases infected with HCoVs subtypes are shown in Table II.

Clinical Comparison According to Primary Outcome

A total of 22 patients required ICU admission, of whom 14 (63.6%) had hypoxia on ICU admission. The presence of hypoxia and underlying diseases were more common in patients who required ICU admission (p=0.003, p=0.004, respectively). In terms of respiratory support, while 21 patients (95.4%) received HFNC/noninvasive MV/invasive MV therapy only one patient had no need for any support, and the difference was statistically significant (p <0.001). When clinical diagnosis, laboratory findings, and the presence of coinfection were compared according to ICU admission, all variables were similar between groups (Table III).

Table II. Clinical comparison between HCoV subtypes.

	NL63 (n=33)	OC43 (n=52)	229E (n=13)	*P* Value
Sex*				0.618
Male	20 (60.6)	35 (67.3)	7 (53.8)	
Female	13 (39.4)	17 (32.7)	6 (46.1)	
Age*				0.552
<5 y	26 (78.8)	45 (86.5)	10 (76.9)	
>5 y	7 (21.2)	7 (13.5)	3 (23.1)	
Season*				**0.001**
Autumn	0 (0)	6 (11.5)	1 (7.7)	
Winter	17 (51.5)	6 (11.5)	4 (30.8)	
Spring	15 (45.4)	26 (50)	7 (53.8)	
Summer	1 (3)	14 (26.9)	1 (7.7)	
Clinical findings*				
Fever	20 (60.6)	31 (59.6)	5 (38.5)	0.434
Cough	21 (63.6)	26 (50)	6 (46.1)	0.295
Wheezing	17 (51.5)	19 (36.5)	9 (69.2)	**0.042**
Rhinorrhea	4 (12.1)	8 (15.4)	2 (15.4)	0.637
Nasal congestion	1 (3)	3 (5.8)	0 (0)	0.520
Hypoxia*	8 (24.2)	21 (40.4)	4 (30.8)	0.474
Prematurity*	4 (12.1)	14 (26.9)	1 (7.7)	0.126
Underlying disease*	18 (54.5)	26 (50)	5 (38.5)	0.617
Clinical condition*				0.420
Upper RTI	14 (42.4)	17 (32.7)	3 (23.1)	
Lower RTI	19 (57.6)	35 (67.3)	10 (76.9)	
Hospitalization*	30 (90.9)	48 (92.3)	12 (92.3)	0.972
ICU admission*	6 (18.2)	15 (48.1)	1 (7.7)	0.203
Treatment*				0.878
No/O_2 need	20 (60.6)	29 (55.8)	7 (53.8)	
HFNC/noninvasive MV/invasive MV	13 (39.4)	23 (44.2)	6 (46.1)	

HCoV: human coronavirus, ICU: intensive care unit, RTI: respiratory tract infection, HFNC: high flow nasal cannula, MV: mechanical ventilator.
*n, %

Clinical Comparison According to Secondary Outcome

Twenty-eight patients (28.6%) did not require any oxygen treatment or respiratory support. Of the patients treated with oxygen, 28 (28.6%) received oxygen with a mask, 22 (22.4%) HFNC, 12 (12.2%) continuous positive airway pressure (CPAP), eight (8.2%) MV support. Cough and nasal obstruction were more frequently seen in the group that did not need oxygen or only received oxygen with a mask (p=0.02, p=0.04, respectively). Fever, wheezing, and nasal discharge were more frequently seen in the group that received high oxygen support (p=0.038, p=0.002, p=0.039, respectively). The presence of hypoxia, infiltration on chest X-ray, and elevated C-reactive protein levels were more frequently detected in patients who received HFNC/noninvasive MV/invasive MV therapy (p=0.001, p=0.036, p=0.004, respectively). The clinical data of the cases according to the oxygen treatment are shown in Table IV.

Table III. Clinical comparison between ICU admission.

	ICU Admission (n=22)	Non-ICU Admission (n=76)	P Value
Sex*			0.122
Male	17 (77.3)	45 (59.2)	
Female	5 (22.7)	31 (40.8)	
Age*			0.907
0-5 y	18 (81.8)	63 (82.9)	
>5 y	4 (18.2)	13 (17.1)	
Season*			0.272
Autumn	3 (13.6)	4 (5.3)	
Winter	3 (13.6)	24 (31.6)	
Spring	12 (54.5)	36 (47.4)	
Summer	4 (18.2)	12 (15.8)	
Clinical findings*			
Fever	16 (72.7)	40 (52.6)	0.150
Cough	9 (40.9)	44 (57.9)	0.051
Wheezing	9 (40.9)	36 (47.4)	0.215
Rhinorrhea	4 (18.2)	10 (13.1)	0.306
Nasal congestion	0 (0)	4 (5.3)	0.166
Hypoxia*	14 (63.6)	19 (25)	**0.003**
Prematurity*	3 (13.6)	16 (21)	0.438
Underlying disease*	17 (77.3)	32 (42.1)	**0.004**
Clinical condition*			0.406
Upper RTI	6 (27.3)	28 (36.8)	
Lower RTI	16 (72.7)	48 (63.1)	
Laboratory findings			
Total WBC (10^3/uL)**	11.595 ±5.715	10.937±6.288	0.661
Platelet counts (10^3/uL)**	357.590±134.204	349.485±182.005	0.847
C-reactive protein (mg/L)***	22.2 (5.1-67.6)	7.4 (2.4-20)	
Procalcitonin (μg/L)***	0.16 (0.03-3.6)	0.13 (0.04-1.23)	
Respiratory viruses*			0.187
Single HCoV detection	9 (40.9)	20 (26.3)	
HCoV + ≥1 detection	13 (59.1)	56 (73.7)	
Infiltration in chest graphy*	13 (59.1)	38 (50)	0.452
Treatment*			**<0.001**
No/O_2 need	1 (4.5)	55 (72.4)	
HFNC/noninvasive MV/invasive MV	21 (95.4)	21 (27.6)	

HCoV: human coronavirus, ICU: intensive care unit, RTI: respiratory tract infection, WBC: white blood cell, IQR: interquartile range, HFNC: high flow nasal cannula, MV: mechanical ventilator, SD: standart deviation.
*n, % **mean±SD *** median (IQR)

Discussion

This study aimed to determine the rate of HCoVs and epidemiological characteristics during a five year period in Turkey. The overall detection rate of HCoVs in our study population was 4.6%, a value that is slightly lower than previously reported rates of 6.7% and 6%, but similar to studies from Turkey and Spain.[2,11,12] However,

Table IV. Clinical comparison between treatment.

	No/O_2 Need (n=56)	HFNC/Noninvasive MV/Invasive MV (n=42)	*p* Value
Sex*			0.147
Male	32 (57.1)	30 (71.4)	
Female	24 (42.8)	12 (28.6)	
Age*			0.218
0-5 y	44 (78.6)	37 (88.1)	
>5 y	12 (21.4)	5 (11.9)	
Season*			**0.045**
Autumn	2 (3.6)	5 (11.9)	
Winter	20 (35.7)	7 (16.7)	
Spring	23 (41.1)	25 (59.5)	
Summer	11 (19.6)	5 (11.9)	
Clinical findings*			
Fever	28 (50)	28 (66.7)	**0.038**
Cough	32 (57.1)	21 (50)	**0.020**
Wheezing	18 (32.1)	27 (64.3)	**0.002**
Rhinorrhea	6 (10.7)	8 (19)	**0.039**
Nasal congestion	3 (5.3)	1 (2.4)	**0.040**
Hypoxia*	10 (17.8)	23 (54.8)	**0.001**
Prematurity*	8 (14.3)	11 (26.2)	0.140
Underlying disease*	24 (42.8)	25 (59.5)	0.102
Clinical condition*			0.050
Upper RTI	24 (42.8)	10 (23.8)	
Lower RTI	32 (57.1)	32 (76.2)	
Laboratory findings			
Total WBC (10^3/uL)**	10.319±5709	12.079±6587	0.246
Platelet counts (10^3/uL)**	345.278±178.479	359.140±164.145	0.458
C-reactive protein (mg/L)***	5.9 (1.5-18.4)	19.3 (6.2-47.4)	**0.004**
Procalcitonin (μg/L)***	0.14 (0.02-1.8)	0.13 (0.05-0.52)	1.000
Infiltration in chest graphy*	24 (42.8)	27 (64.3)	**0.036**

HCoV: human coronavirus, RTI: respiratory tract infection, WBC: white blood cell, IQR: interquartile range, HFNC: high flow nasal cannula, MV: mechanical ventilator, SD: standart deviation.
*n, % **mean±SD *** median (IQR)

in 70% of cases, these respiratory infections were coinfections with other viral agents. It was noticed that the cases were frequently young, and coinfection was associated with increased clinical severity. In a cohort study from Norway, it was found that coinfections were observed at a rate of 68%. HCoVs infection included a significant part of respiratory tract infections requiring hospitalization and was associated with lower respiratory tract infections.[13] Similarly, in a study involving hospitalized children with acute respiratory infections in Guangzhou, China, a significant difference was found between HCoV subtypes in terms of pneumonia.[14] In a Jordan centered study in which hospitalized children under two years of age were examined, the coinfection rate was 75.7%. However, there was no significant difference in clinical diagnosis in this study.[11] According to our results, lower respiratory tract

infections were more common in patients with coinfections.

It has long been established through several observational studies that weather conditions influence the seasonal incidence of many respiratory viruses. Regional and annual variations in the circulation of HCoVs have been described.[15] Epidemiologic studies of HCoVs infections suggest that they exhibit a seasonal pattern. In a temperate climate, HCoVs infections are primarily detected in winter and spring, with low-level circulation throughout the year.[5] The seasonal spread has been compatible with studies conducted in Europe and the USA.[15-20] According to studies from Asian countries, HCoVs can peak in all seasons.[15,21] During the 5-year follow-up period in this study, HCoV-OC43 was mostly seen in patients, similar to studies from Norway and China.[13,14] A survey conducted in Belgium showed that HCoV-OC43 and HCoV-229E were detected in all seasons.[22] On the other hand, HCoV-NL63 was caught mostly in winter and spring, with the number of new cases peaking in January and March.

In our population, one-fourth of children with HCoVs infection were admitted to the ICU. The presence of an underlying disease was associated as a significant risk factor. In a study conducted in New York, 11% of patients hospitalized with HCoV infection were admitted to the ICU. Moreover, the presence of chronic complex underlying conditions, including cardiovascular, genetic, and respiratory diseases, has been associated with increased disease severity.[23] Although the underlying disease was significant in ICU admission in both studies, there is a substantial difference in ICU admission rates. When the subtypes were examined, we could not find a difference in terms of ICU admission.

In our study, the rate of children who received respiratory support was 42%. In the study involving hospitalized children under the age of five, the need for respiratory support was around 14%.[24] In a study examining children under 18 who were hospitalized with HCoVs between January 2013 and December 2014, this rate was 18%. In the same study, children younger than two years old and those with underlying diseases were more likely to receive respiratory support.[20] According to our data, it is not possible to make this implication from our study. Remarkably, that our patients received a high rate of respiratory support compared to other studies. There is a point to consider that some of the reviews consisted of only inpatients and there was a specified age range.[18,25,26]

Antibiotics were administered to 78.6% of our population. This high frequency of antibiotic use is similar to that reported in the Southeast Brazil study.[23] Secondary bacterial infections and long-term respiratory viral panel results were among the factors causing unnecessary antibiotic use. Although viruses are the most common cause of acute respiratory infections, excessive or inappropriate antibiotics have been used in more than 50% of acute respiratory infections worldwide, leading to severe consequences such as drug side effects, high rates of resistance, and multidrug resistance in children with viral infections.[26]

Multiple pathogens detected in nasopharyngeal aspirates of patients with respiratory disease are increasingly recognized. Our study found that the respiratory tract viral panels have been frequently sent from hospitalized cases. Outpatient and asymptomatic cases can also be included in the study to demonstrate the reflections of HCoV on the clinic. However, there are some limitations to this method as well. It is difficult to determine whether these are true coinfections or indicate the continued transmission of pathogens from a previous infection. It has been shown that in 0.4% of patients without symptoms of respiratory tract infection, HCoV can be detected by PCR (polymerase chain reaction), and HCoV RNA is detected in real-time PCR up to 14 days after illness.[23]

This research had some limitations. One of the main limitations was that it was of retrospective

nature, and therefore, we could not access all clinical information of the patients. Due to our hospital's limited facilities, we were able to perform viral tests mainly on inpatients.

In conclusion, our five-year population-based study shows that the HCoV subtypes OC43, NL63, and 229E appear with characteristic outbreak patterns, primarily in the spring and winter. The cases were frequently under the age of five. It was seen that our population presented with wide clinical pictures such as upper respiratory tract infection, croup, bronchiolitis, asthma, and pneumonia. It should be kept in mind that clinical findings may be more severe if HCoVs, which generally cause mild respiratory diseases, are coinfected with another viral agent. Future multicenter studies with large populations involving all age groups are needed to provide additional information on the epidemiology and clinical features of HCoVs.

Ethical approval

Ethics committee approval was obtained for non-interventional clinical research with the 2020/13-15 protocol number.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: AET, EKÖ; data collection: ST, YEK, AŞ; analysis and interpretation of results: GÜ, YD; draft manuscript preparation: NÖY, AKA, DYÇ: 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

Psychiatric symptoms in children with COVID-19, mothers' psychological resilience and related factors: pandemic hospital inpatient experiences

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ABSTRACT

Background. Information on psychological problems and affecting factors in children hospitalized with the suspicion or diagnosis of COVID-19 is limited. We aimed to screen the psychiatric symptoms of children hospitalized with COVID-19 in Ankara City Children's Hospital and evaluate the caregivers' depression, anxiety, stress, and resilience levels during hospitalization.

Methods. Among the children and adolescents hospitalized in Ankara City Children's Hospital between 1 May 2020 and 31 May 2020 due to the diagnosis of COVID-19, those who agreed to participate in the study were included. The Strengths and Difficulties Questionnaire (SDQ), the Depression Anxiety Stress Scale-21 (DASS-21) scale, and the Brief Resilience Scale (BRS) were used to determine the symptoms.

Results. The mean age of study group (n = 49) was 8.7 ± 5.0 years, 59.2% (n = 29) were girls. The mean children's SDQ-externalizing problems scores were higher in the COVID-19 positive mothers group than the COVID-19 negative mothers' group. The SDQ-total score was positively and strongly correlated with the DASS total score, DASS-depression score, and DASS-anxiety score. BRS scale scores were negatively correlated with the SDQ-total, externalizing, and DASS-21scale scores.

Conclusions. COVID-19 positivity in mothers could be associated with externalizing problems in children. The high resilience of caregivers of inpatients seems to be related to less psychopathology in children. During hospitalization, caregivers' psychological evaluation and psychosocial support may be important for preventive child mental health.

Key words: children, COVID-19, COVID-19 survivors, inpatient, caregivers, resilience.

In December 2019, a novel coronavirus was announced in Wuhan, China. The cause of infection was "severe acute respiratory system corona virus-2 (SARS-CoV-2)", and the disease was "Coronavirus Disease 2019 (COVID-19)". As a result of the rapid spread of the virus all over the World, COVID-19 was declared as a pandemic by the World Health Organization (WHO) on 11 March 2020.[1] COVID-19 infection in children has a milder course, and deaths are rarely reported.[2] This situation causes many children to be considered low-risk groups in terms of infectious diseases in the pandemic. However, in a review discussing the effects of the pandemic on mental health in children, it is recommended that children in the COVID-19 pandemic be considered a sensitive group in terms of mental health problems.[3]

In the literature, most studies on COVID-19 infection and its psychiatric effects are conducted with the adult age group. Preliminary results have shown that COVID-19

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infection is associated with delirium, insomnia, internalizing symptoms, post-traumatic stress symptoms, obsessive-compulsive symptoms[4-6], and in a retrospective cohort study, anxiety and depression were the most common psychiatric symptoms in adult patients with COVID-19.[7] The number of publications on psychological difficulties, especially in children hospitalized with COVID-19, is limited. For prevention, support, and treatment plans for children's mental health problems, it is necessary to increase knowledge on this subject. Our study aimed to screen the psychiatric symptoms of children hospitalized with COVID-19 in Ankara City Children's Hospital and evaluate the depression, anxiety, stress, and resilience levels of the caregivers during hospitalization.

Material and Methods

Children with COVID-19 in inpatient pediatric clinics of Ankara City Children's Hospital in May 2020 were included in this study. Inclusion criteria were; being between 2-17 years of age, having COVID-19 PCR positivity, having a mother as a caregiver, having mothers' capacity to understand and fill in the questionnaires. In addition, sociodemographic form, Strengths and Difficulties Questionnaire-parent form to evaluate children's psychiatric symptoms, Depression Anxiety and Stress Scale to evaluate psychiatric symptoms of mothers, and Brief Resilience Scale to evaluate the resilience of mothers were completed by the mothers.

COVID-19 severity: It is categorized as mild, moderate, and severe illness. Individuals having a mild illness were defined as children who had any of the various signs and symptoms of COVID-19 (e.g., fever, cough, sore throat, malaise, headache, muscle pain, nausea, vomiting, diarrhea, loss of taste and smell) but who did not have shortness of breath, dyspnea, or abnormal chest imaging. Moderate illness: was those with evidence of lower respiratory disease during clinical assessment or imaging and having an oxygen saturation (SpO2) ≥94% on room air at sea level. Severe Illness: Individuals who had SpO2 <94% on room air sea level, a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO2/FiO2) <300 mm Hg, tachypnea (children younger than two months old≥ 60/minute, 2-12 months old ≥ 50/minute, 12 months-5 years old≥ 40/minute), or lung infiltrates >50%. (https://www.covid19treatmentguidelines.nih.gov/overview/clinical-spectrum/)

Depression, Anxiety and Stress Scale (DASS-21) is a self-report 4-point Likert-type scale from 0 (did not apply to me at all/ never) to 3 (applied to me very much/ always), including three subscales each consisting of 7 items: Depression, Anxiety, and stress.[8] Higher scores indicate more severe emotional distress. The validity and reliability study of the Turkish version of the DASS-21 showed that the scale was a valid and reliable instrument in assessing depression, anxiety, and stress levels.[9]

Strength and Difficulties Questionnaire-Parent form (SDQ-parent) consists of 25 items related to children's social, emotional, and behavioral functioning across five subscales: Conduct Problem, Hyperactivity, Emotional Symptoms, Peer Problems, and Prosocial Behavior.[10] The reliability and validity of the SDQ-parent form were confirmed for the Turkish population.[11]

Brief Resilience Scale (BRS) is a 5-point Likert-type scale, consisting of 6 items; Items 2, 4, and 6 are reversed coded. Higher scores indicate higher levels of psychological resilience.[12] The Turkish version of the BRS has been validated in a Turkish population study.[13]

In May 2020, 505 children with COVID-19 diagnosis or suspicion were hospitalized by the Ankara Board of Infectious Diseases decision. Of them, 279 children had COVID-19 PCR positivity, 49 children and caregivers fulfilled the inclusion criteria and accepted participating in this study. Ethical approval was obtained from the Local Committee of Ankara City Hospital (Ethical ID: E1-20-630). In addition, written consents were obtained from adolescents and caregivers.

Statistical analyses

SPSS 17.0 (Chicago Inc. 2008) program was used for statistical analyses. Continuous variables were defined as mean, standard deviation, minimum-maximum values, and categorical ones as frequency (n) and percentage (%). The Kolmogorov-Smirnov test examined the normality of the continuous variables. For comparisons of the continuous variables, student t-test and Mann Whitney-U tests were used. Categorical variables were compared with Pearson chi-square and Fisher's exact test. SDQ-total scores' correlation analysis with other variables was carried out with Spearman correlation analysis. For the multivariate analysis, the possible factors identified with univariate analysis (p-value < 0.1) or correlated with the dependent variable were further entered into the lineer regression analysis. A multiple linear regression analysis was conducted to determine the effect of independent variables of age, COVID-19 positivity of caregivers, DASS, and BPS scores on the SDQ-externalizing scale score. Multiple linear regression analysis was applied using the "Enter" method. p<0.05 was accepted as significant.

Results

Forty-nine children (29 girls, 59.2%; mean age 8.7 ± 5.0 years (age range: 2-17 years) and mothers were included in the study. All children had mild COVID-19. 87.7% of children had siblings. The rate of COVID-19 positivity in one of the family members was 93.9 (n=46). 53.1% (n=26) of mothers had COVID-19 PCR positivity and received treatment. The study group was grouped according to COVID-19 PCR positivity of caregiver mothers during hospitalization. The median age of COVID-19 positive mothers was lower than the COVID-19 negative mothers (z = -2.090 p = 0.037). The mean age of children were lower in COVID-19 positive mothers than those in COVID-19 negative mothers (respectively $\chi 2$= 5.024 p = 0.02; t= -2.533 p = 0.01). Length of hospital stay of children was similar between groups. There was no history of psychiatric disorders in children, and all were in regular education. Chronic diseases of mothers were diabetes mellitus and hypothyroidism, and mothers' psychiatric diagnoses were anxiety disorders. None of the patients or the parents of the patients needed psychiatric consultation during their hospitalization. There was no death due to COVID-19 in positive family members. Sociodemographic characteristics and risk factors were given in Table I.

When two groups were compared in terms of children's psychiatric scores, only SDQ-parent form externalizing score was significantly higher in children with COVID-19 positive mothers group than negative group (t = 2.029. p = 0.05). There was no difference in the mothers' DASS-21 scores and BRS scores between the two groups (Table II).

Correlation analysis of all groups showed that SDQ total, externalizing and internalizing scores were correlated moderate to low with DASS-21 total, depression, anxiety, and stress scores. There was a low negative correlation between BRS total score and SDQ total, externalizing scores. BRS total scores negatively correlated with DASS-21 anxiety (r = -0.314 p = 0.038), depression (r = -0.586 p <0.001), stress scores (r = -0.565 p <0.001) and total scores (r=-0.518 p <0.001). Also, there was a low negative correlation between SDQ externalizing score and child's age (Table III).

Multiple linear regression analysis revealed that there was only a significant predictive value of DASS-21 total score in terms of SDQ-externalizing subscale scores (F=7.58 p=<0.001 R^2 =0.44) (Table IV).

Discussion

The current study aimed to investigate the psychiatric symptoms among children hospitalized with acute COVID-19. We also aimed to investigate whether the mental health of these children could be associated with COVID-19 infection, psychological status, and resilience of caregiving mothers.

Table I. Characteristics of children with COVID-19 and their mothers.

		Mothers COVID-19 PCR positivity		Statistics	
	Total group n = 49	Positive n = 26	Negative n = 23	t, z or χ^2	p value
Children's					
Age (years)[a]	8.7±5.0 (2 – 17)	7.1±4.1 (2 – 16)	10.6±5.4 (2 – 17)	-2.533	0.01
Gender, n (%)				0.882	0.35
Girls	29 (59.2)	17 (65.4)	12 (52.2)		
Boys	20 (40.8)	9 (34.6)	11 (47.8)		
Chronic disease, n (%)				0.903	1.00
Yes	1 (2.0)	1 (3.8)	0		
Length of hospital stay (days)[a]	10.5±3.8 (5 – 19)	10.2±3.9 (5 – 19)	11.1±3.8 (6 – 19)	-0.782	0.44
Mothers'					
Age (years)b	32 (22 – 45)	29 (22 – 43)	37 (24 – 45)	-2.090	0.03
Chronic disease, n (%)				0.238	1.00
Yes	3 (6.1)	2 (7.7)	1 (4.3)		
Psychiatric disease, n (%)				2.357	0.21
Yes	2 (4.1)	0	2 (8.7)		
Risk factors					
The number of siblings. n (%)				2.487	0.32
None	6 (12.2)	2 (7.7)	4 (17.4)		
Two	18 (36.7)	12 (46.2)	6 (26.1)		
Three	25 (51.0)	12 (46.2)	13 (56.5)		
Four	0	0	0		
Living with elderly (≥60 y) person, n (%)				1.154	0.47
Yes	1 (2.0)	0	1 (4.3)		
Living with a health worker, n (%)				1.154	0.47
Yes	1 (2.0)	0	1 (4.3)		
COVID-19 (+) in other family members, n (%)				3.612	0.09
Yes	46 (93.9)	26 (100.0)	20 (87.0)		

[a]: Mean ± standard deviation (range); b: Median (range); *: Fisher's exact test; NA: not-applicable

The study group consisted of very mild and mild COVID-19 child cases. Similar to our sample COVID-19 infection in children has a milder course, and COVID-19 related deaths in children are reported to be rare at the time of the study.[2] So, patients with more severe illnesses could not be included in the study group. Although children are considered a group at low risk for infectious diseases, it is recommended to consider them a sensitive group in terms of mental health problems in the COVID 19 pandemic[3], particularly children with COVID-19 infection.[4,14]

Children with COVID-19 infection have some challenges during the hospitalization due to separation from the caregiver.[4] Separation from the parents is one of the most severe traumas a child can experience because it may affect their self-regulation and resilience.[15,16] Children were hospitalized without separation from caregivers during COVID-19 isolation in our pediatric clinics. A study reported that separation anxiety and insomnia were the most common mental health problems in hospitalized COVID-19 infected children.[17] Parental mental health problems of children hospitalized with

Table II. Comparing psychological scores of children and mothers between two groups.

	Total group n = 49	Mothers COVID-19 PCR positivity		Statistics	
		Positive n = 26	Negative n = 23	t, z or χ^2	p value
DASS-21[b]					
Total score	7 (0 – 41)	6.5 (0 – 33)	7 (0 – 41)	-0.235	0.81
Depression	1 (0 – 12)	1 (0 – 11)	2 (0 – 12)	-0.163	0.87
Anxiety	2 (0 – 15)	1.5 (0 – 15)	2 (0 – 11)	-0.390	0.69
Stress	2 (0 – 19)	2 (0 – 12)	3 (0 – 19)	-0.011	0.99
SDQ[a]					
Total score	9.8 ± 4.5	10.2 ± 4.8	9.3 ± 4.2	0.810	0.42
Emotional	2.3 ± 1.8	2.2 ± 1.8	2.4 ± 1.8	-0.444	0.65
Conduct	1.2 ± 1.1	1.4 ± 1.2	1.0 ± 0.9	1.761	0.08
Hyperactivity	3.2 ± 2.1	3.7 ± 2.4	2.7 ± 1.8	1.732	0.09
Peer relations	2.9 ± 1.6	2.7 ± 1.6	3.1 ± 1.5	-0.824	0.41
Prosocial	7.9 ± 1.6	7.9 ± 1.9	7.8 ± 1.4	-0.059	0.95
Externalizing	4.5 ± 2.8	5.2 ± 3.1	3.7 ± 2.2	2.029	0.05
Internalizing	5.2 ± 2.7	5.0 ± 2.7	5.5 ± 2.7	-0.787	0.43
BRS-total[a]	21.4 ± 4.8	22.0 ± 4.6	20.9 ± 5.1	0.978	0.33

DASS-21: Depression, Anxiety and Stress Scale; SDQ: Strength and Difficulties Questionnaire; BRS: Brief resilience scale; [a]: Mean ± standard deviation (range); [b]: Median (range)

Table III. Correlation analysis of the SDQ-parent form scale scores with related variables.

		DASS-21 total	DASS-21 depression	DASS-21 anxiety	DASS-21 stress	BRS total	Child's age	Mother's age
SDQ-total	rho	0.551	0.601	0.506	0.516	-0.420	-0.203	-0.154
	p	<0.001	<0.001	<0.001	<0.001	0.004	0.176	0.307
SDQ-externalizing	rho	0.501	0.506	0.458	0.506	-0.329	-0.328	-0.131
	p	0.001	<0.001	0.002	<0.001	0.008	0.026	0.385
SDQ-internalizing	rho	0.403	0.490	0.389	0.340	-0.279	0.069	-0.128
	p	0.07	0.001	0.009	0.024	0.064	0.647	0.395

SDQ: Strength and Difficulties Questionnaire-Parent form, DASS-21: Depression, Anxiety and Stress Scale DASS-S: BRS: Brief Resilience Scale

Table IV. Multiple linear regression analysis of the SDQ-parent form scale external scores with related variables.

SDQ- externalizing	B	SE	B	T	p	95%CI	
						Lower	Upper
DASS-total	0.108	0.044	0.345	2.441	0.019	-0.018	0.193
BRS-total	-0.159	0.086	-0.269	-1.846	0.073	-0.333	0.015
COVID-19 positivity of caregiver	1.576	0.791	0.272	1.992	0.054	-0.026	3.177
Child's Age	-0.109	0.082	-0.175	-1.332	0.191	-0.237	0.057

SDQ: Strength and Difficulties Questionnaire-Parent form, DASS-21: Depression, Anxiety and Stress Scale DASS-S: BRS: Brief Resilience Scale

COVID-19 were more severe, and their anxiety and depression were more evident than other inpatient children's parental mental health problems.[18]

In our study, 53.1% of mothers were COVID-19 positive and received concurrent treatment with their children during hospitalization. COVID-19 positive mothers' depression, anxiety, and stress levels were similar to the COVID-19 negative mothers' group. Additionally, sociodemographic characteristics and risk factors (chronic disease, healthcare worker in the family, family member over 60 years old) were similar. However, children of mothers with COVID-19 were more likely to have externalizing problems. Moreover, the externalizing scores of the children were positively associated with the mothers' depression, anxiety, and stress scores. In previous studies, parents' mental health has been related to hyperactive behaviors in children.[19] A recent online survey study suggested that higher rates of parental psychological distress were associated with higher levels of hyperactivity/ inattention in children. Also, parental verbal hostility positively mediates this association.[20] Elevated maternal distress may increase children's risk of externalizing problems by problems in parenting and child self-regulation.[21] Consistent with our results, parents' concerns about themselves or family members who had the COVID-19 were related to higher levels of children's conduct disorder.[22]

There were no differences in internalizing problems between the two groups in our study. A recent study indicated no difference in anxiety between the groups (COVID-19-positive children with COVID-19-positive parents and the control group). However, COVID-19-negative children separated from their parents due to parents' COVID-19 infection demonstrated higher anxiety levels than the control group. These results show that children's high anxiety levels are associated with separation from parents rather than COVID-19 positivity.[23] In our study, groups had similar internalizing symptoms' levels. This result may be related to not being separated from their parents in both groups. Childhood is more prone to physical, psychological, and social vulnerabilities during an illness than adulthood.[24] Isolation is a significant stressor for children, and isolation may worsen the course of the disease. Separation also increases maternal stress levels, increasing heart rate and cortisol levels. These factors may worsen the disease course of mothers.[25] Within the context of infectious disease pandemics, including the current COVID-19 pandemic, avoiding the separation of parents and hospitalized children has been recommended.[24] Moreover, additional recommendations have been reported on whether a mother is COVID-19-positive or negative.[24] It is a child's right to access their parents during hospitalization, and parents should have access to their hospitalized children. Separation should only occur in exceptional conditions, e.g., if adequate in-hospital facilities do not accommodate the parent and the child together. Both parents should be allowed access to hospitalized children under strict infection prevention and control measures, including handwashing/sanitization, face masks, and physical distancing.[24] Appropriate physical conditions and infection control measures were available for caregivers to accompany children in our children's hospital, so primary caregivers attended hospitalized children in our pediatric infection clinic.

COVID-19 positive mothers' children were younger than COVID-19 negative mothers' children, and there was a negative correlation between child age and externalization problems. In a recent study, young children are more likely to show irritability during the pandemic.[26] Furthermore, being younger was associated with higher hyperactivity/inattention behavior in an online survey study during the COVID-19 lockdown.[20]

Resilience plays an essential role in the response of individuals under pressure and can help them cope with problems more effectively. There was no difference in parental resilience between the groups. However,

there was a negative correlation between children's psychiatric symptoms and mothers' psychological resilience.[27] The psychological resilience of parents affects parenting style, psychological well-being of parents and child-mother interactions.[28] Lower psychological resilience demonstrating poor coping skills might affect the child-mother relationship and parenting style, and so caused children's psychiatric symptoms in our study. In a recent study evaluating psychological resilience in the COVID-19 pandemic, lower resilience scores were associated with more severe depression and anxiety.[29] Considering that lower mothers' resilience was related to higher depression, anxiety, and stress in mothers in our study, this could affect children's mental health.

Our study had some limitations. This study was conducted in a single center with a small sample. Thus, our findings cannot be generalized to other populations. The psychopathology of the adolescents and their mothers could not be evaluated with standardized face-to-face interviews. Psychiatric symptoms other than depression, anxiety, and stress levels of mothers were not assessed in this study. The children's psychiatric assessments depend on only parent-reported, and the duration of hospitalization was not recorded.

In conclusion, our results demonstrate that maternal depression, anxiety, and stress were related to psychological status in children with COVID-19. COVID-19 positive mothers' children have higher externalizing scores than COVID-19 negative mothers' children. Mothers' and children's psychological problems increasing with the lower scores of the mothers' psychological resilience may also indicate the psychosocial support needs of mothers hospitalized with children COVID-19 positive. A larger sample with a longitudinal design may reveal the effects of COVID-19 positivity on caregiver and child psychopathology in the following studies.

Ethical approval

Was obtained from the Local Committee of Ankara City Hospital (Ethical ID: E1-20-630).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: EÇ, AP, GŞD, ÖŞÜ; data collection: AP, TÖ, BG; analysis and interpretation of results: GŞD, EÇ, EA, ZG; draft manuscript preparation: ÖŞÜ, EÇ, EA. 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

Pediatric head and neck rhabdomyosarcoma; the role of MRI in differentiation from other common pediatric malignant head and neck tumors

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ABSTRACT

Background. The tumor localization/extent and imaging characteristics of rhabdomyosarcomas (RMSs) especially parameningeal type, could overlap with the common tumors of the head and neck (H&N) such as lymphoma and nasopharyngeal carcinoma (NPC). Our goal was to investigate magnetic resonance imaging (MRI) features that could favor the diagnosis of RMS over lymphoma and NPC in H&N region.

Methods. Pretreatment MRI of 42 pediatric patients (mean: 9.7±5.1 years, min-max: 2-18 years) with a recent diagnosis of RMS (n=12), lymphoma (n=14) and NPC (n=16) were retrospectively studied. Tumor localization, extension and spread were evaluated. Signal and enhancement characteristics of the tumors and the presence of necrosis were noted. ADC values were measured by using both the small sample and single slice methods. For comparison of three groups, the Kruskal Wallis test and Pairwise comparisons were used. The intra-class correlation coefficient (ICC) was calculated for the assessment of inter-observer agreement.

Results. Nasopharynx ±parapharyngeal space involvement was detected in 58.3% of RMSs. Rhabdomyosarcoma was more heterogeneous in T2 images compared to lymphoma ($p=0.014$). Rhabdomyosarcoma showed significantly higher frequency of heterogeneous enhancement ($p<0.001$) and necrosis ($p<0.001$) among these tumors. The mean ADC values of lymphoma were significantly lower than the values of RMS ($p<0.001$) and NPC ($p<0.01$) for both observers. The mean ADC values were higher in RMSs than NPCs ($p>0.05$). Intra-class correlation in ADC measurements was higher for the single slice method (ICC=0.997) than the small sample method (ICC=0.989).

Conclusions. Rhabdomyosarcoma tends to have higher ADC values than lymphoma and has a higher frequency of heterogeneous enhancement and necrotic parts than both lymphoma and nasopharyngeal carcinoma. These features could help radiologists to differentiate RMS from the above-mentioned mimickers.

Key words: rhabdomyosarcoma, nasopharyngeal carcinoma, lymphoma, diffusion-weighted imaging, magnetic resonance imaging.

Rhabdomyosarcoma (RMS), as one of the small blue round cell tumors (SBRCTs), is the most common sarcoma of childhood representing 50.9% of all.[1] It is the second most common head and neck (H&N) malignancy after lymphoma in the pediatric age.[2] Rhabdomyosarcoma has a slight male predominance and is usually diagnosed before the age of 10 years; it can also affect older children.[1,3,4] It has embryonic, alveolar, pleomorphic and spindle cell/sclerosing subtypes.[5] The embryonic subtype affects younger patients and has a good prognosis whereas the alveolar subtype affects older children and has a poor prognosis.[3] The pleomorphic subtype is seen only in adults. Spindle cell/sclerosing subtype is rare and most commonly involves paratesticular and H&N regions.[6]

Head and neck RMSs could be divided into subgroups according to the primary location

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such as parameningeal, nonparameningeal, and orbital RMSs.[7,8] Parameningeal RMSs arise in regions adjacent to the meninges such as the nasopharynx, parapharyngeal space, masticatory space, nasal/paranasal regions.[8] Therefore, parameningeal RMSs harbor surgical difficulties and have a poor outcome. Non-parameningeal RMSs tend to be superficial.[8,9]

Signal characteristics of RMS in magnetic resonance imaging (MRI) are not specific; demonstrating intermediate to high signal intensity (SI) on T2 weighted images (WI) and intermediate SI on T1WI compared to muscle.[9] Head and neck RMSs may show various enhancement patterns; most are reported to enhance heterogeneously.[10] Non-enhancing areas corresponding to necrosis or hemorrhage may be seen.[8,10] Similar to other SBRCTs including lymphoma, RMS demonstrates restricted diffusion due to high cellularity.[11-13]

The imaging characteristics of RMSs, especially parameningeal type, could overlap with the common tumors of the H&N region such as lymphoma and nasopharyngeal carcinoma (NPC) including restriction of diffusion.[8-10]

The definition of the site of origin and the differentiation of RMS is not frequently problematic when it is localized in striated muscles with distortion of the adjacent tissues. Nevertheless, RMS originates from primitive mesenchymal cells and it can arise anywhere where striated muscle is not normally found.[1] Furthermore, RMSs generally rapidly grow with adjacent tissue infiltration[14,15] and sometimes it can be difficult to clearly discriminate the tissue compartments on MRI and thus differentiate RMSs using generally known MRI features. Distinguishing these tumors, which is important for determining different treatment management, can be difficult because of the overlapping demographics, tumor location and extent, and imaging features. Our goal was to investigate imaging discriminators that could favor the diagnosis of RMSs over lymphoma and NPC.

Material and Methods

Patients

Institutional Review Board (2020/16-26) approved the study protocol and informed consent was waived by Hacettepe University Institutional Review Board. A retrospective research was performed from July 2014 to June 2020 in Hospital Information System to identify patients with histopathologically proven diagnosis of RMS, lymphoma and NPC. Inclusion criteria were as follows: 1) patients younger than 18 years old; 2) histopathologically proven diagnosis of RMS, suprahyoid lymphoma and NPC; 3) patients with an MRI scan before biopsy and treatment. Exclusion criteria were as follows: 1) orbital lesions (mainly affecting orbits); 2) too small masses with the longest diameter < 1cm on axial T2WI, to ensure an appropriate localization of region of interest (ROI); 3) images with prominent distortion or susceptibility artifacts; 4) MR examination in which diffusion-weighted imaging was not performed.

Imaging

All MR examinations were performed on 1.5 T scanners (Achieva, Philips Healthcare, Best, the Netherlands; Signa, GE Healthcare, Milwaukee, Wisconsin; and Symphony, Siemens, Erlangen, Germany). The imaging protocol was the same for all scans and included coronal, sagittal, and axial T1WI, coronal short tau inversion recovery (STIR) image, axial T2WI with fat saturation, single shot echo planar imaging (EPI) DWI and post-contrast fat-saturated axial and coronal T1WI. Imaging parameters were; T1WI (TR/TE: 410-640/7-15 ms, 3.5-4 mm section thickness, NEX: 1-2, FA: 90°), coronal STIR (TR/TE: 2353-6200/37-60 ms, 3.5-4 mm section thickness, NEX: 1-2, FA: 90°), axial fat-saturated T2WI (TR/TE: 2320-5000/83-90 ms, 3-4 mm section thickness, NEX: 1-2, FA: 90°), DWI (TR/TE: 3400-5674/75-94 ms, and 3-4 mm section thickness, NEX: 2-4, FA: 90°). Post-contrast T1W images were obtained after IV injection of 0.1 mmol/kg gadolinium-based contrast agent.

Single-shot echo-planar imaging (SS-EPI) technique was used for DWI acquisition and performed before the contrast media injection. DWI was acquired with three b-value with values of 0, 500, and 1000 s/mm^{-2}. Apparent diffusion coefficient (ADC) maps were automatically generated.

Image Evaluation

Qualitative Analysis was performed on hospital Picture Archiving and Communication System (PACS) in consensus by two neuroradiologists (SP and EB, 4- and 7-year experience in H&N radiology) blinded to histopathologic diagnosis. Tumor localization and affected spaces were noted. If more than one space were infiltrated by the tumor, involvement was defined as multicompartment involvement. The presence of skull base invasion, intracranial extension, and retropharyngeal and/or cervical lymphadenopathy were also noted. Distant organ metastasis/involvement was determined by cross-sectional imaging (CT, MRI and PET-CT) findings.

Signal characteristics and post-contrast enhancement patterns of tumors were evaluated. Tumor necrosis was defined as the presence of high SI on T2WI, low SI on T1WI, lack of enhancement with high ADC levels.[10,16,17]

The quantitative analysis was performed on hospital PACS independently by two neuroradiologists (SP and EB) blinded to histopathologic diagnosis. ADCs of the tumors were measured by using both the small sample and single slice method. In the small sample method, three separate circular ROI's (with an area of 1cm^2) were placed on the darkest area on ADC maps, which probably represent the highest cellular activity within the tumor. The necrotic areas were avoided. The mean values obtained from these measurements were defined as ADC_{lesion}. In the single slice method, a freehand ROI was drawn outlining the largest cross-sectional area of the tumor (ADC_{area}) from a single slice.

Statistics Analysis

IBM Statistics 23.0 was used for statistical analysis and the *p* value of <0.05 was considered significant. Categorical variables were presented as count and percentage. Mean and standard deviations (SD) and median (Min-Max) values were given for variables with normal distribution and without normal distribution, respectively. For comparison of three groups, the Kruskal Wallis test was used. Pairwise comparisons were made to determine the relationship between groups. The intra-class correlation coefficient (ICC) was calculated for the assessment of inter-observer agreement. In terms of agreement, ICC value was interpreted as poor (< 0.50), moderate (0.50-0.75), good (0.75-0.90), or excellent (>0.90).

Results

The study included 12 patients with RMS (9 parameningeal and 3 nonparameningeal), 14 patients with lymphoma and 16 patients with NPC. A flow chart of the patients enrolled in the study is shown in Figure 1. Seven patients (58.3%) had embryonic, two patients (16.7%) had spindle cell/sclerosing and one patient (8.3%) had alveolar histopathologic subtypes of RMS. In two patients with RMS histopathologic subtype was undetermined.

Demographic features of the patients, tumor localization and extent, signal and enhancement characteristics are summarized in Table I.

The median age of patients with NPC was significantly higher than the median age of patients with RMS and lymphoma (p<0.001 and p=0.002) There was no significant difference between RMS and lymphoma cohorts on age (p>0.05).

Multicompartment involvement was frequent in both NPC (seen in all cases) and RMS (75%) cohorts. Nasopharyngeal carcinoma showed significantly more multicompartment involvement than lymphoma (p=0.003) and RMS

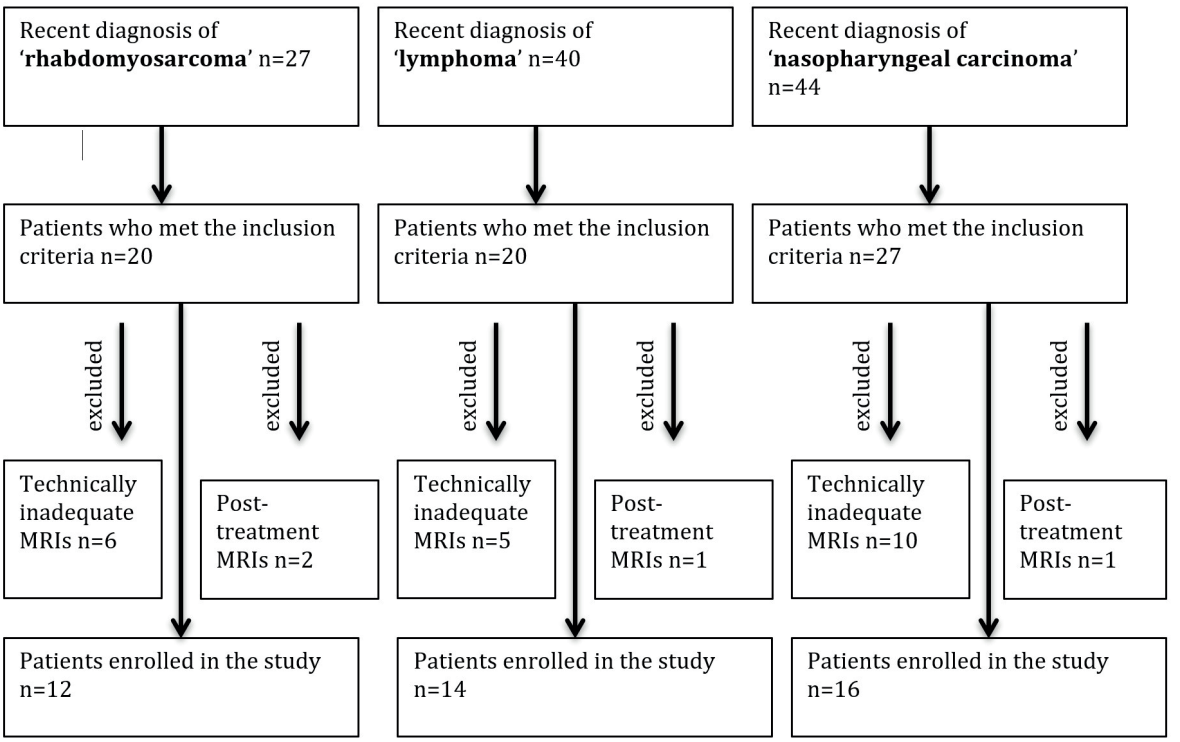

Fig. 1. Flow chart of the patients enrolled in the study.

(p=0.034). There was no significant difference between RMS and lymphoma in terms of multicompartment involvement (p=0.34).

Nasopharyngeal ±parapharyngeal involvement was found as 58.3% in RMS and 57.1% in lymphoma (Fig. 2). Skull base involvement, cervical and retropharyngeal lymphadenopathy were more common in patients with NPC (p<0.001 and p<0.05). In three RMS cases (25%) and in four lymphoma cases (28.6%) tumors tend to encircle vascular structures.

Distant organ metastasis/involvement was seen in five lymphoma patients (p=0.014). Five patients had intraabdominal lymphadenopathy (35.7%), four patients had splenic involvement (28.5%), three patients had hepatic involvement (21%), two patients had intrathoracic lymphadenopathy (14%), two patients had bone marrow involvement (14%). Kidney (n=1), pancreas (n=1) and axillary lymph nodes (n=1) were rare involvement areas.

T2 signal homogeneity was significantly more common in lymphoma (p<0.01). RMS was more heterogeneous in T2 images compared to lymphoma (p=0.014). RMS and NPC were not different in terms of T2 homogeneity (p>0.05). RMS lesions showed a significantly higher ratio of heterogeneous enhancement than lymphoma and NPC (p<0.001). A total of seven patients (six embryonic and one spindle cell/sclerosing subtypes) with RMS (58.3%) had imaging findings compatible with tumor necrosis. None of the lymphoma and NPC had necrotic component (p<0.001) (Fig. 3).

The mean values of ADC_{lesion} and ADC_{area} for each group and the results of the Kruskal Wallis test are summarized in Table II. The mean ADC values were significantly different between groups (p≤0.001).

Through further evaluation with pairwise comparisons, the mean ADC values (ADC_{lesion} and ADC_{area}) were found to be significantly

Table I. Summary of demographics, pathologic and imaging characteristics.

	RMS *n:12*	Lymphoma *n:14*	Nasopharyngeal ca. *n:16*	*p* value
Gender (F/M)	5/7	1/13	2/14	0.088
Median age (years) (min-max)	4 (2-17)	7 (2-15)	14 (11-18)	<0.001*
Multicompartment involvement	75% n:9	57.1% n:8	100% n:16	0.008*
Nasopharynx±parapharyngeal involvement	58.3% n:7	57.1% n:8	100% n:16	0.004*
Skull base involvement	58.3% n:7	35.7% n:5	93.7% n:15	<0.001*
Intracranial extension	8.3% n:1	14.2% n:2	31.2% n:5	0.332
Distant metastasis/distant organ involvement	-	35.7% n:5	-	0.014*
Lymphadenopathy (cervical/retropharyngeal)	41.7% n:5	57.1% n:8	93.7% n:15	0.018*
T2 signal intensity				
Homogeneous	16.7% n:2	64.3% n:9	18.8% n:3	0.006*
Heterogeneous	83.3% n:10	35.7% n:5	81.2% n:13	
Enhancement[a]				
Homogeneous	16.7% n:2	78.6% n:11	81.2% n:13	<0.001*
Heterogeneous	83.3% n:10	21.4% n:3	12.5% n:2	
Presence of necrosis	58.3% n:7	0% n:0	0% n:0	<0.001*

[a] Post-contrast T1WI was not available in a nasopharyngeal carcinoma patient.
*Statistically significance
RMS: Rhabdomyosarcoma

Table II. The comparison of ADC measurements of groups with Kruskal-Wallis Test.

	Rhabdomyosarcoma		Lymphoma		Nasopharyngeal ca.		*p* value	
	Obs1	Obs2	Obs1	Obs2	Obs1	Obs2	Obs1	Obs2
Mean ADC_{lesion} ($x10^{-3}mm^2/s$)	0.907±0.2	0.891±0.21	0.481±0.08	0.456±0.14	0.687±0.11	0.683±0.11	<0.001*	<0.001*
Mean ADC_{area} ($x10^{-3}mm^2/s$)	1.125±0.37	1.135±0.35	0.517±0.08	0.528±0.08	0.740±0.12	0.747±0.15	<0.001*	<0.001*

Values are expressed as mean±SD.
*Statistically significance
Obs1:Observer 1; Obs2: Observer 2

lower in lymphoma than the means of RMS ($p<0.001$ for both observers) and the means of NPC (for ADC_{lesion} $p=0.004$ for obs1; $p=0.001$ for obs2 and for ADC_{area} $p=0.003$ for obs1; $p=0.008$ for obs2) (Fig. 4). The mean values of ADC_{lesion} ($p=0.13$ for obs1; $p=0.21$ for obs2) and ADC_{area} ($p=0.07$ for obs1; $p=0.06$ for obs2) did not show a significant difference between RMS and NPC.

Inter-observer agreement in ADC measurements was higher for the single slice method (ICC=0.997) than the small sample method (ICC=0.989).

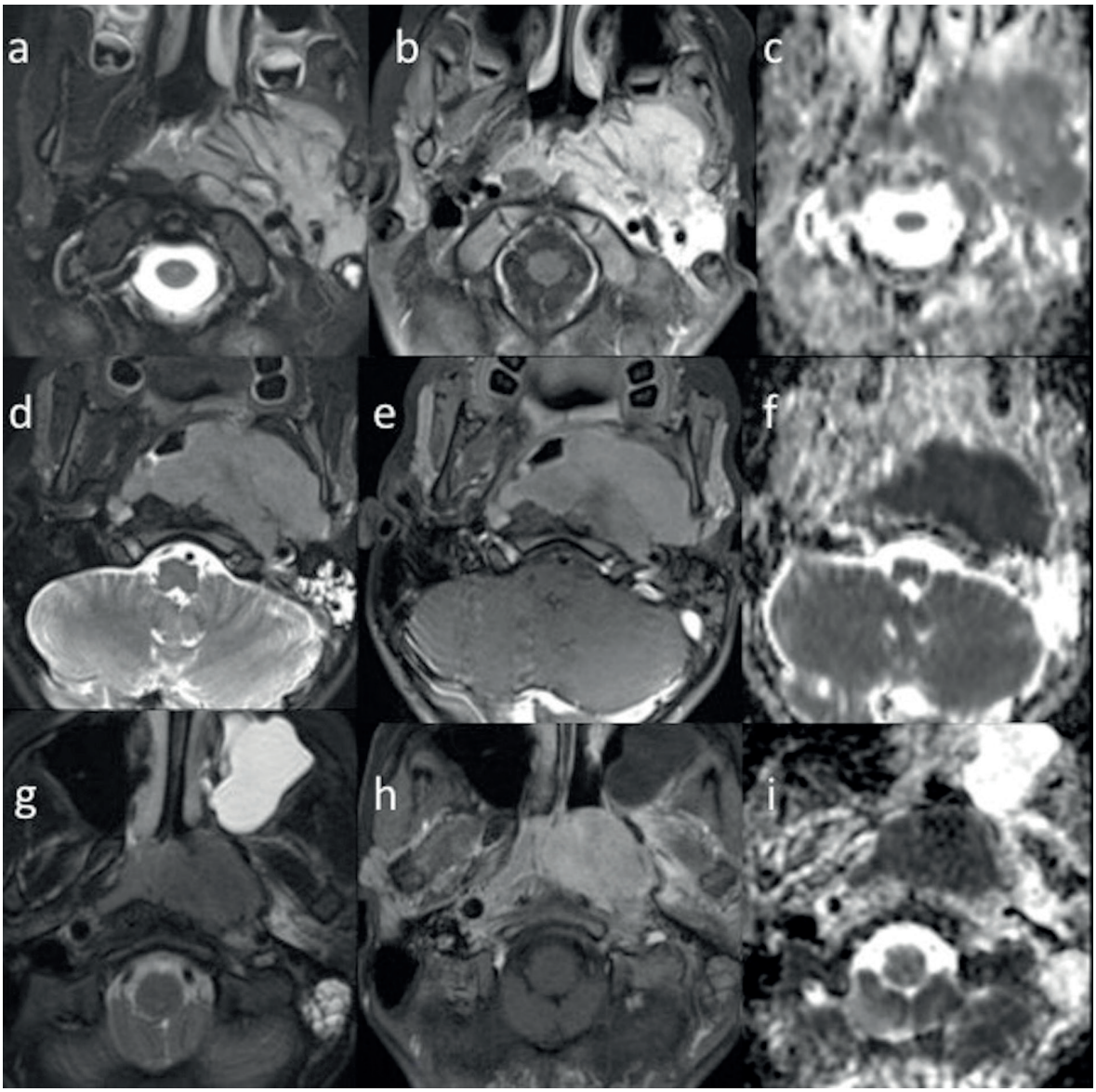

Fig. 2. The MRIs of 6-year-old male patient with RMS (a-c), 12-year-old male patient with lymphoma (d-f) and 13-year-old male patient with nasopharyngeal carcinoma (g-i) with nasopharyngeal and parapharyngeal extension. There is no apparent difference in terms of signal and enhancement characteristics on fat-sat T2WI (a, d, g), and post-contrast fat-sat T1WI (b, e, h). RMS (c) shows higher signal intensity on ADC map compared to lymphoma (f) and nasopharyngeal carcinoma (i).

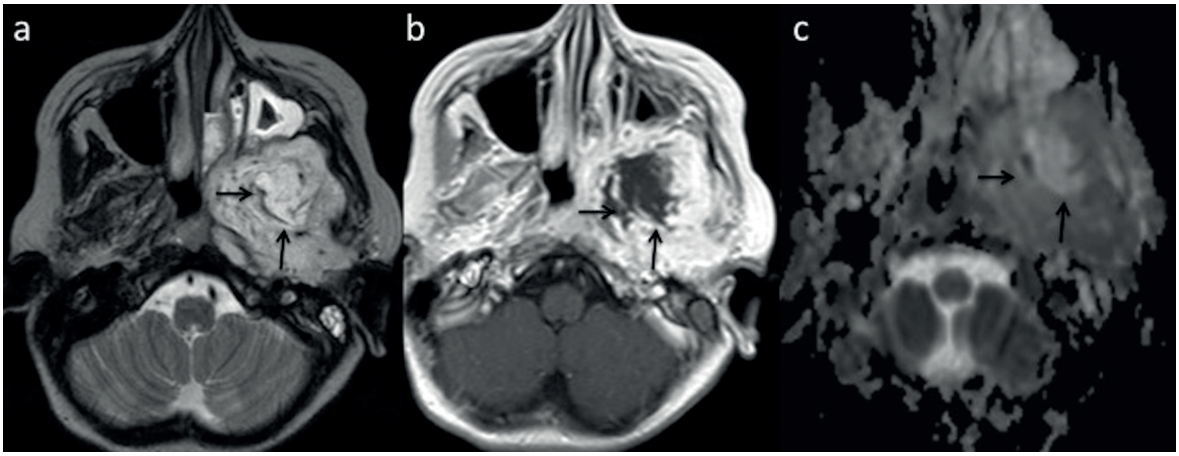

Fig. 3. A 10-year-old female patient with RMS. Necrosis within tumor appears as T2 hyperintense area with lack of enhancement and diffusion restriction (arrows).

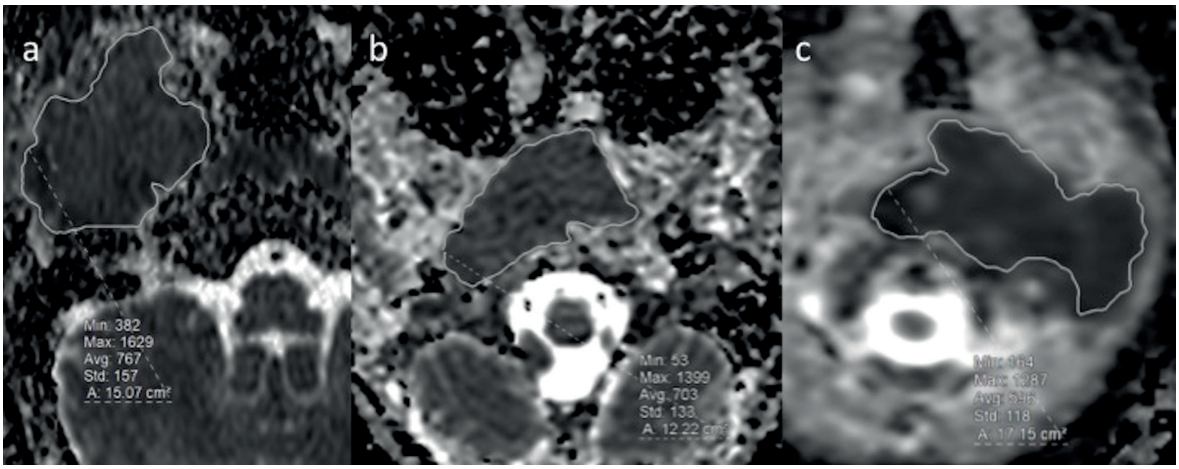

Fig. 4. ADC measurement of the tumors by single slice method; a) 8-year-old male patient with RMS (ADC_{area}: $0.76x10^{-3}$ mm^2/s). b) 15-year-old male patient with nasopharynx carcinoma (ADC_{area}: $0.70x10^{-3}$ mm^2/s). c) 3-year-old male patient with lymphoma (ADC_{area}: $0.59x10^{-3}$ mm^2/s).

Discussion

Parameningeal RMS has overlapping findings with lymphoma and NPC in terms of tumor localization and extent.[9] Nasopharyngeal ±parapharyngeal involvement was frequent in both RMS (58.3%) and lymphoma (57.1%) in our study. Further complicating differential diagnosis, previously reported MRI findings of RMS including high T2 signal intensity, prominent enhancement and restricted diffusion are also seen in the other tumors of concern.[9] To our knowledge, there is no previous study comparing MRI characteristics of these three tumors in the literature. In our study, we found a significant difference in terms of ADC values between RMS and lymphoma; in terms of enhancement pattern and presence of necrosis between RMS and lymphoma or NPC. These imaging findings could be used to help distinguish RMS and could guide effective management of these patients.

The age distribution (median age: 4 years) and a slight male predominance in our RMS cohort were compatible with the literature.[3,4] Although the median age of patients with RMS was significantly smaller than the median age of patients with NPC, there was an overlap between the two cohorts in older ages. The most

common pathologic subtype was embryonic RMS, compatible with the age distribution.

Rhabdomyosarcoma has a locally invasive behavior that is reflected by our high rates of multicompartment involvement (75%) and lack of distant metastasis. Treatment of RMS depends on the histologic subtype, tumor location and stage. It mainly utilizes a multimodality approach that includes systemic chemotherapy and local therapy; consisting of surgery, radiation therapy, or both on a case-by-case basis.[18] Although frequently seen in patients with NPC, skull base involvement was less commonly encountered in RMS (58.3%). In the presence of skull base involvement and intracranial extension, treatment options differ and survival becomes poorer.[19,20] The intracranial extension was rare in RMS (8.3%) and detected more frequently in patients with NPC, although there was no significant difference between the two groups. Cervical and retropharyngeal lymphadenopathies were significantly more frequent in NPC; distant metastasis was seen in lymphoma. These findings could be used adjunctively with the other imaging findings for tumor discrimination.

The signal homogeneity on T2WI was a common finding (%64.3) in lymphoma compared to RMS and NPC (p=0.006). This finding is compatible with the imaging characteristics of sinonasal lymphoma described previously in literature like the other findings in our lymphoma cohort such as frequent homogeneous enhancement (n=11, 78.6%) and lack of necrosis.[21,22] T2 signal heterogeneity and heterogeneous enhancement in pediatric RMS due to necrosis or hemorrhage were also reported previously.[10,23,24] These two findings were frequently detected in our group of RMS (83.3%) and heterogeneous enhancement was significantly more common than both NPC and lymphoma that could be used as a valuable imaging discriminator to favor the diagnosis of RMS.

Tumor necrosis is characterized by the presence of dead cells with preservation of the tissue architecture. It is reported to be an important hallmark of aggressive tumors and associates with hypoxia and angiogenesis.[25,26] In our study, neither lymphoma nor NPC showed necrotic parts despite RMS lesions (58.3%). In literature, embryonal RMS was noted to be more homogeneous with a lower rate of necrosis than alveolar and pleomorphic subtypes.[27] Our findings could be attributed due to the size of the embryonal tumors, because almost all (83.3%) presented as large tumors with multicompartment involvement. Additionally, our small-sized cohort has limitations to reflect the general behavior of this histopathologic type.

Diffusion-weighted imaging is efficiently used to differentiate between benign and malignant H&N tumors and helps in avoiding unnecessary invasive diagnostic procedures or surgery.[11,13,28] In high-grade malignant neoplasms, ADC values are generally low due to high mitotic activity, increase in cell number and size, decrease in the cytoplasm and extracellular matrix. Several studies particularly performed on adults reported that DWI is useful to distinguish lymphoma from NPC.[12,29-31] Our results were compatible; the lymphoma lesions had significantly lower ADC values compared to the NPC and RMS lesions. Since RMS usually presents as a rapidly progressing high-grade tumor, low ADC values could be expected. There are a few studies with a small number of patients that analyzed DWI characteristics of H&N RMSs in literature.[13,28] The ADC values of RMSs in those studies were reported to range from $0.66x10^{-3}$ mm^2/s to $0.91x10^{-3}$ mm^2/s. In the study with the largest number of RMS (n=11) among them, the mean ADC values were found to be $0.78\pm0.07x10^{-3}$ mm^2/s.[13] This value is notably lower than the mean ADC values measured by using the single slice ROI method in our RMS cohort ($1.125\pm0.37x10^{-3}$ mm^2/s for obs1, $1.135\pm0.35x10^{-3}$ mm^2/s for obs2) probably due to differences in the methods of measurement. In that study, the ROIs were placed in a single slice avoiding necrotic areas in contrary to our study in which single slice ROIs were drawn in the largest cross-sectional

area of the tumor including necrotic parts. The contribution of necrotic parts with high ADC values is considered to be responsible for higher ADC values in our RMS cohort measured by the single slice ROI method. The mean ADC values measured with the small sample method in our RMS cohort ($0.907\pm0.20x10^{-3}$ mm^2/s for obs1 and 0.891 ± 0.21 $x10^{-3}$ mm^2/s for obs2) are slightly higher than the mean value reported in that study.

In addition to the single slice method, the mean ADC values measured by the small sample method avoiding necrotic parts were also significantly higher in our RMS cohort compared to lymphoma. This probably reflects the histopathologic features of the embryonic type of RMS that was the most common type in our study. Embryonal RMS contains variable cellularity ranging from poorly differentiated primitive mesenchymal cells to highly differentiated muscle cells within a myxoid matrix.[32] The increasing effect of myxoid matrix on ADC in soft tissue tumor was previously reported.[33] Therefore, higher ADC values detected even in the non-necrotic areas of RMS could be explained by the myxoid matrix of the embryonal type.

Although no statistically significant difference was found, the mean ADC_{area} values have a tendency to be higher in RMS than NPC in our cohorts. This again probably reflects the presence of necrotic parts commonly found in RMS in contrast to NPC. The small size of our cohorts could be the reason for the difference not being statistically significant between the ADC values of these two tumors. Future studies with larger cohorts are recommended for further evaluation.

Long acquisition time, motion artifacts, and frequent need for general anesthesia are the main difficulties of MRI in pediatric patients. Besides, susceptibility artifacts are commonly encountered in DWI of the H&N region due to excessive air-tissue and tissue-bone interfaces. This may affect the correct localization of ROI and estimation of ADC values. ROI method may also affect the ADC measurements; the small sample ROI method has been reported to yield the worst inter-observer correlation in previous studies concerning thyroid nodules[34] and orbital tumors.[35] Our study supports previous reports; the ADC values measured with the single-slice ROI method showed higher inter-observer correlation than the ones measured with the small sample method. Additionally, the single-slice and whole-volume ROI methods are more representative of the histopathologic characteristics of tumors than the small sample ROI method. This is especially important in the discrimination of tumors like RMS with heterogeneous tissue characteristics due to necrosis/cysts or hemorrhage from more homogenous mimickers.

Although relatively small number of the patients with RMS (n=12) is a limitation, our RMS cohort presents the largest RMS series in the literature. The study has also some limitations due to its retrospective design; the scans were acquired using different scanners with different parameters, which could affect image evaluation. We believe the use of different scanners is not a major limitation. However, possible effect of using different scanners on ADC values cannot be completely excluded and more investigation is needed to make a definite assessment.

The data on which specific gadolinium-based contrast agent was used in the studies could not be found. On the other hand, we believe the use of different contrast agents is not a limitation for qualitative evaluation of enhancement patterns (whether homogenous or heterogeneous).

We also could not perform whole-volume ADC histogram analysis due to the software unavailability. Even though this method has a promising ability to predict histopathologic characteristics of lesions, its application is limited in clinical practice. Another limitation due to the retrospective design was the inability to histopathologically confirm necrotic parts defined in MRIs.

In conclusion, RMS tends to have higher ADC values than lymphoma and has a higher frequency of heterogeneous enhancement and necrotic parts than both lymphoma and NPC. These features could help radiologists to differentiate RMS from the above-mentioned mimickers. Future studies with larger cohorts are recommended to validate these findings.

Ethical approval

Institutional Review Board (2020/16-26) approved the study protocol and informed consent was waived by Hacettepe University Institutional Review Board.

Author contribution

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

Ototoxicity and long-term hearing outcome in pediatric patients receiving cisplatin

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ABSTRACT

Background. Hearing is essential in child development. Cisplatin which is a common chemotherapy used in many pediatric solid-tumor protocols cause various degrees of ototoxicity. Several risk factors for cisplatin-induced ototoxicity have been reported, including race and age. This study aimed to evaluate the incidence of ototoxicity and its long-term outcome in Thai pediatric solid-tumor patients receiving cisplatin and to determine the risk factors associated with hearing impairment.

Methods. A retrospective study was conducted in solid-tumor patients <15 years old from 2007 to 2019 at Siriraj Hospital, Bangkok, Thailand. Hearing was evaluated by an audiogram and/or auditory steady-state response and the impairment was graded according to the Common Terminology Criteria for Adverse Events version 5. Grade 2 and above was considered significant hearing loss.

Results. In total, the hearing of 47 patients was evaluated. At the end of treatment, hearing impairment and significant hearing loss were found in 66% and 48.9% of patients, respectively. A high median cumulative cisplatin dose was significantly associated with worse hearing impairment ($p = 0.039$) and a more progressive grading of ototoxicity ($p = 0.005$). A risk factor for significant hearing loss was a cumulative dose ≥400 mg/m^2 ($p = 0.014$). All 9 patients who received a cumulative dose >600 mg/m^2 and 5 patients who received aminoglycoside developed significant hearing loss. One patient had progressive hearing impairment at 8 months after the end of treatment and 1 patient developed grade 3 ototoxicity which required a hearing aid after bone marrow transplantation. The latter patient received a total cisplatin dose of 708.2 mg/m^2 and carboplatin 1400 mg/m^2.

Conclusions. The incidence of hearing impairment in pediatric patients receiving cisplatin is high. Regular hearing evaluation is essential for the early detection of ototoxicity. Long-term follow-up is recommended, especially in patients who have a combination of other risk factors for hearing loss.

Key words: cisplatin, ototoxicity, pediatric, oncology, long-term.

Cisplatin is incorporated in the treatment of many pediatric solid tumors, including hepatoblastoma, neuroblastoma, intra- and extracranial germ cell tumor (GCT), and osteosarcoma. Its mechanism of action is the activation of the apoptosis cascade and generation of reactive oxygen species, leading to cellular toxicity.[1,2] Cisplatin is well known to cause permanent sensorineural hearing loss especially in the high-frequency range. Previous reports of the incidence of cisplatin-induced ototoxicity revealed varying rates from 11-97% depending on the criteria used.[3-8] Several risk factors for cisplatin-induced ototoxicity have been reported, including an increasing cumulative dosage, prior cranial irradiation, co-administration with aminoglycosides, renal insufficiency, race, male gender, age <5 years old, and glutathione S-transferases genetic polymorphisms.[1,3,4,6-8] A previous study in Thai oncology patients showed that 79.5% had

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hearing loss at the end of treatment according to Brock classification, and a cumulative cisplatin dose >400 mg/m^2 was associated with an increased risk of ototoxicity.[6] The audiogram in that study was evaluated only once at the median time of 25.7 months between the last dose of cisplatin and hearing evaluation.[6] However, cisplatin-induced ototoxicity may be progressive if the patient is receiving repeated doses of chemotherapy and can occur up to 5 years later.[5,9]

Since hearing is essential for child development; hearing loss in early life can affect children in many aspects. Impairment of hearing, especially at 500 Hertz (Hz)–4 kiloHertz (kHz), which is the human speech frequency, can cause delayed speech, impaired cognitive function, and affect neurodevelopmental outcomes.[6,9,10] Long-term hearing loss, even if at high frequency, can also affect language development.[9] Therefore, we were interested to analyze Thai pediatric solid-tumor patients receiving cisplatin-based chemotherapy to address the ototoxicity effects of cisplatin, which may provide a recommendation for follow-up in future patients.

Material and Methods

Patients and objectives

This cohort study was a retrospective chart review of pediatric solid-tumor patients, including neuroblastoma, hepatoblastoma, osteosarcoma, and GCT, who were <15 years old and had received cisplatin-based chemotherapy at the Department of Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand from January 2007 to December 2019. Their baseline hearing was evaluated by an audiogram and/or auditory steady-state response (ASSR) pre- or within 1 month of the initiation of cisplatin and then followed-up with further hearing tests for at least 2 sequential times. All the patients had an audiogram performed for both ears at the last hearing evaluation to quantify the change in hearing threshold. Patients who had an anatomical defect of the ear and renal dysfunction before treatment, which may be risk factors for hearing loss and cisplatin toxicity, were excluded. The primary objective was to study the effect of cisplatin on the incidence of ototoxicity throughout the course of treatment and after the end of therapy in Thai pediatric solid-tumor patients. The secondary objective was to determine the risk factors that may be associated with hearing impairment. This study was approved by the ethical committee of the Faculty of Medicine Siriraj Hospital (Si 052/2020) and was registered in the Thai Clinical Trial Registry (TCTR20210221001).

Data collection and analysis

Data collection included the patients' gender, diagnosis, age at diagnosis, dose of cisplatin (mg/m^2), co-administration of other chemotherapies and aminoglycoside, complications including infections that required hospitalization, and outcome at the end of treatment. The audiogram and/or ASSR results, including frequency and threshold of hearing, were recorded. The hearing impairment severity was assigned a numeric grade from 0–4 according to the Common Terminology Criteria for Adverse Events version 5.0 (CTCAE v.5) established by the National Cancer Institute.[11] Grade 0 is considered normal hearing. Grades 1, 2, 3, and 4 are considered to indicate mild, moderate and severe hearing loss indicating the need for therapeutic intervention including hearing aids, and very severe hearing loss indicating the need for a cochlear implant, respectively.[11] CTCAE grade 2 and above were considered to impact the hearing function in children because a hearing threshold shift at 4 kHz or less is in the range of human speech frequency.[9] These grading toxicities represented significant hearing loss in this study.

Data were analyzed with descriptive statistics using SPSS Statistics for Windows, version 26.0 (SPSS Inc., Chicago, IL, USA). Data are presented as the mean±SD or median (range) for continuous variables and number (%) for

categorical variables. The Mann–Whitney U test, Chi-square test, and Kruskal–Wallis test were used, as appropriate, to compare and identify the association between factors, such as cisplatin dose and duration of follow-up with the grading of hearing impairment. Kaplan–Meier analysis was performed for each cumulative cisplatin dose range to determine the hearing loss-free duration from the first dose of cisplatin and the significance was assessed using the log-rank test. Univariable logistic regression was employed to analyze the covariables on the impact of hearing impairment. Significant factors were included in the multivariable analysis using a multiple logistic regression method. A p-value of <0.05 was considered statistically significant.

Results

In total, 47 patients were enrolled in this study. The median age at diagnosis was 7.1 (range 1.3–14.8) years old, and about one-third of the patients were <5 years old. Osteosarcoma was the most common disease (36.2 %). One patient with extracranial GCT received autologous bone marrow transplantation (BMT) after relapsed disease and 3 patients with intracranial GCT received cranial radiation (median dose 5,000; range 2,268–5,000 centigray). Infectious complications occurred in 19 patients (40.4%), which consisted of febrile neutropenia in 16, sepsis in 6 (among whom, 2 patients had septic shock), moderate to severe gastroenteritis in 5 (among whom, 1 patient was diagnosed with typhlitis), and urinary tract infection and respiratory syncytial virus pneumonia in 1 patient each. Five patients received aminoglycoside. The disease-free survival rate at the end of treatment was 68.1%. The chemotherapies for each type of malignancy are shown in Table I. The characteristic of the patients and diseases are summarized in Tables II and III. The median cumulative dose of cisplatin for all patients was 423.1 (range 118–1080) mg/m^2. No statistically significant difference in cumulative dose (p = 0.235) was found for each type of malignancy, as demonstrated in Table III. Overall, 28 patients (59.6%) received a cumulative dose of cisplatin ≥400 mg/m^2.

Audiologic evaluation

The results from the baseline hearing evaluation are demonstrated in Table II. Six patients (12.8%) had grade 2–3 hearing impairment at the first hearing evaluation. At the end of treatment, 31 patients (66%) had grade 1 or higher hearing impairment and 23 of them (48.9%) had significant hearing impairment (> grade 2). Patients who received a higher cumulative dose range of cisplatin had significantly worse hearing impairment at the end of treatment (p = 0.001; Table IV) and a significantly more

Table I. Chemotherapies for each type of malignancy in this study.

	Cisplatin	Bleomycin	Carboplatin	Doxorubicin	Etoposide	Cyclophosphamide	Ifosfamide	Methotrexate	Irinotecan	Topotecan	5-Fluorouracil	Vincristine	Remarks
Hepatoblastoma	√√		√	√	√		√				√	√	
Extracranial GCT	√√	√√	√		√√		√						
Intracranial GCT	√√		√		√		√						
Osteosarcoma	√√		√	√√	√		√	√√					√√ = all cases
Neuroblastoma	√√		√	√√	√√	√√	√		√	√		√	√ = some cases

GCT: germ cell tumor

Table II. Characteristics of the patients (n = 47).

Characteristics		Number	Percent
Gender	Female	24	51.1
	Male	23	48.9
Age at diagnosis (years)	0–5	15	31.9
	6–10	15	31.9
	10–15	17	36.2
Hearing grade at baseline evaluation	0	33	70.2
	1	8	17.0
	2	4	8.5
	3	2	4.3
Other medications	Doxorubicin	31	66.0
	Etoposide	29	61.7
	Methotrexate	16	34.0
	Carboplatin	15	31.9
	Ifosfamide	14	29.8
	Cyclophosphamide	10	21.3
	Vincristine	10	21.3
	Other chemotherapies†	17	36.2
	Aminoglycoside	5	10.6
Other treatments	Cranial radiation	3	6.4
	Surgical removal	33	70.2
	Bone marrow transplantation	1	2.1
Complications	Infections/ Febrile neutropenia	19	40.4
Outcome at the last follow-up	Survive without disease	32	68.1
	Palliative/Dead	15	31.9

†Other chemotherapies: 5- Fluorouracil, irinotecan, topotecan, bleomycin.

Table III. Number of patients in each range of cumulative cisplatin doses and median with range doses classified by diagnosis.

Diagnosis	Number of cases for each cumulative cisplatin doses range (mg/m^2)					Cumulative cisplatin doses (mg/m^2)	
	Total	0–200	201–400	401–600	>600	Median	Range
Osteosarcoma	17	1	5	11	0	427.4	118–596
Neuroblastoma	11	0	4	2	5	423.1	286–1080
Hepatoblastoma	8	0	5	0	3	395.5	230–1038
Extracranial GCT	8	1	1	3	3	523.7	172–1040
Intracranial GCT	3	0	2	1	0	384.6	316–411
Total	47	2	17	17	11	423.1	118–1080
						(p-value = 0.235)	

GCT: germ cell tumor.

progressive grading shift of hearing impairment during the course of treatment ($p < 0.001$; Table IV). The median cumulative cisplatin dose was significantly higher in patients who had worse hearing impairment ($p = 0.039$; Fig. 1) and a more progressive grading shift of hearing impairment ($p = 0.008$; Fig. 1). One of the 3 patients who received cranial radiation

Table IV. Number of patients in each range of cumulative cisplatin doses classified by Common Terminology Criteria for Adverse Events version 5.0 (CTCAE v.5) for hearing impairment at the end of treatment (n = 47).

CTCAE v.5 for hearing impairment		Number of cases (%) in each cumulative dose range (mg/m^2)					*p*-value
		Total	0–200	200–400	400–600	>600	
Grading at the end of treatment	0	16	1 (6.3%)	9 (56.3%)	6 (37.5%)	0	
	1	8	0	3 (37.5%)	4 (50%)	1 (12.5%)†	
	2	16	1 (6.3%)	4 (25%)	4 (25%)	7 (43.8%)	
	3	6	0	1 (16.7%)	3 (50%)	2 (33.3%)	
	3 with hearing aids	1	0	0	0	1 (100%)	0.001*
Progression of grading until the end of treatment	Unchanged	25‡	2 (8%)	14 (56%)	7 (28%)	2 (8%)	
	+1	9	0	2 (22.2%)	4 (44.4%)	3 (33.3%)	
	+2	9	0	1 (11.1%)	4 (44.4%)	4 (44.4%)	
	+3	4	0	0	2 (50%)	2 (50%)	< 0.001*

CTCAE: Common Terminology Criteria for Adverse Events.
†At 8 months after the end of treatment, this patient had progressively impaired hearing from grade 1 to grade 2.
‡Six patients who had baseline hearing grade 2–3 showed no change in grading during the treatment course.
* Result statistically significant at P < 0.05.

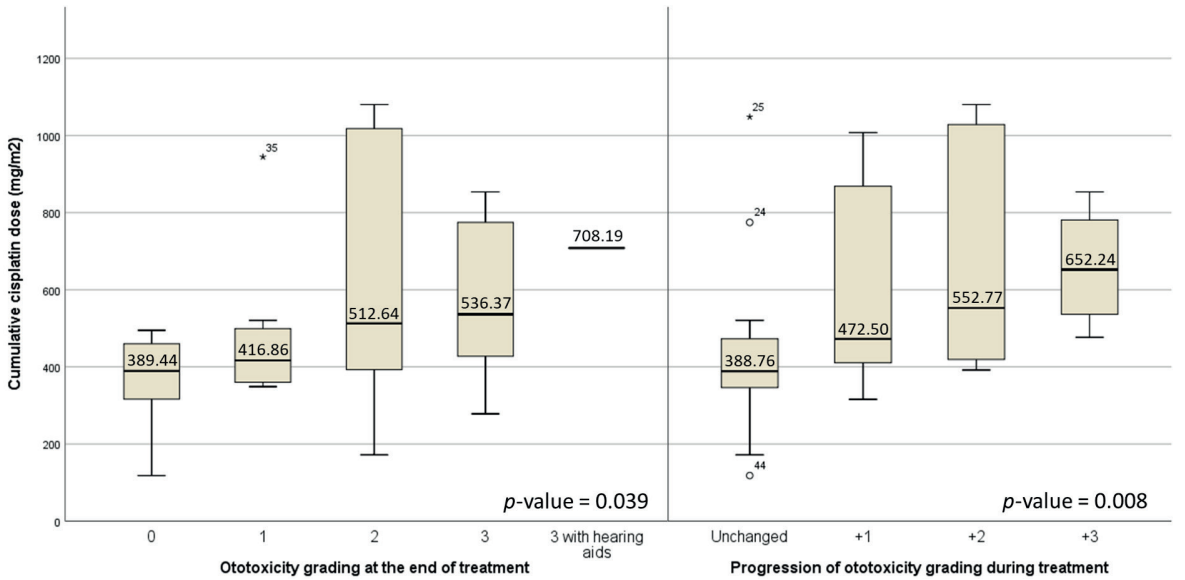

Fig. 1. Median and range of cumulative cisplatin doses classified by Common Terminology Criteria for Adverse Events version 5.0 for hearing impairment (n=47).

had normal hearing until the last evaluation. The other 2 patients had progressed hearing impairment from grade 1 to 2 and grade 0 to 3, respectively. The latter patient had recurrent GCT and received both cisplatin (total 411 mg/m^2) and carboplatin (total 6,570 mg/m^2). The median follow-up time from the first dose of cisplatin to the final hearing evaluation of these 3 patients was 36 (range 27-50) months. One patient who received a cumulative dose of cisplatin 708.2 mg/m^2 had hearing impairment grade 3, which required hearing aids. This patient was diagnosed with stage 1 testicular yolk sac tumor at 1 year 3 months old and total tumor removal was performed without chemotherapy. He had a recurrent tumor at 2 years old and received chemotherapy followed by tandem autologous BMT at 3 years 3 months old. His conditioning regimen consisted of carboplatin 700 mg/m^2 and etoposide 750 mg/m^2

in a 3-day course for a total of 2 courses, 6 weeks apart. During the course of BMT, he had *alpha-hemolytic Streptococci* septicemia but did not receive aminoglycoside. He received 3 cycles of oral etoposide 50 mg/m^2/day for 21 days of a 28-day course after BMT. His hearing evaluation was normal 1 month before BMT. However, his audiogram revealed an impairment >20 dB at 500 Hz–4 kHz 4 months after BMT when he was on oral etoposide (6 months after the last dose of cisplatin) and was classified as grade 3 ototoxicity. He started to use hearing aids 2 years after the BMT.

Risk factors affecting hearing outcome at the last follow-up

Patients were classified into 2 groups, namely grades 0–1 and 2–4 of hearing impairment, according to the clinical implication that a threshold shift >20 dB at 4 kHz and below in at least one ear may affect speech and language development in children.[9,10] Twenty-four patients (51.1%) had grade 2–4 hearing impairment at the last follow-up. Six patients who had baseline hearing grade 2–3 did not show a progression in hearing impairment during the treatment and were excluded from the risk factor analysis. The median cumulative cisplatin dose in these 6 patients was 391.4 (range 172–1048) mg/m^2. None of these patients received aminoglycoside. The association of the patient's characteristics and treatment, such as gender, diagnosis, cranial radiation, chemotherapy except for cyclophosphamide (p = 0.04), and infections that required hospitalization were not found to be statistically significant risks for hearing impairment when comparing grade 0–1 with grade 2–4. An age of ≤5 years old at diagnosis had a significant impact on hearing impairment compared to an older age (Odds ratio 6.67; 95% CI 1.45–30.64, p = 0.015). Patients receiving cumulative doses of cisplatin ≥400 mg/m^2 had a significantly higher risk of developing grade 2–4 hearing impairment compared with patients receiving lower doses (Odds ratio 8.727; 95% CI 1.62–46.93, p = 0.012). All the patients receiving a cumulative dose of cisplatin >600 mg/m^2 (n = 9, excluding 2 patients who had baseline hearing impairment > grade 2) developed significant hearing impairment (grade 2 in 7 and grade 3 in 2 patients; Fig. 2). All the patients who received aminoglycoside (n = 5) developed significant

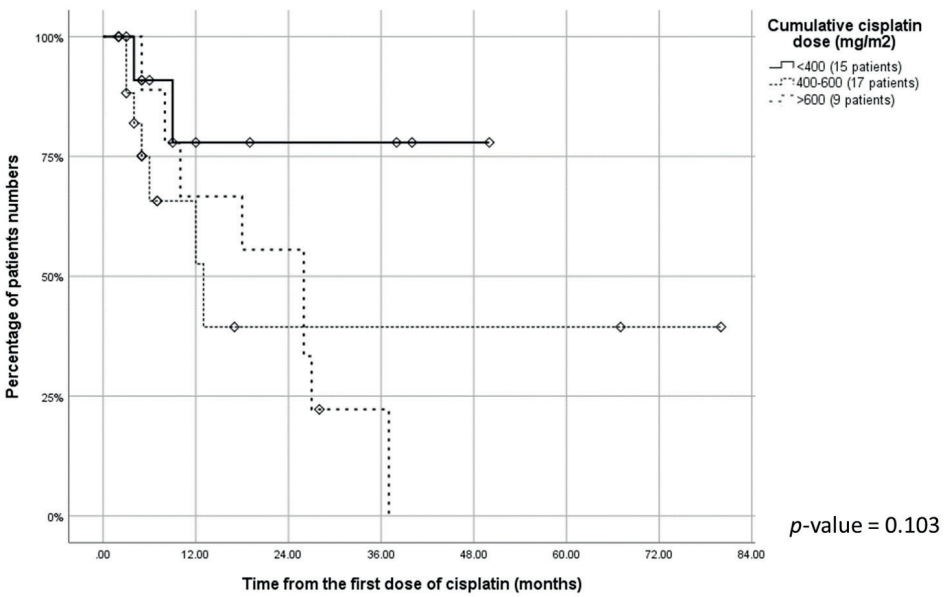

Fig. 2. Percentages of patients who progressed form grade 0-1 to grade 2-4 hearing impairment and duration (months) from the first dose of cisplatin to hearing impairment ≥grade 2 categorized by the cumulative dose ranges of cisplatin (n=41).

hearing impairment (grade 2 in 4 and grade 3 in 1 patient). From the multivariable analysis, the factor that significantly increased the risk of significant hearing impairment was a cumulative dose of cisplatin ≥400 mg/m^2 (Adjusted odds ratio 18.79; 95% CI 1.83–193.19, p = 0.014).

Long-term follow-up of hearing outcome

The median follow-up time from the first dose of cisplatin to the last hearing evaluation for all patients was 17.0 (range 4–121) months. Among the 41 patients who had baseline hearing grade 0–1, 18 (43.9%) progressed to grade 2–4 hearing impairment at the last hearing evaluation. The median time of progression to significant hearing impairment was 9.5 (range 3–37) months. Six patients with grade 2–3 hearing impairment at baseline evaluation had the same grade of hearing impairment until the last hearing evaluation. The median time from the first dose of cisplatin to the last hearing evaluation of these 6 patients was 24.5 (range 8-64) months. Thirty-two patients survived at the end of treatment. Fourteen patients underwent a followed-up audiogram after the end of treatment at a median duration of 35.5 (range 4–67) months. The median cumulative dose of cisplatin in this group of patients was 407.2 (range 172-944) mg/m^2. Thirteen patients did not have a change in hearing grade from the end of treatment to the last hearing evaluation. One patient, who was diagnosed with mediastinal ganglioneuroblastoma and had received a cumulative dose of cisplatin 944 mg/m^2, had hearing impairment grade 1 at the end of treatment but progressed to grade 2 at 8 months later. This patient had 4 episodes of febrile neutropenia, for which he received aminoglycoside at every episode. In total, 24 patients (51.1%) had significant hearing loss at the last hearing evaluation.

Nineteen patients who have hearing impairment received N-acetylcysteine. The time to starting medication and the duration of treatment varied, depending on the physicians' decision and the financial status of the patients, since the cost of N-acetylcysteine was not covered by the national healthcare system in Thailand. Because of the inconsistent data, we did not analyze the effect of N-acetylcysteine on the hearing outcome in this study.

Discussion

Hearing impairment impacts the quality of life of children, especially their speech and language development, which can also affect their academic achievement, cognitive function, and social integration.[9,10] Cisplatin-containing chemotherapy, which can cause ototoxicity and lead to hearing impairment, is incorporated as part of the standard treatment for many types of solid tumors in children. Our hospital applies the CTCAE v.5 classification established by the National Cancer Institute to classify hearing impairment from cancer treatment because each grade of audiological impairment correlates with hearing function and the need for an audiological intervention.[11,12] The reported incidence of hearing impairment from cisplatin depends on the criteria used to classify ototoxicity and the cumulative cisplatin doses received in the studies. Our study reported hearing impairment ≥grade 2 in 12.8% of patients at baseline which is higher than the prevalence of previous reports on hearing impairment in the Thai school-aged pediatric population (3.9-6.1%).[13,14] However, Thai children in rural areas had a higher prevalence of hearing impairment than in the capital city which was caused mainly by a higher incidence of ear infections and impact cerumen.[14] This may explain high prevalence of hearing impairment in our study since Siriraj hospital is a university hospital and a majority of patients were referred from rural areas. Fifty-one percent of our patients had hearing impairment at the last hearing evaluation, which was a similar rate to some previous studies[3,8] but lower than the study by Choeyprasert et al.[6] performed in Thai pediatric patients, which reported grade 2 or worse hearing loss according to Brock classification in 67.6% of their study patients. This discrepancy might be due to the lower doses of cisplatin in

our study than in Choeyprasert et al.'s study[6] (median dose 423.08 mg/m^2 vs. 525.5 mg/m^2, respectively) and the different criteria used for grading hearing impairment.

Risk factors influencing hearing outcome

Cisplatin causes high-frequency hearing loss, which is usually bilateral and permanent. However, higher cumulative doses may also affect hearing thresholds at lower frequencies.[1,2,4] Many risk factors for cisplatin-induced ototoxicity, such as male gender, younger age, high cumulative dose, radiotherapy to the head and neck region, and co-treatment with other ototoxic drugs, have been described.[4,7,15] Our study found that a cumulative cisplatin dose ≥400 mg/m^2 significantly increased the risk of significant hearing impairment. This result was similar to previous studies that demonstrated that a cumulative cisplatin dose >400 mg/m^2 was considered an independent risk factor for moderate to severe hearing impairment.[4,6,7] However, the dose of cisplatin correlated with high-frequency hearing loss was varied in different studies. McHaney et al.[16] reported an 88% incidence of high-frequency hearing loss in patients receiving >450 mg/m^2 cisplatin. The deteriorative effect on hearing ability in our study was noticeable when the cumulative cisplatin dose was >600 mg/m^2 since all the patients who had received this dose developed significant hearing loss. Our patients who received a greater cumulative dose of cisplatin showed a significantly more progressive grading shift of hearing impairment during the course of treatment and had worse hearing impairment at the end of treatment (Table IV and Fig. 1). This result confirms the dose-dependent effect of cisplatin in inducing ototoxicity that has been reported by others.[7,17] The progression of hearing impairment in our study occurred as early as 3 months after the first dose of cisplatin and 43.9% of patients progressed ≥2 grades of CTCAE v.5, which was associated with them having received a higher cisplatin dose. However, the progression of significant hearing impairment in our study occurred as late as 37 months. This emphasizes the importance of early and regular long-term hearing evaluation in this group of patients, especially for those who receive high cumulative cisplatin doses. Another significant risk factor in our study was a young age of ≤5 years old, which correlated with previous literature.[6,7,17] However, the multivariable analysis in our study did not show the younger age as significant. This could be due to the small number of this population (31.9%). Interestingly, all the patients who received aminoglycosides in our study showed impaired hearing function ≥grade 2 even though the statistical risk of this drug could not be calculated, but this should emphasize that patients who receive co-treatment with other ototoxic drugs, such as aminoglycosides, need to be closely evaluated for their hearing. If possible, ototoxic antibiotics should be avoided in patients who receive a high cumulative dose of cisplatin. Infections are common complications during the course of chemotherapy, as in our study, which revealed that 40.4% of patients had significant infections that required hospitalization. Infections that have been reported to cause hearing loss in children include chronic suppurative otitis media, meningitis, mumps, and measles.[18] We did not find that infections were associated with a higher risk of significant hearing loss. This might be because none of our patients had these types of infections. Cyclophosphamide and carboplatin were found to be risk factors for ototoxicity, especially when co-administered with cisplatin in the treatment protocol.[3,9,19-22] However, our study could not find any significant relationship between the co-treatment of carboplatin and cyclophosphamide with cisplatin ototoxicity. Other risk factors, such as male gender and cranial irradiation reported in previous literature, had no significant association with ototoxicity in our study.[4,22] This could be due to the small number of patients in our study.

Long-term follow-up of hearing outcome

Cisplatin-induced ototoxicity has mostly been reported to be irreversible[5,9,23,24], although some

studies have observed an improvement in hearing impairment over time post-therapy.[3,25,26] Our study revealed no improvement in hearing impairment after the cessation of therapy. All except one patient did not improve or show progress in their grading of hearing impairment after the end of treatment evaluation. This patient received a high cumulative dose of cisplatin (944 mg/m^2) and had a progression of hearing impairment from grade 1 to grade 2 at 8 months after discontinuing cisplatin. A similar progression of hearing impairment was reported by Bertolini et al.[20], whereby 120 pediatric patients with solid tumors showed no improvement of hearing impairment over the follow-up time after receiving cisplatin and/or carboplatin containing chemotherapy. On the contrary, a worsening or progression of hearing loss at lower frequencies was detected, and 5% of audiograms showed toxicity ≥grade 2 according to Brock's grading scale before the end of therapy. This grading of hearing impairment was observed in 11% of the early post-therapy evaluations and progressed to 44% after more than 2 years of follow-up.[20] Of note, one patient in our study who had recurrent GCT and had received a cumulative high dose of cisplatin (708.2 mg/m^2) followed by a cumulative carboplatin dose of 1,400 mg/m^2 in the BMT course developed grade 3 ototoxicity, which required hearing aids during follow-up after BMT. This finding correlated with the study by Parsons et al.[21] which reported that 9 out of the 11 study children with advanced stage neuroblastoma who underwent autologous BMT (82%) had evidence of speech-frequency hearing loss post-BMT. This group of patients received a high dose of carboplatin >2 g/m^2. This high dose of carboplatin is ototoxic, particularly in patients who have previously received cisplatin therapy or other ototoxic agents. All of these results emphasize the importance of the long-term evaluation of hearing function in pediatric patients who have received cisplatin-based chemotherapy.

In conclusion, this retrospective study demonstrated cisplatin toxicity, in terms of causing irreversible high-frequency hearing loss. A significant risk factor for grade 2-4 hearing loss was found to be a cumulative cisplatin dose ≥400 mg/m^2. Early and regular hearing evaluation during cisplatin treatment is essential for the early detection of hearing impairment. Long-term follow-up is recommended, especially in patients with a high risk of hearing impairment who have been receiving a high cumulative dose of cisplatin, have a young age at diagnosis, and their treatment involves the co-administration of other ototoxic drugs, such as aminoglycoside and high-dose carboplatin.

Our study has several limitations including its retrospective nature, which might lead to some missing data especially data on the ototoxicity of drugs other than aminoglycoside, carboplatin and cyclophosphamide; the limited study number, which might have prevented some parameters from achieving statistical significance in the analyses; and the lack of genetic study for analyzing the association with cisplatin-induced ototoxicity. However, our study highlighted the importance of early and regular audiologic monitoring during and long-term after cisplatin treatment, especially in high-risk patients. Further studies are needed to minimize this complication by the early detection of ototoxicity and the use of cooperative potential otoprotective medication to observe the benefit of hearing impairment recovery and to prevent the progression of this complication.

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Ethical approval

This study was approved by the ethical committee of the Faculty of Medicine Siriraj Hospital (Si 052/2020) and was registered in Thai Clinical Trial Registry (TCTR20210221001).

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: TS, KT, KS; data collection: TS; analysis and interpretation of results: TS, KS, KT, KP; draft manuscript preparation: TS, KS, KP, NV, JB. 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

Prevalence of inguinoscrotal pathologies and risk factors in a cohort of 388 children with spina bifida

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ABSTRACT

Background. There is limited information about the prevalence and risk factors of inguinal hernia and undescended testis in patients with spina bifida (SB). The aim of this study was to identify the properties and prevalence of inguinoscrotal diseases in these patients.

Methods. A questionnaire was completed by parents of patients with the diagnosis of SB in our center. Together with demographic data, presence an of inguinal hernia, side, operation history, presence of ventriculoperitoneal (VP) shunt, type of SB aperta or occulta, recurrence and presence of undescended testis were questioned. Patients were grouped into 2 as SB aperta and occulta. The prevalence of these pathologies and their clinical properties were evaluated.

Results. In this study, 388 patients were evaluated. Of these, 238 patients had SB aperta and 150, SB occulta. There was no significance in comparison of gender. The prevalence of inguinal hernia was 12.6% in general. A hernia was noted in 37 SB aperta patients (15.6%) whereas this was seen in 12 of the SB occulta patients (8%) (p=0.029). When there was a VP shunt, hernia prevalence was 21.5% and when there was no shunt, this ratio was 7.1% (p=0.0001). Prevalence of inguinal hernia was 21.8% in males and 3.2% in females (p=0.0001). When there was a VP shunt with SB aperta the prevalence was 21.9% and when a VP shunt was present with SB occulta, this number was found to be 13.3% (p=0.006). The prevalence of undescended testis was 17.7% and there was no difference between SB aperta and occulta patients.

Conclusions. Inguinal hernia and undescended testis are more frequent in SB patients when compared to the normal population. VP shunts and male gender may be risk factors for inguinal hernia in these children. These findings may imply neurological factors in the etiology of inguinal hernia and undescended testis.

Key words: inguinal hernia, undescended testis, spina bifida, children.

Spina bifida (SB) is a developmental anomaly of the neural tube when there is incomplete closure of this fetal structure. This maldevelopment results in neural injury and various neurological deficits related to the level of the lesions. Two major forms of the disease have been named as SB aperta (SBA) and SB occulta (SBO) according to the closure of the skin over the vertebral defect. In a wide study in the United States, the overall prevalence of this anomaly has been found to be 3.1 in 10000 live births.[1] In a recent meta-analysis, the pooled prevalence of SB has been shown to be 4.76 cases in 10000 births including live births, stillbirths and termination of pregnancies.[2]

Inguinoscrotal pathologies, including inguinal hernia (IH) and undescended testis (UDT) are the most commonly encountered group of diseases in pediatric surgical practice.

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IH and UDT are linked together in their etiopathogenesis. The processus vaginalis is expected to obliterate after the testes complete their descent and failure of obliteration causes IH. The incidence of IH is quite high, as much as 1-4%, equivalent to 10-20 cases per 1000 live births.[3] The incidence of UDT varies according to the gestational age of the newborn and is accepted to be 1-4.6% in full-term and 1.1-45% in preterm male babies.[4] There are various theories about how UDT develops in children.[5,6]

The association of UDT and SB had not been understood until 1981.[7] Various studies have reported an incidence of UDT with SB to be between 1-25.6%, afterward.[8-10] In general, the incidence of UDT has been accepted to be higher in patients with SB in comparison to the general population. On the other hand, the association of IH and SB has not been delineated, yet and the factors affecting the occurrence of IH in these patients have not been fully demonstrated.

The aim of this present study was to identify the prevalence of IH and UDT in a large patient cohort with SB as well as the factors that might affect its coexistence. To the best of our knowledge, the present study is the largest cohort in the literature on this subject and the only study that questions the risk factors for IH in this patient group.

Material and Methods

Patients who were admitted to our SB center with the diagnosis of SBA and SBO were included in the study. Ethical approval was obtained from the institutional review board for the study (İstanbul Medeniyet University Göztepe Prof. Dr. Süleyman Yalçın City Hospital Ethical Review Board, IRB No:2020/0618). Clinical data for SBA and SBO were collected retrospectively. The clinical information regarding the type of spinal anomaly and past medical history were also collected from the patient files. All parents of the patients were interviewed prospectively to note the information for IH and UDT. Together with demographic data, presence of an inguinal hernia, laterality, presence of ventriculoperitoneal (VP) shunt, type of SB (aperta or occulta), recurrence of IH and presence of UDT were questioned. Patients with noncommunicating hydrocele were not included in the study and communicating hydrocele was accepted in the spectrum of IH due to the persistence of the processus vaginalis.

Patients were grouped as SBA and SBO. Clinical data as well as the prevalence of IH were questioned as per the diagnosis, presence or absence of VP shunt and combined prevalence according to the diagnosis and VP shunt, together. Thus, the factors that affect the generation of IH were sought.

Statistical Analysis

The statistical analyses were done with the statistical program SPSS 22.0 (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL, USA). Numeric values were given as mean ± standard deviation (SD) and categorical values in percentage. Firstly, all relations between numeric and categorical variables in the groups were investigated by using univariate tests (i.e. Pearson chi-square test and Student's t-test). After univariate evaluations, statistically significant p values lower than 0,05 were selected for multivariate binary logistic regression analysis to evaluate the major factors influencing IH and UDT formation. Results of multivariate binary logistic regression were given with a p-value, odds ratio (OR) and 95% confidence interval (CI) for Exp B. One sample t test for proportions was used to analyze the prevalence of IH in this study with an estimated prevalence of 3% in the general population. A p-value below 0.05 was considered to be statistically significant.

Results

A total of 388 patients who were admitted to our SB center were evaluated. Of these, 238 patients had SBA and 150 patients had SBO. The mean age of the patients was 5.56±4.29 years (between 2 months and 18 years of age) and 207 of them

were males and 181 females. In the SBA group, there were 123 males and 115 females and in the SBO group there were 80 males and 70 females. The comparison of gender distribution was not significant (p=0.613). The mean age in SBA group was 5.09±3.89 years whereas the mean age in SBO group was 6.33±4.71 years (p=0.008).

Prevalence of IH was 12.6% in general including all the SB patients. This ratio was statistically significant when compared to the estimated prevalence in the population (p<0.001). Hernia was noted in 37 SBA patients (15.6%) whereas this was seen in 12 of SBO patients (8%) (p=0.029). Hernia was bilateral in 22 patients (44.9%), right sided in 14 (28.6%), left sided in 12 (24.4%) and undefined in one patient. Recurrence was noted in 9 patients (18.4%). When there was a VP shunt, hernia prevalence was 21.3% (35 patients among 164 patients with VP shunt) and when there was no shunt, this ratio was 7.1% (15 patients among 211) (p=0.0001). The prevalence of IH was 21.8% in males and 3.2% in females (p=0.0001). When there was a VP shunt with SBA the prevalence was 21.9% and when a VP shunt was present with SBO, this number was found to be 13.3% (p=0.006) (Table I). The prevalence of UDT was 17.7% and there was no difference between SBA and SBO patients.

Table I. Summary of major clinical factors affecting inguinal hernia development.

	Presence of IH (%)	*p**
Gender		
Male	21.8	0.0001
Female	3.2	
SBA+VP	21.9	0.006
SBO+VP	13.3	
VP(+)	21.3	<0.0001
VP(-)	7.1	
SBA	15.6	0.029
SBO	8	

IH: inguinal hernia, SBA: spina bifida aperta, SBO: spina bifida occulta, VP: ventriculoperitoneal shunt
*The *p* values give the comparison of each parameter with the data one line under.
Pearson chi-square test.

Cross comparisons were done to evaluate the risk factors for IH. Of the 165 patients with a VP shunt, 140 (84.8%) were males in this cohort whereas this figure was 15.2% (n:25) for females (p<0.0001). Male SBA patients with IH were compared with male SBO patients with IH in terms of VP shunt presence. Of the 31 male SBA patients with IH 27 (87%) had a VP shunt while only 16.7% of male SBO patients with IH (n:2 among 12 patients) had this intervention for hydrocephalus (p<0.0001).

Multivariate binary logistic regression analysis was done to identify the risk factors in the development of IH and UDT in this patient group. Male gender (p<0.0001, OR=0.107, 95%CI for ExpB 0.045-0.254) and presence of a VP shunt (p<0.0001, OR=0.185, 95%CI for ExpB 0.083-0.408) were found to be significant risk factors in the formation of IH in patients with SB. Then, we analyzed the prevalence of SB patients with IH but without a VP shunt. There was a total of 15 patients (7.1%) with IH but without a VP shunt. When we compared this ratio with an estimated prevalence of 3% in the general population with one sample t test for proportions, the difference was statistically significant (p=0.017). No significant risk factor

Table II. Clinical data of the patients according to the type of the spinal dysraphism.

	SBA	SBO	*p**
Patients, n	238	150	
Age (years)	5.09±3.89	6.33±4.71	**0.008**
Gender, n			
Male	123	80	0.613
Female	115	70	
VP(+), n (%)	148(62.2%)	17(11.3%)	**<0.0001**
IH(+), n (%)	37(15.6%)	12(8%)	**0.029**
UDT(+), n (%)	24(19.7%)	12(14.8%)	0.375

SBA: spina bifida aperta, SBO: spina bifida occulta, VP: venriculoperitoneal shunt, IH: inguinal hernia, UDT: undescended testis
‡122 patients in SBA group had data for testicular position and 81 patients in SBO group. 24 patients in SBA group (19.7%) had UDT and 12 patients (14.8%) in SBO group had UDT.
*Age comparison with Student's t test, other comparisons with Pearson chi-square test.

was found in the development of UDT in SB. The comparisons between groups are summarized in Table II.

Discussion

The development of inguinoscrotal pathologies can be better explained with the descent of the testis. After the migration of primitive germ cells to the gonadal ridge at about the 6th week of gestation, the differentiation of the gonad, either as a male or a female, takes place. During the elongation of the fetus, the testes are attached to the internal ring. Later, the gubernaculum is formed at the caudal portion of the testes and the peritoneum bulges towards the inguinal canal to form the processus vaginalis. After the 7th month of gestation, the testicular descent happens and the processus vaginalis obliterates. Failure of the obliteration of the processus vaginalis causes an IH and hydrocele in about 1-3% of children with a male predilection of 6:1.[3,11,12] This value of a maximum 3% was used as an estimated ratio of IH in the general population in this study. Many risk factors have been defined in the generation of IH but SB as a risk factor has not been delineated, yet.[11,12] In females, ovaries descend similarly but this descent stops in the pelvis. The cranial portion of the gubernaculum forms the ovarian ligament and the distal portion, the round ligament. The gubernaculum seems to be important in the descent of ovaries as well and the rudimentary development of the processus vaginalis forms the canal of Nuck, with its persistence, causing the inguinal hernia.[12]

SB is a neurodevelopmental anomaly of the neural plate closure during fetal life. Due to spinal cord injury in the course of the disease, it causes various clinical problems like hydrocephalus after Arnold Chiari Type 2 malformation, lower urinary tract and gastrointestinal dysfunction, motor and sensorial problems of lower extremities and erectile dysfunction.[13] The prevalence of this condition has dropped from 1/1000 live births to 3.1-4.6/10000 in the United States after food fortification with folic acid and early termination of pregnancy.[1,2,13] However, the prevalence is still high in other regions of the world.[2]

The true prevalence of IH in patients with SB has not been clearly defined in the English literature. In a large, nationwide study on the co-occuring malformations seen with SB, the patients were defined as isolated and non-isolated, the non-isolated cases were those with associating anomalies involving other organ systems. In this study, 1170 patients were included between 1976 and 2011. Only UDT was mentioned in this study among genital defects but IH has not been evaluated.[8]

In the current study, we observed an increased prevalence of IH in patients with SBA. VP shunt was also an associating risk factor with male gender. When we compared SBA patients with VP shunts and SBO patients with VP shunts, the prevalence was 21.9% in SBA vs. 13.3% in SBO. Whereas, when we evaluated the prevalence of IH patients without a VP shunt among all SB patients (7.1%), this ratio was still high in comparison with the estimated IH ratio in the general population. To the best of our knowledge, SB itself has not been previously reported as a potential risk factor for IH in children.

VP shunting is a classically known etiological factor for IH in children.[11,12] Increased intraabdominal pressure and high patency rates of the processus vaginalis in children have been accepted to be the main reason for this finding.[14,15] In a large cross-sectional study in Taiwan, medical records of 675 children with a VP shunt and 6704 children without this intervention had been followed up for 8 years. After 8 years, IH had been observed in 4.1% of control patients whereas 13.3% of patients with a VP shunt developed IH ($p<0.001$).[16] In other studies, the same prevalence was observed to be between 15 to 23.8% after VP shunting.[14,15,17,18]

The coexistence of IH with VP shunting has a direct relationship with the patency of the processus vaginalis. This patency is believed to be present in 70-80 % of babies at birth, dropping to 30-40% by 3 to 4 years of age.[15,19,20] Weaver et al.[21] followed 1548 children after laparoscopy was done for various surgical indications. They detected 308 patients with asymptomatic patent processus vaginalis. After a median of 8.1 years, 13% of the patients were seen to develop IH.[21] In this current study, 29.7% of the SB patients with a VP shunt were found to have an associating IH giving a slightly higher prevalence than the general information in the literature.

IH is 5-10 times more common in males than females. The nuck canal, equivalent to the processus vaginalis, obliterates earlier at 7 months of gestation in females than in males and this factor is believed to be the cause of this gender predilection.[22] Similarly, the prevalence of IH was 21.8% in males with SB and 3.2% in females with statistical significance, in this study.

Until the observation of Kropp and Voeller[7] in 1981, UDT had been evaluated as a coincidence rather than an association with SB. Several studies have demonstrated this relationship with a prevalence of 1-25.6% among children with SB significantly higher than the general population.[7-10,22] This figure was 17.7% in this study with no difference between SBA and SBO patients. Neurological functional impairment in nerve fibers of the genitofemoral nerve in SB is believed to be the cause of this high prevalence.[9] The genitofemoral nerve originates from L1-L2 nerve roots and the level of the vertebral defect in SB has been shown to affect the prevalence of UDT, being about 19% in low lesions and about 36% in high lesions.[9] The genital branch of the genitofemoral nerve innervates the gubernaculum with the cremasteric muscle and the gubernaculum is an important factor in testicular descent. Beasley et al.[23] in their experimental study, had divided the genitofemoral nerve of newborn rats below two days of age and had demonstrated that denervation of the gubernaculum results in UDT. This explanation has merit to describe the association between UDT and SB but no information for IH has been found in English literature describing how IH associates with SB other than patients with VP shunts. This current study demonstrated that IH prevalence in our SB patient group is significantly higher than the estimated prevalence of IH in the general population.

Tanyel has a different explanation for the spectrum of inguinoscrotal pathologies including IH, hydroceles and UDT. According to his theory, a transient decrease in sympathetic tonus coupling with increased parasympathetic tonus initiates apoptosis of the smooth muscles surrounding the processus vaginalis after testicular descent. The smooth muscles of the processus vaginalis are necessary to propel the testis to the scrotum. After the completion of descent, this programmed cell death causes obliteration of the processus vaginalis. Aberrations in timing, intensity or sustainance of this activity cause hernia, hydrocele and UDT. If no necessary autonomic activity happens after the descent of the testis, no obliteration of the processus vaginalis occurs and this ends up with IH. If this activity happens earlier than expected, early apoptosis of the smooth muscle of the processus vaginalis causes an inability of testes to be propelled to the scrotum. This autonomic activity which is called central catecholaminergic activity is mediated by the central nervous system and spinal cord towards autonomic ganglions.[6] When we consider the high prevalence of IH and UDT in patients with SB, Tanyel's theory may explain why we observed an increased prevalence of these inguinoscrotal pathologies when compared to the general population and this theory covers both the explanation for IH and UDT. However, this explanation should be thoroughly investigated in this patient group to increase knowledge.

An age difference was found between the SBA and SBO patients in this study. This may be attributed to the late presentation of SBO patients for surgery. IH was found to be

bilateral in nearly half of the patients and thus, contralateral exploration should be considered in these patients.

There are certain limitations of the current study. This study was an observational, descriptive study without any data of neurological examination and the etiological explanations are only hypothetical. The data derived from the questionnaire relies on the declaration of the parents and patients. A detailed neurological examination with the information of spinal defect level might support the etiological explanations and should be planned for prospective studies. The processus vaginalis samples taken from the children with SB and IH should be investigated to question Tanyel's theory and if possible together with magnetic resonance tractography for the tracts of central catecholaminergic activity.

In conclusion, IH hernia and UDT are seen more frequently in children with SB when compared to the general population. VP shunting and male gender seem to be independent risk factors for IH in these children. The findings of the current study may support the impaired neurological activity as an etiological explanation for the generation of IH and UDT in the normal population, as well. Further clinical studies are necessary to test this hypothesis.

Ethical approval

This study was conducted in compliance with the ethical principles according to the Declaration of Helsinki, and it was approved by the Institutional Review Board of İstanbul Medeniyet University Göztepe Prof. Dr. Süleyman Yalçın City Hospital (IRB No: 2020/0618).

Author contribution

The authors confirm contribution to the paper as follows: ŞKÖ, study design, analysis, interpretation of results and draft manuscript preparation, MAK, DÖÖ, VÖ, data collection, HC, İA, interpretation of results. 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

Is there a role of viral infection in cystic fibrosis exacerbation in children?

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ABSTRACT

Background. Cystic fibrosis (CF) is a degenerative disease distinguished by progressive epithelial secretory gland dysfunction associated with recurrent respiratory tract infections. Despite that bacteria have previously been studied as the main cause of CF airway damage, a strong effect of respiratory viral infections is also now recognized. We aimed to detect the relationship between viral infection and exacerbation in children with cystic fibrosis.

Methods. This is a cross sectional observational study recruiting 60 patients diagnosed as CF following in Cystic Fibrosis Clinic, Children's Hospital, Cairo University, throughout a period of 7 months. Their age ranged from 6 months to 13 years. Patients had nasal swabs and sputum samples obtained when they developed respiratory exacerbations. Multiplex PCR (polymerase chain reaction) technique was used to detect respiratory viruses from nasal swabs.

Results. We detected viruses in 48 patients during exacerbation (80%), the most common virus was rhinovirus in 43.4% of patients, followed by bocavirus in 20%, adenovirus in 13.3%, enterovirus in 10% and human metapneumovirus in 6.7%. Co-infection with double viruses was detected in 10 patients. Bacterial infection was present in 56.7% of patients; the most common organism was *Pseudomonas* in 20% of patients, followed by *Staphylococcus aureus, methicillin resistant Staphylococcus aureus, Klebsiella and Haemophilus influenzae*. CRP was positive in 53.3% of patients. There was a significant relationship between sputum positive bacterial culture and each of influenza A virus, enterovirus and human metapneumovirus.

Conclusions. This study demonstrated that exacerbation in cystic fibrosis may be exaggerated by viral infections such as influenza A and enterovirus necessitating hospitalization which shows the important protective role of vaccination. Also, a strong relationship was detected between some viruses such as enterovirus, human metapneumovirus and influenza and between bacterial infection.

Key words: cystic fibrosis, exacerbation, respiratory viruses, rhinovirus.

Cystic fibrosis (CF) patients frequently describe that colds and other upper respiratory tract infections are the prevalent cause of exacerbations of their respiratory symptoms. Despite this, there is lack of sufficient data in the literature on the role of respiratory viruses in CF lung disease and treatment options for these pathogens.[1]

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The role of bacterial pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* have been greatly investigated for many years whereas the respiratory virus pathogenesis is still not well studied. The availability of new diagnostic molecular tests to detect viral infections has recently expanded the interest in evaluating their effect in some diseases such as CF in children and in adults.[2]

The prevalence of viral respiratory infections varies significantly in the literature, ranging from 5 to 68%.[3]

It is highly detected in children, although a significant number of adult patients with CF also have viral infections, mainly rhinovirus.[4]

Although the specific mechanism by which viral infections may provoke a pulmonary exacerbation in children with CF has not yet been identified, viruses likely play a major role and have to be considered when investigating exacerbations in children.[5]

In this study we aimed to assess the relationship between viral infection and exacerbation in children with cystic fibrosis.

Material and Methods

This was a cross sectional observational study recruiting 60 patients diagnosed as CF, based on clinical manifestations and confirmed by a positive sweat chloride test, coming in acute exacerbation to the Cystic Fibrosis Clinic in Children's Hospital, Cairo University. The study was approved by the Institutional Ethics Committee of Kasr Al Ainy (I-300314) and informed consents were obtained from study subjects and/or their legal guardians before starting.

Inclusion criteria: age range from 6 months to 13 years old, both genders were included.

Full medical history and clinical evaluation with special stress on respiratory manifestations were recorded (fever, exaggerated cough, frequency of exacerbation, need for oxygen, need for hospital admission and intensive care unit admission, type of breathing and oxygen saturation).

Laboratory investigations included C-reactive protein, sputum culture, nasopharyngeal swab.

Blood was collected by venipuncture, allowed to clot and serum was separated by centrifugation at room temperature and was frozen at -20°C. The analysis of all samples was carried out at the laboratory of the Department of Clinical Pathology, Kasr Al Aini Hospital, Cairo University.

The sputum was collected in sterile ice - cream cups after instructing the patient to rinse his/her mouth thoroughly with water and cough forcefully to bring out the mucous from the tracheobronchial tree. Suction was used to collect a sputum sample in patients who were unable to cough. A soft, flexible tube was inserted through the nose and down the throat; and suction was applied for up to 15 seconds to collect the sample. The sputum sample was placed in a container with a growth medium or culture medium. Bacteria that grow on media were detected under a microscope or by chemical tests.

Nasopharyngeal swab samples were in the form of nasopharyngeal swabs taken on viral transport media (VTM).

Nasopharyngeal swab was taken as recommended through inserting the flocked flexible dacron or nylon fiber swab into one of the nostril. The swab is inserted till it reaches half of the distance between the ear lobule and the ala of the nose. The swab is left for few seconds then withdrawn to be put in the VTM containing tube labelled with the patient unique ID. The VTM is prepared inhouse using bovine albumin, HEPES buffer, penicillin and streptomycin in HANK's balanced salt solution. stored in the -70 freezer until processed.

PCR testing for respiratory viruses:

- Viral Nucleic Acid Extraction: Viral nucleic acid extraction was done after centrifugation of the sample for 10-20 min and 200 μl from the sediment taking as starting material using the Biospin Virus RNA Extraction kit (cat number BSC62M1 from Bioflux), according the manufacturer's instructions.
- CDNE was done manually by cDNA Synthesis Premix (SGRT801) from Seegene.
- PCR was done for the following viruses: adenovirus, influenza A virus, influenza B virus, parainfluenza viruses 1–4, rhinovirus, respiratory syncytial virus, bocavirus, human metapneumovirus,

coronavirus 229E, coronavirus NL63, coronavirus OC43, and enterovirus. PCR was done by real-time multiplex PCR using AnyplexTM II RV16 Detection (v1.1) (cat. no. RV7G01Y) supplied by Seegene, operated on a CFX96TM Real-Time PCR Detection System (Bio-Rad).

- Interpretation of the results was done according to the manufacturer's instructions, in addition to automatic analysis using the Seegene viewer software after exporting the run data to it.

Chest X-ray was done for all patients during exacerbation of cystic fibrosis.

Statistical analysis

Data were coded and entered using the statistical package SPSS (Statistical Package for the Social Sciences) version 24. Data were summarized using mean, standard deviation, median, minimum and maximum in quantitative data and using frequency (count) and relative frequency (percentage) for categorical data. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney U tests. For comparing categorical data, chi square (χ^2) test was performed. Exact test was used instead when the expected frequency was less than 5. P-values less than 0.05 were considered statistically significant.

Results

Our study included 38 males (63.3%) and 22 females (36.7%) with a median age of 4 years and range from 6 months to 13 years. Demographic data are shown in Table I.

The most common presentation was exaggerated cough present in all patients, followed by increased wheezing. Sputum culture showed mixed flora in 43.3 % of patients. *Pseudomonas* was the most common organism detected followed by *Klebsiella*. CRP was positive in 53.3% of patients (Table II).

Abnormal chest X-Ray (CXR) was detected in most of the patients in the form of consolidation in 20%, hyperinflation in 23.3%, increased bronchovascular markings in 26.7% and air bronchogram in 10%, while 20% of patients had normal CXR (Table II).

The most common virus detected was rhinovirus in 43.4% of patients, followed by bocavirus in 20%, adenovirus in 13.3%, enterovirus in 10% and human metapneumovirus in 6.7%. Detection of viruses required a semi quantitative method to assess the viral load in the sample (+, ++, +++ as shown in Table III). Double virus was detected in 10 patients (16.7%). None of the included patients had history of influenza vaccination.

A significant relationship was detected between positive bacterial culture and certain viruses as influenza A virus (p value <0.001), enterovirus (p value 0.002) and human metapneumovirus (p value 0.002), but no relationship was noted between double virus and bacteria with a p value of 0.08 (Table IV).

Many viruses were detected in CF patients during exacerbation necessitating hospital admission including: rhinovirus, influenza A, enterovirus, bocavirus and adenovirus. But a significant relationship was only detected between hospital admission and influenza A and enterovirus (Fig 1).

A significant relationship was detected between the presence of influenza A and enterovirus and the need for oxygen (with p values 0.004 and 0.02, respectively).

No significant relationship was detected between the need for intensive care unit (ICU) admission and the 2 viruses detected (rhinovirus and bocavirus) with p values 0.730 and 0.101, respectively.

Statistically significant differences were noted between different age group and influenza A, rhinovirus, enterovirus and adenovirus (with p values 0.036, 0.011, 0.002, 0.018 respectively) (Fig 2), but no relationship was detected in case of bacterial infection (p=0.137).

Table I. Demographic data of the study population (N=60).

Characteristic	n	%
Age groups		
<2 years	18	30.0
2 years to 6 years	24	40.0
>6 years	18	30.0
Gender		
Male	38	63.3
Female	22	36.7
Weight percentile		
Below 5th percentile	44	73.3
From 5th to 95th percentile	16	26.7
Height percentile		
Below 5th percentile	32	53.3
From 5th to 95th percentile	28	46.7
Frequency of exacerbation		
Twice per year	6	10.0
≥3 times per year	12	20.0
≥Once per month	42	70.0
Viral detection: positive	48	80.0
Viral detection: negative	12	20.0
	Mean ± SD	Median (Range)
Age (months)	58.7±41.11	49.5 (6-156)
Weight (kg)	12.73±6.84	11.52 (3-28)
Height (cm)	93.03±23.5	89.5 (55-140)
Age at diagnosis (months)	30.7±39.34	10 (2-132)
Body mass index (kg/m^2)	13.81±1.30	14 (11.2-15.6)
Sweat chloride test (mEq/L)	100.6±24.32	98 (67-155)
Duration of hospitalization (days)	11.14±6.62	8 (3-20)

SD: standard deviation

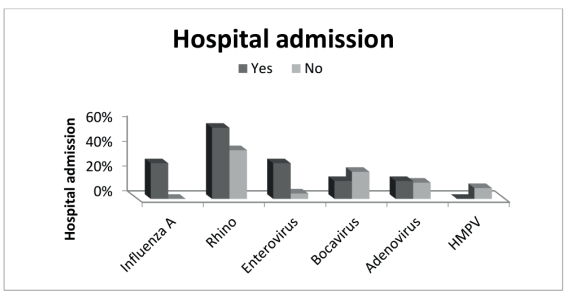

Fig. 1. Relation of viruses with hospital admission.

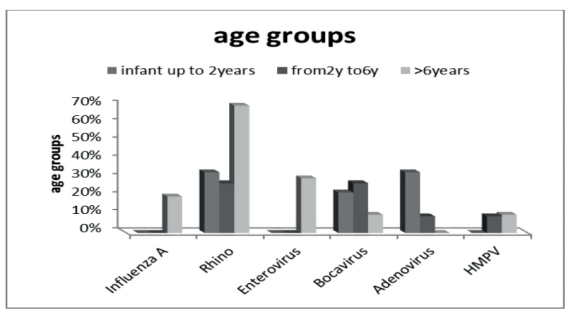

Fig. 2. Relation of age group with virus.

Table II. Clinical, laboratory and radiological parameters of patients during exacerbation (N=60).

Clinical presentation		n	%
Fever		50	83.3
Exaggerated cough		60	100.0
Hospital admission		14	23.3
Need for nasal oxygen		16	26.7
Need for intensive care unit		4	6.7
Increase wheezes		58	96.7
Increased crepitations		22	36.7
Laboratory tests			
Sputum culture	*Staphylococcus aureus*	6	10.0
	Pseudomonas	12	20.0
	MRSA	2	3.3
	Haemophilus influenzae	4	6.7
	Klebsiella	8	13.3
	E. coli	2	3.3
	Mixed flora	26	43.3
CRP	Positive	32	53.3
	Negative	28	46.7
Chest X-ray	Air bronchogram	6	10.0
	Consolidation	12	20.0
	Hyperinflation	14	23.3
	Increased broncho-vascular markings	16	26.7
	Normal	12	20.0

CRP: C-reactive protein, MRSA: methicillin resistant S. aureus

Discussion

In our study, we detected rhinovirus in 43.4% of CF patients during exacerbation. Similarly, it was reported as the most common respiratory virus in exacerbations of CF in many other studies.[2,3,6,7]

In our study, influenza A virus was detected in only 6.7% of patients although no one had a history of influenza vaccination. Similarly, a French study found that its prevalence was 9%.[2]

Also, Asner et al.[8] detected a low proportion of pH1N1 (5.3%) even though it was performed during the larger second wave of the 2009 influenza with a modest pH1N1 vaccine uptake rate of 46.5%.

On the contrary, Wat et al.[9] conducted a study during a typical influenza season and reported higher influenza A and B detection rates (25%) despite a high influenza vaccination uptake of 70%.

We detected adenoviruses in 13.3% of patients. Many studies have reported similar results.[2,8]

Bocavirus has seldom been screened in CF, but in our study, it was detected in 20% of patients. de Almeida et al.[10] and Keravec et al.[11] detected its prevalence to be about 5%.

We detected human metapneumovirus in 6.7% of patients. Another cohort study of older CF children hospitalized for exacerbation, showed that its prevalence reached up to 47.6%.[12]

In addition to rhinoviruses, other enteroviruses can be detected in the airways, leading to respiratory symptoms similar to rhinovirus. In our study, we detected enterovirus in 10% of patients, while in studies by de Almeida et al.[10] and Esposito et al.[13,14] enterovirus prevalence

Table III. Viral load in cystic fibrosis patients.

Virus	Load	n	%
Influenza A	Undetected	56	93.3
	Detected +	2	3.3
	Detected ++	2	3.3
	Detected+++	0	0.0
Influenza B	Undetected	60	100.0
Rhinovirus	Undetected	34	56.7
	Detected +	4	6.7
	Detected ++	22	36.7
	Detected+++	0	0.0
Respiratory syncytial virus-A	Undetected	60	100.0
Respiratory syncytial virus -B	Undetected	60	100.0
Enterovirus	Undetected	54	90.0
	Detected +	2	3.3
	Detected ++	4	6.7
	Detected+++	0	0.0
Bocavirus	Undetected	48	80.0
	Detected +	8	13.3
	Detected ++	4	6.7
	Detected+++	0	0.0
Adenovirus	Undetected	52	86.7
	Detected +	4	6.7
	Detected ++	2	3.3
	Detected+++	2	3.3
Human metapneumovirus	Undetected	56	93.3
	Detected +	0	0.0
	Detected ++	4	6.7
	Detected+++	0	0.0

mostly ranged between 3.2 and 7.75%, but can reach 29.4% or 35%, as reported in two 5-month studies.[8,15]

Viral coinfection is quite common in the CF respiratory tract, it involves at least two viruses, and sometimes more. We detected viral coinfection in 16.7% of patients. All of them were double viruses (rhinovirus with enterovirus, rhinovirus with bocavirus, rhinovirus with adenovirus and adenovirus with bocavirus). Other studies showed that viral coinfection rates ranged from 0 to 34.6%.[4,8] In Miró-Ca˜nísa et al.[16] study, the most common virus coinfection was rhinovirus plus adenovirus (6/20) and rhinovirus plus enterovirus (4/20).

A statistically significant relationship was noted between different age groups and some viruses, but no relation was detected in case of bacterial infection. Similarly, Asner et al.[8] detected that virus positive patients during CF pulmonary exacerbation were significantly younger than virus negative patients.

We detected a relationship between bacteria and each of influenza virus, enterovirus and human metapneumovirus which highlight the possibility that respiratory virus infections could increase the severity of infection.

Wat et al.[9] didn't find statistical differences between the viral and non-viral groups for bacteria isolation (p=0.909). Charles and

Table IV. Relationship between viral and bacterial culture.

	Sputum culture														P value
	S. aureus (n=6)		*Pseudomonas* (n=12)		*MRSA* (n=2)		Mixed flora (n=26)		*Klebsiella* (n=8)		*H. influenzae* (n=4)		*E. coli* (n=2)		
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	
Influenza A (n=4)	2	33.3	-	-	-	-	-	-	-	-	-	-	2	100.0	<0.001
Rhinovirus (n=26)	2	33.3	4	33.3	-	-	12	46.2	4	50.0	2	50.0	2	100.0	0.169
Enterovirus (n=6)	-	-	4	33.3	-	-	-	-	-	-	-	-	2	100.0	0.002
Bocavirus (n=12)	-	-	-	-	-	-	10	38.5	2	25.0	-	-	-	-	0.427
Adenovirus (n=8)	-	-	4	33.3	-	-	4	15.4	-	-	-	-	-	-	0.721
Human metapneumovirus (n=4)	-	-	2	16.7	2	100.0	-	-	-	-	-	-	-	-	0.002

MRSA: methicillin resistant S. aureus

Esther's study[17], did not detect a relationship between respiratory virus pathogen status and the frequency of typical CF pathogens such as *Staphylococcus aureus* or *Pseudomonas aeruginosa*.

Many viruses were detected in CF patients during exacerbation necessitating hospital admission. Armstrong et al.[18] demonstrated that respiratory viruses play a key role in CF hospitalizations and they were associated with acquisition of *Pseudomonas aeruginosa* although viral infections were self-limited. Wang et al.[19] have shown the association between viral infection and bacterial respiratory exacerbation, and hospital admissions. Another study detected respiratory viruses in 36% of CF patients during acute pulmonary exacerbations and showed that viral infection increases the prevalence of bacterial infection of specific pathogens such as *Haemophilus influenzae* and *Staphylococcus aureus*.[6] Also, Garcia et al.[12] has shown that *human metapneumovirus* behaves similarly to respiratory syncytial virus in CF and leads to an increased risk of hospitalization and exacerbation.

No significant relationship was detected between the need for ICU admission and the 2 viruses detected (rhinovirus and bocavirus). A study detected that the only discriminatory feature of the children who were rhinovirus positive was lower oxygen saturation.[3]

In conclusion, exacerbation in cystic fibrosis may be exaggerated by viral infections such as influenza A and enterovirus necessitating hospitalization which demonstrate the important role of vaccination and the role of awareness of treating the concomitant viral and bacterial infection. Also, a strong relationship was detected between some viruses such as enterovirus, human metapneumovirus and influenza and between bacterial infection.

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We are really grateful for Dr. Mona El Falaki for her continuous guidance and support throughout the study.

Ethical approval

The study was approved by the Institutional Ethics Committee of Kasr Al Ainy on March 2016 (I-300314) and informed consents were obtained from study subjects and/or their legal guardians before starting.

Author contribution

The authors confirm contribution to the paper as follows: study design: DHH, MSS, MMEA; data collection: OSE; analysis and interpretation of the results: MSS, draft manuscript preparation: DHH, OSE, MMEA. 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 case of familial recurrent 17q12 microdeletion syndrome presenting with severe diabetic ketoacidosis

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ABSTRACT

Background. Heterozygous intragenic mutations of the hepatocyte nuclear factor 1 homeobox b gene (*HNF1B*) located on chromosome 17 and microdeletion of 17q12 region (17q12MD) leads to the complete loss of this gene, which causes renal cystic disease, diabetes mellitus (MODY5), hypomagnesemia, hyperuricemia, liver enzyme abnormalities, genital tract abnormalities and exocrine pancreatic insufficiency. In addition, patients with 17q12MD also have facial dysmorphism, neuro-developmental and neuropsychiatric disorders.

Case. A 16-year-old girl with obesity and mild facial dysmorphism was admitted to the hospital with symptoms of diabetes that started two days prior to her admission. She was diagnosed with severe diabetic ketoacidosis and treated accordingly. She had been followed up with the diagnoses of multicystic renal disease, hydronephrosis, hepatosteatosis, hypomagnesemia and hyperuricemia since the age of six. She had mild intellectual disability. Her menarche started two months ago. Cranial magnetic resonance imaging revealed mild diffuse cerebral and cerebellar atrophy and a partial empty sella. Her mother had diabetes, hypomagnesemia and mild intellectual disability and her maternal grandfather and uncle had diabetes. Her grandfather also had renal cystic disease. All of them are on oral antidiabetic medication. The genetic analysis of the patient and her mother revealed a loss of 1.6 megabases in chromosome 17q12.

Conclusions. MODY5 should be kept in mind in patients with diabetes who present with extra pancreatic findings, especially with renal cystic disease, more over, a genetic analysis including the study of 17q12MD should be carried out in patients who present with additional neuropsychiatric findings. Ketoacidosis can be seen in patients with MODY5. Ketoacidosis and renal anomalies and dysfunction are factors that increase and affect the severity of each other in these patients.

Key words: MODY5, renal cyst, 17q12 microdeletion, ketoacidosis, intellecual disability.

The hepatocyte nuclear factor 1 homeobox gene (*HNF1B*) located on chromosome 17 encodes a transcription factor that plays a role in the development of the kidneys, pancreas, liver, thymus, lungs, intestine and gonads.[1,2] The clinical presentation of *HNF1B* related disease emerges as a result of heterozygous *HNF1B* intragenic mutations and deletions. Most of these patients have structural and functional kidney abnormalities characterized by renal cystic disease. In addition, these patients also present with varying frequencies of diabetes [maturity-onset diabetes of the young (MODY5)], hyperuricemia and early onset gout, hypomagnesemia, liver enzyme abnormalities, genital tract abnormalities (Mayer-Rokitansky-Küster-Hauser syndrome, bicornuate uterus, hemiuterus, cryptorchidism, absence of vas deferens and epididymal cysts) and exocrine pancreatic insufficiency.[1-4] The phenotypic findings of *HNF1B* related disease are also observed in the recurrent 17q12 microdeletion syndrome (17q12MD), which includes the whole-gene loss of *HNF1B* gene. In addition,

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the main findings of 17q12MD which are not observed in *HNF1B* molecular defects are facial dysmorphism, neuro-developmental and neuropsychiatric disorders.[5-7] These two genetic abnormalities are inherited autosomal dominantly. However, there are cases that include no family history and de novo mutations are detected in the majority of these cases.[3-5,8-10] Phenotypic heterogeneity has also been reported in patients with these genetic abnormalities. Thus, diagnostic scoring systems consisting of the phenotypic findings have been developed in order to select patients who need *HNF1B* gene analysis.[2,3]

We present a case of a patient who was admitted with severe diabetic ketoacidosis (DKA) while being followed up with multicystic renal disease, hypomagnesemia and was later found to have 17q12MD herself and in her mother.

Case Report

A 16-year-old girl was admitted with complaints of polydipsia, polyuria, difficulty in breathing and impaired speech that started two days prior to admission. She was admitted to the intensive care unit with the diagnosis of severe DKA. She was being followed up by the pediatric nephrology department since the age of six with the diagnosis of multicystic renal disease, hydronephrosis and was also being followed up because of hypomagnesemia which was detected seven years ago and she was on oral magnesium treatment. Hyperuricemia was detected during her follow-ups. She had mild intellectual disability and learning difficulty and was on a special education programme. There was no consanguinity between her parents. Her menarche started two months ago. Her 42 year old mother had diabetes for five years and mild intellectual disability. Her 47 year old maternal uncle had diabetes for seven years and her maternal grandfather had cystic renal disease and diabetes for ten years. All of them were on oral antidiabetic medication.

During her physical examination; she was confused, lethargic and disorientated. The score of Glascow coma scale (GCS) was: 9, body weight was: 79 kg (>97 p), height was: 162 cm (25-50 p), body mass index was: 30.1 kg/m². She had kussmaul breathing, coarse face, sunken eyeballs, high arched eyebrows, tubular nose, long philtrum, dark circles under the eyes, mild prognatism and decreased skin turgor. Acanthosis nigricans was not seen.

In her laboratory work up analysis, urine analysis showed glucose: +4, protein: +3, ketone: +3 and serum biochemistry showed glucose: 1463 mg/dL and concurrent C-peptide and insulin were; 1.63 ng/mL, 4.4 uU/mL, respectively, sodium: 129 mEq/L, potassium: 3.5 mEq/L, chloride: 84 mEq/L, calcium: 9.79 mg/dL, phosphorus: 6.5 mg/dL, alkaline phosphatase: 224 U/L, magnesium: 0.51 mmol/L, BUN: 73.5 mg/dL, creatinine: 2.58 mg/dL, uric acid: 24.2 mg/dL, AST: <5 U/L, ALT: 9 U/L, HbA1c: 13.5%, fT4: 1.07 ng/dL, TSH: 4.19 uIU, cortisol: 25ug/dL, CRP: 127.5 mg/L, myoglobin: 281 ng/mL and CK-MB: 2.23 ng/mL, blood ketone was: +3. Blood gas analysis showed pH: 6.94, HCO3: 6.3 mmol/L, pCO2: 22 mmHg, BE: -27.3, lactate: 2.8 mmol/L. Fractional magnesium excretion was: 3.39%, spot urine calcium/creatinine was: 0.02 and exrection of protein in her 24-hour urine was calculated as: 18 mg/m²/hour. COVID PCR were: negative. Her urine culture showed a growth of *Klebsiella pneumoniae*. No steatorrhea was detected.

Abdominal ultrasonography (USG) revealed grade 1 hepatosteatosis, grade 1 renal echogenicity on the left side and grade 2 hydronephrosis and 4-5 cystic structures in the right kidney, the largest of which was 10 cm. Ecocardiography and pelvic USG were normal.

The findings of our patient are shown in Table I.

During her clinical follow-up; intravenous fluid-electrolyte replacement, NaHCO3, antibiotic and insulin infusion (0.1 unit/kg/hr) were initiated in the intensive care unit. Oral thiamine treatment was started due to persistence of hyperglycemia, acidosis and high lactic acid level at the 20th hour her admission. The insulin infusion dose was gradually increased to 0.3

Table I. Clinical features of our patient.

Gender	Female
Age (year)	16
Family history	No consanguinity Mother had diabetes mellitus, hypomagnesemia, hepatosteatosis, and mild intellecual disability Maternal grandfather had diabetes mellitus and renal cyst Maternal uncle had diabetes
Clinical Findings	Facial dysmorphism Obesity Steatohepatitis Diabetes mellitus İntellectual disability Cerebral and cerebellar atrophy Partial empty sella Hyperuricemia Multicystic dysplastic kidney Hydronephrosis Renal failure
Genetic analysis	A loss of 1.6 megabases was detected in the 17q12 region in the patient and her mother

units/kg/hour at the 28th hour. During the 32nd hour continuous renal replacement therapy (CRRT) was applied for 10 hours because of persistence of acidosis, presence of oliguria, the unconsciousness and the possibility of uremic encephalopathy (urea level above 171 mg/dL). Acidosis resolved at the 4th hour of the CRRT treatment. Serum glucose began to be regulated during the 48th hour of her hospitalization. Although the fundus examination and cranial computed tomography (CT) were normal, cerebral edema was considered clinically, since she had a Glaskow coma scale score of 9, did not respond to painful stimuli and was unconscious during the recovery phase of acidosis, thus treatment was given for cerebral edema. Subcutaneous intensive insulin regimen was started during the 66th hour of hospitalization, upon the recovery of ketosis, acidosis and hyperglycemia. Before discharge, serum urea and creatinine levels returned to normal. However, glomerular filtration rate (GFR) was 37 mL/min/1.73m^2. The patient had poor mild eye contact and communication. Mild intellectual disability was diagnosed with the Porteus Labyrinths Performance test. Cranial magnetic resonance imaging (MRI) revealed mild diffuse cerebral and cerebellar atrophy and findings of partial empty sella were seen which was previously unreported (Fig. 1). Her mother had hepatosteatosis and didn't have renal dysfunction and any anomaly on her renal USG. The patients mothers laboratory work up

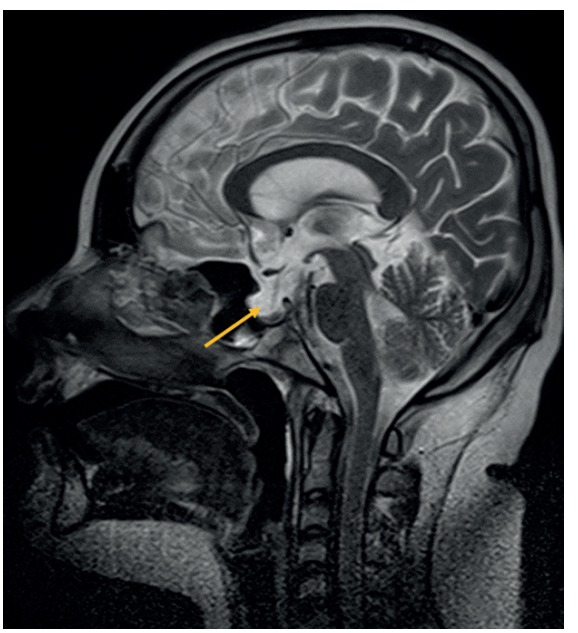

Fig. 1. Cranial MRI findings of our patient; partial empty sella and cerebral atrophy.

showed serum magnesium level of 0.4 mmol/dL, fractionated magnesium excretion of 6%, spot urine calcium/creatinine was 0.08, serum glucose was 172 mg/dL, insulin level was 4.35 uU/mL, C-peptide was 1.68 ng/mL and HbA1C was 8.7%.

Diabetes and extrapancreatic findings in patients were compatible with *HNF1B*-related renal cyst and diabetes syndrome (OMIM# 137920). But since facial dysmorphism, cranial MRI findings and intellectual disability were additionally found, chromosome 17q12MD syndrome (OMIM# 614527) was thought as more accurate for diagnosis. Than microarray analysis was planned instead of *HNF1B* gene sequencing. Array-CGH +SNP analysis (SurePrint G3 Human Genome CGH+SNP Microarray Kit, 4×180K, Agilent Inc, USA) revealed 1,637.567 kb deletion in 17q12 band including *HNF1B* locus (Fig.2) and was reported as arr[GRCh37] 17q12 (34611352-36248918X1). 2019 ACMG criteria was implemented for clinical classification of the deletion.[11] Hence patient's aberration was compatible with recurrent 17q12MD syndrome's critical region and included all morbid genes (*HNF1B, LHX1, ACACA*) associated with recurrent 17q12MD syndrome, the aberration was classified as "pathogenic". Genetic counselling was given to patient's family and segregation analyses were planned. Segregation analyses showed her mother had also the same abberation.

Written informed consent was obtained from the parents to publish this case report.

Discussion

Our patient was being followed up with a diagnosis of cystic renal disease and hydronephrosis since the age of six years and was found to have hypomagnesemia and hyperuricemia during her follow ups. The sudden onset of diabetes and presentation of severe ketoacidosis suggested type 1 diabetes, while the presence of obesity, hepatosteatosis, endogenous insulin release and family history of diabetes made us consider type 2 diabetes in the patient. However, a diagnosis of MODY5 was made due to the presence of extrapancreatic findings in the patient and her family. A genetic analysis was carrried out, and recurrent 17q12MD was detected in the patient. The presence of similar phenotypic findings in other family members indicated that this disease was inherited autosomal dominantly.

MODY5 occurs as a result of heterozygous *HNF1B* mutations and deletions.[1-3,5] *HNF1B* molecular defects are investigated especially in cases that present with renal cysts and diabetes.[2-4,8,12,13] Dotto et al.[13] detected heterozygous *HNF1B* whole-gene deletion in one of 28 patients with renal cysts and prediabetes/diabetes, body and tail pancreatic agenesis and *HNF1B* mutation

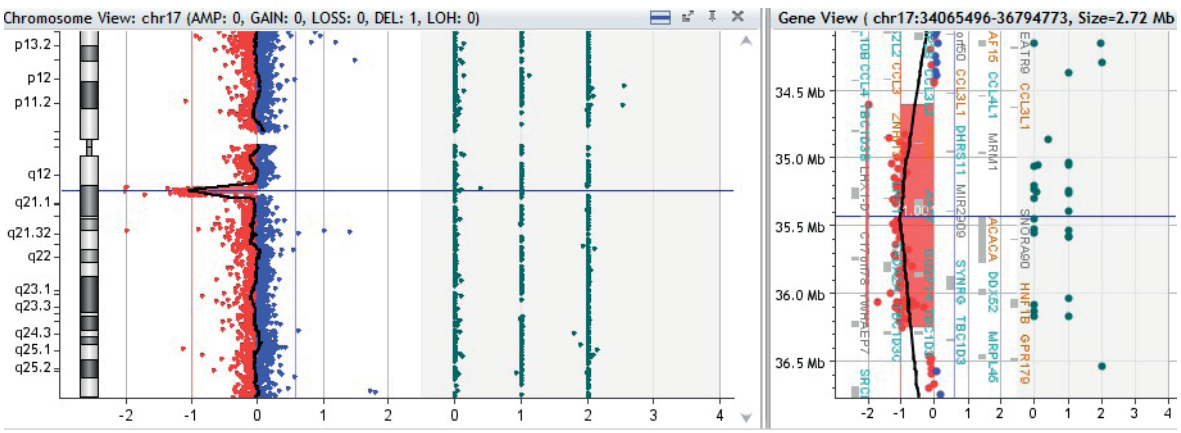

Fig. 2. SNP-Microarray analysis shows the 1.6 megabase deletion in 17q12 locus includes the *HNF1B* gene which is the responsible for MODY5 phenotype.

and hypomagnesemia in the other. Edghill et al.[8] reported that 11 of 23 patients with renal cystic disease and *HNF1B* mutations had diabetes and 7 of them had de novo mutations. Roehlen et al.[5] first found heterozygous *HNF1B* deletion in a patient with MODY5. Later, they noticed that the patient had liver enzyme elevation, pancreatic atrophy, renal cystic disease, facial dysmorphism and mild intellectual disability and detected 1.58 Mb 17q12 MD by microarray tests in repeated genetic analysis.

Inadequate insulin secretion of pancreatic beta cells is responsible for the diabetes in these patients. But, in some patients pancreatic atrophy, hypoplasia of pancreatic trunk, tail and neck were observed.[1-5,13,14] Hepatic insulin resistance was also shown in these patients.[15] Pancreatic imaging was not performed in our patient, whose diabetes etiology was considered to be MODY. On the other hand, insulin resistance develops in patients with renal failure.[16] It is thought that insulin resistance also contributed to the etiopathogenesis of diabetes in our patient, who was obese and had renal dysfunction.

It has been reported that the age of diagnosis of diabetes in *HNF1B* molecular defects is >25 years.[4] Bingham et al.[1] reported that 58% diabetes, 4% impaired glucose tolerance test in *HNF1B* mutation carriers and diabetes were diagnosed at the mean age of 26 years (10-61 years). However, the age of diabetes in a patient with *HNF1B*-related disease was 6 years.[17] In 17q12MD, it was found that 63% of patients had diabetes and the age of diagnosis was <40 years in the review of literature by Roehlen et al.[5] However, the study reported a patient with 17q12MD, in which diabetes was diagnosed at the age of 14. Warncke et al.[18] reported that the average age of diabetes was 13.5 years in 35 MODY5 patients, most of whom had 17q12 MD. The age of diabetic diagnosis of our patient was 16 years of age.

Diabetes is not observed in every patient with *HNF1B* mutations or 17q12MD.[2,3,5,6,9-11] Ulinski et al.[9] reported that none of 25 patients with renal developmental anomalies and *HNF1B* mutations had diabetes.

In terms of the treatment of diabetes in this disease, not all diabetic patients are insulin dependent. There are phenotypic differences between families with *HNF1B* mutations or within the same family; some patients use insulin, and some use oral antidiabetics.[4,5,7,10-13,18-20] Warncke et al.[18] reported that 65.7% of 35 MODY5 cases used insulin, 8.6% used oral antidiabetic drugs and 40% of them were accompanied by extrapancreatic findings. Dubois-Laforgue et al.[4] reported that 159 of 201 adult patients with *HNF1B* molecular defects had diabetes and 122 had renal cysts. 29 patients were using oral antidiabetic drugs, 111 were using insulin. They found that 79% of these patients were insulin dependent during a ten-year follow-up and the frequency of insulin use was higher in those with deletions compared to *HNF1B* mutations. They found diabetic retinopathy and nephropathy in 46 out of 114 patients. It has been reported that the patients with 17q12MD initially used oral antidiabetic drugs and then they eventually became insulin dependent.[5] This heterogeneity observed in the treatment of diabetes in these patients was explained by the expressivity variability of the genetic defects.[1,5,10,12,19]

Diabetic ketoacidosis has been rarely reported in MODY5 cases.[19] Despite having the same genetic defect as her mother, our patient presented with severe ketoacidosis and then an intensive insulin regimen was started. Diabetes developed in other family members at an older age and they were treated by oral antidiabetic drugs.

Developmental and functional abnormalities of the kidneys are common findings of *HNF1B*-related disease and 17q12MD syndrome, and are observed in most patients. Renal multicystic dysplasia is the main finding and the other abnormalities detected are isolated renal cortical cysts, renal hyperechogenicity, renal

hypoplasia, single kidney, ureteral dilatation, horseshoe and duplex kidneys, urinary tract abnormalities, oligomeganephronia, and bilateral hydronephrosis.[1-3,5,10,12] It is possible to diagnose patients by showing bilateral hyperechogenic kidneys in the prenatal period.[2,3,5,10] It has been found that these patients develop severe renal failure, which can lead to dialysis/transplantation at a very high rate.[1-3,5,9,10] It was found that the prognosis of renal disease was worse in those with *HNF1B* mutations than those with deletions.[4] Our patient was being followed up with a diagnosis of multicystic kidney and hydronephrosis since the age of six. We thought that in our case, the coexistence of diabetes and congenital renal anomalies might cause more severe DKA. Cystic kidney disease was also detected in the grandfather. Her mother had no renal abnormalities and dysfunction.

In this group of patients, hypomagnesemia is detected with a varying frequency.[2,3,5,20,21] The *HNF1B gene* plays a role in the genetic regulation of sodium-potassium ATPase, which is critical for magnesium reabsorption in the distal convulated tubule of the kidney.[20,21] Van der Made et al.[21] reported that hypomagnesemia was the first presentation in a patient with heterozygous *HNF1B* deletion and in a patient with 17q12MD. Hypomagnesemia and increased fractional magnesium excretion were detected in both our patient and her mother.

Neurodevelopmental and neuropsychiatric diseases such as autism spectrum disease, agression, anxiety, hyperactivity, developmental delay, cognitive impairment, learning impairment, and seizures are the characteristic symptoms of 17q12MD syndrome. It has also been reported that these patients have facial dysmorphism, growth retardation and skeletal problems.[5,7,17,22-25] These neuropsychiatric abnormalities are not observed in intragenic *HNF1B* pathogenic variants.[3,5] Dixit et al.[7] reported that two of three patients aged <12 years with a history of neonatal transient hypercalcemia, renal cystic disease, no diabetes and 17q12MD between 1.6-2.07 Mb had speech difficulties, autism and mild learning difficulties, one had severe facial dysmorphism and one had hypospadias. An adult woman with 17q12MD without diabetes and cognitive impairment, had right kidney aplasia, left kidney dysplasia, renal failure from infancy, Mayer-Rokitansky-Kuester-Hauser syndrome, congenital joint laxity, kyphoscoliosis and bilateral hip dysplasia.[22] Loirat et al.[25] reported three patients aged 3-9 years, who had no diabetes, had cystic or hyperechogenic kidneys and were diagnosed with 17q12MD (1.49-1.85 Mb). These patients had autism spectrum disease, intellectual disability, social interaction impairments, verbal and non-verbal communication deficits and stereotyped behaviors. Roberts et al.[6] detected de novo 17q12MD including 28 genes in a 17-year-old boy with tall stature, facial dysmorphism, joint laxity, small pancreas, splenomegaly, mild pectus deformity, behavioral changes and cognitive impairment (schizophrenia, autism spectrum disease, developmental delay, intellectual disability, anxiety, and hyperactivity). This patient did not have diabetes, renal or liver pathology. Bernardini et al.[26] reported that two patients with Mayer-Rokitansky-Kuster-Hauser syndrome and one patient with renal cystic disease and mild facial dysmorphism which were all psychomotorly normal.

As seen, although the critical deletion region (1.4 Mb) is the same in all cases of microdeletions, phenotypic heterogeneity from case to case is detected in 17q12MD. It has been suggested this may be related to the extent of the deletion emcompassing the *HNF1B* gene and the other neighbooring genes located in this region, and the number and haploinsufficiencies of the other genes may be responsible for the phenotypic differences. It has been suggested that the loss of *ACACA* and *LHX1* genes may be related to neurodevelopmental and neropsychiatric problems observed in these patients.[5,19,23,24] In our

patient and mother who both had intellectual disability, a 1.6 Mb deletion was detected in the 17q12 region, including morbid genes such as *HNF1B*, *LHX1*, and *ACACA*.

Our patient and some of her family members had phenotypic findings of recurrent 17q12MD. MODY5 should be kept in mind in cases with extrapancreatic multisystem findings especially renal cystic disease; however, genetic analysis should be performed in patients with neuropsychiatric findings with a method that will detect 17q12MD. Genetic counseling should be given to cases related to autosomal dominant inheritance.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: CA, BK; data collection: CA, BK; analysis and interpretation of results: CA, BK, EK, ES, AKT, NÇ, CY, SK; draft manuscript preparation: BK. All authors reviewed the results and approved the final version of the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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Case Report

A rare case of antileucine-rich glioma-inactivated 1 encephalitis in a 14-year-old girl

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ABSTRACT

Background. Autoimmune limbic encephalitis in children occurs most frequently in those with antibodies against the N-methyl-D-aspartate glutamatergic receptor. We report the case of a 14-year-old girl who was diagnosed with antileucine-rich glioma-inactivated protein 1 limbic encephalitis.

Case. A fourteen years old, previously healthy girl applied to the emergency department with suspicion of dystonic seizure, ataxia, gait disturbance and speech disorders. Serum sample of the patient was positive for leucine-rich glioma inactivated protein 1 IgG.

Conclusions. Although it is a rare disease in childhood, in the presence of new onset psychotic symptoms or altered mental state, concomittant hyponatremia and unique type of seizures, anti leucine-rich glioma inactivated protein 1encephalitis should be considered in differential diagnosis.

Key words: leucine-rich glioma-inactivated 1 encephalitis, pediatric, faciobrachial dystonic seizure, autoimmune encephalitis.

Limbic encephalitis (LE) is an autoimmune neurological disorder associated with antibodies against various antigens.[1,2] In childhood, most patients with autoimmune LE develop with antibodies against the *N*-methyl-D-aspartate (NMDA) glutamatergic receptor.[3] The most common type of autoimmune encephalitis in adults is leucine-rich glioma-inactivated protein 1 (LGI-1)-associated encephalitis. Leucine-rich glioma-inactivated proteins 1–4 take a serious role in synaptic maturation, transmission, and myelination. One of the new autoantigens in autoimmune encephalitis is LGI-1.[4] The median age of patients with LE associated with the LGI-1 antibody is 60 years.[5,6] The clinical presentation is variable, including severe short-term memory loss, psychiatric disturbances, and various types of seizures such as faciobrachial dystonic seizures (FBDS).[5,7] Frequent focal seizures were found in LGI-1-IgG-positive pediatric patients.[7] The condition may be complicated by severe hyponatremia in approximately 60% of cases.[8] Patients usually respond well to immunotherapy.[9] In this paper, we report the case of a 14-year-old girl who initially presented with ataxia and dysarthria, and was diagnosed with anti-LGI-1 LE.

Case Report

A 14-year-old, previously healthy girl was brought to the emergency department because of suspected dystonic seizure, ataxia, gait disturbance, and speech disorders. The additional symptoms reported by the parents were personality changes, visual hallucination, intermittent disorientation, balance disorders, hyperanxiety, intermittent headaches, and vomiting for approximately one month.

On admission to the intensive care unit, a detailed neurological examination revealed dysarthria and 4/5 muscle strength in both the

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lower and upper extremities. Other components of the neurological examination were normal, mental orientation was good (Glasgow Coma Scale Score:15), and the cranial nerves remained intact. No signs of meningeal irritation were found. Other systemic examination results of the patient were normal. Except for hyponatremia (serum Na concentration, 125 mmol/L), no abnormalities were found in other laboratory tests neither in cerebrospinal fluid (CSF) examination.

Computed tomography (CT) of the head revealed a slightly hypodense area involving the left corpus striatum (Fig 1A). Consecutive magnetic resonance imaging (MRI) of the brain revealed mild hyperintensity on the left corpus striatum and insula on diffusion-weighted imaging (Fig 1B) but no true restricted diffusion on the corresponding apparent diffusion coefficient map. Multifocal T2 fluid-attenuated inversion-recovery (FLAIR) hyperintensities and expansion were observed in the left frontal cortex, right medial temporal lobe, left insula and corpus striatum, right inferior frontal region, and left temporal lobe. We observed no hemorrhage on susceptibility-weighted images or contrast enhancement after administration of contrast media (Fig. 1).

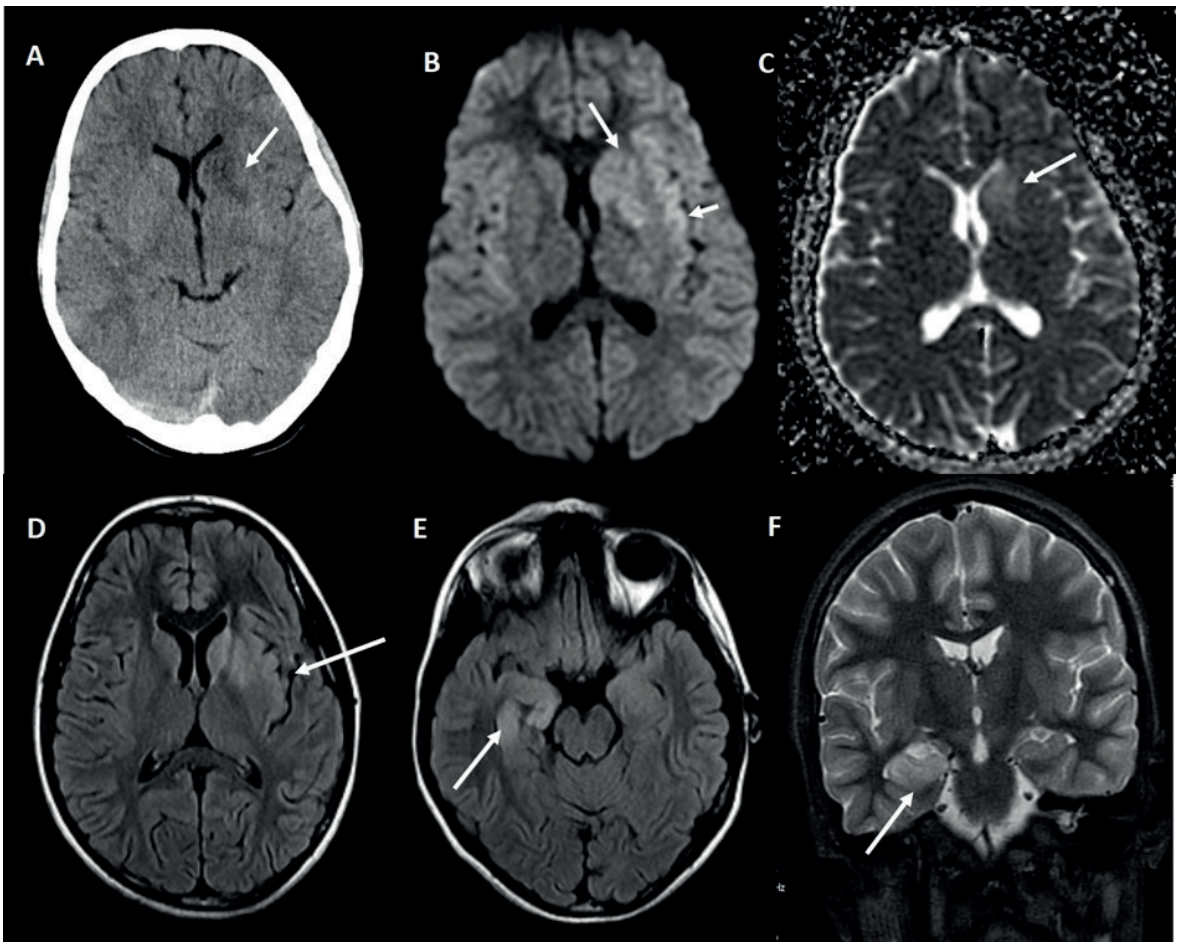

Fig. 1. On non enhanced CT scan (A) slightly hypodense area involving left corpus striatum is seen (arrow). MR imaging of the brain demonstrates: mild hyperintensities in left corpus striatum (long arrow) and insula (short arrow) on DWI (B), but no restricted diffusion on the corresponding ADC map (C). On axial FLAIR images; hyperintensities and mild expansion involving left insula and corpus striatum (D), right medial temporal lobe, left temporal lobe and right inferior frontal lobe (E) is demonstrated. On coronal T2 image hyperintensity and expansion of right hippocampus is better visualized (F).

The typical involvement pattern on neuroimaging studies and clinical and laboratory findings were highly suspicious for autoimmune encephalitis. Although basal ganglia were involved and no hemorrhage was detected on the susceptibility-weighted images, which are useful for distinguishing from herpes simplex encephalitis, viral encephalitides were also included in the differential diagnosis because of similar appearance.

In accordance with the symptoms presented, administrations of maintenance fluid and sodium-correcting fluid, ceftriaxone (maximum, 4 g/d), acyclovir (maximum 1500 mg/d), and levetiracetam (20 mg/kg/d) were initiated. Administrations of pulse methylprednisolone were started at 30 mg/kg (maximum, 1 g/d) for five days. During the follow-up, the sodium level of the patient improved appropriately. The CSF culture and polymerase chain reaction (PCR) against herpes simplex type I and II viruses were negative. A serum sample was submitted for an autoimmune encephalitis panel and was positive for LGI-1 IgG (reference value: negative). Intravenous immunoglobulin (IVIG) treatment (2 g/kg for two days) was also given owing to the persistence of the brachial dystonic seizures and ataxia. The electroencephalogram performed on the patient was found to be normal. In addition, abdominal and thoracic imaging examinations were performed to rule out a paraneoplastic form of autoimmune LE.

The patient's symptoms completely improved, but short-term brachial dystonia recurred intermittently. The patient was discharged from the hospital with oral steroid therapy.

Written informed consent was obtained from the parents of the child.

Discussion

Autoimmune encephalitides (AEs) are infrequent and various neurological diseases characterized by immune-mediated inflammation of the brain. AE phenotypes have mainly been reproduced from an antigen location.[2,10] With increased research on autoimmunity, several new autoantibodies have been discovered and that expanded AE subtypes spectrum.[10] Antibodies targeting the extracellular matrix-associated components of the voltage-gated potassium channel complex (VGKC) can include those that target the proteins. Some of these proteins which coassociate directly or indirectly with the VGKC, are contactin-associated proteinlike 2 (CASPR2), contactin 2, dipeptidyl aminopeptidase-like protein 6 (DPPX), ADAM 22, ADAM 23, and LGI-1.[4,11] The VGKC is present on the membrane of neurons in the central and peripheral nervous systems. Leucine-rich glioma-inactivated protein 1 and CASPR2 are the proteins most frequently associated with the VGKC.[4,10,12] Leucine-rich glioma-inactivated protein 1 is mainly present in the hippocampus and temporal cortex.[4,12] LGI-1 LE has been reported predominantly in adults with a mean age at onset of approximately 63 years.[6] Our patient had one of the rare cases of LE with anti-LGI-1 antibodies in children in literature.

Patients with anti-LGI-1 encephalitis has often been misdiagnosed as a mental disorders since the disease progresses with acute or subacute onset of cognitive dysfunction. Its clinical manifestations are FBDS, cognitive disorder (mainly recent memory deterioration), epilepsy, mental disorder, hyponatremia, autonomic dysfunction, psychosis, hallucinations, emotional disturbances, spatial disorientation, and sleep disorders.[6,9] Our patient's initial symptoms were personality changes, hyperanxiety, and intermittent headaches. However, she was admitted to the hospital only when symptoms of dystonic seizure, ataxia, gait disturbance, and speech and balance disorders occurred, approximately one month after the onset of the initial symptoms.

In adults, an association between LGI-1 protein disturbances and abnormal seizure activity has been found in clinical and genetic studies.[13,14] Patients usually present with multiple seizure types, including the characteristic FBDS, focal tonic or clonic seizures, and myoclonic

seizures.[6,9,15] Focal seizures are common in a limited number of pediatric patients.[7] Faciobrachial dystonic seizures are present in almost two-thirds of patients with LGI-1 encephalitis and are characterized by focal seizures with or without loss of awareness, mostly involving the face and arms. Faciobrachial dystonic seizures are frequent but brief seizures lasting less than 5 seconds that may be associated with vocalization, fear, automatism, or loss of consciousness.[16] They can also present unilateral or bilateral arm posturing with facial grimacing lasting less than 3 seconds without loss of consciousness.[17] Our patient had brachial dystonic seizures lasting less than 5 seconds without loss of consciousness.

Lumbar puncture is essential in the diagnosis and exclusion of encephalitis. Autoantibodies to LGI-1 and VGKC can be detected in both CSF and serum, but serum tests are more sensitive.[7,9] Using both serum and CSF may increase the sensitivity of the test. In our patient, the lumbar puncture sample showed no abnormalities. However, we could not examine the antibodies in the CSF.

In several studies, hyponatremia has been observed in approximately 40%–60% of patients.[6,9,12] Serum sodium abnormalities correlate with the disease condition.[8] Our patient also had significant hyponatremia at the first admission.

Magnetic resonance imaging commonly, but not always, reveals hippocampal T2 hyperintensity.[12] Magnetic resonance imaging findings may be unilateral but are more commonly bilateral.[18] Neuroimaging findings may be absent in the early stages of the disease. Most patients with prolonged symptoms showed medial temporal MRI changes.[18,19] Furthermore, T1 hyperintensities in the basal ganglia have also been reported.[7] Positron emission tomography could be more sensitive than MRI and may be useful in the early diagnosis of the disease.[6] In our patient, MRI revealed T2-FLAIR hyperintensities and a mild expansion involving the left frontal cortex, right medial temporal lobe, left insula and corpus striatum, right inferior frontal region, and left temporal lobe. No hemorrhage as well as contrast enhancement or a true diffusion restriction were observed.

The use of antiepileptic drugs alone for seizures and antipsychotic drugs for psychiatric symptoms is not generally effective. First-line treatment includes methylprednisolone and immunoglobulins, and a combination of these has been shown to be better. Patients show an excellent response to immunotherapy.[9,12] When methylprednisolone and immunoglobulins or plasmapheresis do not produce the desired therapeutic effect, the inclusion of pharmacotherapy in second-line therapy with rituximab or cyclophosphamide is recommended.[20] The condition usually has a good prognosis, with almost 70% of patients doing well after two years of follow-up. Disease recurrence, which can occur mostly within the first six months, accounts for 30% of patients. The major remaining symptoms are amnesia, spatial disorientation, and insomnia.[6] However, no clear data are available on the prognosis in childhood. In our patient, we used pulse methylprednisolone and IVIG therapy. Her symptoms completely improved, but short-term brachial dystonia recurred intermittently. Follow-up was continued with oral steroid therapy.

Consequently, though a rare disease in childhood, anti-LGI-1 encephalitis should be considered in the differential diagnosis in patients with new-onset psychotic symptoms or altered mental state, concomitant hyponatremia, and unique types of seizures.

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Author contribution

The authors confirm contribution to the paper as follows: study conception and design: GÖ;

data collection: GÖ, TK, DBK; analysis and interpretation of results: GÖ, TK, DBK; draft manuscript preparation: GÖ, TK, DBK. All authors reviewed the results and approved the final version of the manuscript.

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Case Report

Lethal encephalitis in a pediatric patient with SARS-CoV-2

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ABSTRACT

Background. SARS-CoV-2 mostly affects the respiratory system. Some studies have reported neurological disorders associated with SARS-CoV-2. Despite an increase in reported instances, encephalitis caused by COVID-19 infection is still poorly understood.

Case. We reported a rare presentation of SARS-CoV-2 in a 15-year-old patient. He had a fulminant course with encephalitis. He had mild symptoms of a COVID-19 infection five months ago and recovered without any sequel. Despite appropriate treatment, the patient had a devastating course.

Conclusions. This was a severe presentation of SARS-CoV-2 with central nervous system manifestations.

Key words: encephalitis, status epilepticus, SARS-CoV-2, childhood.

Encephalitis is an inflammation-related neurological illness affecting the brain parenchyma.

It is characterized by focal brain changes, with or without meningeal involvement, and can be caused by a variety of factors (infectious, inflammatory, autoimmune, paraneoplastic, etc).[1-3] Viral encephalitis is the most common infectious cause of encephalitis. It presents with fever, headache, clouding of consciousness, seizures, personality change, focal neurologic deficits, coma, and death.[1,2]

SARS-CoV-2 manifests as fever, cough, fatigue, and pneumonia. Studies have shown that SARS-CoV-2 can cause central nervous system manifestations such as seizures, altered levels of consciousness, cerebral ischemia, and encephalitis.[4]

Patients with SARS-CoV-2 encephalitis may have mild respiratory symptoms at the beginning; later on clinical findings may progress to deterioration and loss of consciousness progressing to confusion.[5] Here we represent a pediatric case of SARS-CoV-2 encephalitis with a devastating course.

Case Report

A 15-year-old male patient was admitted to an outpatient emergency clinic at a local hospital. He had a headache, sore throat, loss of appetite, malaise, and vomiting for one week. He had a history of a COVID-19 infection five months ago. Three days before admission, he had vertigo, clumsiness, and drop attacks. He had used antihistamine without a prescription. Afterwards, he had a tendency to sleep. The physician who had examined him had attributed this sleep event to the antihistamine drug. Brain computed tomography (CT) revealed normal findings. On the seventh day of initial symptoms, his parents took him to the hospital when they could not awaken him. The patient's consciousness ameliorated. He was hospitalized at the local hospital. On the first morning of admission at the local hospital, he became comatose with a Glasgow coma scale (GCS) of 3, he was intubated and referred to our hospital. Physical examination on admission

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was as follows: temperature: 39°C, blood pressure: 110/70 mmHg, pulse rate: 90 bpm, and respiratory rate: 20 bpm; he had a GCS of 3, no lateralization, or neck stiffness. He had an extensor plantar response. His laboratory findings were normal. On the third day of admission, D dimer (1.14 mg/L), Troponin I (150 pg/ml), and BNP (38.2 pg/ml) increased.

Blood culture and sputum culture revealed no organism. A thorax CT revealed bilateral infiltrations in the lower segments, as well as subpleural atelectasis in the posterior lower segment of the left lung. Brain CT was normal, and brain magnetic resonance imaging (MRI) did not reveal any abnormality in axial brain MRI images T1W, T2W, FLAIR, or post contrast T1W images, but there was significant diffusion restriction at frontotemporoparietal cortical areas bilaterally representing cytotoxic edema in diffusion-weighted images (DWI) (Fig. 1).

In the light of his physical examination, clinical findings, and brain MRI findings, the patient was considered to have encephalitis. Other infectious agents could not be ruled out due to laboratory insufficiencies. The lumbar puncture could not be done because the GCS was 3 and the parents did not provide informed consent. Vancomycin, ceftriaxone, favipiravir, and acyclovir treatment were initiated. Because of the severe involvement of the central nervous system and GCS of 3 on admission, daily intravenous immunoglobulin (1 gram/kg/day IVIG for 2 days) was added to his regimen. The patient developed status epilepticus within 6 hours of admission after the first dose of IVIG. We obtained a non-enhanced CT, which revealed cortical sulci effacement, representing brain edema (Fig. 1). Mannitol, dexamethasone, and 3% NaCl were added to the patient's treatment. We started plasmapheresis and discontinued after the third session due to hypotension. The patient deteriorated despite treatment. He had non-reactive pupils in the midline, an absent gag reflex, oculovestibular, oculocephalic, and cornea reflexes, showing that he was brain dead 48 hours after admission. Carotids Doppler ultrasonography (USG) revealed disruption of both internal carotid artery (ICA) and middle cerebral artery (MCA) arterial flow forms with a pattern of high- resistance short-duration reverse flow. The patient died on the eighth day of admission.

Discussion

SARS-CoV-2 has potential neurovirulence and can cause neurological disorders in a short or long-term span due to inadequate immune responses and/or viral propagation in the central nervous system (CNS).[6,7] Neurological complications vary from mild to severe such as headache, anosmia, disturbance of consciousness, seizures, and paralysis. Neurological symptoms can follow respiratory symptoms with a delay.[8] In a recent study from France, 58 of 64 COVID-19 patients had neurologic problems, including encephalopathy, agitation, and confusion.[9] The pathophysiology of SARS-CoV-2-associated encephalitis is not completely known. The pathogenesis of encephalitis as a COVID-19 consequence has been proposed in three ways: molecular mimicry, direct invasion of the neurological system, and systemic inflammation.[10-13] Numerous penetration routes have been proposed, including the hematogenous route and trans-synaptic transmission. Disruption of the blood-brain barrier (BBB) may be a hallmark of SARS-CoV-2 neuropathogenesis. By regulating the entry of immune cells or viruses into the CNS, the BBB plays an important role in the pathogenesis of neurotropic viruses. By interacting with angiotensin converting enzyme-2 (ACE-2) on neurons and glia, the virus might start a cycle of viral budding and further harm neuronal tissue once it has access to it.[14] On the other hand, if the innate immune system fails to cope with SARS-CoV-2, the adaptive immune system, which is systemic, and virus-specific, will be activated, resulting in immunological memory stimulation. Cell-mediated immunity and humoral immunity are frequently part of the adaptive immune response. Nonetheless, if a virus escapes the immune system and causes

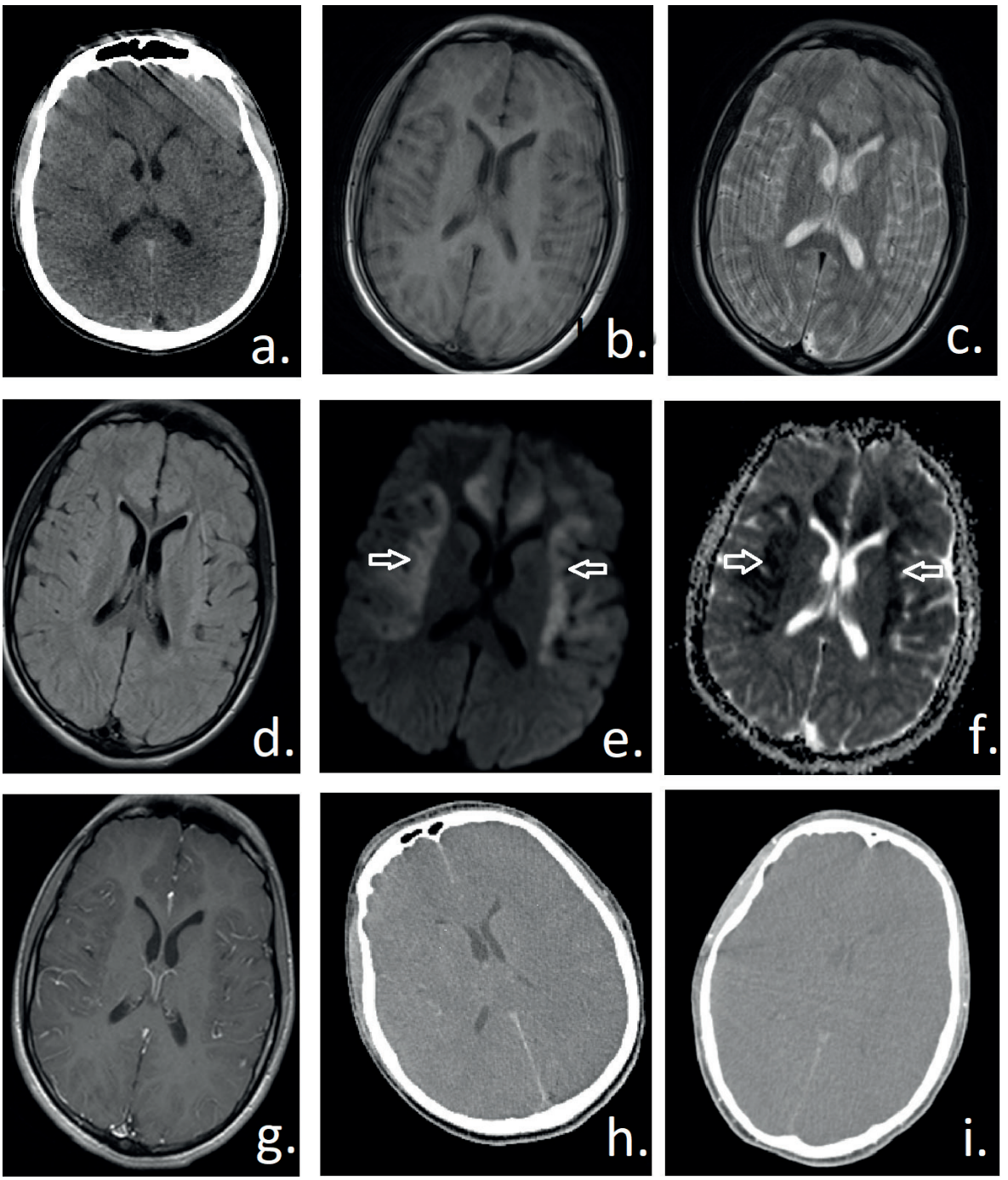

Fig. 1. Radiologic features with severe brain involvement.

Axial non-enhanced brain CT (a), axial brain MR images T1W (b), T2W (c), FLAIR (d), post contrast T1W (g) images show no significant abnormality. In diffusion-weighted images (e) and ADC (f), significant diffusion restriction is seen in frontotemporoparietal cortical areas bilaterally (arrows), representing cytotoxic edema. 16 hours later, a second axial non-enhanced brain CT (h) was obtained and cortical sulci effacement was seen, representing brain edema. A third post-contrast brain CT (i) was obtained 14 hours after the second brain CT and significant brain edema with loss of brain perfusion is seen.

enhanced viral replication or over reactive innate immune responses, viral infections can spread to all CNS regions. Following the activation of glial cells by SARS-CoV-2 viruses, various inflammatory chemokines and cytokines are produced. Increased inflammatory infiltrates can exacerbate neuroinflammation and cause neuronal damage.[14] Molecular mimicry is a third proposed mechanism for encephalitis as a COVID-19 complication.[10] The increase in the host antibodies and lymphocytes occurs in response to infection with the SARS-CoV-2 virus. Although these immune molecules are expected to be specific for antigens of the SARS-CoV-2 virus, some of them are cross-reactive and attack self-antigens.[10] Although virus isolation is essential for a definite diagnosis of viral encephalitis, it is problematic with COVID-19 because SARS-CoV-2 transmission is transitory and the CSF titer may be exceedingly low and it may not be positive in some circumstances.[5,7] The neurological symptoms are seen in severely affected patients.[15] Our patient did not have severe respiratory symptoms at the beginning; he had neurological symptoms with dizziness, headache, and finally loss of consciousness. He had normal laboratory values except for the positive viral PCR. Rapid deterioration might be due to the former SARS-CoV-2 which he had five months ago. The adaptive immune system might be activated, resulting in immunological memory stimulation and resulted in extensive damage in the CNS.

Imaging in viral encephalitis shows focal or diffuse altered cerebral signal intensity, cerebral edema, diffusion restriction, hemorrhages, necrosis, and enhancement.[16] With the invasion of viruses, signal changes in DWI are divided into the acute stage, which constitutes congestion, perivascular cell infiltration, and thrombus formation; late acute and early subacute stages that constitute vasculitis. Perivascular cell infiltration decreases, leading to a decrease in the severity of diffusion restriction at this stage.[17] We had detected significant diffusion restriction at the frontotemporoparietal cortical areas bilaterally representing cytotoxic edema in diffusion-weighted images supporting acute encephalitis. Our patient did not have a relapsing-remitting or progressive course of neurologic symptoms after the first COVID-19 infection. He had an acute onset of neurologic symptoms when he was re-infected. Our patient's symptoms were devastating within seven days. We accept neurological involvement as a para-infectious phenomenon with acute symptoms and MRI findings.

Treatment of SARS-CoV-2 is largely supportive.[5] COVID-19 related inflammatory CNS diseases such as encephalitis were successfully treated with a combination of intravenous immunoglobulin and corticosteroids, of these patients 11 of the 12 had recovered.[18] Plasmapheresis was also demonstrated to be beneficial in a case series of six severely ill COVID-19 encephalitis, with five of them recovering enough to be discharged from the ICU to a regular ward after plasmapheresis.[19] We preferred IVIG and plasmapheresis. We did not add high dose corticosteroids to the treatment because we could not rule out any other infective causative agents.

SARS-CoV-2 can invade the nervous system. Encephalitis may be devastating. Brain MRI may show early findings in the diffusion-weighted series. The other series of MRIs may be normal. We recommend MRI with diffusion-weighted images early in the neurologic disturbance to rule out lesions causing cytotoxic edema. Despite proper timing of antiviral and other supportive treatments for CNS involvement, the clinical outcome may be poor. More cases are needed to understand the neurologic involvement with SARS-CoV-2.

Author contribution

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

Conflict of interest

The authors declare that there is no conflict of interest.

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Case Report

Steroid-resistant peripheral neuropathy in a child: a rare finding in immunoglobulin a vasculitis

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ABSTRACT

Background. Immunoglobulin A vasculitis (IgAV; Henoch-Schönlein purpura) is the most common vasculitis of childhood, affecting the small vessels with systemic involvement, especially the skin, joints, gastrointestinal system and kidneys. Peripheral neuropathy is very rare. Herein, we present a patient who was diagnosed as IgAV and developed refractory peripheral neuropathy in the course of disease.

Case. An 11-year-old boy was admitted to our clinic with pain and swelling in both ankles and symmetric palpable purpura extending from the knees to the dorsum of his feet. IgAV diagnosis was established and outpatient follow-up was started. On the 18th day of follow-up, he was admitted with widespread palpable purpura, myalgia and edema in the lower extremity, abdominal pain and left scrotal swelling. Intravenous prednisolone 2 mg/kg/day was started, all his symptoms improved and edema was resolved, but on the third day of the prednisolone therapy, the patient suffered from numbness in the left foot. Electromyoneurography showed moderate to severe axonal degeneration of the left tibial nerve. The symptoms of patient didn't improve with bolus methylprednisolone and intravenous immunoglobulin therapy. All of the patient's neurological complaints and signs regressed significantly within one week after bolus cyclophosphamide therapy. His oral prednisolone was gradually tapered and stopped at the end of the third month. After a follow-up period of six months, the patient had no complaints.

Conclusion. Peripheral neuropathy is a rare complication of IgAV and occasionally it could be severe. Cyclophosphamide therapy should be kept in mind in patients with refractory neuropathy due to IgAV.

Key words: IgA vasculitis, cyclophosphamide, pediatric patient, peripheral neuropathy, steroid.

Immunoglobulin A vasculitis (IgA vasculitis [IgAV]; formerly known as Henoch Schönlein purpura [HSP]) is one of the most common systemic vasculitic diseases in childhood. IgAV is an immune-mediated leukocytoclastic small vessel vasculitis characterized by non-thrombocytopenic, palpabla purpura on the lower extremities. It is a multisystem disease that typically affects the skin, joints, gastrointestinal tract and kidneys.[1] Nervous system involvement is uncommon. Headaches, seizures, visual changes and reduced conscious levels are the most frequent neurologic symptoms. Peripheral nervous system dysfunction has been reported more rarely.[2] Here we report an 11-year-old boy who was diagnosed as IgAV and developed axonal degeneration of the left tibial nerve during follow-up.

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Case Report

An 11-year-old boy was admitted to our clinic with pain and swelling in both his ankles as well as rashes. His past and family history

were not remarkable. His blood pressure was normal. Physical examination revealed edema and tenderness in both ankles and symmetric palpable purpura extending from the knees to the dorsum of his feet. Laboratory findings were as follows: hemoglobin 13 g/dl, white blood cell count 13,000/mm^3, platelet count 251,000/mm^3, erythrocyte sedimentation rate 13 mm/hour, C-reactive protein 12.5 mg/L, and ASO 467 IU/ml. A throat culture was negative for group β-hemolytic *Streptococcus*. Urinalysis, blood biochemistry, serum immunoglobulin levels, C3 and C4 levels were normal. An investigation of hepatitis and viral markers, antinuclear antibody, anti-double stranded DNA and antineutrophil cytoplasmic antibody (ANCA) were all negative.

IgAV diagnosis was established according to European League Against Rheumatism (EULAR)/Paediatric Rheumatology International Trials Organisation (PRINTO)/ Paediatric Rheumatology European Society (PRES) criteria with palpable purpura and arthritis.[3] A skin biopsy was performed; it showed leukocytoclastic vasculitis, with IgA deposition. Bed rest and non-steroidal anti-inflammatory treatment was recommended and out patient follow-up was started. On the 18th day of follow-up, he was admitted with widespread palpable purpura, myalgia and edema in the lower extremity, abdominal pain and left scrotal swelling. The patient was hospitalized and intravenous prednisolone 2 mg/kg/day was started due to scrotal and abdominal involvement. All symptoms improved and edema was resolved, but on the 3rd day of the prednisolone therapy, the patient suffered from numbness in the left foot. Neurological examination revealed hypoesthesia in the soles of the left foot and the thumb. Deep tendon reflexes were normal and he had no motor abnormalities. Electromyoneurography (EMNG) was performed; it showed moderate to severe axonal degeneration of the left tibial nerve. Bolus methylprednisolone (30 mg/kg/day) was given for 3 consecutive days, followed by oral prednisolone, but the hypoesthesia in his left foot continued. Although intravenous immunoglobulin (IVIG) 1 g/kg/day for 2 consecutive days was given, the patient's symptoms didn't improve. Cyclophosphamide bolus was given (500 mg/m^2) seven days after IVIG treatment. All of the patient's neurological complaints and signs regressed within one week and he was discharged. His oral prednisolone was gradually tapered and stopped at the end of the third month. After a follow-up period of 1 year, the patient had no complaints. Control EMNG was not performed due to improvement of clinical findings after treatment. The patient had no history to suggest familial Mediterranean fever (FMF) and *MEFV* gene analysis was found to be normal.

A written informed consent was obtained from the patient.

Discussion

Immunoglobulin A vasculitis is the most common systemic vasculitis of childhood. The diagnosis of IgAV is based on clinical criteria and histopathological findings.[4] At the first admission, our patient was diagnosed as IgAV with typical palpable purpura and arthritis according to the EULAR/PRINTO/ PRES classification criteria which were validated specifically for childhood-onset disease.[3] Approximately 3 weeks later, while he was hospitalized on steroid treatment for gastrointestinal and scrotal involvement, peripheral neuropathy developed which is a finding that is also very rarely reported in IgAV.[5] Other systemic vasculitis should be considered in differential diagnosis of a patient with purpura and neuropathy. Skin biopsy findings are also important to exclude other forms of vasculitis such as ANCA-associated vasculitis.[4] Our patient's skin biopsy revealed in leukocytoclastic vasculitis, with IgA deposition. Moreover, negative ANCA tests, absence of renal involvement, no relapse during follow-up after discontinuation of steroid treatment all ruled out especially the diagnosis of microscopic polyangiitis.

Belman et al.[5] reported that nervous system involvement in IgAV is usually underestimated. Garzoni et al.[2] reviewed nervous system dysfunction in IgAV and showed that peroneal neuropathy, peripheral facial palsy, Guillain–Barre´ syndrome, brachial plexopathy, posterior tibial nerve neuropathy, femoral neuropathy, ulnar neuropathy and mononeuritis multiplex were reported as cranial or peripheral neuropathy conditions. It has been suggested that deposition of circulating immune complexes containing IgA on myelin sheaths could cause the peripheral demyelination in IgAV.[6] Furthermore, systemic vasculitis may cause infiltration of the vasa nervorum or the epineural arteries by inflammatory cells resulting in ischemia and thrombosis of the peripheral nerves. However, sometimes mechanical compression of the nerve because of local edema and joint effusion may cause peripheral neuropathy.[7] Although our patient's edema was resolved with steroid treatment, his neurological complaints continued. Inflammatory infiltration of small vessels and ischemia probably resulted in axonal degeneration of the left tibial nerve of our patient.

There is no a clear treatment recommendation for peripheral neurological involvement in IgAV. Patients were reported to improve following bolus methylprednisolone administration and IVIG treatment should be given in IgAV complicated by acute inflammatory neuropathy like Guillain-Barre syndrome.[8,9] Our patient's clinical course didn't get better after bolus corticosteroid treatment and IVIG was administered. Therefore, for the possibility of a permanent sequelae in our patient we gave a single dose of bolus cyclophosphamide treatment and the symptoms began to improve and disappeared within one week.

Although peripheral neuropathy is a rare complication of IgAV, it may have a resistant course. Cyclophosphamide therapy should be kept in mind in patients who don't respond to intensive steroid therapy and IVIG.

Author contribution

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

Conflict of interest

The authors declare that there is no conflict of interest.

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Case Report

Primary renal aspergillosis in a newborn: a case report and review of the literature on children

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ABSTRACT

Background. Primary renal aspergillosis is uncommon and mainly affects people with immune system impairment and/or genitourinary disease.

Case. We report the case of a male newborn with Down syndrome and congenital heart disease, who underwent surgery for anorectal malformation and presented persistent fever and impaired kidney function secondary to kidney abscesses due to *Aspergillus*. The patient responded favorably to antifungal treatment and percutaneous drainage but died following heart surgery.

Conclusions. To the best of our knowledge, only seven cases of renal aspergillosis have been reported in children worldwide, this being the second in a newborn. *Aspergillus* species must be considered among the fungal etiological agents of genitourinary tract infections in order to establish adequate antifungal treatment to achieve therapeutic success against filamentous fungi.

Key words: *Aspergillus*, aspergillosis, kidney, child, pediatrics.

Aspergillosis is a major fungal infection in severely immunocompromised adults and children.[1] The estimated annual incidence of aspergillosis is 437 cases/100,000 (0.4%) in high-risk immunocompromised children, including those undergoing allogeneic hematopoietic stem cell transplantation, and children with hematological malignancies such as acute myeloid leukemia or primary immunodeficiencies including chronic granulomatous disease, among others.[1,2]

Aspergillosis is rare in non-immunocompromised children, including neonates and premature infants.[3] It is unusual for *Aspergillus* species to primarily affect extrapulmonary organs, such as the genitourinary tract[4], being secondary to invasive and/or disseminated forms in most cases.[5]

We present a case of primary renal aspergillosis in a newborn without comorbidity of the genitourinary tract and apparently not immunocompromised.

Case Report

A male newborn diagnosed with Down syndrome, congenital heart disease (ventricular septal defect and patent ductus arteriosus), and anorectal malformation without fistula was referred to our institution for surgical management. After surgery for the anorectal malformation, the infant presented severe sepsis, observing the presence of *Acinetobacter iwofii* and *Escherichia coli* in blood cultures. The patient progressed with clinical deterioration and hemodynamic instability, requiring the use of vasopressors, ventilatory support, and broad-spectrum antibiotic treatment with meropenem

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and vancomycin. Although subsequent blood cultures were negative, there was persistence of fever, altered inflammatory markers (C-reactive protein: 74 mg/dl and procalcitonin: 0.7 ng/ml), and an increase in serum creatinine to 0.63 mg/dl. On suspicion of invasive fungal infection, fluconazole was administered for 10 days, and then amphotericin B was continued. Complementary studies including echocardiography, fundoscopy, abdominal ultrasound, and computed tomography urography were performed. The results of echocardiography and fundoscopy showed no evidence of vegetations or signs of fungal endophthalmitis, respectively. Abdominal ultrasound showed altered bilateral renal echogenicity, whereas the tomography showed obstruction of the pelvis and left renal calyces due to renal abscesses (Fig. 1). A left nephrostomy was performed and 15 ml of purulent material was obtained from the drainage of the renal abscesses. Culture with Sabouraud agar showed growth of thick septate hyphae with dichotomous branching compatible with *Aspergillus* spp. Amphotericin B treatment was suspended and voriconazole treatment was started at a dose of 9 mg/kg/dose every 12 hours, with favorable clinical, laboratory, and imaging evolution of the patient (Fig. 2). Urine cultures were always negative; however, urinary sediment before initiating voriconazole treatment showed 50 leukocytes per field, positive leukocyte esterase, and 30 red blood cells per field. These findings normalized 2 weeks after treatment (3 leukocytes per field, negative leukocyte esterase, and 5 red blood cells per field). Additionally, venereal disease research laboratory studies and tests for the diagnosis of human immunodeficiency virus (HIV) and hepatitis B virus were performed, all with negative results. Immunoglobulin levels were within normal limits: IgA 50 mg/dl (value normal: 0-83), IgG 353 mg/dl (value normal: 232-1411), IgM 48 mg/dl (value normal:

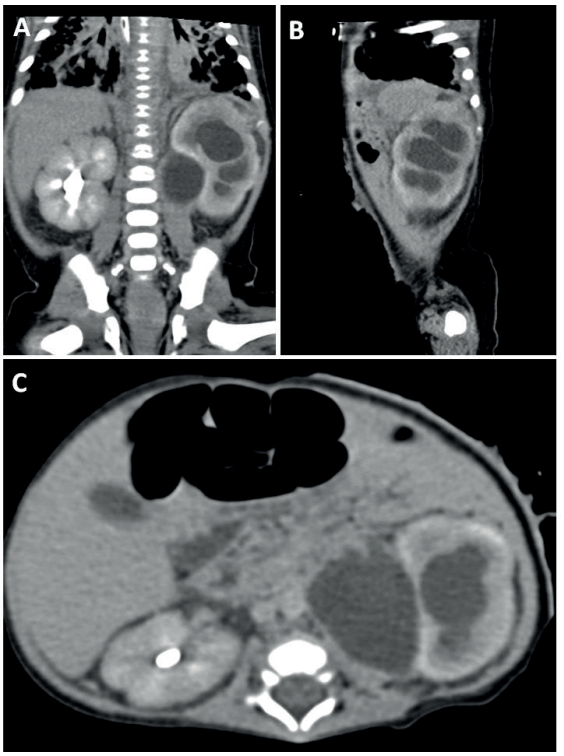

Fig. 1. Computed tomography at the time of diagnosis suggestive of stenosis of the left pyeloureteral junction, which is associated with the presence of renal abscesses.

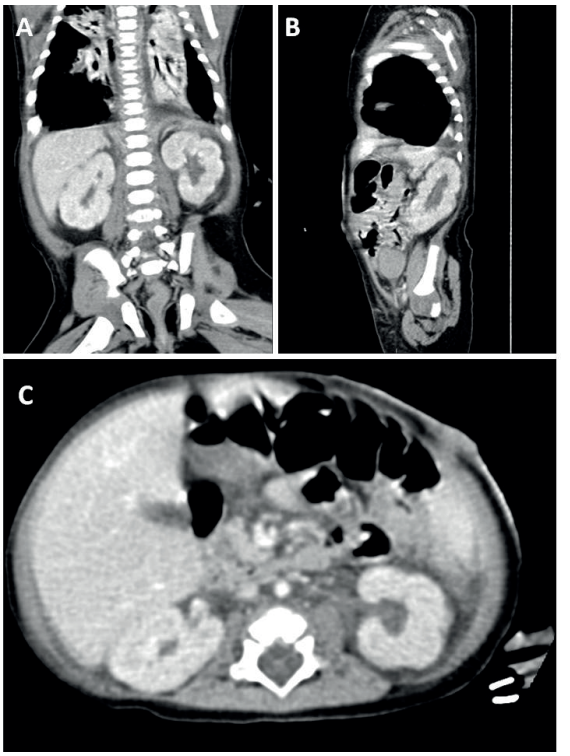

Fig. 2. Computed tomography after drainage of kidney abscesses and specific antifungal therapy, showing evidence of resolution of the findings described.

0-145). The patient completed three months of antifungal treatment and underwent surgery for congenital heart disease using the aorto-pulmonary banding technique plus patent ductus arteriosus closure. The evolution of the patient following surgery was unfavorable, with the presentation of respiratory and hemodynamic deterioration, followed by death.

The case report was approved by the Institutional Review Board of the Instituto Nacional de Salud del Niño San Borja (INSN-SB), under the institutional code PI-257, and that participation involved informed consent.

Discussion

Infection of the genitourinary tract by *Aspergillus* species is an uncommon event that can occur by hematogenous spread in immunosuppressed patients, or by ascending infection from the lower urinary tract.[6] The latter is a rare clinical condition (primary renal aspergillosis), described mainly in adult patients with certain predisposing factors, such as kidney transplantation, HIV infection, diabetes mellitus, obstructive pathology of the genitourinary tract, chronic corticosteroid therapy, use of intravenous drugs, prolonged use of broad-spectrum antibiotics, or the use of invasive devices.[5-9] Including our case, only 7 cases of primary renal aspergillosis have been reported in children worldwide.[9-12] The characteristics of these children are described in Table I. All these cases were male patients, with half being diagnosed with immunodeficiency. Our patient had undergone surgery for anorectal malformation as a comorbid condition similar to the case reported by Martinez-Pajares et al.[9] in a newborn. However, the patient also suffered from bladder exstrophy and epispadias, anatomical alterations of the genitourinary tract that could increase the risk of infection.[13] In addition, Down syndrome, present in our case, has been associated with defects of the immune system such as T and B cell lymphopenia, a decrease of naive lymphocytes, impaired mitogen-induced T cell proliferation, and defects of neutrophil chemotaxis.[14]

The presence of persistent fever and a decrease in the glomerular filtration rate are the main manifestations described in these cases.[15] Urological imaging studies, such as ultrasound or tomography, are important to determine the presence of fungal kidney abscesses or other conditions of the genitourinary tract, which allow establishing an adequate diagnostic and therapeutic plan.[9] Likewise, microbiological identification of the *Aspergillus* species, as well as the performance of antifungal susceptibility tests are crucial to provide targeted antifungal treatment, since antifungal susceptibility patterns differ among the different species.[16,17]

In our report, the culture of the renal abscess was essential to establish the etiological diagnosis. However, it was not possible to perform the antifungal susceptibility test because it was not available at the institution at the time of the present case. In addition to culture, there are

Table I. Characteristics of the seven cases of renal aspergillosis in children reported in the literature.

N° / Year of publication	Age	Sex	Predisposing factor	Location	Aspergilloma
1 / 1977 [10]	6 years	Male	Chronic granulomatous disease	Unilateral	No
2 / 1984 [10]	6 years	Male	Acute lymphoid leukemia	Unilateral	No
3 / 1985 [10]	5 years	Male	Acute lymphoid leukemia	Unilateral	No
4 / 2009 [9]	Newborn	Male	Surgery for anorectal malformation, bladder exstrophy and epispadias	Bilateral	Yes
5 / 2014 [12]	2 years	Male	-	Bilateral	Yes
6 / 2015 [11]	6 years	Male	Burkitt lymphoma	Unilateral	Yes
7 / Present case	Newborn	Male	Surgery for anorectal malformation	Unilateral	Yes

other diagnostic tests for aspergillosis that are based on the detection of components of the fungal cell wall, such as galactomannan (GM). The GM test is primarily useful for the diagnosis of invasive aspergillosis (angioinvasive form) in neutropenic patients.[3,4] However, its sensitivity in non-neutropenic patients is low due to the ability to clear the fungal mannan components mediated by mannosyl macrophage receptors in the bloodstream.[18] Park et al. reported that the serum GM test has a low sensitivity in patients with pulmonary aspergilloma without vascular compromise.[19] Additionally, in children, the presence of false-positive results could be observed due to a cross reaction with lipoteichoic acid from *Bifidobacterium bifidum pennsylvanicum*, a bacterium that is part of the intestinal microbiota, especially in neonates.[3]

Regarding treatment, the guidelines suggest that for the management of renal aspergillosis, as well as in other extrapulmonary aspergillosis, it is important to consider adjuvant surgery (such as ureteral stenting, percutaneous nephrostomy, and even nephrectomy) in addition to antifungal therapy.[17,20] The antifungal therapy of choice in renal aspergillosis is voriconazole. However, although it is used in different age groups in some centers, the use of voriconazole in children under 2 years of age is not authorized and therapeutic drug monitoring should be used to guarantee adequate exposure to the drug.[21] Likewise, if creatinine clearance levels are less than 50 ml/min, intravenous administration should be avoided, due to the possible accumulation of the solubilizing sulfobutyl ether cyclodextrin, which would further affect kidney function.[15] In these cases, one could choose to administer voriconazole orally or switch to another antifungal with a spectrum against *Aspergillus*, such as itraconazole or liposomal amphotericin B.[17,20] In some cases, the use of combined antifungal therapy has even been described.[11] In our case, despite the patient being under 2 years of age and having no results for the antifungal susceptibility test or *Aspergillus* speciation, a favorable response was obtained with voriconazole, observing a decrease in fever, imaging resolution in relation to the presence of kidney abscesses and normalization of urinary sediment (mainly leukocyturia and hematuria), respectively (urine culture was always negative). However, three months after completing the voriconazole treatment, the patient underwent cardiac surgery and died after progressing unfavorably.

In conclusion, although there are few reported cases of primary renal aspergillosis in children, for the diagnosis of primary fungal infections it is important to consider not only yeast fungi, such as Candida species, but also filamentous fungi, such as *Aspergillus*, especially in immunosuppressed children and in those with intra-abdominal malformations (mainly of the genitourinary tract). In addition, we highlight the importance of drainage as a diagnostic and therapeutic method that allows identification of the fungus and the reduction of fungal load. In turn, identification allows establishing a timely and appropriate treatment to achieve therapeutic success.

Ethical approval

The case report was approved by the Institutional Review Board of the Instituto Nacional de Salud del Niño San Borja (INSN-SB), under the institutional code PI-257, and that participation involved informed consent.

Author contribution

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

Autosomal recessive hypophosphatemic rickets type 2; a novel mutation in the ENPP1 gene

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ABSTRACT

Background. Hypophosphatemic rickets (HR) is a rare disease caused by several genetic mutations in factors that cause an increase in fibroblast growth factor 23 (FGF23), and renal phosphate transporters. ENPP1 (ectonucleotide pyrophosphatase / phosphodiesterase 1) mutations cause autosomal recessive inheritance hypophosphatemic rickets type 2.

Case. In our study, we present a novel mutation in the ENPP1 gene detected in 4 siblings in a single family.

Conclusion. Our findings can be applied to further understand molecular pathogenesis and to establish a correlation between genotype and phenotype for HR.

Key words: hypophosphatemic rickets, ENPP1 gene, novel mutation.

Hypophosphatemic rickets (HR) is a disorder marked by renal phosphate wasting. It is caused by several genetic mutations in renal phosphate transporters and in pathways leading to increased fibroblast growth factor 23 (FGF23) signaling or secretion. The most common inherited form of HR is X-linked HR (XLH; OMIM: # 307800).[1] HR is a rare disease with a prevalence of 3.9 per 100,000 live births.[2] Hypophosphatemia occurs with kidney phosphate loss, which causes bone mineralization defects such as rickets and osteomalacia.

FGF23, the most important phosphaturic agent, is produced by osteocytes and osteoblasts.[3] FGF23 inhibits renal phosphate reabsorption by decreasing the expression of phosphate transporters in proximal renal tubules.[4] It also inhibits 25-OH vitamin D-1-hydroxylase and activates 25- hydroxyvitamin D-24-hydroxylase. This results in reduced 1,25 (OH) 2D levels and increases 24,25-dihydroxyvitamin D (24,25 (OH) 2D) levels. The most common cause of HR is an X-linked PHEX gene mutation. HR is also caused by autosomal dominant FGF23 gene mutations, DMP1 mutations, which give rise to autosomal recessive HR type 1, and ENPP1 mutations, resulting in autosomal recessive HR type 2.[3]

The majority of ENPP1 mutations cause either generalized arterial calcification of infancy (GACI). Myointimal proliferation is a component of GACI.[5] ENPP1 mutations trigger HR through a pathway that is yet to be discovered.[6]

ENPP1 plays an important role bone mineralization, soft tissue calcification, and regulation of pyrophosphate levels by producing inorganic pyrophosphate (PPi). Mineral accumulation in bones is determined by the ratio of phosphate to PPi regulated by ENPP1. When ENPP1 is mutated, it causes hypophosphatemic rickets due to high FGF23 levels. However, this mechanism is not fully understood.[5,6]

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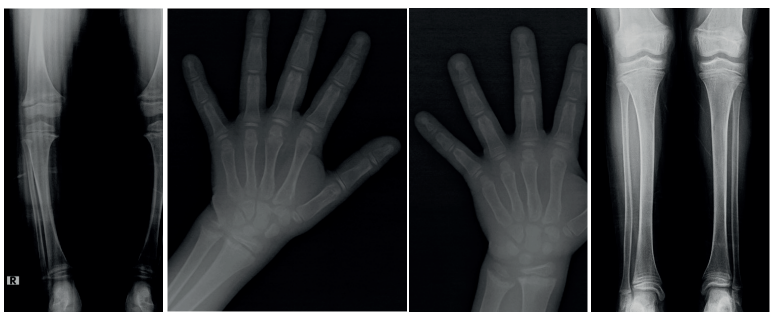
This paper describes a novel mutation in the ENPP1 gene that was observed in four siblings diagnosed with autosomal recessive HR type 2.

Cases Report

Three siblings (P2,P3,P4) had previously been admitted to another hospital due to bowing of legs and short stature. They had been treated irregularly for HR, and had not received treatment for the previous two years. The other sibling (P1) had bowing of legs and short stature but had not been previously diagnosed. This patient was evaluated and diagnosed with HR.

The age, gender and anthropometric measurements of the patients are given in Table I. The patients were born full term and had normal birth weights. After they started walking, bowing and short stature were noticed in the legs. All patients had disproportionate short stature. They received vitamin D prophylaxis until the age of one and HR treatment (calcitriol and phosphorus) irregularly. Their parents were cousins and there were no similar diseases observed in any other members of the family. Maternal height was 160.4 cm (-0.46 SDS), paternal height was 165 cm (-1.82 SDS). The family had a total of five children. Three of

Table I. Physical examination and test results of the patients.

Patient(P)	P1	P2	P3	P4
Gender	Female	Female	Male	Female
Age(years)	5.0	9.08	10.8	12.3
Height (SDS)	-3.08	-3.17	-3.73	-5.19
Weight (SDS)	-1.5	-1.12	-2.65	-2.67
Upper-to-lower body segment ratio (SDS)	1.4 (> +2)	1.3 (> +2)	1.1 (+2)	1.1 (+1)
Physical examination findings	Short stature Genu varum	Short stature Genu varum	Short stature Genu varum	Short stature Genu valgum Hypoplastic teeth and caries
Radiography (First application)				
Renal US	Normal	Urinary nephrocalcinosis	Urinary nephrocalcinosis	Normal
Renal Doppler US	Normal	Normal	Normal	Normal
Carotid Doppler US	Normal	Normal	Normal	Normal
Echocardiography	Normal	Minimal Tricuspid valve insufficiency, Minimal Mitral valve insufficiency, Minimal Aortic valve insufficiency	Minimal Aortic Valve Insufficiency	Pulmonary Valve Insufficiency, (Pulmonary Balloon Valvuloplasty performed)

the children were treated irregularly for HR, one child had bowing of legs and had not been diagnosed, and the other child was completely normal (Fig. 1). There were no findings in the parents either.

On the first admission (after two years without treatment), calcium (Ca), phosphorus (P), PTH, alkaline phosphatase (ALP), and 25-hydroxy vitamin D (25(OH)D) levels were measured. Tubular phosphate reabsorption (TRP), and urinary calcium and creatinine (U Ca / Cr) ratios were also calculated. The results of these measurements are summarized in Table II. Standard deviation of ALP are given.[7] Renal function was normal in all patients, and tubular dysfunction (except phosphaturia) was absent in all patients. Carotid and Renal Doppler ultrasonography (US) imaging was performed on all patients. On imaging, carotid and renal arteries appeared normal. Patients P2 and P3 had nephrocalcinosis and there were valve anomalies in the echocardiogram results of patients P2, P3, and P4. Patient P4 underwent pulmonary balloon valvuloplasty due to pulmonary valve insufficiency. There was improvement in valve insufficiency. Existing pathologies are summarized in Table I.

All patients had hypophosphatemia, hyperphosphaturia, elevated ALP levels, mild elevations in PTH levels, normocalcemia, normocalciuria, and rickets (enlargement and irregularity in epiphyseal plates, especially bowing of the bones in leg radiographs). Calcitriol (15 ng/kg/d) and oral phosphate (40 mg/kg/d) treatment was initiated following the diagnosis of HR. Biochemical values

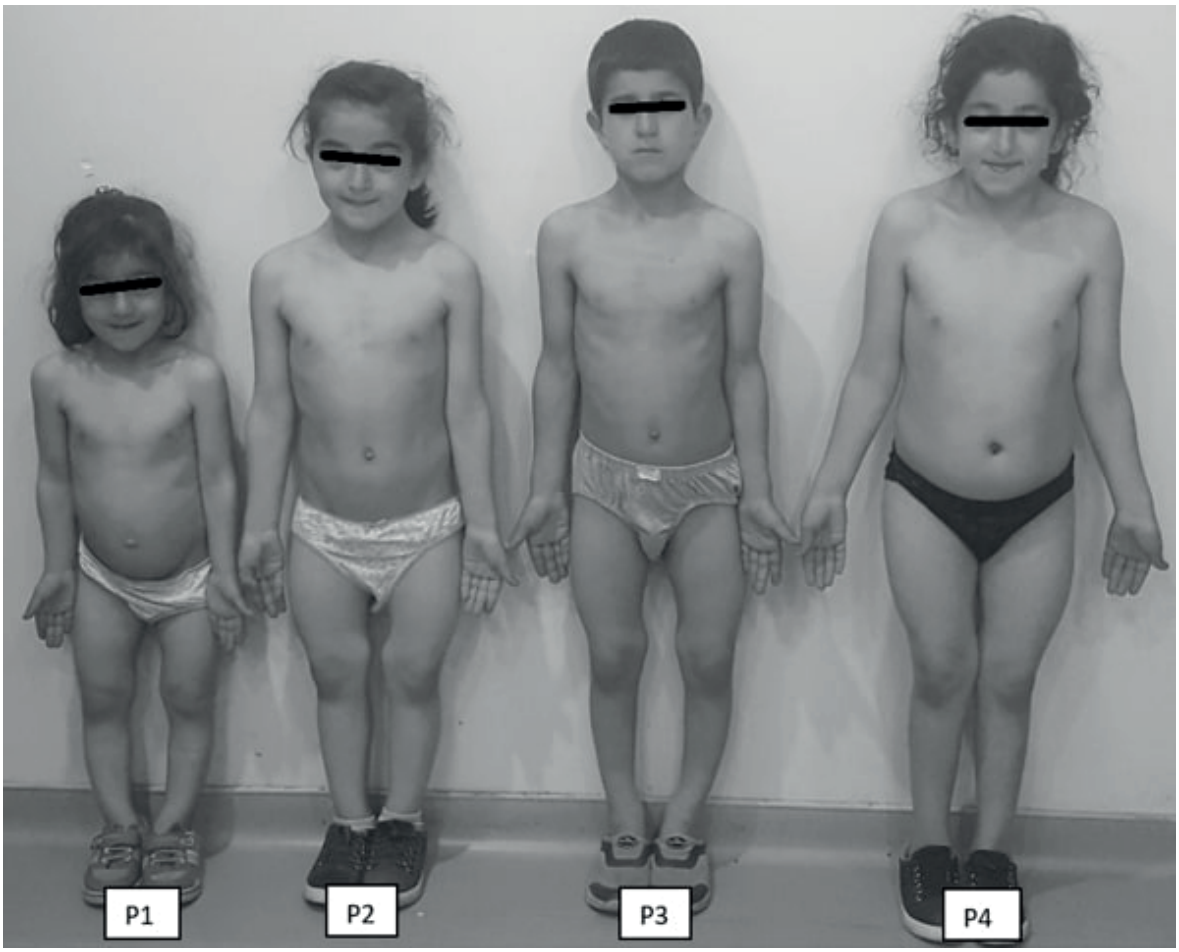


Fig. 1. Phenotype of patients.

Table II. Laboratory results of patients.

Patients	Age (years)	Ca (mg/dL), range (8.4-10)	P (mg/dL), range (3.4-6.2)	ALP (U/L), (-2SD , + 2SD)	PTH (pg/mL) range (15-68.3)	25 OHD (ng/mL), range (20-60)	TPR	U Ca / Cr
P1	5.0	9.6	2.5	562 (294-483)	136.4	31	%76	<0.01
P2	9.08	9.3	2.5	881 (326-769)	89.7	24	%75	<0.1
P3	10.8	9.7	2.4	1015 (400-938)	110.1	30.4	%74	<0.03
P4	12.3	9.6	2.6	960 (204-924)	99	27.8	%76	0.07

Ca: calcium, P: phosphate, ALP: alkaline phosphatase, PTH: parathyroid hormone, 25OHD: 25-hydroxyvitamin D, U Ca / Cr: urine calcium: creatinine ratio, TRP: tubular phosphate reabsorption

of the unaffected sibling and parents were normal. Since the parents were relatives and phenotypically normal, genetic mutations known to cause autosomal recessive HR were studied first. No mutations were detected in the DMP1 genes of the four affected siblings. The ENPP1 gene was examined in only one (P2) of the siblings and a homozygous c.1092-2A> C mutation was found (Fig. 2). Although the mutation detected in the ENPP1 gene did not lead to any change in the protein sequence, it is a splice site mutation that acts at the exon-intron boundary that can cause the formation of a non-functional protein. Four of the in silico algorithms (DANN, EIGEN, FATHMM-MKL and MutationTaster) that are used to predict the effects of mutations classified as pathogenic by the American College of Medical Genetics and Genomics (ACMG) were evaluated as pathogenic. The possibility of a pathogenic effect of the mutation, which has never been shown before in the literature, has been emphasized. Later, in the genetic analysis performed by the parents, heterozygous c.1092-2A> C genetic changes in the ENPP1 gene were detected in both (Fig. 2). Written consent was obtained from the family of the patients.

Genetic Analysis

Genomic DNA was extracted from the patients' peripheral blood lymphocytes using a QIA amp DNA mini kit (Qiagen, 51304, Duseldorf, Germany) according to the manufacturer's protocol and sequenced that comprised all exons and exon-intron junctions of ENPP1 and DMP1 genes on the Illumina-MiniSeq sequencing platform. The ACMG standards and reference to the Varsome, NCBI, ClinVar, and HGMD databases were used to classify the mutations into five categories: pathogenic, likely pathogenic, variants of uncertain significance, likely benign, or benign.

Discussion

Here, we present four cases of autosomal recessive HR type 2 caused by mutations in the ENPP1 gene in individuals from the same family. In the genetic examination of the patients, we found a novel mutation in ENPP1, which was not previously shown in the literature. The frequency of autosomal recessive HR type 2 is extremely rare. In a multi-center study in Turkey involving 166 patients with HR, 75 patients were genetically analyzed and none of these patients had a mutation in the ENPP1 gene.[1] There have not been any previously reported cases of ENPP1 gene mutations in Turkey prior to this case study.

Genetic disorders associated with the ENPP1 gene show phenotypic heterogeneity depending on the type and location of the mutation. GACI and HR result from biallelic mutations and exhibit recessive inheritance patterns. The nuclease domain or phosphodiesterase domain that mediates ENPP1 catalytic activity is affected by most mutations that cause ectopic calcification.[8]

With dominant or recessive inheritance, biallelic or heterozygous ENPP1 gene mutations have been shown to cause Cole's disease (irregular hypopigmentation, cutaneous calcifications, and punctate keratoderma), a

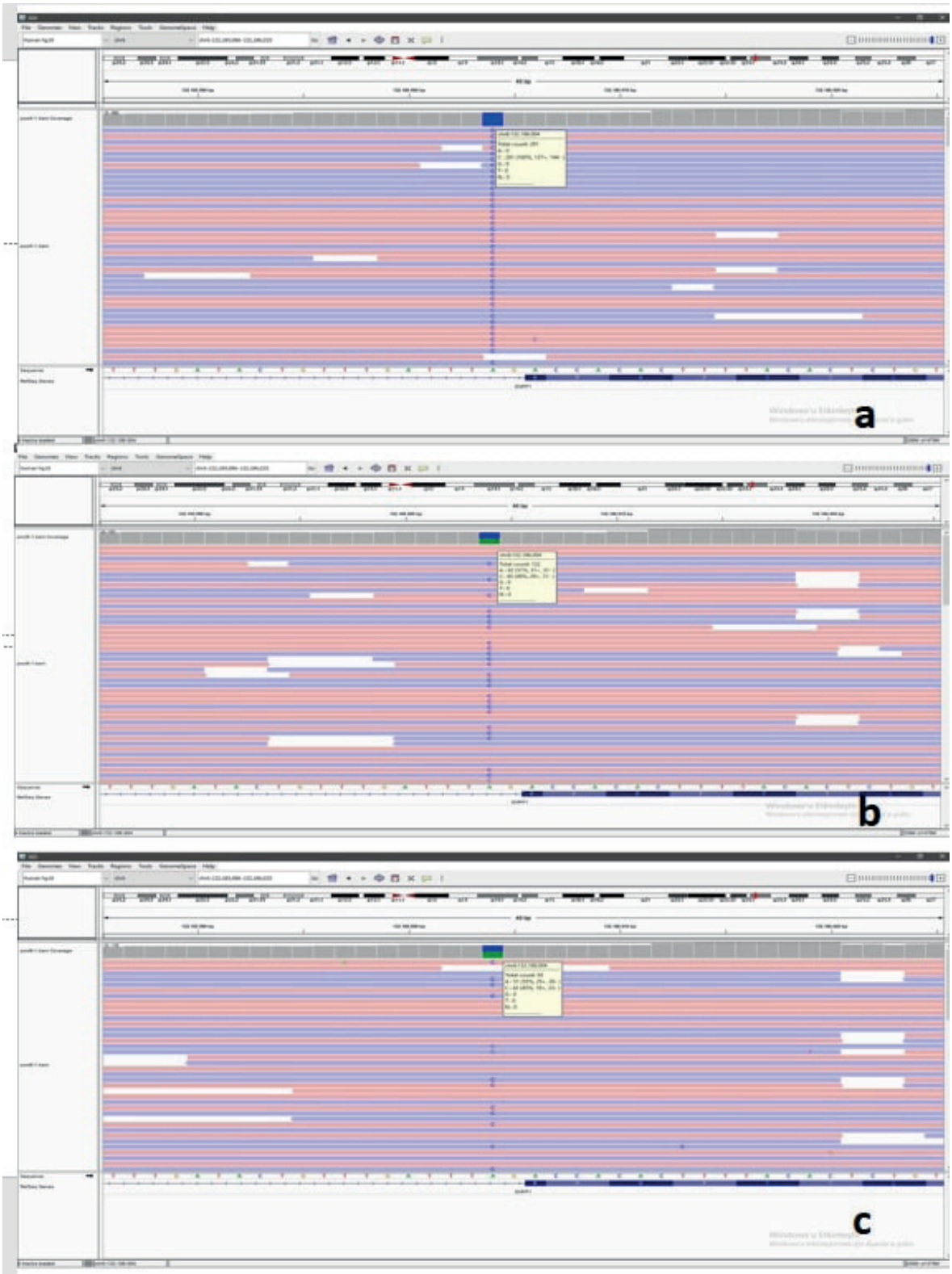


Fig. 2. ENPP1 Gene c.1092-2A> C Homozygous mutation (Patient P2 (a)), ENPP1 Gene c.1092-2A> C Heterozygous mutation (Mother (b) and Father (c)).

rare genodermatosis.[9] Brunod et al.[10] reported that some cases of GACI were associated with hypophosphatemia and even HR. It has been found that hypophosphatemia is associated with a milder phenotype and a better prognosis.[10] Physiopathology is not well understood. It is assumed that hypophosphatemia in GACI may occur through a physiological compensatory mechanism rather than the primary defect.[5] However, patients with mutations in the ENPP1 gene have an autosomal recessive phenotype for HR without any arterial calcification, suggesting a different pathway in the development of the disorder.[10] Mild pulmonary stenosis and thickening of the aortic valves have been observed in HR patients with ENPP1 gene mutations. The resulting changes in the heart are thought to occur due to arterial calcifications. Current studies show that these patients are susceptible to heart valve anomalies.[6] The patients in this study had no evidence of carotid and renal arterial calcification, but the changes observed in heart valves may be a result of arterial calcification. However, we think that this situation should be supported by studies with more patients.

There are two mechanisms that may explain why loss-of-function mutations in ENPP1 result in HR in some patients and GACI in others. The first explanation is that affected individuals exhibit various disease phenotypes based on genetic and environmental factors. This explanation has been proposed because patients showing a mild GACI phenotype accompanied by arterial calcifications, hypophosphatemia and hyperphosphaturia have been identified.[10] In addition, two members of the same family carrying the same homozygous ENPP1 mutation had HR without arterial calcification in one and GACI with hypophosphatemia in the other case. The other explanation is that different mutant alleles are associated with functional differences. There is no evidence for different isoforms of the ENPP1 protein and mutations are spread throughout the gene in both diseases. However, the effect of mutations at the protein level may be different for the two phenotypes.[6]

Although the change we detected in the ENPP1 gene does not lead to any change in the protein sequence, it is a splice site mutation that can affect the exon-intron segment and cause the formation of a non-functional protein.

GACI is an autosomal recessive disease and has a hypermineralizing phenotype. This disease is known to be caused by inactivating mutations in the ENPP1 gene. That both GACI and hypophosphatemic rickets are caused by loss-of-function mutations is most strongly supported by the observation reported in a single family, in which father suffered from and his son suffered from GACI plus hypophosphatemia, although both had the same homozygous ENPP1 mutation in the same family.[6] However, individuals with the same homozygous loss-of-function ENPP1 mutation in our patients had the same clinical findings.

Although the mechanism by which mutations in the ENPP1 gene increase FGF23 levels and cause hypophosphatemic rickets cannot be fully explained, there have been studies showing that loss of function mutations in this gene reduce ENPP1 activity and lead to hypophosphatemic rickets with an increase in FGF23 levels.[6] However, FGF23 levels could not be determined in our cases.

In summary, we uncovered a novel mutation in the ENPP1 gene that occurred in siblings diagnosed with autosomal recessive HR type 2. Our findings can be applied to further understand molecular pathogenesis and to establish a correlation between genotype and phenotype for HR.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: ECB; data collection:ECB, HSA; analysis and interpretation of results: ECB, HSA; draft

manuscript preparation: ECB, HSA. All authors reviewed the results and approved the final version of the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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Case Report

Recurrent painful ophthalmoplegic neuropathy: a report of two new pediatric cases

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ABSTRACT

Background. Recurrent painful ophthalmologic neuropathy (RPON), formerly known as ophthalmoplegic migraine, is characterized by repeated attacks of one or more ocular cranial nerve palsies with an ipsilateral headache. While steroid therapy has been reported to be beneficial for attacks, no clear consensus on prophylactic treatments exists. We present two cases emphasizing the diagnostic significance of the loss of enhancement during the symptom-free period and valproate as a beneficial option in prophylaxis.

Case 1. A 4-year-old girl presented with a one-week right frontal headache, vomiting and photophobia. Neurological examination revealed ptosis, oculomotor nerve paresis, and delay in light reflex in the right eye. Brain magnetic resonance imaging (MRI) revealed a 5.5 mm nodular enhancement in the cisternal part of the 3rd cranial nerve in the right premesencephalic area. The enhancement regressed after a 6-month symptom-free period. While propranolol, topiramate and flunarizine were inefficacious in prophylaxis, the patient responded to valproate prophylaxis and benefited from the administration of steroids for one week during the attacks.

Case 2. A 7-year-old girl presented with a ten-day right-sided, throbbing headache in the frontal region, one-day eye deviation and double vision. Neurological examination revealed inward gaze restriction and ptosis in the ipsilateral eye to the headache. Brain MRI revealed a 4.5 mm, enhancing, nodular lesion in the 3rd cranial nerve lodge in the right perimesencephalic area. Her symptoms regressed in one week with dexamethasone and she received prophylactic propranolol. Neuroimaging findings disappeared after a 3-month symptom-free period. After valproate was added because of a relapse, she did not experience any further attacks.

Conclusions. RPON is an uncommon disease in childhood with unknown etiology. On brain MRI with contrast during the symptom-free period, regression of the enhancement or complete resolution of the lesion are guiding features in the diagnosis. Valproate may have beneficial effects on RPON treatment.

Key words: ophthalmoplegia, cranial nerves, neuropathy, headache, valproate.

Recurrent painful ophthalmoplegic neuropathy (RPON), previously known as ophthalmoplegic migraine, is a rare syndrome characterized by repeating attacks of one or more ocular cranial nerve palsies with an ipsilateral headache, in which secondary causes have been excluded.[1] The first contributions to the literature on this disease date back to the 19th century with Note in 1854 and Gubler in 1860, but Charcot named it "migraine ophthalmology" for the first time in 1890. It was thought to be a migraine variant in the early years when the definition emerged as it is associated with one or more ocular cranial nerve palsies, most often the oculomotor nerve and a migrainous headache. The original International Classification of Headache Disorders (ICHD) classified this disorder as a migraine variant, but the ICHD 2 named this disorder "ophthalmoplegic migraine" and classified it as a cranial neuralgia in 2004. It was reclassified as a cranial neuropathy in the ICHD 3-beta by the International Headache Society in 2013, and the term "RPON" was established.[2] The exact prevalence of RPON, a

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disease in which pediatric patients account for the majority of reported cases, is unknown, but it is estimated to be 0.7 per million.[3] Since RPON is a rare disease, there are few publications in the literature, and here we aimed to present two pediatric patients who were diagnosed with RPON according to the ICHD-3 criteria and emphasize the diagnostic significance of the loss of enhancement or lesion during the symptom free period and discuss valproate as a useful prophylactic alternative.

Case 1

A four-year-old girl presented with a one-week right-sided frontal throbbing headache, vomiting and photophobia which occurred every 1-2 months for the last year. Previous episodes of headache were unrelated to infections and had lasted for 2-3 days with vomiting 10-15 times a day. The patient's medical history apart from headache episodes was unremarkable. There was no family history of neurological disease except for a history of migraines in the patient's aunt. Neurological examination was normal. Brain magnetic resonance imaging (MRI) performed a year ago was normal. After 3 days, the patient presented with ptosis, ophthalmoparesis with impaired adduction and slowed pupillary light reflex in the right eye. Brain MRI revealed 5.5 mm contrast enhanced nodular lesion on the right at the level of the ambient cistern belonging to the cisternal part of the 3rd cranial nerve (Fig.1a). Magnetic resonance angiography (MRA) revealed reduced flow at P1 segment of the right posterior cerebral artery (PCA), moderate narrowing of the P1 segment posteriorly, compared to the left P1 segment, secondary to the lesion originating from the 3rd cranial nerve (Fig.1b). Two days later, the findings had spontaneous regression (normal light reflex, partial regression in the others). Propranolol prophylaxis was started and the patient was followed up with the prediagnoses of RPON and trigeminal nerve schwannoma. The patient had many further attacks and prophylaxis of propranolol, topiramate and flunarizine were used, respectively, at the maximum effective doses. Although headache was observed in all of these attacks, ophthalmoparesis accompanied only the first one. This was attributed to starting steroid therapy as soon as the headache occurred, without waiting for the development of ophthalmoparesis. After a 5 month attack-free period -under flunarizine, the patient presented with a similar attack. Valproate was added to flunarizine. After 6 months without attacks under dual therapy, the valproate dose was gradually decreased. In the 41st month, when the last attack was 6 months ago, the patient was asymptomatic, the size of the lesion was 4 mm and there was no contrast enhancement on brain MRI (Fig.1c). Repeated MR angiography was normal (Fig.1d). However, the patient had a relapse at the 42nd month, and the dose of valproate was increased again. The fact that the lesion did not show progression made the diagnosis of trigeminal nerve schwannoma unlikely and loss of enhancement in the symptom free period was thought to be in favor of the diagnosis of RPON. Headache and ophthalmoplegia episodes regressed with methylprednisolone therapy used for 5-10 days without any sequelae. The only time the patient was not given steroids was on the last attack because she presented after the attack was over. The patient is currently in the 54th month of the follow-up and has been symptom free for 12months.

Case 2

A previously healthy seven-year-old girl presented with a right-sided frontal headache persisting for the last 10 days, with the addition of limited inward ocular movement and double vision for the last day. The patient described previous similar headache episodes that occurred almost every day for 3-4 months, lasting for 1-4 hours. The attacks restricting daily activity, became more pronounced with physical activity, benefited from analgesics, and were not accompanied by photophobia, phonophobia, nausea, vomiting and ocular symptoms. The patient presented with a similar

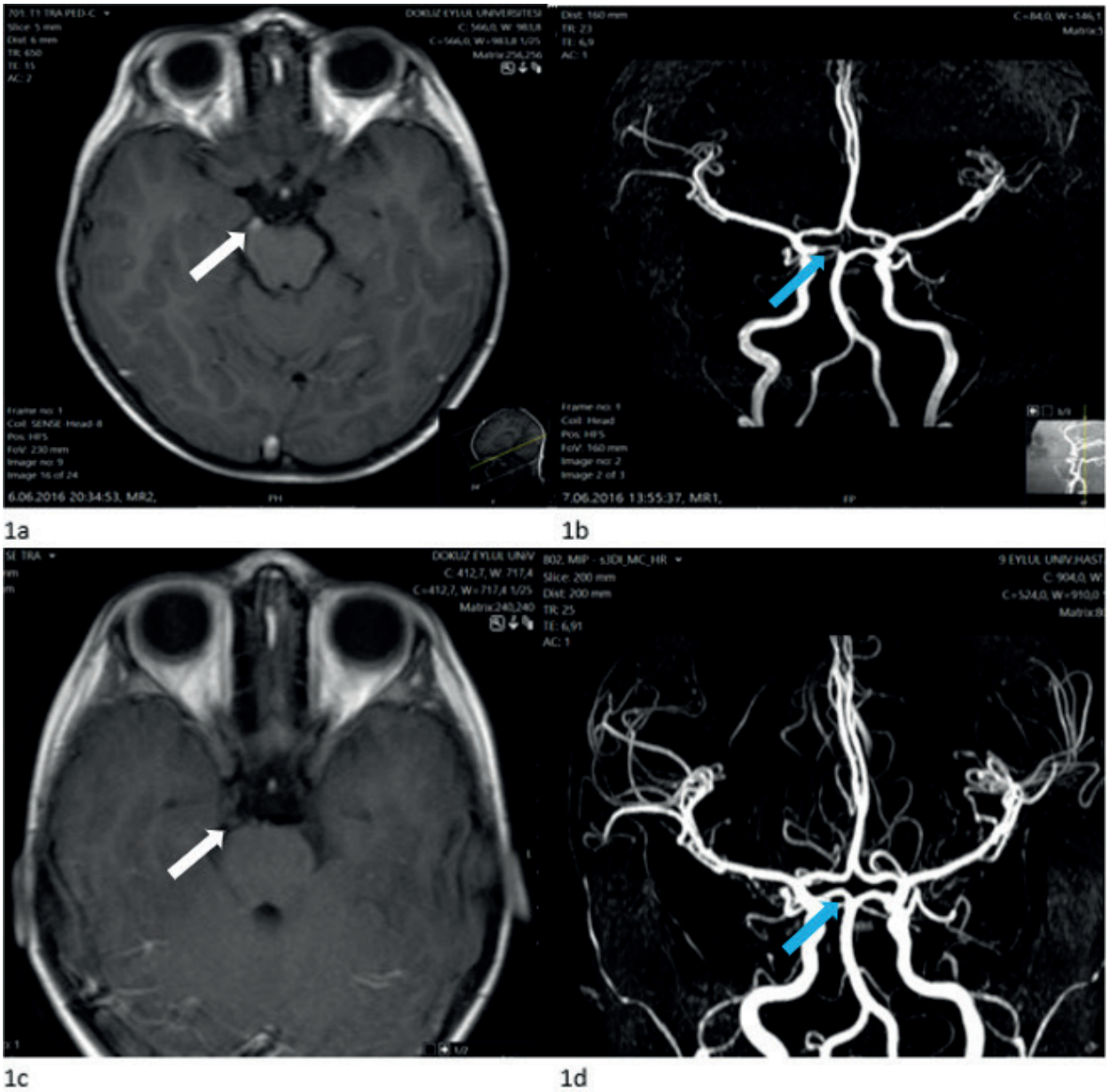
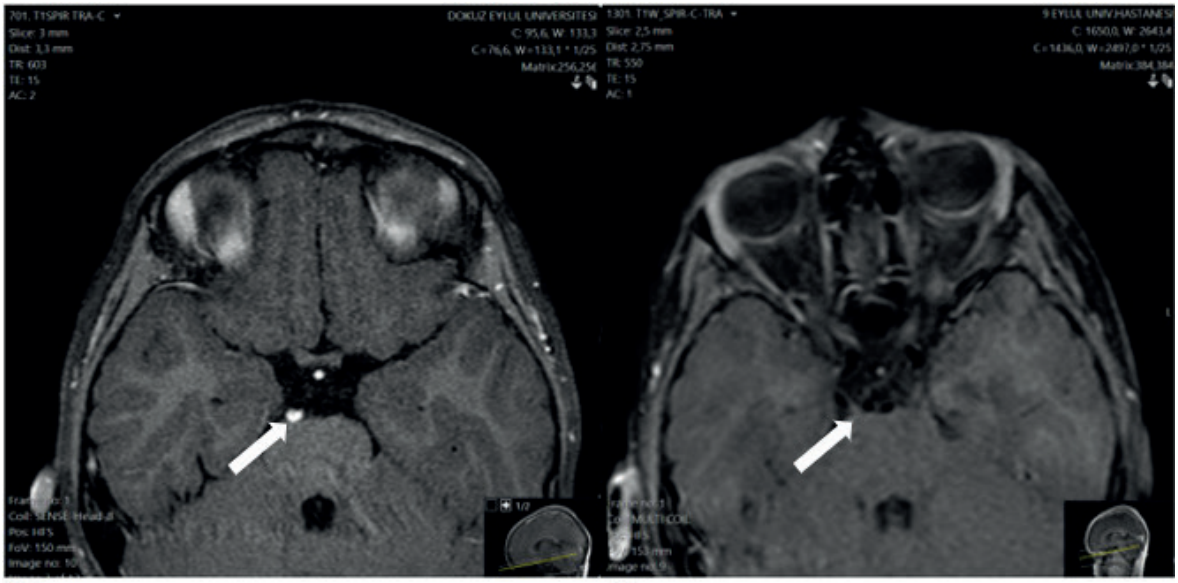


Fig. 1. (a) Axial postcontrast T1 images show thickening and increased enhancement 5.5 mm nodular lesion on the right at the cisternal portion of the 3rd cranial nerve. **(b)** Reduced flow at right P1 segment of posterior cerebral artery secondary to lesion. **(c)** After 6 months, the lesion size decreased to 4 mm and there was no enhancement on postcontrast T1 weighted image. **(d)** Repeated MR angiography study was normal.

headache persisting for the last 10 days, with the addition of impaired adduction and double vision within the last 24 hours. The patient had an unremarkable family history. Neurological examination was normal except for limited inward gaze and ptosis in the ipsilateral eye. Brain computed tomography (CT) was normal. Brain MRI revealed a 4.5 mm, enhancing, nodular lesion in the 3rd cranial nerve lodge in the right perimesencephalic area (Fig. 2a). Brain CT angiography was performed to rule out vascular causes and was normal. Dexamethasone was initiated with pre-diagnoses of trigeminal nerve schwannoma and RPON. Headache regressed in 8 days and ophthalmoplegia in 6 days. Propranolol prophylaxis (1 mg/kg/day) was started. The patient had a second attack in the 9th month of the follow-up. Headache and ophthalmoplegia in this attack had the same characteristics as in the previous one.



2a 2b

Fig. 2. (a) Nodular lesion in the right 3rd cranial nerve at the perimesencephalic area show prominent contrast enhancement. **(b)** 12th month follow up study shows no lesion and no abnormal enhancement of the right oculomotor nerve at postcontrast T1 image.

The patient received only analgesic treatment as the patient presented after the symptoms regressed. The propranolol dose was increased to 1.5 mg/kg/day. Brain MRI revealed similar lesion size and contrast enhancement. No lesions and enhancement in the cranial nerve lodge were observed in the brain MRI in the symptom-free period (12th month) (Fig.2b). At the 13th month of follow-up, the patient had an attack (headache without ophthalmoplegia) lasting 7 days but steroid treatment was not given owing to improvement of headache with symptomatic treatment. Valproate was added to the propranolol prophylaxis. The patient is currently in the 18th month of the follow-up (5-month symptom free). Table I shows the clinical findings and neuroimaging results of the cases. The parents gave their informed consent for this publication.

Discussion

In the literature, the age of onset of RPON ranges from 3 months to 74 years. Liu et al.[3] showed that although the average age of onset for this disorder was 22.1years, 65.8% of the cases were in the pediatric age group. The patients we reported were 4 and 7 years old. While cases with predominantly female gender were reported, some publications stated that the frequency of male cases was higher, and in some others no difference between the sexes was found.[4,5] Liu et al.[3] reported the female: male ratio as 1.4: 1 in their review in 2020. Our cases, which support this rate in the literature, were female. In the literature, a family history of migraine was reported as 34.5%.[3] Case 1's aunt suffered from migraines, while the family history of the other members was unremarkable.

The differential diagnosis for cranial neuropathy is extensive, and all possibilities should be considered in the diagnosis of RPON. Exclusion of orbital, parasellar, or posterior fossa lesions is essential.[1] Apart from excluding these lesions, another advantage of brain MRI is to demonstrate the affected cranial nerves. Nerve thickening and/or gadolinium enhancement of the affected nerve can be seen using brain MRI during an attack of RPON, whereas negative findings are highly common during the symptom-free period.[1,6-8] Although the most commonly involved nerve is the 3rd cranial nerve,

Table I. The clinical findings and neuroimaging results of the cases.

	Number of episodes	Number of episodes with ophthalmoplegia	Number of episodes with spontaneous regression	Duration of episodes (days)						MRI findings							
				Before steroid therapy		After steroid therapy		With spontaneous regression		Mean duration of steroid therapy (days)	During attacks			During symptom free periods			
				Headache	Ophthalmoplegia	Headache	Ophthalmoplegia	Headache	Ophthalmoplegia		Number	Lesion	Contrast enhancement	Number	Lesion	Contrast enhancement
Case 1	16	3		8.6 (1-25)	1.5 (1-2)	3 (1-7)	1	1.7 (1-4)	12	6 (3-10)	6	(+)	(+)	1	(+)	(-)
Case 2	3	2	2	1	10	1	8	6	5.5 (4-7)	1	6	2	(+)	(+)	1	(-)	(-)

abducens and trochlear nerve involvement have been reported in the literature. Our patients also presented with 3rd cranial nerve involvement. Similar to our cases, cranial nerve involvements have been reported as contrast enhancing nodular lesions.[8,9] Hashimoto's encephalopathy and myelin oligodendrocyte glycoprotein-associated disease, which may present with recurrent ophthalmoplegia, were not considered in our patients with long-term follow-up due to their clinical and radiological features. Imaging findings are more commonly seen in pediatric cases and the disappearance of lesion usually takes 12 weeks.[10] Since Case 1 experienced frequent attacks, contrast regression on brain MRI occurred in the 41st month after the first attack (24th week after the last attack). In Case 2, the lesion completely regressed in the brain MRI and this regression was observed 12 months after the first attack (12 weeks after the last attack), similar to the literature. Although brain CT findings are generally normal, abnormalities in the involved nerve have been rarely reported in RPON patients.[11] Brain CT was performed in one of our cases and was normal. Also, angiographic evaluations revealed right-sided fetal-type PCA endorsing the compressive/ischemic hypothesis which remarks that edema of the wall of the internal carotid artery or PCA could block the arterial supply for cranial nerves or compress the cranial nerves in RPON.[5,7,12] Concerning pathophysiological considerations, in favour of a migrainous, not neuropathic etiology, several mechanisms have been discussed to explain how migraine may induce ophthalmoplegia. Migraine-related intumescence of the vessel walls of the PCA has been proposed to cause occlusion of arterial branches supplying the cisternal portion of the oculomotor nerve. Ischemic havoc of the blood-nerve barrier would cause vasogenic edema, explaining ophthalmoplegia, thickening, and contrast enhancement of the nerves.[13] Shin et al.[14] have shown reversible, ipsilateral ischemia in the areas of perforating branches of the PCA by brain technetium Tc 99m ethyl cysteinate dimer single photon emission computed tomography

(SPECT), in two patients with oculomotor nerve involvement. Reverting to normal levels of regional cerebral blood flow has been demonstrated on a follow-up SPECT during the symptom-free period suggesting reversible ischemia in the territories of the branches of the PCA may be a possible pathophysiological mechanism for this disease. In Case 1, the angiographic evaluation revealed reduced flow right PCA (P1 segment), moderate narrowing of the P1 segment posteriorly, secondary to the lesion originating from the 3rd cranial nerve. This image supported the ischemic hypothesis similar to those reported in the literature. In our study, the headaches were localized to the frontal region. While the location of headache in RPON patients was 48.3% orbital-related, frontal headaches were detected as 11.6% in the literature.[3] The time interval between headache and ophthalmoplegia was reported ≤1 week in 95.7% of the patients.[3] We found that this interval was 3-5 days (mean 4 days) in Case 1 and 3-9 days (mean 6 days) in Case 2, similar to the literature. Cranial nerve palsies may continue for a few days to weeks, but the headache resolves completely with or without any special treatment.[3,15-17] Headaches in our patients regressed in an average of 9.2 days (1-32 days). The spontaneous regression rate of headache was 31.5%. Ophthalmoplegia in our patients regressed in an average of 5 days (1-12 days). The spontaneous regression rate of ophthalmoplegia was 40%. In attacks without spontaneous regression, the duration of symptom relief with treatment was between 1-8 days (mean 3.4 days) for headache and 1-6 days (mean 2.6 days) for ophthalmoplegia.

Since RPON is a very rare disease, no treatment trials or guidelines for RPON exist. Observational studies are the only publication that can make recommendations on effective treatments. In the review by Liu et al.[3] in 2020, it was reported that 47.3% of the patients received corticosteroid treatment and 96.2% of them benefited from this treatment within 1 hour-8 weeks. In our study, while 3-10 days of steroid treatments in Case 1 regressed the headache between 1-7 days (mean 3 days), ophthalmoplegia regressed in 1 day. In Case 2, steroid treatment (6 days) was given in one of the attacks; where headache regressed in 8 days and ophthalmoplegia in 6 days. Indomethacin, intravenous immunoglobulin, nonsteroidal anti-inflammatory drugs, ergotamine and furosemide are other agents used in the treatment of attacks. Other prophylactic agents with variable efficacy are flunarizine, propranolol, verapamil, valproate, cyproheptadine, gabapentin, pizotifen, imipramine, and amitriptyline.[16] In the literature, pregabalin was found to be useful in a patient whose headache and ocular paralysis regressed with steroid therapy but recurred with gradual dose reduction, and who was also unresponsive to prophylaxis with beta-blockers, calcium channel blockers, and topiramate.[18] Wang et al.[19] reported an adult patient who did not experience an attack with the combination of valproate and flunarizine. Margari et al.[7] reported that valproate was used in a pediatric patient with a history of focal seizures and epileptic abnormalities on electroencephalography, and both electroencephalography findings and the attack frequency decreased. In our study, when Case 1 continued to have frequent attacks with propranolol, topiramate and flunarizine, valproate was initiated. While valproate decreased the attack frequency and enhancement in MRI, reduction of its dose caused a relapse. In Case 2, there was no attack with valproate, which was initiated after the attacks continued while using propranolol.

In, conclusion, the changes in the naming and classification of RPON for nearly two decades indicate the ongoing debate around this rare disorder. A brain MRA during initial work-up and a brain MRI with contrast after a symptom-free period of at least 3 months may be helpful in both the differential diagnosis and diagnostic challenges of RPON. A combined treatment approach with acute attack and prophylactic treatments can manage acute symptoms as well as minimize recurrence for which valproate may be an effective prophylaxis option.

Author contribution

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

Conflict of interest

The authors declare that there is no conflict of interest.

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Intrapancreatic accessory spleen in child

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ABSTRACT

Background. Intrapancreatic accessory spleen is a congenital abnormality with duplicated splenic tissue located in ectopic sites.

Case. We report a case of 10-year-old male patient with an infrequent finding of intrapancreatic mass. The examination of complete blood count, biochemistry, tumor markers were within the normal reference ranges. Imaging series found an intrapancreatic mass without wash-out effect on contrast-enhanced ultrasound, MRI-intensity equal to splenic one and no increased glucose metabolism on PET/CT. Follow-up of the patient did not demonstrate progression of the size or change of ultrasound characteristics of the lesion.

Conclusions. Intrapancreatic accessory spleen is an asymptomatic lesion and without the need of surgical therapy. It is important to differentiate it from pancreatic malignant tumors.

Key words: pancreatic tumor, accessory spleen, contrast-enhanced ultrasound, CEUS, PET/CT.

Intrapancreatic accessory spleen (IPAS) is a congenital abnormality with duplicated splenic tissue located in ectopic sites. The most common locations for accessory spleens are the hilum of the spleen, followed by being adjacent to the tail or other parts of the pancreas. The patients usually present with no clinical symptoms.[1,2]

Case Report

A 10-year-old male patient was referred to the Department of Pediatrics with a complaint of constant eructation during the day. There were no complaints when the patient was asleep. He had undergone an appendectomy three weeks earlier. The onset of belching was several days after the operation. Thalassemia minor was established at 1 year of age. An off-patient consultation was done due to eructation and a tumor mass was found in the pancreas.

The physical examination and laboratory data showed no abnormalities. Pancreas enzymes were normal. Tumor markers, including carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA 19-9) were within reference ranges.

Abdominal ultrasound of the pancreas appeared to be normal in size, without any remarkable findings in the parenchyma and ducts. In between the head and body of the pancreas a hypoechoic mass was visualized with dimensions 13 x 8 mm. On Doppler examination no vascularization of the mass was found. (Fig. 1)

The other parenchymal organs remained normal in size and echogenicity. No pathological findings were registered on Doppler examination of the major abdominal vessels. No free fluid was found in the abdominal cavity.

On contrast enhanced ultrasound (CEUS) the lesion appeared to be hypoenchanced during the early dynamic phase (Fig. 2) and isoenchanced

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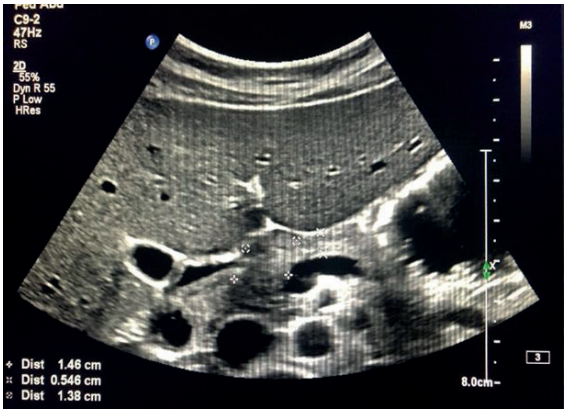

Fig. 1. B-mode ultrasonography examination presenting an intrapancreatic mass, located in the body of the pancreas.

during the late venous phase. No wash-out was observed (Fig. 3).

On gadopentetic-acid-enhanced MRI the lesion appeared round and localized on the ventral surface of the pancreas. The mass demonstrated relative hyperintensity in conventional T2-weighted images and isohypointensity in T1-weighted images. The lesion showed hyperintensity on diffusion-weighted imaging at b=0 and b=500 s/mm^2. After contrast application a formation increased in intensity characteristics equal to splenic parenchyma (Fig 4).

On fludeoxyglucose Positron Emission Tomography / Computed Tomography (18 F-FDG PET/CT) in the pancreas a mass was observed with unremarkable delineated borders and without increased glucose metabolism in the lesion. The standardized uptake value (SUVmax) was measured < 2.0. The conclusion was the absence of PET/CT evidence of malignant lesions. (Fig. 5)

The 6-month follow-up of the patient showed no increase of the lesion in size or change of ultrasound pattern. During work-up for intrapancreatic tumor, the eructation stopped.

Based on clinical and imaging features, our working diagnosis was accessory spleen in pancreas. No invasive treatment was planned. The initial complaint was considered as functional.

This case is an illustration that appropriate use of imaging series can avoid the need of invasive procedures and surgery in patients with intrapancreatic tumors.

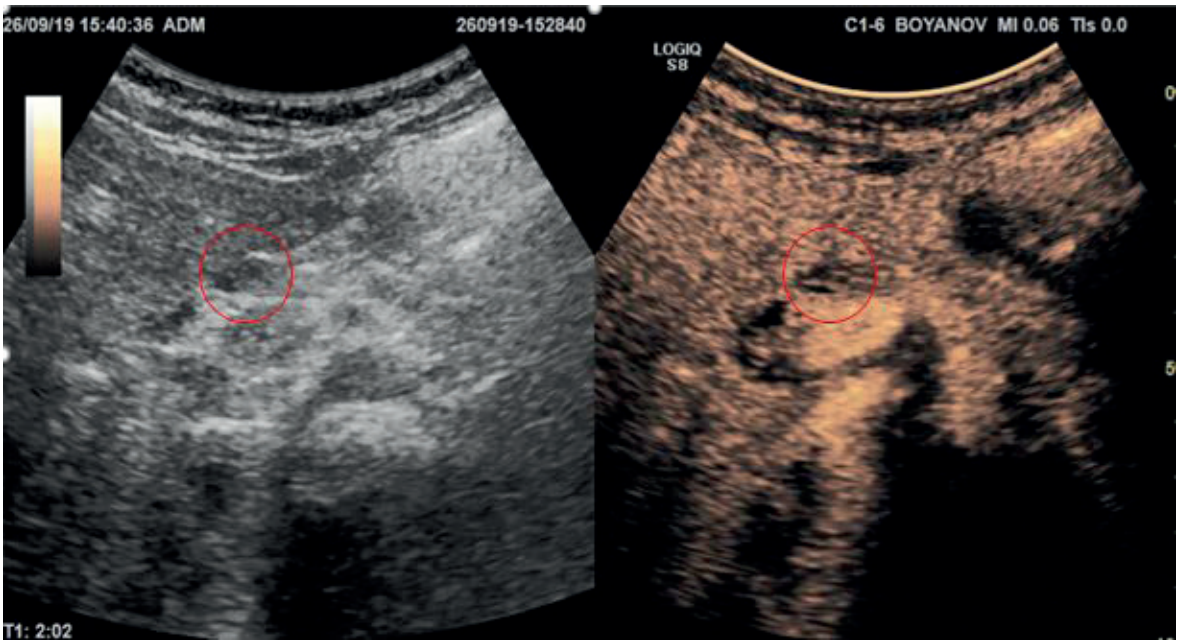

Fig. 2. Arterial phase of CEUS, presenting a hypoenhanced lesion at the site of the intrapancreatic mass.

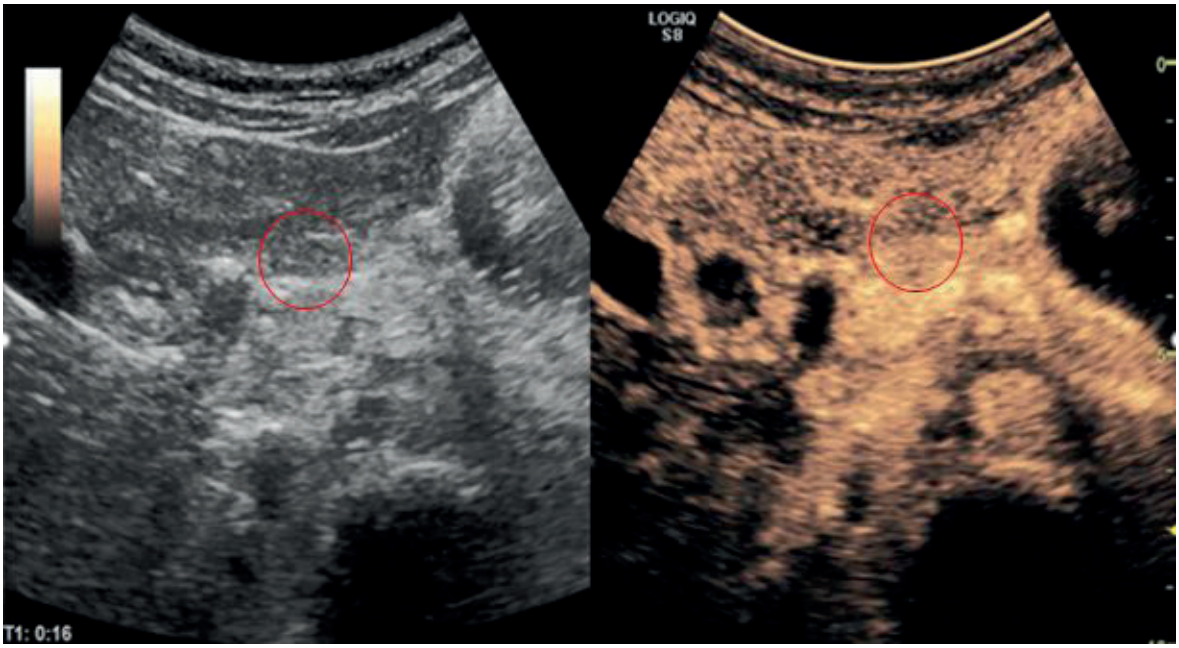

Fig. 3. Venous phase of CEUS presenting that lesion remain isoenhanced to the pancreatic parenchyma.

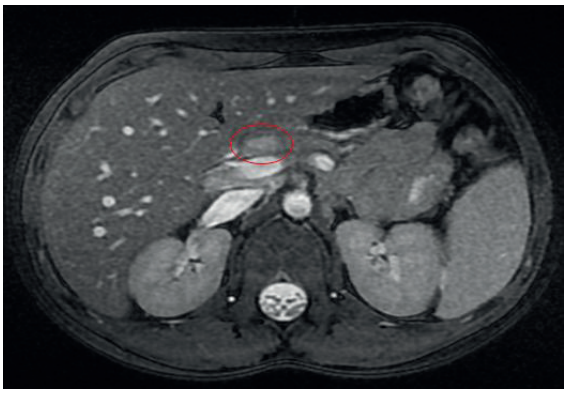

Fig. 4. Contrast-enhanced MRI hyperintense lesion at the site of intrapancreatic mass.

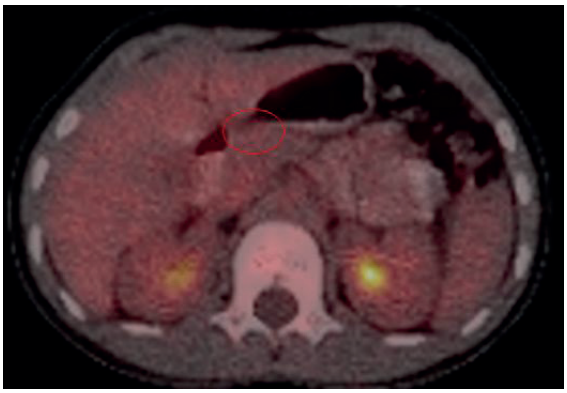

Fig. 5. PET/CT presenting absence of metabolic activity at the site of intrapancreatic mass.

Written informed consent was obtained from the patients'parents.

Discussion

When the solid mass was first discovered we primarily discussed the possibility of a neuroendocrine tumor (NET) with pancreatic localization. Johnson reported 60% inactive tumors in a studied cohort. They are usually large in size and often the metastases appear at diagnosis.[3] Stawarski et al. reported similar results.[4] Symptomatic tumors are most often insulinomas and present with hypoglycemia. Other possible manifestations are redness of the face and neck, tachycardia, dizziness, excessive sweating, diarrhea, bronchospasm, abdominal pain, weight loss, lack of appetite, and vomiting.[3,4] Due to the patient's lack of complaints and signs, a functionally inactive tumor was considered.

The second possibility was a benign mass. Usually, ectopic spleens (up to 80%) are located close to the splenic hilum, and 16% are in the tail of the pancreas. IPAS in other parts of the

pancreas is an occasional finding. In general, IPAS is an asymptomatic lesion and it does not need surgical therapy.[2]

Laboratory diagnosis of NET includes the examination of chromogranin A and chromogranin B.[3] The assay demonstrates high specificity, but low sensitivity and a high probability of interference. In the respective types of symptomatic NET, the level of the respective hormone can be examined, and various functional tests can be performed, as well.[3,4] After consultation with a pediatric endocrinologist, a possible inactive tumor was considered in our patient. During the follow-up, the child showed no complaints.

Sofuni et al. as well as the PAMUS study, concluded that ultrasound examination visualized well tumor formation and hypervascular enhancement pattern. CEUS demonstrated an excellent capability for the differentiation of the solid pancreatic masses.[5,6] D'Onofrio et al. reported that ultrasound techniques allow the differentiation of benign from malignant lesions in the pancreas, as well as solid from cystic lesions. CEUS pattern of NET is described as a rapid, intense enhancement of the lesion resulting in a hyperechoic appearance in the early contrast phases due to hypervascularity of the lesion. In the late phase NET appear hypoechoic due to the rapid wash-out effect. The minority of NET do demonstrate described characteristics.[7,8]

Rinzivillo et al.[9] reported that ^{18}F-FDG PET findings strongly correlate with disease behavior. In patients with unknown disease status, ^{18}F-FDG PET can provide relevant clinical information in patients with a positive examination. Calabrò et al.[10] concluded that ^{18}F-FDG PET is indicated in patients with high-grade NET or in those with the suspicion of rapid progression and for prognosis of the tumor stratification.

During the CEUS examination a hypoenchanced lesion in the early dynamic phase and an isoenchanced in the late phase is a typical US pattern. The IPAS is normally seen in magnetic resonance of hyperintense diffusion weighted in T2 and hypointense weighted in T1 compared to normal pancreatic tissue. The ^{18}F-FDG PET/CT or ^{68}Ga-DOTA-TOC PET/CT has a high specificity to differentiate IPAS from pancreatic adenocarcinoma or neuroendocrine tumors.[11,12]

A small proportion of cases of IPAS may occur secondary to other diseases, including epidermoid cyst and inflammatory pseudotumor.[13-15] In adults, IPAS needs histopathological conformation. In children, tissue material is usually obtained during surgical operations. Our patient demonstrated clinical and imaging presentation of a benign solid intrapancreatic lesion. That's why we accepted a "wait and watch" approach, but fine needle biopsy or laparoscopic examination remain options, in case of growth of the tumor or appearance of hypervascular pattern.

The diagnosis of IPAS may be difficult secondary lesions are present. Imaging studies certainly have an important role in IPAS diagnosis. Ultrasound examination, combined with CEUS, MRI and PET/CT revealed to be highly accurate methods in the diagnosis and differentiation of IPAS and small solid pancreatic tumors.[16-20]

IPAS is an asymptomatic lesion and surgical treatment and histopathological confirmation may not be needed if diagnostic imaging highly suggests IPAS in children. There is significant importance in differentiating IPAS from pancreatic malignant tumors. This diagnosis must be considered before surgery of solid pancreatic masses to avoid unnecessary pancreatic resections.

Author contribution

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

Conflict of interest

The authors declare that there is no conflict of interest.

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