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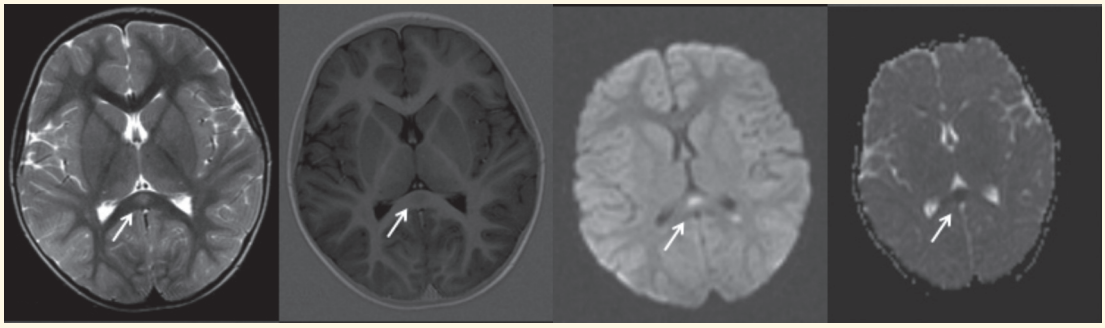

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Editorial

Dear Readers,

Our struggle with the new coronavirus disease (COVID 19), which first appeared in December 2019 in the city of Wuhan, China, as a mysterious respiratory disease of unknown origin and turned into a pandemic after it began to threaten public health globally, continues.

While there have been positive developments regarding the vaccine, on the anniversary of the first appearance of the disease, the UK became the first country to raise the alarm for a rapidly multiplying Covid-19 mutation. Other variants announced after that (South Africa, Brazil, California etc.) also caused worldwide concern. Finally, the delta variant, which was first seen in India and spread rapidly to other countries, causing a significant increase in the number of cases despite widespread social vaccination, caused the quarantine measures to come up again. Despite all the devastating effects of the pandemic, the mild course of the disease in vaccinated individuals, the higher need for hospitals and intensive care units in unvaccinated individuals, the initiation of adolescent vaccines and the acceleration of vaccination studies in young age groups gave rise to great hopes. Vaccine is our greatest weapon against this global threat. Raising awareness of the society about the importance and necessity of the vaccine, and producing solutions for the factors that cause vaccine instability should be our biggest task in the fight against the covid 19 pandemic.

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I would like to thank all our colleagues who contributed to the publication of our latest issue with their scientific articles, the JPR family, which I am very happy and proud to be a member of, and the Galenos Publishing House.

Sincerely yours,
Dr. Gülhadiye Avcu

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Original Article

Transition Time to Full Oral Feeding Skill and Its Determinants in Very Preterm Infants: A Single Center Experience

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ABSTRACT

Aim: Transition from tube to full oral feeding (FOF) represents an important milestone for very preterm infants, and can be affected by various factors. This study aimed to evaluate transition time to FOF in very preterm infants and to identify the factors affecting this ability.

Materials and Methods: In this 4-year study, infants' characteristics, feeding steps, and comorbidities were retrospectively evaluated. Infants were categorized into two groups based on FOF timing and comparisons were made. Logistic regression analysis was used to examine any affecting factors.

Results: Transition from tube to FOF occurred on a median of 20 days. There were 48 and 52 neonates in the ≤20 days and >20 days groups, respectively. Gender, delivery type, and antenatal history were similar between the groups. The majority were supported with formula. More infants were at >30 weeks of gestational age, and had a birth weight >1.47 kg in the ≤20 days group. A lesser proportion of these required invasive interventions, and had comorbidities. In this study, the requirement of non-invasive ventilation ≤3 days, receiving kangaroo mother care, the promotion of non-nutritive sucking within the first week, and the achievement of full enteral feeding in ≤14 days were associated with a positive effect on the transition to FOF in ≤20 days. However, a gestational age of ≤30 weeks and diagnosis of bronchopulmonary dysplasia had a negative effect.

Conclusion: This was one of the few studies to investigate the timing of transition to FOF in very preterm infants as well as its affecting factors. Further studies are required to provide guidance on interventions to shorten FOF time and to provide kangaroo mother care, non-nutritive sucking and breastfeeding in the very preterm population.

Keywords: Very preterm infants, full oral feeding, affecting factors

Introduction

Survival in very preterm (VPT) infants born at <32 gestational weeks has improved significantly owing to advances in perinatal interventions and neonatal intensive care practices which could have led to airway and feeding morbidities (1). Transition from tube to full oral feeding (FOF), which is a major discharge criterion indicating the maturity and health of the preterm, represents an essential milestone in the feeding process for these infants (2,3).

Swallowing is already present in the fetus by week 16 of gestation and is expected to be functional at around 34-36 gestational weeks. VPT infants usually experience

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difficulties in coordinating sucking, breathing, and swallowing, and thus are tube-fed for some duration (4,5). However, the developmental stages of oral feeding skills have not been very well characterized in prematurely born infants (6,7). Furthermore, the feeding process may be influenced by a number of factors including the infants' own characteristics, receiving kangaroo mother care (KMC), and the promotion of non-nutritive sucking (NNS), as well as by the presence of neonatal comorbidities such as hemodynamically significant patent ductus arteriosus (hsPDA), necrotizing enterocolitis (NEC), infections, and bronchopulmonary dysplasia (BPD) (6,8). It is known that nutrition is a crucial component of care in VPT infants. Feeding difficulties leading to inadequate nutrition during this critical period have been associated with the duration of hospital stay as well as growth retardation, and adverse neurodevelopmental outcomes in the later stages of life (9).

In this retrospective study, we aimed to evaluate the time to FOF and identify the factors affecting transition to FOF in preterm infants born at <32 weeks of gestation and followed up in the neonatal intensive care unit (NICU) of our hospital.

Materials and Methods

Study Design

In this study involving infants born at <32 weeks of gestation between January 2016 and December 2019 at Kocaeli Derince Training and Research Hospital, the following data retrieved from the medical records were retrospectively evaluated: Perinatal, demographic and clinical characteristics including maternal age, maternal disease, antenatal steroid use, delivery mode, APGAR score, clinical risk index-II score (CRIP-II score predicting neonatal mortality at admission), sex, gestational age (GA), birth weight (BW), percentile for GA on the Fenton curve (10), surfactant treatment, duration of intubation/non-invasive ventilation (NIV)/oxygen support, caffeine use, duration of hospitalization; feeding steps; and comorbidities. Infants with malformations of the head, neck, and gastrointestinal (GI) tract; genetic abnormalities; and BW of <10th percentile for gestation on the Fenton curve were excluded. The study protocol was approved by the University of Health Sciences Turkey, Kocaeli Derince Training and Research Hospital, Clinical Research ethics committee (no: 2020/72).

The feeding steps defined in study were as follows: In addition to parenteral nutrition (PN), colostrum was started on the first day of life directly by mouth or through orogastric tube. After minimal enteral feeding (EF) for 3-5 days, EF was increased in accordance with GA/BW by increments of either 20 mL/kg/day or 30 mL/kg/day at 2-3 hour intervals. Feedings consisted of either fortified mother's milk or preterm formula. Fortification was initiated at an EF dosage of 50 mL/kg/day. When cardiorespiratory stability was attained, even if it was with NIV support, KMC and NNS (via mostly an expressed breast or via a suitable pacifier) were initiated. While PN was gradually reduced, progression to full EF (FEF) was carried out based on GI tolerance. At this stage, infants were transitioned to oral feeding, when they exhibited oral-motor cues and coordinated sucking, breathing, and swallowing. The tube was removed when the infant could receive more than 80-85% of FEF via breast or bottle. Target weight gain was set at a rate of 15-20 g/kg/day (8,11,12). Once FOF was achieved, ad libitum feeding was started before discharge. Time to FEF and transition to FOF were recorded for each infant. FEF was defined as an enteral volume of at least 150 cc/kg/day containing 120 calories/kg/day by gavage feeding alone (8,11).

Feeding intolerance was defined as the presence of one or more of the following: a) vomiting more than 3 times in any 24-hour period; b) any episode of bile-stained vomiting; c) abdominal wall erythema/tenderness and/or decreased bowel sounds; d) gross/occult blood in stools; e) milky gastric residue >50% of the previous feed volume (residue was checked only in cases of clinical suspicion). These criteria for feeding intolerance also comprised indications for temporary cessation of feeds (8,13,14).

Co-morbid factors identified in the study population included hsPDA, NEC, late-onset sepsis, and BPD. hsPDA was defined as a ductus arteriosus diameter of ≥1.5 mm and/or a left atrium/aortic root ratio ≥1.5, as assessed by Doppler echocardiography within the first 48-72 hours of life (15). NEC was suspected in infants with abdominal/systemic symptoms and signs, and they were categorized using modified Bell's classification (14). Sepsis with and without positive blood cultures after 72 hours of life was defined as late-onset culture proven and clinical sepsis, respectively (16). BPD was defined as the requirement for more than 28 days of supplemental oxygen between birth and 36 weeks of corrected GA (17).

Statistical Analysis

Statistical analyses were performed using IBM-SPSS for Windows version 20.0 (IBM Corp., Armonk, NY, USA). Kolmogorov-Smirnov test was used to test the normality of the data distributions. Continuous variables were expressed as mean (±standard deviation) and median (25th-75th percentiles). Categorical variables were expressed as counts

(percentages) or percentages. Intergroup comparisons of normally and non-normally distributed continuous variables were performed using Student's t-test and Mann-Whitney U test, respectively. Yates' chi-square and Monte Carlo chi-square tests were used for intergroup comparisons of categorical variables. A two-sided p-value of <0.05 was considered statistically significant.

To investigate the variables that had an effect on feeding in VPT infants, median time of FOF was chosen as the cutoff point to divide the participants into comparable groups, in line with the published literature (18,19). For other factors, mean/median values were used to compose the subgroups. Subsequently, binary logistic regression analysis was used to determine those variables with a significant effect on feeding status.

Results

Baseline Characteristics of the VPT Infants

A total of 100 infants (53 males) born at <32 weeks of gestation who were admitted to the NICU and met the inclusion criteria were included in this 4-year study. The GA and BW of the infants were 30 (28.4-31) weeks and 1.47±0.34 kg, respectively. Transition from FEF to FOF occurred on day 20 (10-35), at 32.6 (32.4-33.5) postnatal weeks. The infants were discharged at 34.5 (32.5-37.2) weeks of age, with a weight of 2.01±0.20 kg, and all of them were on ad libitum feeding. The baseline characteristics of the study population are presented in Table I. The majority were supported with formula during hospitalization. The percentage of those exclusively fed with mother's milk (via breast or bottle) was 16% at discharge.

Table I. The baseline data of the study, %/mean ± SD/median (25^{th}-75^{th})

Female/male, %	47/53
Vaginal delivery/cesarean, %	31/69
Birth weight (kg), mean ± SD	1.47±0.34 (minimum 0.65 - maximum 1.85)
Gestational age, median (25^{th}-75^{th})	30 (28.4-31) (minimum 25 - maximum 31.4)
Maternal age (yrs), median (25^{th}-75^{th})	28 (22-34)
Ratio of maternal PPROM*, %	61
Ratio of antenatal steroid, %	42 (42)
APGAR score at 5 min, median (25^{th}-75^{th})	8 (7-9)
CRIP[&] score, median (25^{th}-75^{th})	6 (2-8)
Ratio of surfactant administration, %	69 (69)
Duration of intubation (days), median (25^{th}-75^{th})	0 (0-2)
Duration of NIV support (days), median (25^{th}-75^{th})	3 (1-8)
Ratio of caffeine receivement, %	83 (83)
Day of first KMC and NNS, median (25^{th}-75^{th})	7.5 (4-20)
Duration of using PN[£] (days), median (25^{th}-75^{th})	10 (5-14)
Day of achieve to FEF[β] (days), median (25^{th}-75^{th})	14 (10-18)
Day of transition to FOF[€] (days), median (25^{th}-75^{th})	20 (10-35)
Number of epizodes of feeding cessation, median (25^{th}-75^{th})	3 (0-5)
Ratio of hsPDA[$], %	16
Ratio of NEC[₭] ± clinical/proven sepsis, %	10
Ratio of late-onset neonatal sepsis (clinical/proven), %	28/22
Ratio of BPD[₹] diagnosing (mild/moderate), %	19/9
Ratio of abnormal cranial USG findings, %	15
Duration of hospitalization (days),median (25^{th}-75^{th})	30 (16.3-47.8)
Weight at discharge (Kg), mean ± SD	2.01±0.20
Postnatal weeks at discharge, median (25^{th}-75^{th})	34.5 (32.5-37.2)

*: Preterm premature rupture of membrane, ¢: Non-invasive ventilation, ∞: Kangaroo mother care and non-nutritive sucking, &: Clinical Risk index II score, £: Parenteral nutrition, β: Full enteral feeding, €: Full oral feeding, $: Hemodynamically significant patent ductus arteriosus, ₭: Necrotizing enterocolitis, ₹: Bronchopulmonary dysplasia, SD: Standard deviation, APGAR: Appearance, pulse, grimace, activity, and respiration, PPROM: Preterm premature rupture of membrane, CRIP: Clinical risk index, NIV: Non-invasive ventilation, KMC: Kangaroo mother care, NNS: Non-nutritive sucking, PN: Parenteral nutrition, FEF: Full enteral feeding, FOF: Full oral feeding, hsPDA: Hemodynamically significant patent ductus arteriosus, NEC: Necrotizing enterocolitis, BPD: Bronchopulmonary dysplasia, USG: Ultrasonography

Comparison of Neonates with FOF ≤20 and >20 Days

There were 48 and 52 neonates with a GA of 31 (30.4-31.2) weeks and 28 (27-29.3) weeks, respectively, in the two study groups defined on the basis of a median time to FOF of ≤20 days and >20 days, respectively.

In the comparison of both groups, more infants in the FOF ≤20 days group were >30 weeks of GA, had BW >1.47 kg, and had a CRIP score ≤6. A smaller proportion of those in this FOF ≤20 days group received surfactant, were supported with NIV >3 days, needed caffeine/PN; and a higher proportion received KMC and were provided with NNS within 7.5 days of life. Additionally, time to FOF of ≤20 days was associated with no or reduced hsPDA + NEC+/-sepsis (all cases with NEC had stage 1-2 disease), a lesser occurrence of mild/moderate BPD, having no or ≤3 episodes of feeding cessation, an absence of abnormal cranial ultrasound findings at admission, and higher rates of FEF ≤14 days and discharge within 30 days. The groups were similar in terms of gender, delivery type, antenatal and maternal history, and APGAR score distribution (p>0.05). Table II shows the comparison of these two groups.

Table II. The comparison of ≤20 days and >20 days FOF groups, n (%)

	≤20 days FOF group, (n=48)	**>20 days FOF group, (n=52)**	**p-value¥**
Gender			
Female	26 (56.2)	21 (40.4)	0.238
Male	22 (45.8)	31 (59.6)	
Style of delivery			
Vaginal	14 (24.2)	17 (32.7)	0.869
Cesarean	34 (70.8)	35 (67.3)	
Birth weight (kg)			
≤1.47 kg	10 (20.8)	39 (75)	<0.001
>1.47 kg	38 (79.2)	13 (25)	
Gestational age			
≤30 weeks	12 (25)	44 (84.6)	<0.001
>30 weeks	36 (75)	8 (15.4)	
Maternal age			
≤28 years	24 (50)	24 (46.2)	0.854
>28 years	24 (50)	28 (53.8)	
Maternal disease			
PPROM*	26 (54.2)	35 (67.3)	0.254
Preeclampsia, others	22 (45.8)	17 (32.7)	
APGAR score at 5 minute			
≤8	9 (18.8)	11 (21.2)	0.960
>8	39 (81.2)	41 (78.8)	
CRIB& score			
≤6	38 (79.2)	14 (26.9)	<0.001
>6	10 (20.8)	38 (73.1)	
Antenatal steroid			
None/incomplete	29 (60.4)	29 (55.8)	0.789
Completed	19 (39.6)	23 (44.2)	
Surfactant administration			
Not administered	24 (50)	7 (13.5)	<0.001
Via INSURE€ method	10 (20.8)	15 (28.8)	
Remaining intubated	14 (29.2)	30 (57.7)	
Duration of NIV¢ support			
≤3 days	43 (89.6)	16 (30.8)	<0.001
>3 days	5 (10.4)	36 (69.2)	
Caffeine			
Not received	17 (35.4)	0 (0)	<0.001
Received	31 (67.6)	52 (100)	
Receiving KMC&NNS∞			
≤7.5 days	39 (81.2)	10 (19.2)	<0.001
>7.5 days	9 (18.8)	42 (80.8)	

Table II. continued

Duration of PN[£] ≤10 days >10 days	 39 (81.2) 9 (18.8)	 7 (13.5) 45 (86.5)	 <0.001
Achieving FEF[β] ≤14 days >14 days	 43 (89.6) 5 (10.4)	 7 (13.5) 45 (86.5)	 <0.001
Number of episodes of feeding cessation None - ≤3 >3	 41 (85.4) 7 (14.6)	 13 (25) 39 (75)	 <0.001
Comorbidities None hsPDA[§]+NEC[ĸ]±sepsis Sepsis (clinical/proven)	 24 (50) 5 (10.4) 19 (39.6)	 0 (0) 21 (40.4) 31 (59.6)	 <0.001
BPD[ŦŦ] Mild/Moderate None	 4 (8.3) 44 (91.7)	 24 (46.2) 28 (53.8)	 <0.001
Cranial ultrasound findings at admission Normal Abnormal[ø]	 48 (100) 0 (0)	 37 (71.2) 15 (28.8)	 <0.001
Duration of hospitalization ≤30 days >30 days	 37 (77.1) 11 (22.9)	 6 (11.5) 46 (88.5)	 <0.001

¥: Chi-square test
*: Preterm premature rupture of membrane, &: Clinical risk index II score, ₡: INtubate-SURfactant administration and extubate, ¢: Non-invasive ventilation, ∞: Kangaroo mother care and non-nutritive sucking, £: Parenteral nutrition, β: Full enteral feeding, §: Hemodynamically significant patent ductus arteriosus, ĸ: Necrotizing enterocolitis, Ŧ: Bronchopulmonary dysplasia, øGrade 1 or 2 intraventricular hemorrhage, and grade 1 periventricular leukomalacia (according to Papile)
FOF: Full oral feeding, PPROM: Preterm premature rupture of membrane, APGAR: Appearance, pulse, grimace, activity, and respiration, CRIB: Clinical risk index for babies, NIV: Non-invasive ventilation, KMC: Kangaroo mother care, NNS: Non-nutritive sucking, PN: Parenteral nutrition, FEF: Full enteral feeding, hsPDA: Hemodynamically significant patent ductus arteriosus, NEC: Necrotizing enterocolitis, BPD: Bronchopulmonary dysplasia

In the groups of FOF ≤20 days and FOF >20 days, the proportions of those fed exclusively mother's milk and exclusively formula were 12% and 4% in the former and 2% and 5% in the latter. The rest were fed both, but mostly formula fed rather than mother's milk. The main mode of feeding was via bottle in both groups (75% and 90% in FOF ≤20 days group and FOF >20 days group, respectively).

Logistic Regression Analysis for Factors that Affect the Transition to FOF at ≤20 Days

Based on the results of binary regression analysis, the factors associated with an increased likelihood of transition to FOF in ≤20 days included GA of >30 weeks, requirement of NIV support ≤3 days, receiving KMC and the promotion of NNS within the first 7.5 postnatal days, achievement of FEF in ≤14 days, and an absence of BPD (Table III). Other factors were not found to have a significant effect on the transition to FOF (p>0.05).

Discussion

Although the prevalence and long-term outcomes of feeding difficulties in premature infants are well-known, the time to attain oral feeding skills as well as the factors that influence this process have not been very well characterized (6,7,9). This study revealed that VPT infants with >30 weeks of GA, BW of >1.47 kg, reduced need for interventions, and a lower number of co-morbid conditions were more likely to have time to FOF of ≤20 days. The requirement for NIV support ≤3 days, receiving KMC and the promotion of NNS within the first week, and the ability to achieve FEF in ≤14 days had positive effects on the transition to FOF. Conversely, GA of ≤30 weeks and a diagnosis of BPD prolonged this transition process.

GA, neurodevelopmental maturity level, the quality of care in the NICU, and the facilitation of cue-based oral feeding are among the reported determinants of oral feeding ability in preterm infants (20). When compared to term infants, oral feeding in preterm infants is complicated by innate differences in muscle tone and independent or interdependent suck, swallow and breathe coordination (2,3,5,21). Prematurity can further impair brain development and contribute to poor feeding skills by leading to decreased myelination and white matter disturbances as well as

Table III. The results of binary logistic regression analysis of factors affecting transition to FOF in ≤20 days

	β	SE	Wald	p-value	OR (%95 CI)
Birth weight of >1.47 kg	0.445	0.945	0.228	0.633	1.56 (0.25-16.69)
Gestation age of >30 weeks	-2,364	0.745	2,988	0.012	4.34 (0.64-6.66)
Caffein and surfactant treatment	18,880	-	0.000	0.998	-
Requirement to NIV¢ support ≤3 days	2,844	1,123	3,413	0.011	5.87 (1.90-8.51)
Receiving KMC and NNS∞ within first 7.5 days	2,404	0.867	3,686	0.006	7.11 (2.02-9.57)
Achievement of FEFβ ≤14 days	3,138	1,042	3,075	0.003	8.10 (4.99-10.49)
Presence of hsPDA§+NEC₭+/-sepsis	18,988	-	0.000	0.998	-
Diagnosis of BPD₮	-3,847	1,807	4,531	0.033	11.61 (6.66-15.36)
No or ≤3 episodes of feeding cessation and duration of PN ≤10 days	1,511	0.871	2,006	0.083	4.53 (0.82-24.99)
CRIB& score ≤6 and absence of abnormal cranial ultrasound findings⌀ at admission	18,880	-	0.000	0.998	-

&: Clinical risk index II score, ¢: Non-invasive ventilation, ∞: Kangaroo mother care and non-nutritive sucking, £: Parenteral nutrition, β: Full enteral feeding, §: Hemodynamically significant patent ductus arteriosus, ₭: Necrotizing enterocolitis, ₮: Bronchopulmonary dysplasia
⌀Grade 1 or 2 intraventricular hemorrhage, and grade 1 periventricular leukomalacia (according to Papile)
FOF: Full oral feeding, OR: Odds ratio, SE: It is similar to a standard deviation to a mean, NIV: Non-invasive ventilation, KMC: Kangaroo mother care, NNS: Non-nutritive sucking, FEF: Full enteral feeding, hsPDA: Hemodynamically significant patent ductus arteriosus, NEC: Necrotizing enterocolitis, BPD: Bronchopulmonary dysplasia, PN: Parenteral nutrition, CRIB: Clinical risk index for babies

by delaying the development of a specialized neural circuit known as the suck central pattern generator (9,22). Additionally, maturational delays in GI motility and/or different comorbidities affect feeding skills during the postnatal period (6). Although a variety of tools to assess the readiness for sucking in pre-terms have been proposed, the 2016 Cochrane meta-analysis suggested a lack of evidence to estimate their effects on the time to establish FOF (7).

To best of our knowledge, only a limited number of previous studies have investigated time to attain oral feeding skills in VPT infants as well as the factors or comorbidities that affect this process (6,19,23,24). In a study by Jadcherla et al. (6) where three strata were defined based on GA at birth (i.e., <28, 28-32 and 32-35 weeks), the impact of prematurity and comorbidities on feeding milestones were extensively explored for the first time. Regardless of GA, most neonates were discharged on FOF at <37 weeks' gestation in that study. However, in addition to having prolonged hospitalization, compared with infants >28 weeks of GA, those with <28 weeks of GA had significant delays in feeding with respect to FEF and FOF. A correlation was also found between postmenstrual age (PMA) of FEF and FOF (6). Similarly, in this study, infants born at <30 weeks of GA had significant delays in terms of achieving FEF and FOF, compared to infants born at >30 weeks of GA. Furthermore, achieving FEF within 14 days was associated with an 8.1-fold increased likelihood of achieving FOF within 20 days. Time to FOF in our overall study group occurred between 32.4-33.5 postnatal weeks. Patients were discharged at an average 34.5 (32.5-37.2) weeks of age on ad libitum feeding. In Giannì's (23) study, PMA at time of FOF achievement and length of hospital stay in VPT infants were 36.7±3.68 weeks and 66.3±44 days, respectively. Additionally, in a recent study by Brun et al. (19), time to FOF in VPT infants occurred at 36.6 (35.6-39.2) weeks PMA, and was found to be associated with their duration of hospital stay. The differences in FOF times of these studies may be partially explained by fact that Giannì et al. (23) and Brun et al. (19) also included those infants who were small for GA in their studies.

Lower GA, hypotension, the presence of gastroesophageal reflux, and prolonged ventilation and CPAP were reported to delay time to FOF in Jadcherla's (6) study ($p<0.05$). Also, each 1-week increment in GA was associated with a 1.25-fold increased chance of achievement of oral feeding. In addition, independent of the presence of comorbidities, apnea, BPD and sepsis were negatively associated with FOF at discharge. According to their findings, a ventilation duration of >10 days led to a delay of 1.2 weeks in achieving FOF (6).

In VPT infants, Hwang et al. (24) reported that low BW, moderate-severe BPD, NEC and PDA were predictors for the

PMA of FOF. Similarly, Giannì et al. (23) reported that low BW, BPD, GI surgery, and having neurosensory disease were independently associated with higher PMA at time of FOF. In the study by Brun et al. (19), duration of NIV and oxygen therapy, BPD, and PDA were associated with an older age at FOF. Moreover, in that study, BPD emerged as the single most important predictor. Consistent with previous reports, we also found that GA ≤30 weeks, the requirement for NIV support >3 days, and a diagnosis of BPD were associated with an increased risk of delay for FOF.

Despite some discrepancies between studies, probably due to differences in patient characteristics, management strategies, or analysis methods, BDP appears to be a common risk factor identified in those above-listed studies. Several plausible explanations have been put forward concerning the association of respiratory support with delayed FOF. For instance, infants receiving any type ventilation support could be exposed to nociceptive stimuli in the naso/oro-facial region, which could lead to altered processing of sensory information (25). Additionally, infants are not offered oral feeding mainly due to a fear of aspiration during the treatment of respiratory distress in most NICUs (19). However, BPD and delayed FOF were found to be independently associated with delayed brain maturation at term-equivalent age in a population of VPT infants (26).

In contrast with previous publications, provision of KMC and NNS within the first week and their effects on time to FOF in VPT infants have been specifically evaluated in our study. Preparation of the GI tract with human milk immediately after birth decreases the time required to achieve FEF (8,11,12). At the same time, positive stimulation should be provided by allowing KMC and NNS as often as possible, once the infant has become hemodynamically stable (3,8,11,12). A meta-analysis showed that KMC in low BW infants was associated with reduced mortality, sepsis, hypothermia, length of hospitalization, and increased weight, length, head-circumference, and breastfeeding (27). In a previous study including infants born <37 weeks without any comorbidity, BW>2 kg as well as receiving KMC within the first 3 days was associated with 6-fold and 5-fold increases in the likelihood of early initiation of oral feeding, respectively (28).

NNS has been observed during intrauterine life as early as 15 weeks, reported to be rhythmical by 20 weeks, and continues to improve throughout gestation (29). During KMC, a drop of milk may prompt the infant to lick the nipple and then to suck on it over time. Since it provides nutritive suction, NNS represents an essential component of the early stage of infant feeding, whether it involves the mother's expressed breast or a suitable pacifier. It further contributes to physiological stability, enhances nutrient absorption and GI track functioning. These facilitate the process of transition from tube to oral feeding due the acceleration of maturation (7,30,31). In the 2016 Cochrane meta-analysis involving infants born <37 weeks of GA, it was shown that NNS shortened the transition from gavage to FOF by 5.51 days and reduced hospitalization by 4.59 days (7). Noori et al. (32), in their study with infants born at 26-34 weeks of GA, showed that NNS via the mother's finger sped up time to FOF and reduced hospitalization in intervention groups in comparison to control groups; 22±14.51 vs 30.05±18.58 days, and 31.26±16.89 vs 41.82±23.07 days, respectively. The findings of our study in infants born at <32 weeks of GA showed that KMC and NNS provision within the first week was associated with a 7.1-fold increased likelihood of transition to FOF within 20 days, and similar to the previous study by Noori et al. (32), these infants were discharged from hospital within 30 days.

Not surprisingly, the findings of this study are in agreement with the existing evidence indicating that younger neonates with comorbidities require additional time to consistently exhibit oral feeding skills. On the other hand, it is interesting to observe that until now, few studies have explored the effects of comorbid conditions on the feeding process among VPT infants (6,19,23,24). The present study appears to be the first to evaluate and demonstrate the effects of KMC and NNS provision on time to FOF. However, the current study has several limitations. This was a single center study with a retrospective design, which aimed at examining well-known factors in neonates. There may be other variables not evaluated in this study that possibly affect FOF. Also, it is known that the type/mode of feeding has an impact on the feeding process. Unfortunately, due to the fact that the majority of our study subjects received nutritional support with formula and/or bottles, it was not possible to determine the type/mode of feeding which has a possible influence on the time to achieving FOF.

Conclusion

In conclusion, this is one of the few studies to demonstrate the timing of transition to FOF and its determinants in VPT infants. Further multicenter studies with further infants are required to provide guidance on interventions aimed at shortening time to FOF as well as to provide more insights into the roles of KMC, NNS and breastfeeding on the VPT population.

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Ethics

Ethics Committee Approval: The study protocol was approved by the University of Health Sciences Turkey, Kocaeli Derince Training and Research Hospital, Clinical Research ethics committee (no: 2020/72).

Informed Consent: Retrospective the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Design: İ.E., A.G., Data Collection: İ.E., A.G., Analysis or interpretation: İ.E., A.G., Literature Research: Writing: İ.E., A.G.

Conflict of Interest: The authors declared no conflict of interest.

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Original Article

The Validity and Reliability Study of the Turkish Version of the Preterm Oral Feeding Readiness Assessment Scale (T-POFRAS)

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ABSTRACT

Aim: To test the validity and reliability of the Turkish version of the Preterm Oral Feeding Readiness Assessment Scale (T-POFRAS) in order to add this scale to the literature.

Materials and Methods: A methodological study was conducted with 90 pre-terms in a neonatal intensive care unit in a state hospital.

Results: The best cut-off score value was 29 from the receiver operating characteristics analysis. For the 1st observer, 100% sensitivity and 95.7% specificity were seen at a cut-off score of 29, whereas for the 2nd observer, 95.5% sensitivity and 97.8% specificity were seen. The inter-rater agreement was quite high when the two observers were divided into groups according to their cut-off scores (Kappa=0.933; p=0.0001). The inter-rater agreement was 96.7% (in 87 preterms).

Conclusion: This study showed that the validity of the Turkish version of T-POFRAS was acceptable.

Keywords: Preterm infant, reliability and validity, feeding behavior, enteral nutrition

Introduction

Oral feeding of preterm infants is a complex and dynamic process that consists of the interaction of oral-motor, neurological, cardiorespiratory, and gastrointestinal systems (1). However, preterm infants encounter a variety of difficulties in the first weeks of their lives including neuro-developmental retardation as well as physiological and behavioral irregularities (2).

Achievement of oral feeding is explained with the synactive theory. This theory proposes that three subsystems (autonomic, motor, and behavioral state) of preterm infants are compliant with each other and coordination of these subsystems occurs from behavioral organization by the infant during potential maturation and normal development. These three systems affect each other, and disorganization of any system affects the function of the other systems. Thus, achievement of oral feeding requires the normal functioning of these systems (3,4).

Ensuring normal growth and development of preterm infants is closely related to feeding. Some studies

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have examined the feeding of preterm infants from a multidimensional perspective and reported on the negative effects of feeding problems on the growth and development of infants (5-7). The criteria determined by the American Academy of Pediatrics for the hospital discharge of high-risk neonatal infants include "oral feeding sufficient to support appropriate growth" (8).

Previous studies also report that various factors play a role in a preterm infant's readiness for being fed. These factors include neurological maturation, severity of any disease, and the infant's ability to reorganize the autonomic, motor, and behavioral state systems between two feeding periods (1,3,8). In this context, there are often difficulties regarding a successful start of oral feeding and the achievement of full oral intake. Although there are several universally recognized oral feeding practices for premature infants (for example, feeding is not preferred for extremely premature infants in the first weeks or when intubated orally), individualized oral feeding protocols are required for the majority of premature infants (8).

Studies on preterm infants have also addressed the type of nutrition as a nutrition strategy in three stages: Parenteral Nutrition, Parenteral Nutrition + Enteral Nutrition, and Enteral Nutrition (9-11). National and international guidelines agree (12-16).

A standard method is not followed for every baby in the transition of preterm babies from parenteral nutrition to breastfeeding. It is not always possible to know for certain whether a preterm infant is ready for oral feeding. The infant who is thought to be ready sometimes fails and oral feeding is delayed (17-19). However, there are cue-based feeding models and scales in the literature evaluating an infant's readiness for oral feeding that assess all physiological systems during their development (17-19). In the cue-based feeding model, oral feeding of the baby should be started and feeding should be terminated if the baby shows signs of stress (20). These cues; responding to the gentle touches on the baby's face, tolerating full enteral feeding, licking their lips with their tongue, opening their mouth, turning to a stimulus close to their mouth, showing sucking behavior, bringing their hand to their mouth, oxygen saturation at normal values during feeding, the baby maintaining a state of alertness during the feeding process, keeping their body in a flexed posture, lowering their tongue to take the pacifier and licking the bottle teat (20-22).

In Cochrane Review (Instruments for assessing readiness to commence suck feeds in preterm infants: effects on time to establish full oral feeding and duration of hospitalization), a preliminary search revealed three instruments designed to aid neonatal care providers in determining preterm infants' readiness to commence feeding (23).

The Preterm Infant Nipple Feeding Readiness scale is a 10-item scale that scored variables such as gestational age, post-conceptual age, color and activity, state regulation, hunger cues and tone. Subsequently, this instrument was renamed the Feeding Readiness and Progression in Preterms scale (24). The second instrument found was the Early Feeding Skill (EFS) assessment tool, which not only aims to assess feeding readiness but also feeding skill and feeding recovery. The feeding readiness section of the EFS consists of five items that assess an infant's readiness to commence oral feeds by observing their tone, energy level, state of arousal and oxygen saturation (25). The Neonatal Oral Motor Assessment scale (NOMAS) measures infants' nutritive sucking behaviors. Some studies have investigated the NOMAS psychometric characteristics within a healthy preterm population (26).

Lastly, the Preterm Oral Feeding Readiness Assessment scale (POFRAS) was developed and tested. POFRAS is an 18-item preterm infant oral feeding readiness instrument consisting of items in relation to corrected gestational age, behavioral state, global posture and tone, gag reflex, tongue movement and cupping, jaw movements and maintenance of an alert state. Each item was scored from 0 to 2 with a possible maximum score of 36 (27,28).

As stated in the POFRAS study, health care professionals have difficulty determining the appropriate and safe time to start oral feeding in preterm infants (27-29). Moreover, when preterm infants switch from gastric tube feeding to oral feeding, it is one of the biggest concerns of health care professionals, and therefore these professionals require an objective criterion to support the start of this process (22,27,29). The POFRAS scale is an objective scale that can be easily used by healthcare professionals. Scoring requires as little as a few minutes. Preterm babies whose physiological stability is considered to be suitable for oral feeding are determined to be ready to be fed orally by use of this scale.

The scale ensures an objective assessment of readiness for oral feeding and can have a positive effect on recognition of and support for oral feeding readiness, shorter hospitalization, and a reduction in health expenses. Supporting evidence-based oral feeding through a meticulous assessment or using evidence-based guidelines maximizes the infants' and caregivers' hospital experience, and can increase parent-infant bonding and parent satisfaction (29).

No measurement tool is known to be available in Turkey to assess the readiness of preterm infants for oral feeding. This study tested the validity and reliability of the Turkish version of POFRAS in order to add this scale to the literature.

Materials and Methods

Participants

This methodological study was conducted in a neonatal intensive care unit of a state hospital in a province located in western Turkey. The population of the study included preterm infants who were admitted to the neonatal intensive care unit, whose corrected gestational age was ≤ 36 weeks + 6 days, and who could not be fed orally. The infants had no face deformity and no respiratory, cardiovascular, gastrointestinal, or neurological disorders or syndromes that would prevent or aggravate oral intake.

The sample of the study included 90 preterm infants who matched these criteria, which was five times higher than the 18 items included in the scale. The sample size was determined based on the number of items included in the scale (30).

Instruments

The study data were collected using a "Preterm Infant Introductory Information Form" and the "POFRAS". The introductory information form for preterm infants had seven questions including the infant's age, gestational age, corrected gestational age, age in days, birth weight, current weight, and problems experienced during labor. POFRAS is an observational scale developed by Fujinaga et al. (28) and a pilot study of this scale with 10 preterm infants and the original study with 30 preterm infants were conducted with individuals who fitted the following inclusion criteria: Corrected gestational age <36 weeks and 6 days; clinically stable; absence of facial deformities; an absence of respiratory, cardiovascular, gastrointestinal and neurological disorders or syndromes that prevent or make oral feeding difficult; and not having received oral feeding of milk. The scale includes five categories (corrected gestational age, behavioral organization, oral posture, oral reflexes, and non-nutrive sucking) with a total of 18 items and it assesses preterm infants' readiness for oral feeding. Each items is scored from 0 to 2, and the maximum score of the scale is 36. The cut-off score to switch a preterm infant to oral feeding is 30 (28). The Kappa coefficient calculated to evaluate the inter-rater agreement was very good (>0.85).

Data Collection

The data were collected by two neonatal intensive care unit nurses who were the observers. The preterm infants were evaluated using POFRAS 15 minutes before feeding time. The observers did not orally communicate with each other. First, one of the observers placed the infant in an incubator in a lateral decubitus position and awakened the infant through gentle tactile touching or calling the infant by their name. Following this, both of the researchers simultaneously observed the behaviors of the infant. Biting, sucking reflex, and non-nutrive sucking included in the scale items were evaluated twice by the observers who were wearing gloves and using the second finger. Non-nutritive sucking was evaluated over a one-minute period. After both observers gave scores using the scale, the researcher fed the infant via the finger feeding method. Finger feeding is an alternative feeding method and provides a temporary feeding method for preterm infants (31,32). The researcher allowed the preterm infant to suck 5ml of breast milk using a 5ml non-piston injector by fixing a 6 French feeding tube which was 40 cm long to the second finger of the gloved hand with medical tape. Milk flow was maintained from the injector which was at the same level as the infant during feeding by the help of the preterm infant's sucking pressure; therefore, milk flowed into the oral cavity when the infant sucked.

Breastfeeding should be stopped if the presence of sucking does not occur within five minutes or symptoms that damage the stability of the preterm infant emerge (apnea, bradycardia, cough, saturation decrease, change in skin color, nasal flaring, hiccup, gagging, etc.) (25,33,34). Studies regard an infants' ability to be fed with 5ml of breast milk as the "gold standard" (28,35).

Data Analysis

The data obtained from the study were analyzed using the statistical package for the social sciences version 17 software package. The significance level was $p<0.05$. The data were analyzed to test validity including linguistic validity, content validity, and criterion validity. For criterion validity, the cut-off score of the POFRAS was compared with global accuracy, sensitivity, and specificity using the gold standard, the receiver operating characteristics (ROC) curve. The Kappa coefficient for inter-rater reliability, intra class correlation (ICC), and Kappa agreement for each scale item were calculated within the scope.

Ethical Considerations

To test the validity and reliability of the POFRAS in Turkish, permission was obtained via e-mail from the

authors who developed the scale. Following this, approval was obtained from the ethics committee of non-invasive clinical trials of the Pamukkale University (January 16th, 2018), and then legal permission was obtained from the state hospital where the study was conducted. Written informed consent was obtained from the parents of the preterm infants within the study after informing them about the study aims.

Results

Descriptive Characteristics of Preterm Infants

The study found that 58.9% of the infants included in the study were female and 41.1% were male. Of the problems experienced during birth, 84.4% were respiratory distress (RD) and 15.6% were RD + small for gestational age. The mean gestational age was 33.70 (±1.80) weeks, the mean corrected gestational age was 34.49 (±1.56) weeks, the mean age of the infants was 5.86 (±5.47) days, their mean birth weight was 2,061.25 (±404.51) grams, and their mean current weight was 2,024.90 (±370.93) grams (Table I).

Validity

Language and Content Validity

The linguistic validity was tested firstly for the validity practices of the scale. The scale was translated from English to Turkish by a pediatrician, a nurse academic, and a translator, all of whom have an advanced command of English. These three versions of translation were combined into one and finalized by the researchers. Following this, the resulting form was translated back from Turkish to English by a neonatologist, a translator, and a pediatrician, all of whom write and speak both languages very well and who had never seen the scale before. Following this, the translation was compared with the original scale by the researchers and it was finalized. Regarding content validity, eight specialists including three nurse academics in pediatric nursing, one neonatologist, and four pediatricians were asked to score from 1 to 3 using a triple scoring system (1=essential, 3=not necessary) to assess the applicability of the scale items.

Lawshe's (36) technique ranks the opinions of the specialists as (a) "Essential," (b) "Useful, but not essential," and (c) "Not necessary." In the present study, the content validity ratio (CVR) was 0.75 and the content validity index (CVI) was 1. Since CVI was higher than CVR, the content validity of the scale was statistically significant. This result indicates the comprehensibility of the scale items (36).

Criterion Validity

Figure 1 shows the area under curve (AUC) values calculated on the ROC curve for each observer.

The best cut-off score based on "Youden index" values was 29 from the ROC analysis. For the 1st observer, 100% sensitivity and 95.7% specificity were seen at a cut-off score of 29, whereas for the 2nd observer, 95.5% sensitivity and 97.8% specificity were seen (Table II).

The inter-rater agreement was quite high when the two observers were divided into groups according to their cut-off scores (Kappa=0.933; p=0.0001). The inter-rater agreement rate was 96.7% (in 87 people) (Table III).

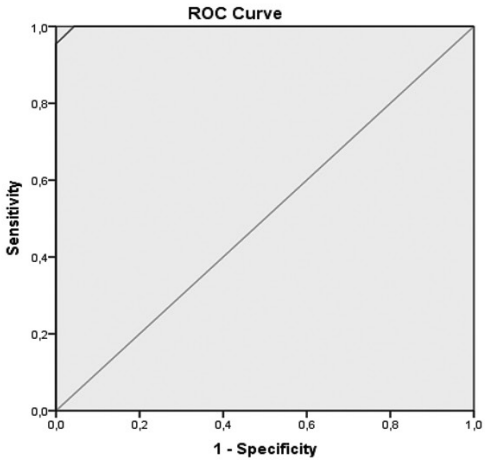
Reliability

The Kappa agreement of the scale items was fair only for three items (16.6%), whereas it was very good for fourteen items (83.3%) (Table IV).

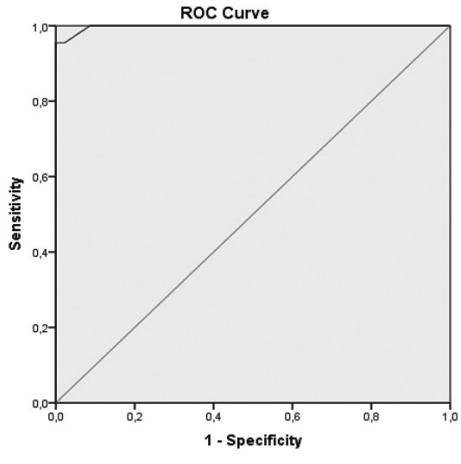
Table I. Descriptive characteristics of neonatal preterm infants

Descriptive characteristics		**Mean ± SD**	**Min. - max.**
Gestational age (weeks)		33.70±1.80	(29-36)
Corrected gestational age (weeks)		34.49±1.56	(29.4-36.6)
Days of life		5.86±5.47	(1-35)
Birth weight (grams)		2061.25±404.51	(1,290-2,950)
Current weight (grams)		2024.90±370.93	(1,230-2,830)
		Number	**Percentage (%)**
Gender	Female	53	58.9
	Male	37	41.1
Problems at birth	RD	76	84.4
	RD/SGA	14	15.6

SD: Standard deviation, Min: Minimum, Max: Maximum, RD: Respiratory distress, SGA: Small for gestational age



1st Observer AUC=0.999 (95% CI=0.996-1)



2nd Observer AUC=0.998 (95% CI=0.993-1)

Figure 1. Receiver operating characteristics curve (Global accuracy)
ROC: Receiver operating characteristics, AUC: Area under curve, CI: Confidence interval

Table II. Youden index values and Kappa coefficients for both observers				
	Sensitivity (%)	**Specificity (%)**	**Kappa**	**p-value**
1st observer	100	95.7	0.956	0.0001*
2nd observer	95.5	97.8	0.933	0.0001*

Table III. Distribution by the inter-rater cut-off scores		
Cut-off score	**1st observer (n)**	**2nd observer (n)**
≤29	44	47
≥28	46	43
Total	90	90

The ICC value was ICC=0.997 [95% confidence interval (CI)=0.996-0.998] and statistically significant.

Discussion

In this study, highly significant values as a result of the reliability and validity analysis of the Turkish version of the scale were found, in parallel with the original version of the POFRAS. The CVR was 0.75 using Lawshe's (36) technique. This result indicates the comprehensibility of the scale items in terms of linguistic validity.

The most commonly used method to determine the appropriate cut-off score with the highest accuracy is the ROC curve method. To use this method, a reference reported as the "gold standard" is required. The area below the curve is the AUC (AUC; 0.5<AUC<1) (37,38). The reference determined as the "gold standard" in the present study was the "Finger Feeding" nutrition method.

In this study, the cut-off score was 29 from the ROC analysis. Of the three cut-off scores (28, 29, and 30) specified in the original scale, the highest (30) was taken. It is compatible with the cut-off score specified in the present study. Having a cut-off score makes a scale objective, which makes the scale user-friendly, fast, and practical for health care professionals to determine readiness for oral feeding. The AUC score in the original scale was significant with a value of 0.5<AUC, as it was in the present study.

Regarding reliability, inter-rater agreement was calculated using the Kappa coefficient. Fleiss classified the agreement levels of a Kappa score of 0.75 or higher as very good, 0.40-0.75 as fair, and lower than 0.40 as poor (39). The inter-rater Kappa coefficient of agreement in the present study (0.93) showed a very good agreement as in the original version of the scale (0.85). Furthermore, the majority of the scale items (82.3%) showed a very good

Table IV. Inter-rater Agreement-Kappa (K) on POFRAS items for 90 infants

Scale items	Kappa	Qualitative Assessment
Corrected gestational age	1.00	Very good
Behavioral state	0.96	Very good
Global posture	0.97	Very good
Global tonus	1.00	Very good
Lips posture	0.95	Very good
Tongue posture	1.00	Very good
Rooting reflex	1.00	Very good
Sucking reflex	0.95	Very good
Biting reflex	0.74	Fair
Gag reflex	0.75	Very good
Tongue movement	0.95	Very good
Tongue cupping	1.00	Very good
Jaw movement	0.97	Very good
Sucking strain	0.87	Very good
Sucking and pause	0.70	Fair
Maintenance of sucking/pause	0.90	Very good
Maintenance of alert state	0.86	Very good
Stress signs	0.70	Fair

POFRAS: The Preterm Oral Feeding Readiness Assessment scale

Kappa agreement. In addition, the inter-class ICC score was highly significant at the 95% CI.

The POFRAS scale can be used in Turkish culture by health care professionals (physicians, nurses, etc.) who work in neonatal intensive care units. It can be used to determine the readiness of preterm infants who do not have a feeding barrier for oral feeding.

Preterm infants' readiness for oral feeding is a longer and more complex process compared to those who are full-term. This process is dependent on the physiological development of the infant. If a full-term infant does not have any pathology to prevent it, it can be fed orally immediately after delivery because it has completed its developmental processes and its body systems are coordinated. However, this situation is more complex for preterm infants. The infant needs a suitable period of time to be ready for oral feeding and to strengthen its physiological stability. The coordination of all the body systems prior to the transition to oral feeding makes the feeding process faster and easier. Therefore, cardiorespiratory and neurological (motor, autonomic, and behavioral) systems, not just a single system, should work in harmony prior to oral feeding.

To improve physiological stability, some interventions are required for preterm infants. These include feeding the infant either with a parenteral or a gastric tube or providing a mixed diet (parenteral and gastric feeding together) until the infant gains spontaneous breathing ability. As the infants' body systems are developing and improving, their abilities are expected to change over time. The change in the infant's skills over time should be evaluated so the interventions meet the infant's needs. The suction reflex of the infant who can achieve spontaneous breathing can be strengthened by non-nutritive suction methods. During this time, the preterm infant improves their suction reflex and learns to regulate their suction pressure and rhythm.

Those scales that evaluate readiness for oral feeding are guidelines that provide a reliable and valid way to systematically monitor and assess the development of skills for feeding. They function as a guide in the selection of interventions to best support the skills required for oral feeding. After individualized interventions for specified goals are determined, ensuring the infant's ability for spontaneous nutrition, coordinating breathing, coordinating breathing and swallowing, regulating and managing milk flow, and maintaining stable physiological interactions during feeding must be assessed continually.

For example, a score less than 29 in POFRAS shows that the infant is not ready for oral feeding. If an infant has a score greater than 29, oral feeding methods may be applied.

Study Limitations

The scale is only suitable for healthy preterms. It is not to be used for term babies and those preterm babies who have pathologies that can prevent feeding.

Conclusion

The Turkish Version of the Preterm Oral Feeding Readiness Assessment scale [T-POFRAS (Appendix 1)] was analyzed for reliability and validity, and the study concluded that the validity of the inter-rater agreement was high, the inter-rater coefficients of consistence were fair, and the Kappa agreement of the scale items was very good in general. Health care professionals who work in neonatal intensive care units are recommended to use the T-POFRAS to determine neonatal preterm infants' readiness for oral feeding. This version will enable health care professionals to assess preterm neonatal infants' readiness for oral feeding, and then objectively determine the process of determining when to switch to oral feeding.

Ethics

Ethics Committee Approval: The study approval was obtained from the ethics committee of non-invasive clinical trials of the Pamukkale University. Research ethics committee (approval no: 60116787-020/4304, date: 16.01.2018).

Informed Consent: Written informed consent was obtained from the parents of the preterm infants within the study after informing them about the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Z.Ç., B.Ç., Data Collection or Processing: Z.Ç., B.Ç., Analysis or Interpretation: Z.Ç., B.Ç., Literature Search: Z.Ç., B.Ç., Writing: Z.Ç., B.Ç.

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Appendix 1. Turkish version of the Preterm Oral Feeding Readiness Assessment Scale (T-POFRAS)

Corrected gestational age	**(2) ≥34 weeks**	**(1) Between 32 and 34 weeks**	**(0) ≤32 weeks**	
Behavioral organization				
Behavioral state	(2) Alert	(1) Drowsy	(0) Sleep	
Global posture	(2) Flexed	(1) Partly flexed	(0) Extended	
Global tonus	(2) Normotonia	(0) Hypertonia	(0) Hypotonia	
Oral posture				
Lips posture	(2) Closed	(1) Half-open	(0) Open	
Tongue posture	(2) Flat	(0) Elevated	(0) Retracted	(0) Protruded
Oral reflexes				
Rooting reflexes	(2) Present	(1) Weak	(0) Absent	
Sucking reflexes	(2) Present	(1) Weak	(0) Absent	
Biting reflexes	(2) Present	(1) Exacerbated presence	(0) Absent	
Gag reflexes	(2) Present	(1) Present in anterior region	(0) Absent	
Non-nutritive sucking (The test should take 1 minute)				
Tongue movement	(2) Adequate	(1) Altered	(0) Absent	
Tongue cupping	(2) Present		(0) Absent	
Jaw movement	(2) Adequate	(1) Altered	(0) Absent	
Sucking strain	(2) Strain	(1) Weak	(0) Absent	
Sucking and pause	(2) 5 to 8	(1) >8	(0) <5	
Maintenance of rhythm	(2) Rhythmic	(1) Arhythmic	(0) Absent	
Maintenance of alert state	(2) Yes	(1) Partial	(0) No	
Stress signs	(2) Absent	(1) Up to 3	(0) More than 3	
Saliva accumulation	( ) Absent	( ) Present		
Nose wings trembling	( ) Absent	( ) Present		
Skin color change	( ) Absent	( ) Present		
Apnea	( ) Absent	( ) Present		
Tonus variation	( ) Absent	( ) Present		
Posture variation	( ) Absent	( ) Present		
Tongue or jaw tremors	( ) Absent	( ) Present		
Hiccupping	( ) Absent	( ) Present		
Crying	( ) Absent	( ) Present		
Maximum score: 36				

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Original Article

Association of Vitamin D Status with Morbidity in Children with Sickle Cell Disease in Tertiary Care Hospital

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ABSTRACT

Aim: Children with sickle cell disease (SCD) are at high risk of vitamin D deficiency (VDD). The prevalence of VDD in different countries is between 65-100% in these patients. The present study was undertaken to find the prevalence of VDD among sickle cell children and to assess the association of co-morbidities with VDD.

Materials and Methods: Total 89 children who were attending a sickle cell clinic/admitted to paediatric wards of a Tertiary Care Centre were enrolled in this study. After their history, clinical examination and anthropometry were investigated, samples were taken for serum 25-hydroxy vitamin D ng/mL and calcium level assessment. The outcome was morbidity in sickle cell children in terms of frequency of pain episodes, number of febrile episodes, number of blood transfusions and total number of admissions.

Results: Out of 89 cases, 58 (65.17%) cases were deficient in vitamin D (<20 ng/dL), 22 (24.72%) cases had insufficiency (20-30 ng/dL) and 9 (10.11%) cases had normal vitamin D levels (>30 ng/dL). The mean vitamin D level was 19.42ng/dL. Morbidity in SCD was more in VDD children compared to vitamin D sufficient children with significance in the number of pain episodes and the total number of hospital admissions but not in the number of admissions for acute febrile illness or the total number of blood transfusions.

Conclusion: VDD was prevalent in 65.17% of children with SCD. Children between 4-12 years were more affected with a male predominance. As this study involved children with SCD alone, future studies need to be carried out involving children without SCD to establish a better possible link between vitamin-D and SCD morbidity.

Keywords: Sickle cell, vitamin D, deficiency, co-morbidities, anthropometry, prevalence

Introduction

Sickle cell disease (SCD) is the most common hemoglobinopathy worldwide. It is caused by a mutation resulting from an exchange of nitrogenous bases in the sixth codon of the beta-globulin haemoglobin gene, generating abnormal haemoglobin called haemoglobin S (HbS) (1). SCD has a high prevalence in India, especially in the central and western regions, and has a considerable health burden (2). As per an Indian Council of Medical Research survey, about 20% of children with SCD expired by the age of two and 30% of children with SCD among the tribal community die before they reach adulthood (2,3).

Children with sickle cell anaemia (SCA) have a higher risk of developing nutritional deficiencies due to reduced

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appetite (4), poor dietary intake of nutrients and infectious complications, (5) which demand greater attention from health care professionals. Among the vitamins, vitamin D must be carefully evaluated in children with SCA because these patients are more likely to develop vitamin D deficiency (VDD) when compared to healthy controls (6). This is due to the high concentration of melanin in the skin, low levels of physical activity and low food intake (5). Calcium and vitamin D are important for bone metabolism, and low calcium intake leads to a reduction in the ideal bone mass peak in children and adolescents with SCA, which leads to growth failure (7). VDD, in turn, is associated with increased respiratory infections, muscle weakness and increased risk of falls and micro lesions (8).

In addition, SCD is a genetic disorder with various life-threatening organ-system complications like periodic vaso-occlusive crises, chronic haemolysis, jaundice, infarcts and acute chest syndrome (1). Vaso-occlusive crises are the result of interactions between sickle erythrocytes, inflammatory cytokines and endothelium. VDD, which has effects on endothelial dysfunction and cytokines, has possibly contributed to the pathogenesis of SCD (6). Patients exhibit elevated leukocyte counts, abnormal activation of granulocytes, monocytes, and endothelial cells, and increased levels of multiple inflammatory mediators. Finally, SCD is an inflammatory condition and vitamin D binding protein has been shown to decline in inflammatory conditions. New literature suggests there is an association between VDD and increased anaemia in patients with chronic anaemia (9). Patients with SCD are susceptible to all of these complications, although it is unclear to what extent VDD is a contributing causal factor.

VDD is seen frequently in patients with SCD and it has emerged as a public health focus in recent years for its contribution to adverse skeletal and extra-skeletal manifestations (10). However, the relationship between inflammation and VDD in SCD pathogenesis has not been investigated to date. VDD is common in Indian children and there is well documented literature showing vitamin D in SCD is lower than in the general population (6). VDD can be reliably and inexpensively treated making it a prime intervention to potentially improve health outcomes among those with SCD. To date, prophylactic vitamin D supplementation is not practiced by a majority of physicians. Despite this evidence, VDD remains both under-recognized and under-treated in patients with SCD. Studies from other countries have documented that SCD children are 5.3 times more likely to be deficient in vitamin D compared to healthy controls (6). However, limited data is available from India with respect to the potential association between VDD and its association with the co-morbidities observed in SCA. Hence, the present study was conducted to study the association of vitamin D status with morbidity in children with SCD.

Materials and Methods

The present cross-sectional study was conducted on 89 children of both sexes, aged between 2-18 years with SCD with "SS" and "SF" pattern, diagnosed by high-performance liquid chromatography, who were attending a sickle cell clinic or admitted to the paediatric wards of a Tertiary Care Centre in central India during a period of 2 years from September 2016 to August 2018. Children on hydroxyurea therapy, sickle cell children with chronic diseases or on vitamin D supplementation were excluded from this study. Ethical clearance and approval was obtained from Institutional Ethical committee and written informed consent was taken from the parents of the patients.

A detailed history, clinical examination and anthropometry were taken and data was collected in structured data collection forms. Blood samples were drawn in plain tubes for 25-hydroxyvitamin D [25(OH)D] and serum calcium. The estimation of vitamin D was done by radio-immune assays using chemiluminescent protein binding assay because of unavailability and cost factors. The classification of VDD based on serum levels of vitamin D according to Indian paediatrics guidelines (11) are as follows; 1) deficiency <20 ng/dL, 2) insufficiency 20-30 ng/dL and 3) Sufficiency >30 ng/dL. Calcium was estimated by CALC - Arsenazo III method. The outcome for morbidity in sickle cell children was determined in terms of the frequency of pain episodes, the number of admissions for acute febrile illness, the number of blood transfusions and the total number of admissions. Data for morbidity was obtained from the records in the sickle cell clinic and from a timeline which was given for sickle cell children.

Statistical Analysis

Statistical analysis was done by using the software, STATA, version 10.1; 2011. Descriptive statistics like mean, and standard deviation were used for quantitative measures while frequency and percentages were used to summarize qualitative measures. Inferential statistics included hypothesis testing procedures like Pearson's chi-square test for assessing differences in proportions and testing associations. Two-independent samples t-test was used for assessing differences in the means of groups. P-value less than 0.05 was considered statistically significant.

Observations and Results

A total of 89 children with SCD (SS-88 children and SF-1 child) were enrolled in this study, of whom 50 (56%) were males and 39 (44%) were females. The majority of cases were in the age group of >4-8 years (40.45%) followed by >8-12 years (34.83%), 2-4 years (13.48%) and >12-18 years (11.24%). The mean age of patients was 7.32±3.27 years, ranging from 2.5-16 years.

VDD was reported in 58 (65.17%) children with SCD, 22 (24.72%) cases had insufficiency and 9 (10.11%) cases had normal vitamin D levels as depicted in Figure 1. The mean vitamin D level was 19.42 ng/dL, ranging from 5.12 to 62.05 ng/dL.

Table I shows the comparison of vitamin D levels with age and sex, which also shows no relationship of vitamin D levels with age and sex.

Nutrition status is classified into undernourished and normal based on WHO and CDC classification criteria. Out of 89 cases, 26 (29.21%) children had normal nutrition and 63 (70.79%) children were undernourished. VDD was more among undernourished than children with normal nutrition, with a p-value of 0.001, which was statistically significant (Figure 2), (Table II).

1. Vitamin D deficiency in SCD children was associated with increased pain episodes compared to vitamin D sufficient children (p=0.001).

2. Number of hospital admissions for acute febrile illness had an inverse relationship with vitamin D levels with a p-value of 0.152 which was not statistically significant. Thus, VDD was associated with an increased risk for admissions for acute febrile illness but not significantly.

3. Number of blood transfusions had an inverse relationship with vitamin D level with a p-value=0.728 which was not statistically significant.

4. Total number of hospital admissions had an inverse relationship with vitamin D levels. Decrease in vitamin D levels increases the number of total admissions in deficient group > insufficient group > normal vitamin D level with a p-value=0.018.

The comparisons of mean values of co-morbidities (number of pain-crises, acute febrile illness, blood transfusions and total number of admission) between VDD and vitamin D sufficiency groups are shown in Table III. The mean number of pain episodes, admissions for acute febrile

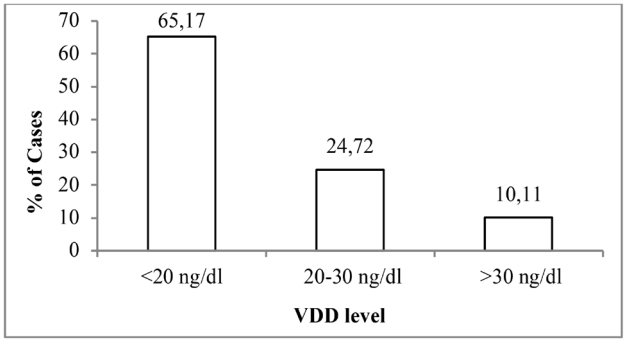

Figure 1. Distribution of cases according to vitamin D deficiency levels in SCD

SCD: Sickle cell disease, VDD: Vitamin D deficiency

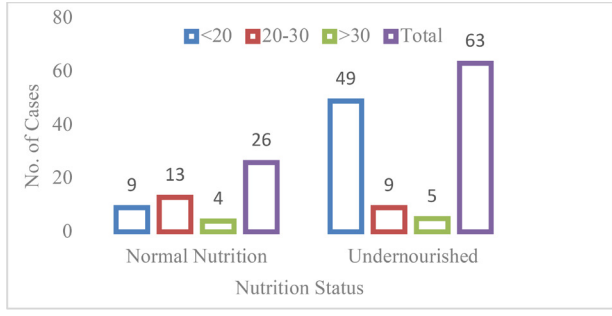

Figure 2. Association between vitamin D levels and nutrition status

Table I. Comparison of vitamin D levels with age and sex

Vitamin D level in ng/dL	No. of cases (%)	Age group in years								Total		p-value
		>2-4		>4-8		>8-12		>12-18				
		M	F	M	F	M	F	M	F	M	F	
<20	58 (65.17%)	3	2	13	12	12	10	5	1	33 (66%)	25 (64.1%)	Chi-square (2)=2.42 p=0.29
20-30	22 (24.72%)	2	2	6	3	5	2	1	1	14 (28%)	8 (20.51%)	
>30	9 (10.11%)	1	2	1	1	1	1	0	2	3 (6%)	6 (15.38%)	
Total	89 (100%)	6	6	20	16	18	13	6	4	50 (56.17%)	39 (43.82%)	
p-value	Chi-square (6)=3.92, p-value=0.688											

M: Male, F: Female

Table II. Association of co-morbidities with vitamin D status in children with SCD

Vitamin D level in ng/dL	Pain episodes category				p-value
	0-3	>3-6	>6-9	>9	
<20	9 (27.27%)	34 (82.93%)	13 (92.86%)	2 (66.67%)	0.001
20-30	16 (48.48%)	6 (14.63%)	1 (7.14%)	1 (33.33%)	
>30	8 (24.24%)	1 (2.44%)	0 (0.0%)	0 (0.0%)	
Vitamin D level in ng/dL	**Hospital admissions for acute febrile illness**				
	0-2	**>2-4**	**>4-6**	**>6**	
<20	19 (51.35%)	29 (78.38%)	5 (62.5%)	5 (71.43%)	0.152
20-30	14 (37.84%)	4 (10.81%)	3 (37.5%)	1 (14.29%)	
>30	4 (10.81%)	4 (10.81%)	0 (0%)	1 (14.29%)	
Vitamin D level in ng/dL	**Number of blood transfusions**				
	0-4	**>4-8**	**>8-12**	**>12**	
<20	51 (65.38%)	6 (66.67%)	0 (0.00%)	1 (100%)	0.728
20-30	19 (24.36%)	2 (22.22%)	1 (100%)	0 (0.00%)	
>30	8 (10.26%)	1 (11.11%)	0 (0.00%)	0 (0.00%)	
Vitamin D level in ng/dL	**Hospital admissions category**				
	0-5	**>5-10**	**>10-15**	**>15**	
<20	11 (37.93%)	34 (80.95%)	9 (69.23%)	4 (80%)	0.018
20-30	12 (41.38%)	6 (14.29%)	3 (23.08%)	1 (20%)	
>30	6 (20.69%)	2 (4.76%)	1 (7.69%)	0 (0.00%)	

SCD: Sickle cell disease

Table III. Comparison of mean values of co-morbidities between vitamin D deficiency and vitamin D sufficiency group

Co-morbidities	VDD group	Vitamin D sufficiency group	p-value
Number of pain episodes	5.60±3.25	2.22±1.64	0.01
Number of admissions for acute febrile illness	3.48±1.76	2.66±1.93	0.09
Number of blood transfusions	3.00±5.25	1.55±1.81	0.4
Total number of hospital admissions	9.01±5.30	5.11±3.01	0.02

VDD: Vitamin D deficiency

illness, blood transfusion and total admissions was more in the VDD group than the vitamin D sufficiency group.

Discussion

In the present study, the most common age group of SCD patients was 4 to 8 years, similar to that reported in the Garrido et al. (12) study. A male predominance was observed, which may be due to the fact that, in India, there is a lower healthcare utilization rate among females (13). Children with SCD are more prone to nutritional deficiency. VDD is one of the most common nutrient deficiencies among children with SCD. The prevalence of VDD in young children is around 50-90% in the Indian subcontinent (14). In the current study, VDD was found in 58 (65.17%) children, insufficiency in 22 (24.72%) and sufficient in 9 (10.11%) children with SCD. Thus, the study shows that 90% of children were below 30 ng/dL and 65.17% of children were deficient. These results correlated well with previous studies (12,15,16). Rovner et al. (6) defined VDD as a vitamin D level less than 11 ng/mL and insufficiency as a vitamin D level between 11 to <30 ng/mL. They found that 33% of children with HbSS were deficient, compared with only 9% of healthy children. The current study found that only 20% of children had VDD <11 ng/dL, so VDD is highly prevalent

in children with SCD. The mean vitamin D level was 19.42 ng/dL, which is comparable with the study done by Winters et al. (17) where they found the mean [25(OH)D] level was 17.2±9.5 ng/dL.

There was no correlation between vitamin D level and sex. There was an inverse relationship between age and vitamin D level but it was not statistically significant (p=0.688). Similar results are reported in the study conducted by Adewoye et al. (18) and AlJama et al. (19). VDD was more in undernourished children than in children with normal nutrition. Jackson et al. (20) reported vitamin D levels were not significantly correlated with body mass index (BMI) percentile (p=1.00)/BMI Z-score (p=0.53). Ozen et al. (21) found vitamin D levels were significantly lower in children whose height and/or weight were >2 standard deviation below the mean.

There was low vitamin D level found in an existing study which was associated with an increase in the frequency of painful crises. Vaso-occlusive crises are the result of interactions between sickle erythrocytes, inflammatory cytokines and endothelium. VDD, which has effects on endothelial dysfunction and cytokines, has possibly contributed to the pathogenesis of SCD (7). Serum [25(OH) D] ng/mL is a negative acute phase reactant, which has implications for acute and chronic inflammatory diseases. Serum [25(OH)D] ng/mL is an unreliable biomarker of vitamin D status after acute inflammatory insult (22). This may be the reason behind the increased severity of bone pain and hospitalisation due to vaso-occlusive crises. Lee et al. (16) found similar findings that serum [25(OH) D] ng/mL was associated with pain, but no significant association between serum [25(OH)D] ng/mL and acute chest syndrome. Osunkwo et al. (23) also found a significant association between VDD and painful crises. Shams et al. (24) found vitamin D administration was associated with lower postoperative analgesia requirement and postoperative complication. There was an increased risk of admissions in vitamin D deficient sickle cell children compared to vitamin D sufficient children but this was not statistically significant (p=0.220). In children with low levels of vitamin D, there was an increase in the number of blood transfusion but this was not statistically significant (p=0.728). These findings are in accordance with earlier studies (25,26). Total number of hospitalizations was inversely proportional to the vitamin D level (p=0.018). McCaskill et al. (27) also found similar results that vitamin D serum levels are inversely associated with medical record reported hospitalizations visits (p=0.04).

Study Limitations

The seasonal variation of vitamin D levels was not considered in the present study. The presence of sickle cell nephropathy, which could significantly contribute to development of VDD, was not evaluated. We also did not determine the prevalence of lactose intolerance, the dietary intake of vitamin D or other life style factors which could affect the development of VDD in this population. In order to understand the relationship between vitamin D and co-morbidities, a molecular study needs to be carried out in patients with SCD to observe changes occurring in the presence of decreased levels of vitamin D.

Conclusion

Vitamin D was one the most common nutrient deficiencies encountered in sickle cell children with a prevalence of 65.17% in the present study. Therefore, the authors suggest regular monitoring of serum vitamin D levels in patients with SCD and, subsequently, supplements to relieve pain, enhance efficiency, reduce the development of anaemia and reduce the requirement of hospital admission in patients with insufficient vitamin D. As this study involved children with SCD alone, a future study needs to be carried out involving children without SCD to establish a better possible link between vitamin D and SCD morbidity.

A clinical trial with vitamin D supplementation is planned. This trial will help us to prove the hypothesis that there is indeed a relation between vitamin D and sickle cell anaemia.

Ethics

Ethics Committee Approval: The study was given the Institutional Ethical committee approval from Government Medical College, Nagpur Maharashtra, India (protocol no: 201627, date: 14.10.2016).

Informed Consent: Written informed consent was obtained from the parents.

Authorship Contributions

Concept: D.J., Design: D.J., H.K., Data Collection or Processing: D.J., H.K., Analysis or Interpretation: D.J., H.K., Literature Search: D.J., H.K., Writing: D.J., H.K.

Conflict of Interest: There is no conflict of interest is declared by the authors.

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Original Article

Prevalence, Determinants and Impact of Haemoglobin Phenotype Misdiagnosis Among Parents of Children Living with Sickle Cell Disease in Nigeria

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ABSTRACT

Aim: Nigeria has the highest number of children with sickle cell disease (SCD) with a consistent prevalence of 2 to 3% despite increased awareness and widespread premarital screening. In our practice, we observed that complaints of haemoglobin phenotype misdiagnosis are common among parents of children living with SCD. The present study was thus carried out to identify the prevalence, determinants, as well as the perceived impacts of haemoglobin phenotype misdiagnosis in Lagos, Nigeria.

Materials and Methods: This study included the parents of children with SCD aged below 18 years. Interviewer administered questionnaires were used to obtain relevant biodata, sociodemographic data and an assessment of the perceived impact from both caregivers and their children, between May and July 2019.

Results: Fifty-nine (32.4%) out of the 182 parents recruited had a previous haemoglobin phenotype misdiagnosis. Misdiagnosis was significantly more in individuals in the upper social class and those that had their tests performed in private laboratories. Clinical, psychosocial and economic impacts of having an affected child with SCD were reported.

Conclusion: The frequency of wrong haemoglobin phenotype diagnosis is alarmingly rampant amongst the parents of children with SCD. This is potentially devastating to these families and to society; hence, the government needs to act by auditing and enforcing regulatory oversight of laboratories as well as instituting a nationwide new-born screening programme to replace the existing widespread use of haemoglobin electrophoresis for the diagnosis of SCD.

Keywords: Misdiagnosis, haemoglobin-phenotype, sickle cell disease, laboratory, Nigeria

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Introduction

Nigeria has the highest number of persons with the sickle cell trait and sickle cell disease (SCD) in the world (1). Yearly, about 150,000 babies are born with SCD while a quarter of Nigerians carry the sickle cell trait (1). SCD can be prevented by the early identification of carriers, genetic counselling and prenatal diagnosis (2).

A noteworthy but unfortunate factor that is contributing to the public health burden of this disorder in Nigeria is the wrong diagnosis of haemoglobin phenotypes (3). In practise, we have encountered many partners that married on the basis of compatible haemoglobin phenotypes only to be faced later with the shocking reality of both being sickle cell carriers after the birth of a child discovered to have SCD during protracted or recurrent illnesses, or even after debilitating complications like cerebrovascular accident. Such incidents have resulted in broken homes or marital distrust out of the misconception that one partner was dishonest about their haemoglobin phenotype status.

Alkaline haemoglobin electrophoresis is currently the most widely used method of haemoglobin phenotype testing in Nigeria and is the sole technique used by most laboratories despite its limitations (4). Aside from being the only method of diagnosis, inappropriate practices such as the use of inferior cellulose acetate electrophoresis paper and machine and also the absence of control samples may account for these misdiagnoses. The standard recommended practice, however, is to perform sickling/solubility test alongside alkaline haemoglobin electrophoresis in order to confirm samples with Hb S and ultimately to rule out the presence of haemoglobins G and D which could be found in our population and usually co-migrate with Hb S in alkaline electrophoresis (5,6). The routine use of HPLC, the gold-standard diagnostic modality, is rare in Nigeria.

Although public sensitization programmes have considerably increased the populace's awareness of sickle cell disorders, as well as the utility of screening procedures such as premarital haemoglobin phenotype testing, the expected consequent reduction in the prevalence of SCD, especially in children, is yet to be seen (7-10).

Therefore, this study investigated the burden, determinants and impact of misdiagnoses of haemoglobin phenotypes so that this knowledge may guide specific interventions that could curb or mitigate the role of misdiagnosis in the persistently high endemicity of SCD in Nigeria.

Research Questions

1. What proportion of parents of children (below 18 years) with SCD had haemoglobin phenotype testing pre-maritally?

2. How many parents knew their haemoglobin phenotype results before the birth of any of their children with sickle cell disorder?

3. How many parents of children with SCD had at least one previously wrong genotype diagnosis?

4. What are the factors associated with wrong genotype diagnosis (place of testing, number of times testing was done, socio-economic class of the primary caregiver)?

5. What was the perceived impact of the misdiagnosis of sickle cell disorder on the child, couple and family?

Aim and Objectives

General Aim: To determine the prevalence, determinants and impact of wrong haemoglobin genotype results among the parents of children living with sickle cell disorders.

Specific Objectives

1. To determine the prevalence of wrong haemoglobin genotype results among the parents of children living with sickle cell disorders.

2. To determine the determinants of wrong haemoglobin genotype results among the parents of children living with sickle cell disorders.

3. To determine the impact of wrong haemoglobin genotype results among the parents of children living with sickle cell disorders.

Materials and Methods

Study Design

The current study was a cross sectional, questionnaire-based and qualitative study, involving the parents of children with sickle cell disorder that attended the Sickle Cell Foundation, Idi-Araba, Lagos State, Nigeria between May and July 2019. The Sickle Cell Foundation is a non-governmental organization that provides services such as genetic screening, medical care, research, counselling and training related to sickle cell disorder. It receives individuals with sickle cell disorder from Lagos state and its neighbouring states due to its highly subsidized and expert care.

Study Setting, Population and Recruitment

An unknown prevalence of 50% was used in determining the sample size in the current study (11). Subjects who met

the inclusion criteria were subsequently recruited until the desired sample size was met.

Government hospitals, also called public hospitals, were defined as hospitals established and funded by the government (12).

The inclusion criteria were parents of children who had previously been diagnosed with SCD by electrophoresis and who agreed to participate in the study. These parents were recruited until the desired sample size was reached.

Those excluded from the study were parents of subjects with unknown haemoglobin phenotype, parents with no knowledge of when/where their genotype test was done and caregivers other than the biological parents.

Ethical Approval

Approval for this study was obtained from the Health Research Ethics Committee of Lagos State University Teaching Hospital (NHREC04/04/2008) and all the participants gave written informed consent while those children aged seven years and above gave their assent.

Data Collection Method

A self-designed questionnaire was used to obtain information from the study participants. The biodata and questions about time of diagnosis, place of diagnosis, and any previous wrong diagnosis in either or both parents were obtained. The impact of having a child or children with SCD on the family (parents and children) was ascertained using a previously created tool by Brown et al. (13).

Statistical Analysis

The data were analysed using Statistical Package for Social Sciences (SPSS) version 20.0. Descriptive statistics were presented as medians with interquartile range for skewed data. Continuous variables were compared between two groups using the student's t-test, categorical variables were compared between two or more groups. Statistical significance was set at p-value<0.05.

Results

A total of 190 parents and child sets were recruited, out of which 8 were excluded (4 cases of unknown genotype of parents, 2 cases of relatives as primary caregivers and 2 cases of previous recruitment of siblings). The majority of the study children were homozygous for SCD - Hb SS (n=178, 97.8%)- and 4 (2.2%) were compound heterozygous HbSC. Thus, a total of 182 children aged 6 months to 204 months with SCD and their parents' responses were analysed. The median age of children with SCD was 84 months [interquartile range (IQR)=60-123]; boys and girls had similar ages at 84 months (IQR=60-132) vs. 96 months (IQR=48-120), z=-0.494, p=0.621). A total of 103 (56.6%) of the children with SCD were males with a male-to-female ratio of 1.6:1.

As shown in Table I, about 80% of the children with SCD were less than 10 years of age and about 90% had both biological parents as primary caregiver. The majority of the mothers and fathers were in the third and fourth decade of life, respectively. About one-third of the children were from upper-income, one-third from middle-income and one-third from lower-income families.

There is significant association between the time of testing and social class. A larger proportion of those parents in the upper social class had haemoglobin phenotype tests done before marriage compared to those in the middle and lower social classes as shown in Table II.

The majority of the parents (father-mother pairs) were of AS-AS phenotypes (Figure 1). Only 9 (4.9%) couples had other sickle cell traits or homozygous sickle cell anaemia in one member.

Table I. Socio-demographic characteristics of subjects

Age group	**Frequency**	**Percentages**
≤5 years	60	33.0
6-10 years	77	42.3
11-18 years	45	24.7
Mothers' age groups	**Number of subjects**	**Percentages**
<20	1	0.5
20-29	19	10.4
30-39	101	55.5
40-49	59	32.4
≥50	1	0.5
Unknown	1	0.5
Fathers' age groups	**Number of subjects**	**Percentages**
20-29	2	1.1
30-39	44	22.9
40-49	109	56.8
≥50	34	17.7
Unknown	3	1.6
Marital status of caregivers	**Frequency**	**Percentages**
Single	3	1.6
Separated/divorced	10	5.5
Married	162	89.0
Widowed	7	3.8
SEC	**Frequency**	**Percentages**
Upper	57	31.3
Middle	63	34.6
Lower	62	34.1

SEC: Socio-economic class

Table II. Relationship between time of testing and social classes				
Time of testing	**Upper (%)**	**Middle (%)**	**Lower (%)**	**p-value**
Before marriage	53 (93.0)	45 (71.4)	43 (69.4)	χ^2=11.516, p=0.003*
After marriage	4 (7.0)	18 (28.6)	19 (30.6)	
Total	57 (100.0)	63 (100.0)	62 (100.0)	
Parental knowledge of genotype after child's diagnosis				
No	40 (70.2)	35 (55.5)	30 (48.4)	χ^2=5.956
Yes	17 (29.8)	28 (44.4)	32 (51.6)	p=0.05
Total	57 (100.0)	63 (100.0)	62 (100.0)	
*: Significant p-value				

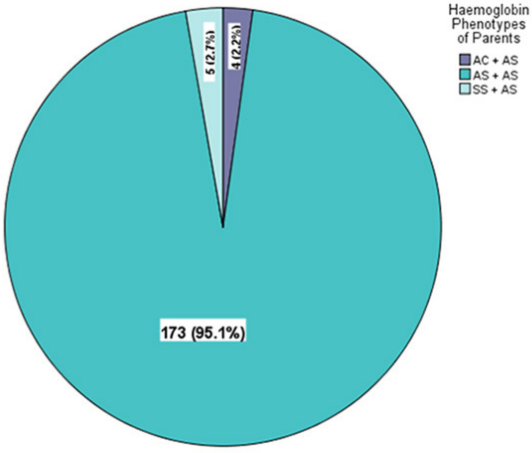

Figure 1. Haemoglobin phenotypes of the parents

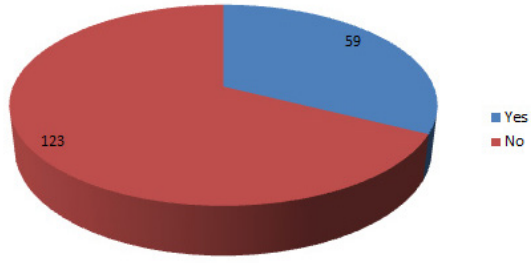

Figure 2. Frequency of haemoglobin phenotype misdiagnosis

Frequency of Wrong Haemoglobin Phenotyping

Almost one-third (n=59/182, 32.4%) of either parent had been previously mistyped as HbAA (Figure 2). Three of the four mothers with HbAC had been previously misdiagnosed as HbAA.

Factors Associated With Misdiagnosis

Those parents who had had multiple haemoglobin phenotype tests done had a lower occurrence of misdiagnosis compared to those who had done the test only once [37 (62.7%) vs 22 (37.3%); χ^2=49,860, p=0.0001].

In all, 62 (34.1%) and 63 (34.6%) parents are in the lower and middle social class respectively. Individuals in the upper social class had a significantly higher proportion of misdiagnosis compared to those in the middle and lower social classes [26 (44.1%) vs 23 (39.0%) vs 10 (16.9%); χ^2=12,520, p=0.002].

A significant proportion of parents who had haemoglobin phenotype misdiagnosis had the investigation done at various private laboratories and private hospitals compared to those done in government hospitals as shown in Table III.

Perceived Impacts of Misdiagnosis

Among the caregivers with a previous misdiagnosis of haemoglobin phenotype, about two-thirds (n=44/59, 74.6%;), one-fifth (n=13/59, 22.0%) and less than a tenth (n=2/59, 3.4%) had one, two and three children with SCD, respectively. Figure 3 lists the various clinical and psychosocial impacts of having a child with SCD identified in subjects with previous wrong genotype testing.

Discussion

As high as 42.3% of the parents in the present study realised they had a sickle cell trait after the birth of an affected child. This is similar to previous report by Ezenwosu et al. (14) in the South-Eastern part of Nigeria. A new-born screening programme for SCD is currently not available in Nigeria and very few health facilities have the highly specific diagnostic methods such as high-performance liquid chromatography (HPLC), which is very expensive. Haemoglobin electrophoresis machines are relatively cheaper, widely available in many centres and thus remain the most commonly used method for the diagnosis of SCD in Nigeria (14). In the last few decades, Nigeria has witnessed increased awareness of primary prevention of SCD among its populace such that premarital haemoglobin

Table III. Relationship of genotype test results to the place of testing

Previous wrong haemoglobin genotype testing			
Place of testing	**No (%)**	**Yes (%)**	**Total (%)**
Government hospitals	45 (83.3)	9 (16.7)	54 (100)
Private hospitals	42 (66.7)	21 (33.3)	63 (100)
Private laboratories	36 (55.4)	29 (44.6)	65 (100)
Total	123 (67.6)	59 (32.4)	182 (100)
$\chi 2$=10,553, p=0.005			

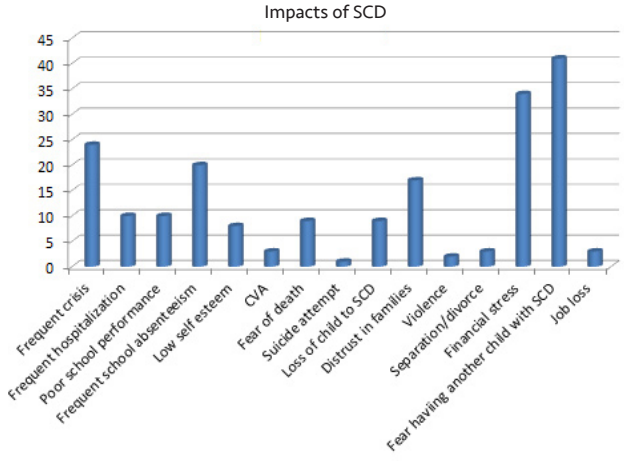

Figure 3. Impact of SCD on the affected child and caregivers in misdiagnosed caregivers
SCD: Sickle cell disease, CVA: Cerebrovascular accident

phenotyping is currently encouraged and practised by the many major religious groups in Nigeria as a measure to forestall births of children with SCD through informed marital decisions. Unfortunately, however, misdiagnosis is still highly prevalent as observed in our study; a large number of the parents we sampled married on the premise on the premise of compactible haemoglobin phenotype with no risk of having a child with HbSS.

Persons in the high social class had more phenotype testing done before marriage/child birth compared to individuals in the lower social classes in our cohort. There has been an increased dissemination of information in schools on SCD and the need for testing in recent times and this could have contributed to the awareness and earlier screening in persons in the high social class.

Three out of four parents with the HbAC phenotype in the current study were previously diagnosed as HbAA, and almost a third of participants had been wrongly diagnosed in the past. There are many sources of error in haemoglobin phenotype diagnosis as it is currently done in most diagnostic centres in Nigeria. Some could include the non-use of positive control samples (AS and AC), the use of inferior electrophoresis set-up and low-quality cellulose acetate paper, the re-cycling of acetate papers in order to cut cost as well as the use of unqualified personnel in the conduct of haemoglobin phenotyping (15).

In the present study, we observed that a significantly higher proportion of misdiagnosis occurred in private laboratories compared to government or private hospitals. An even more disturbing finding was that misdiagnosis was still high in subjects who had testing done more than once. Perhaps this suggests that poor quality results are widely prevalent. Mitigating these dangerous practices requires that the relevant agencies guiding the medical laboratory practice in Nigeria ensure strict monitoring and compliance of these laboratories in order to reach the recommended standard operating procedures. The Medical Laboratory Science Council of Nigeria regulates, inspects and accredits medical laboratories in Nigeria (16). However, "quack" laboratory scientists are rampant in our environment and the use of non-accredited, and hence unregulated, private laboratories by some citizens may account for this high frequency of misdiagnosis. In addition to curb this menace, the populace needs more education on the existence and potential dangers of such laboratories as well as the means of identifying adequately accredited ones.

Our finding suggests that errors in the diagnosis of the haemoglobin phenotype is less prevalent in public-owned compared to private laboratories. This may be a reflection of the comparatively higher statutory regulatory oversight in government-owned facilities. However, long waiting-times may discourage the use of public-owned laboratories despite being relatively cheaper than the private ones. Perhaps, this may explain why a larger proportion of those of higher social class had more misdiagnoses than those of lower status since they may be able to afford the private services with shorter waiting-times.

The standard practice of haemoglobin phenotype determination should also include a qualitative test widely known as sickling or solubility test, which helps to provide additional information that may not be available in the alkaline haemoglobin electrophoresis method. For instance, Haemoglobins S, D, and G migrate together in the same lane in alkaline electrophoresis. As a result, Hb D or G in a heterozygous individual will appear as Hb S; but in the sickling/solubility test, only Hb S forms precipitate showing a positive sickling/solubility test, while Hb D and G remain soluble, indicating a negative sickling/solubility test (5,6).

The use of hemolysate, instead of whole blood, for electrophoresis should be encouraged to ensure a more accurate diagnosis since other possible interfering proteins would have been washed off during hemolysate preparation. It is important to note that where appropriate diagnosis cannot be made with the use of both alkaline electrophoresis and sickling/solubility test, wrong assumptions must be avoided. Such blood samples should be sent to a better equipped laboratory that can offer definitive haemoglobin phenotype diagnosis. In such cases, a complete blood count, HPLC, and acid electrophoresis might provide some useful hints in cases of the presence of unusual haemoglobin variants.

However, the most effective lasting and long-term solution to the problem of haemoglobin diagnosis is to institute a nationwide new-born screening programme to replace the existing widespread use of haemoglobin electrophoresis with the more sensitive HPLC which gives a more accurate result as well as the early identification of carriers and infants with SCD.

The clinical and psychosocial impacts of having at least one child with SCD were identified in parents with wrong diagnosis in the current study. Parental fear of having another "sick" child, the financial stress and frequent crises of affected children were the most commonly reported effects. Some individual responses are given below:

A respondent: I was very angry when I realized my son has sickle cell anaemia and I went to that private laboratory to fight. My wife knew she is HbAS and I went to this lab before marriage and was given a result of HbAA. I realised I am HbAS after re-testing following my son's (who is the second and last child) frequent ill health that led to his diagnosis. There has been a lot of pressure from my parents for us to have more children but I cannot handle another child with SCD.

Another respondent: We realized my son has sickle cell anaemia after he had a stroke. I told the doctor this was not possible. I am educated and I did my test in a government hospital which was AA. The doctor advised me to repeat it and I got another result of AS. Currently my son is yet to speak, he is on physiotherapy and the financial implication is huge.

In Nigeria, where an out-of-pocket financing system is still mostly practiced, the financial burden of SCD on families is enormous, as was previously reported by Olatunya et al. (17). The marked effect of SCD on families in our environment is significant and having these effects following a misdiagnosis is quite disturbing and devastating to a family, especially in a society where family ills may be readily attributed to the woman's fault. This further emphasises the need to ensure compliance with standards in phenotype diagnoses and the institution of effective measures to curb laboratory practices by unqualified or incompetent doctors.

Conclusion

In conclusion, the misdiagnosis of the haemoglobin phenotype is rampant in our environment, especially in private laboratories. There is a need for effective and prompt measures to curtail this menace. More importantly, the need for effective nationwide new-born screening for SCD cannot be overemphasized.

Ethics

Ethics Committee Approval: Approval for this proposed study was obtained from the Health Research Ethics Committee of Lagos State University Teaching Hospital (NHREC04/04/2008).

Informed Consent: All the participants gave written informed consent while the children from seven years and above gave their assent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: M.O.A., P.O.U., Design: M.O.A., P.O.U., Analysis or Interpretation: M.O.A., P.O.U., Literature Search: M.O.A., P.O.U., Writing: M.O.A., O.O., A.A., P.O.U., F.L.

Conflict of Interest: No conflict of interest was declared by the authors.

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Original Article

Healthcare-associated Infections at a Tertiary Level Pediatric Intensive Care Unit From Turkey

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ABSTRACT

Aim: Healthcare-associated infections are important conditions, as they may increase morbidity and mortality, prolong hospital stay and increase costs. A higher incidence of healthcare-associated infections has been reported in developing countries, but data on its epidemiology in pediatric patients are limited. The aim of this study was to determine the rate and distribution of health care-associated infections and antimicrobial susceptibility patterns in a pediatric intensive care unit.

Materials and Methods: Demographic and clinical details, microbiological findings, antibiotic susceptibility results and the outcomes of all hospitalized pediatric intensive care unit patients were collected for one year (September 2017 to September 2018).

Results: The health care-associated infections rate was 5.6 per 100 admissions and the incidence density was 7.2 per 1,000 patient-days. Bloodstream infections (50%) were the most common type and *Klebsiella* species (40.9%) was the most common cause of health care-associated infections. All of the *Klebsiella* spp. were resistant strains producing extended-spectrum beta-lactamases (77.7%) and the remaining were resistant to carbapenem. *Acinetobacter* species and colistin resistance was not detected in any isolates.

Conclusion: This study demonstrated a low prevalence of health care-associated infections but a high rate of antibiotic resistance in *Klebsiella* species in a pediatric intensive care unit. In addition to improved surveillance, consultation with infectious disease specialists will allow the development of interventions to reduce healthcare-associated infections, in order to regulate both empirical treatment and ongoing management, and also to provide appropriate targeted therapy.

Keywords: Healthcare-associated infections, antimicrobial resistance, children

Introduction

Healthcare-associated infections (HAI) continue to be an important source of morbidity and mortality worldwide, but especially in developing countries such as our country (1). They cause a major public health problem with prolonged hospitalization, broad spectrum antibiotic requirement, increased resistance patterns and consequent additional healthcare costs. Although intensive care units (ICUs) account for fewer than 10 percent of the total beds in most hospitals, the incidence of HAI is higher in ICUs than any other hospital units. More than 20 percent of all nosocomial infections are acquired in ICU (2,3). Patients hospitalized in an ICU are more prone to HAI due to their severe clinical conditions and the consequent invasive procedures (4).

Although the rate of HAI in pediatric intensive care units (PICUs) in developed countries is lower than in adult ICUs, this situation is different in developing countries (1). Also, the rate of HAI is higher in PICUs than in other children's units. In a multicenter study conducted in Europe, the incidence of HAI in general pediatric units was reported to

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be 1% while it was 23.6%. in PICU (5). In a study from our country, the HAI rate was reported to be 22.24% in a PICU (1).

Infections and sepsis are the leading cause of death in non-cardiac ICUs. The most important nosocomial infections in ICUs are bloodstream infections (BSIs), ventilator-associated pneumonia (VAP), and urinary tract infections (UTIs).

In this study, we aimed to determine the incidence of HAIs, the distributions of the pathogens and the antimicrobial susceptibility in a PICU, in a city hospital in Turkey.

Materials and Methods

Balıkesir City Hospital is a non-tertiary state hospital which was opened with the aim of providing health service to a wide region. The hospital started admitting patients in 2017. The pediatric ICU, which is the only one in the area, consists of nine tertiary level beds. Each nurse has to care for two patients. Two pediatric infectious diseases specialists work and participate in the active surveillance of HAI with the infection control team.

In this study, the data of the patients, aged 1 month to 18 years and hospitalized between September 2017 and September 2018 in the PICU, were collected retrospectively. The information of the patients (age, gender, disease, period of hospital stay, ventilator or device use, antibiotic treatment, clinical and laboratory findings) and their culture results were investigated. The definition of HAI was made according to the "Centers for Disease Control and Prevention" criteria (6), a nd HAI was defined as an infection occurring 48 hours after hospitalization or 10 days after discharge. The rate of HAI was calculated according to the following formula; the number of HAIs/the number of all hospitalized patients x100.

The incidence density was calculated according to the formula; the number of HAIs/ total patient-days x 1,000 in a given period. This research was made with the approval decision of Balıkesir University Clinical Research Ethics Committee dated 05.12.2018 and numbered 2018/199.

Statistical Analysis

The study was registered with IBM SPSS 20.0 for Windows. In statistical analysis; for the analysis of numerical data, arithmetic mean ± standard deviation with minimum and maximum values were used while in categorical data, the number (n) and percentage (%) are given.

Results

During the one-year study period, 398 patients were hospitalized in the tertiary level PICU and HAI developed in 22 of these patients. Only two of them died because of underlying chronic diseases, namely severe hydrocephalus and cerebral palsy. The mean age of the patients was 81.9 months (range: 3-190 months). Half of the patients who developed HAI (n=11, 50%) were female. When the patients were evaluated according to their primary diagnosis, neurological disorders were the largest group (81.8%) with 18 patients (11 cerebral palsy, 3 epilepsy, 2 hydrocephalus, 2 spinal muscular atrophy). Two (9%) had genetic syndrome with tracheostomy, 1 (4.5%) patient had acute diarrhea-dehydration and 1 (4.5%) patient had glioma (Table I).

The HAI rate was 5.6 per 100 admissions and the incidence density was 7.2 per 1,000 patient-days. The most frequently detected HAI types were BSI (50%), VAP (40.9%) and UTI (9.1%), respectively. *Klebsiella* species (40.9%) was the most common cause of HAI, followed by *Candida* species (18.1%), *Pseudomonas aeruginosa* (13.6%), *Serratia marcescens* (9.1%), *Enterobacter cloacae* (9.1%), *Stenotrophomonas maltophilia* (4.5%) and coagulase-negative *Staphylococcus* (methicillin-resistant) (4.5%) (Table II). Four of the BSIs were central line-associated BSI (CLABSI); It was detected in patients who had been hospitalized for more than 30 days and diagnosed with cerebral palsy. The microorganisms were determined to be *Candida* spp. in three cases, while in one case it was *Stenotrophomonas maltophilia*. All of the isolated *Klebsiella* species were resistant strains; 77.7% of them produced Extended spectrum betalactamases (ESBLs) and the remaining were resistant to carbapenem. Carbapenem susceptibility was detected in 66.6% of

Table I. Demographic characteristics of the patients

Gender	
Female	11 (50%)
Male	11 (50%)
Median age	81.9 months (3-190 months)
Underlying disease	18 (81.8%)
Neurological disorders	
Cerebral palsy	11 (50%)
Epilepsy	3 (13.6%)
Hydrocephalus	2 (9%)
Spinal muscular atrophy	2 (9%)
Genetic syndrome	2 (9%)
Glioma	1 (4.5%)
Acute gastroenteritis	1 (4.5%)

Pseudomonas aeruginosa and 100% of *Serratia marcescens* and *Enterobacter* spp. (Table III). Colistin resistance was not detected among the microorganisms produced in cultures. The only coagulase-negative *Staphylococcus* was methicillin-resistant.

Discussion

HAIs in PICUs are an important problem resulting in prolonged hospital stay, increased medical costs, and increased morbidity and mortality (7). Most of the HAIs occurring in ICUs are associated with the use of invasive devices such as central line or mechanical ventilators (8). In developing countries, it has been reported that the incidence of HAI is higher than in developed countries due to the high number of patients, limited staffing and poor compliance with infection control measures (9-10). In developed countries, HAI rates are lower among children than among adults. In the United States, 5-10% of hospitalized adult patients are reported to have HAIs, while the rate is 1.5% to 4% for ten-year-olds and 7% -9% for infants younger than 1 year old (5). In our country, it was reported that the rates of HAIs in pediatric and adult patients varied between 1.3% and 16% in 2009 (10).

HAIs from PICU are reported to range 6% to 13.7% according to one study (11). Some previous studies have shown that the prevalence of PICU-acquired HAIs ranged from 9.1% to 42.5% (11-13). In our study, the HAI rate was lower than most of the similar reports with a rate of 5.6 per 100 admissions.

The incidence of the HAI type may differ according to the characteristics of the department, hospital and region. In the United States, the most common HAI types were reported to be BSI (28%), pneumonia (21%) and UTI (15%) in PICUs (14). In our study, the most common HAI type was BSI (50%), VAP (40.9%) and UTI (9.1%). Similarly, from our country, Atici et al. (1) reported that the most commonly observed HAIs were BSI (37.5%), pneumonia (21.4%), and UTI (20.5%) while Kepenekli et al. (15) reported that the most common HAIs were pneumonia (55%), BSI (27%) and UTI (7%), respectively. In another study with HAI ratios between 1.4% and 2.4%, the most commonly observed infections were UTI, surgical site and BSIs, and the most frequently isolated pathogens were *E. coli*, *Klebsiella pneumoniae*, *Enterococcus* spp. and *Staphylococcus aureus* (16).

In previous years, gram-positive factors were the most common causes of hospital infections. Today, *Pseudomonas, Klebsiella* and *Acinetobacter* species are among the leading factors in both adult and pediatric patients, in addition to coagulase-negative *Staphylococcus* strains (17,18). *Pseudomonas aeruginosa* is reported to be the most common agent of infections in PICUs in Europe (5). In our study, consistent with the literature, gram-negative agents were the most common agents in HAIs. *Klebsiella* spp. were the most often isolated microorganisms in all types of HAIs including BSI and VAP. *Acinetobacter* species, which are a common and important agent of HAIs in the world, were not detected in our study but interestingly, *Candida* species were the second most common, especially in BSIs. This was due to long-term hospitalized patients with cerebral palsy and respiratory failure, who had central venous catheter and a history of treatment with broad spectrum antibiotics.

Table II. Distribution of the healthcare-associated infection (HAI) pathogens

Microorganism	BSI n (%)	VAP n (%)	UTI n (%)
***Klebsiella* spp.** *K. pneumoniae* *K. oxytoca*	**3 (13.63)** **-**	**5 (22.7)** **-**	**-** **1 (4.55)**
Pseudomonas aeruginosa	**1 (4.55)**	**2 (9.09)**	**-**
***Candida* spp.** *C. parapsilosis* *C. albicans*	**2 (9.09)** **1 (4.55)**	**-**	**-** **1 (4.55)**
***Enterobacter* spp.** *E. cloacae* *E. aerogenes*	**1 (4.55)** **1 (4.55)**	**-**	**-**
Serratia marcescens	**1 (4.55)**	**1 (4.55)**	**-**
Stenotrophomonas maltophilia	**1 (4.55)**	**1 (4.55)**	**-**
Coagulase-negative *Staphylococcus*	**1 (4.55)**	**-**	**-**
Total	**11 (50)**	**9 (40.9)**	**2 (9.1)**

BSI: Bloodstream infection, VAP: Ventilator-associated pneumonia, UTI: Urinary tract infection

Table III. Distribution of antibiotic susceptibility of the gram-negative pathogens

Pathogens	n (%)
***Klebsiella* spp.**	
ESBL (+)	7 (77.7)
CRE (+)	2 (22.3)
Pseudomonas aeruginosa	
Carbapenem susceptibility (+)	2 (66.6)
Carbapenem susceptibility (-)	1 (33.4)
***Enterobacter* spp.**	
Carbapenem susceptibility (+)	2 (100)
Carbapenem susceptibility (-)	0 (0)

ESBL: Extended spectrum beta-lactamases, CRE: Carbapenem resistant *Enterobacteriaceae*

The resistance characteristics of microbiological agents responsible for hospital infections also vary over the years (19). There has been a rapid rise in the rate of resistance among bacterial pathogens in ICUs. The widespread use of antibiotics is associated with the development of resistance to antimicrobial agents. An international, multicenter study reported that the 78% of *Klebsiella pneumoniae* isolates produce ESBLs (17). In a study from our country, 50% of *Klebsiella* spp. and *E. coli* isolates were reported to be ESBL positive (1). The resistance rates found in our study were consistent with the literature; 77.7% of *Klebsiella* spp. produced ESBLs and 22.3% were resistant to carbapenem. In some previous studies, it was reported that the susceptibility rate to carbapenem ranged from 48% to 71% (20,21). It was reported to be 63% among *Pseudomonas aeruginosa* isolates in a national study (1). In our study, carbapenem susceptibility was detected in 66.6% of *Pseudomonas aeruginosa* and colistin resistance was not detected in any isolate.

The low number of patients and the retrospective nature are the most important limitations of our study.

Conclusion

In conclusion; we report a low rate of HAIs in our study but most were resistant bacteria. The low rate may be due to the unit being newly opened and the good condition of the hospital as well as the daily intensive care visits provided by pediatric infectious diseases specialists. Active surveillance is required for effective infection control, and pediatric infectious disease consultation leads to appropriate antibiotic use, reduced HAI rates, and finally improved PICU patient outcomes.

Ethics

Ethics Committee Approval: This research was made with the approval decision of Balıkesir University Faculty of Medicine Clinical Research Ethics Committee dated 05.12.2018 and numbered 2018/199.

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: G.A., Design: G.A., Data Collection or Processing: G.A., B.Y.A., Analysis or Interpretation: G.A., Literature Search: G.A., B.Y.A., Writing: G.A.

Conflict of Interest: No conflict of interest was declared by the authors.

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Original Article

Evaluation of Children Receiving Tissue Plasminogen Activator Therapy for Thrombosis: Single Center Experience

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ABSTRACT

Aim: In this retrospective study, our objective was to evaluate children with arterial or venous thromboembolism, who were treated with tissue plasminogen activator (tPA) in our hospital.

Materials and Methods: The medical records of 56 tPA treatments administered to 53 patients with thrombosis in the paediatric intensive care unit and paediatric clinic at Çukurova University, Balcalı Hospital between September 2013 and August 2018, were investigated retrospectively.

Results: Thirty-three of the patients were males (58.9%). The median age was 13.5 months (0-203 months). Fifty-two of the patients received low-dose tPA treatment (91.2%) and the mean treatment duration was 63.8±43.3 hours (3-192 hours). Thrombolytic treatment was administered to 38 patients (67.8%) with catheter-related arterial thrombus, to 8 patients (14.3%) with intracardiac thrombus, to 4 patients (7.2%) with pulmonary arterial thrombus, and to 6 patients (10.7%) with deep venous thrombus. No complication was observed in 47 treatments (83.9%). However, 7 patients had minor (12.5%) and 2 patients had major bleeding (3.6%). Recanalization could not be achieved in 8 cases (14.3%) and 4 patients underwent thrombectomy. The use of anticoagulant treatment with tPA did not change the complication rate or the success rate of the recanalization.

Conclusion: We determined that low-dose tPA treatment was effective in the treatment of life-, limb- or organ-threatening arterial and venous thromboembolism in children. However, randomized studies with larger sample sizes and control groups are required.

Keywords: Alteplase, tissue plasminogen activator, thrombosis, venous, arterial

Introduction

Thrombosis is rare in childhood; however, the rate of diagnosis arterial thromboembolism (ATE) and venous thromboembolism (VTE) has increased as a result of the early diagnosis by using the latest imaging techniques (1). It is known that the risk of thromboembolism increases in cases of cardiac, oncological/haematological diseases, central or arterial catheterization, or underlying predisposition to thrombosis (2,3). There are studies showing that the incidence of venous thrombosis among children was between

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18.8 and 58 per 10,000 hospital admissions (4,5). Arterial thromboembolism is less common than VTE in childhood. In a retrospective cohort study, the reported incidence of arterial thrombosis in the extremity was 2.35/10,000 (6). The incidence of peripheral arterial thrombosis was between 1.2% and 3.2% in critically-ill children (3,7). It was reported that the risk of arterial thrombosis increases in children depending on their young age and low body weight and the incidence of arterial thrombosis might increase by up to 11.4% after cardiac catheterization (8).

Streptokinase, urokinase, and tissue plasminogen activator (tPA) are the main agents used in thrombolytic therapy. tPA is the most preferred medical treatment in children. The relatively shorter half-life (approximately 5 minutes) and the lower anaphylactic reaction risk in repeated use are the advantages of tPA. The success rate of thrombolytic treatment differs in arterial and venous thrombosis (9). Guidelines based on experience in the treatment of the adults are used during the therapeutic approach to childhood thrombosis. Although the use of tPA has increased in paediatric patients as a thrombolytic agent, there are conflicting data in the literature about the optimal dosing and treatment duration.

In this study, the aim was to evaluate the efficacy and safety of tPA treatment in paediatric patients, who were under tPA treatment or under follow-up for ATE or VTE with various diagnoses.

Materials and Methods

Patient Population

Following the approval of the Ethics Committee, the medical records of 56 treatments administered to 53 patients, who were treated with tPA due to thrombosis in the paediatric intensive care and paediatric clinic between September 2013 and August 2018 in the Çukurova University Hospital, were investigated retrospectively. Regarding the diagnosis and follow-up, echocardiography was used for intracardiac thrombus and Doppler ultrasonography for intravascular thrombus. Computed tomography was used in cases of suspected pulmonary embolism. The location of the thrombus was classified as: (1) Catheter-related arterial thrombus including secondary to cardiac catheterization and arterial pressure monitoring; (2) pulmonary artery; (3) intracardiac thrombosis including atrial and ventricular; and (4) deep venous thrombosis (DVT) of the femoral vein or portal vein.

Treatment

The tPA doses and concomitant anticoagulant treatment options were administered according to the recommendations of the clinicians. Plasminogen levels could not be measured in this hospital. During the tPA transfusion, the prothrombin time/international normalized ratio, activated partial thromboplastin time and fibrinogen level were assessed before tPA treatment, 4 hours after the start of the infusion, and with 6-8-hour intervals after infusion. If the fibrinogen level decreased below 100 mg/dL, 10 mL/kg fresh frozen plasma (FFP) was administered to preserve a plasminogen level of 100 mg/dL. Platelet count was measured twice a day in order to maintain a platelet level above $100x10^9$/L. In cases of major or minor bleeding, tPA treatment was discontinued and FFP was administered. Antifibrinolytic treatment was used in cases of major bleeding. Daily Doppler ultrasonography or echocardiographic examination was performed in the 6^{th} hour of the treatment and during the follow-up in patients receiving tPA treatment. In those patients who received tPA treatment due to thrombus in the extremity after cardiac catheterization, the treatment was terminated if the circulation and pulse examination were normal or recanalization was observed during Doppler ultrasonography. The treatment was also terminated if recanalization was determined with Doppler ultrasonography or echocardiography in patients without cardiac catheterization.

The patients were evaluated for age, sex, primary diagnosis, comorbidities, thrombolytic treatment indication, thrombotic vessels, anticoagulant treatment concomitant to tPA treatment, tPA dose and duration, complications related to thrombolysis, duration of hospitalization, thrombectomy, and amputation. Vital organ haemorrhage and any event requiring discontinuation of thrombolytic therapy are described as major and mucosal bleeding or bleeding from any skin insertion site described as minor complications. The patients were divided into two subgroups according to the tPA dosage, namely low-dose (<0.1 mg/kg/hour) or high-dose (≥0.1 mg/kg/hour) (10).

Statistical Analysis

SPSS v20.0 software package was used for the statistical analysis. Categorical measurements are given as numbers and percentages, and numeric measurements as mean and standard deviation values (if necessary, median and minimum/maximum values were also referred).

Table I. Characteristics of the patients	
	n (%)
Sex	
Male	33 (58.9)
Female	23 (41.1)
Primer diagnosis	
Cardiological	34 (60.7)
Nephrological	11 (19.6)
Hemato-oncological	4 (7.2)
Others	7 (12.5)
Comorbidity	
Infection	11 (19.6)
Respiratory	6 (10.7)
Cardiac	5 (8.9)
Hepatic	2 (3.6)
Indication of tPA	
Arterial thrombosis (catheter related)	38 (67.8)
Intracardiac thrombosis	8 (14.3)
Central venous thrombosis	6 (10.7)
Pulmonary thrombosis	4 (7.2)
Therapy	
tPA	14 (25.0)
tPA+Low-molecular-weight heparin	27 (48.2)
tPA+Unfractionated heparin	15 (26.8)
Complication	
None	47 (83.9)
Minor	7 (12.5)
Major	2 (3.6)
Recanalization	
Not achieved	8 (14.3)
Partial	18 (32.1)
Complete	30 (53.6)
Surgery/thrombectomy	4 (7.1)
Amputation	2 (3.6)
tPA: Tissue plasminogen activator	

Results

Fifty-six tPA treatments from a total of 53 cases were included in this study. Thirty-three of the participants were male (58.9%). The median age was 13.5 months (0-203 months). Thirty-four of the patients (60.7%) had cardiological, 11 (19.6%) nephrological and 4 (7.1%) haemato-oncological diseases (Table I). The most common comorbidity was infection (19.6%).

Thrombolytic treatment was administered to 38 patients (67.8%) with catheter-related arterial thrombus, to 8 patients (14.3%) with intracardiac thrombus, to 4 patients (7.2%) with pulmonary arterial thrombus, and 6 patients (10.7%) with DVT. Catheter-related arterial thrombus was associated with younger age (p=0.001). No significant difference was found between the groups in terms of tPA dosage and tPA infusion time (p=0.46 and p=0.44, respectively).

The mean value of the tPA dose in all treatments was 0.07±0.09 mg/kg/hour (range: 0.02-0.5 mg/kg/hour) and the duration of the tPA treatment was 63.8±43.3 hours (3-192 hours). Minor and major bleeding emerged in 7 (12.5%) and 2 cases (3.6%), respectively, and no complication was observed in 47 treatments (83.9%). The tPA dose and treatment duration in patients with or without bleeding are listed in Table II.

Complete, partial and no recanalization was achieved in 30 (53.6%), 18 (32.1%) and 8 (14.3%) treatments, respectively. The patients were evaluated in two groups as complete recanalization and partial/no canalization. There was no significant relationship between age, primary diagnosis, tPA dosage, and the duration of the tPA treatment. Four patients underwent thrombectomy due to thrombosis in the femoral artery (n=1), in the external iliac vein (n=1), in the portal vein (n=1), and the pulmonary artery (n=1). Two of 38 patients (5.2%) who had thrombus following the arterial catheterization in the extremity did not respond to the medical treatments and underwent amputation. Two patients with DVT, who were unresponsive to tPA treatment, were discharged with low molecular weight heparins (LMWH) treatment. Fourteen of the treatments consisted of only tPA (25.0%), 27 (48.2%) had concomitant LMWH and 15 (26.8%) had concomitant unfractionated heparin. The median value of the LMWH and unfractionated heparin doses were 2 mg/kg/day and 20 units/kg/h, respectively. The use of anticoagulant treatment with tPA did not alter the complication rate or the success rate of recanalization (p>0.05).

tPA dose was evaluated in two groups, namely, low-dose (<0.1 mg/kg/hour) or high-dose (≥0.1 mg/kg/hour). The duration of the tPA treatment was 69.3±41.5 hours (3-192 hours) and 8.6±3.6 hours (6-13 hours) in the low-dose and high-dose groups, respectively. Complications were observed in two patients in the high-dose group (40%) and seven patients in the low-dose group (13.7%). The characteristics of the patients with low-dose and high-dose tPA treatment are listed in Table III. Statistical analysis could not be done due to the low sample size in the high-dose group.

Discussion

The aim of the treatment in thromboembolism is the prevention of growth of the clot and embolism, the restoration of circulation, the limitation of long-term sequel, and a decrease in recurrence risk. Anticoagulant agents, such as unfractionated heparin, low-molecular-weight heparin, vitamin K antagonists, or thrombolytic

Table II. Characteristics of the patients with and without bleeding complications

	All patients (n=56)	Patients without bleeding complication (n=47)	Patients with bleeding complication (n=9)	p-value
Age (months)	57.2±68.7 13.5 (0-203)	62.3±70.5 18.0 (1-203)	30.8±54.7 6.0 (0-166)	0.07
tPA dosage (mg/kg/h)	0.07±0.09 0.05 (0.02-0.5)	0.05±0.04 0.05 (0.02-0.25)	0.14±0.19 0.06 (0.03-0.50)	0.35
tPA infusion time (hours)	63.8±43.3 67.5 (3-192)	70.1±42.5 75.0 (6-192)	31.2±32.4 16.0 (3-96)	0.01

Table III. Characteristics of the patients with low-dose and high-dose tissue plasminogen activator treatment

	Low-dose tPA (n=51) mean±SD median (min-max)	High-dose tPA (n=5) mean±SD median (min-max)
Age (months)	59.8±70.2 17.0 (0-203)	31.2±49.8 12.0 (5-120)
tPA dosage (mg/kg/h)	0.05±0.01 0.05 (0.02-0.06)	0.30±0.18 0.25 (0.12-0.50)
tPA infusion time (hours)	69.3±41.5 72.0 (3-192)	8.6±3.6 6.0 (6-13)
Complication, n (%) None Minor Major	 33 (86.3) 6 (11.8) 1 (2.0)	 3 (60.0) 1 (20.0) 1 (20.0)
Recanalization, n (%) Not achieved Partial Complete	 5 (9.8) 17 (33.3) 29 (56.9)	 3 (60.0) 1 (20.0) 1 (20.0)

tPA: Tissue plasminogen activator

agents (streptokinase, urokinase, and tPA) may be used in the treatment of children with thromboembolism (11-13). While the anticoagulant treatment prevents the growth of thrombus, decreases the risk of embolism, it also enables the shrinkage of the thrombus by its inherent fibrinolytic mechanism. Although the use of tPA has increased in paediatric patients as a thrombolytic agent, general contraindications to thrombolysis include active bleeding, concurrent bleeding diathesis, recent major surgery or trauma, intracranial haemorrhage, and extreme prematurity (14,15).

Although the guidelines do not recommend thrombolysis in paediatric patients with DVT in most cases of the clinical thrombosis, it should be considered in cases of pulmonary embolism with hemodynamic compromise or venous thrombus that may lead to irreversible organ or extremity damage (16,17). The goal of thrombolysis is the dissolution of the vascular occlusion caused by thrombus with the help of the activation of the fibrinolytic system. The primary indication for thrombolysis is limb- or life-threatening acute or subacute occlusive venous or arterial thrombosis. Strong indications for thrombolysis include pulmonary embolism with hypotension or shock, superior vena cava syndrome, bilateral renal vein thrombosis, congenital heart disease with shunt thrombosis, large (>2 cm) atrial thrombus, and cerebral sinovenous thrombosis with neurologic impairment (16). In this study, 6 patients with DVT (10.7%) received tPA treatment. Severe acute abdominal pain developed in two of these patients and magnetic resonance examination revealed portal vein thrombosis. Due to the development of hemodynamic imbalance, acute hepatic failure, and acute kidney failure, tPA treatment was initiated to prevent progress towards chronic liver failure. Doppler ultrasonography showed that flow was restored in the portal vein of the patient, whose clinical condition improved. The remaining four patients had diffuse thrombosis starting from the popliteal vein, extending to the superior iliac vein, and had the risk of extremity loss. In one of these four patients who received tPA due to a risk of extremity loss, a resolution could not be achieved and surgical thrombectomy was necessary. These patients had risk factors, such as immobilization, nephrotic syndrome, and previous abdominal surgery, which may cause thrombophilia.

A literature review evaluated 413 children who received thrombolytic treatment with streptokinase, urokinase, or tPA. It was reported that a complete recanalization was achieved in 53%, 43%, and 69% patients, respectively (18). There are also studies where low-dose heparin infusion or low-molecular-weight heparin were added to the thrombolytic treatment (10,19). Concomitant use of anticoagulation with systemic thrombolysis is recommended to prevent new thrombus formation during thrombolysis, as clot lysis releases active thrombin which bonds to thrombi (10). In this study, 14 patients (25.0%) received only tPA, and LMWH and unfractionated heparin

was added to the tPA treatment in 27 (48.2%) and 15 (26.8%) patients, respectively. The use of anticoagulant agents did not alter the complication rate or the success rate of the recanalization. Although it may be concluded that anticoagulant treatment concomitant to tPA did not cause any significant difference regarding the resolution, a definitive conclusion was not possible because of the small sample size of this study.

The rate of bleeding complications and the success rate of the thrombolytic treatment in children differs between health centres. The rate of complete and partial recanalization with thrombolytic treatment was observed to be between 26-88% (18-22). In this study, the rate of complete and partial recanalization was 85.7%. In eight patients, recanalization could not be achieved and four patients underwent thrombectomy. Two patients did not respond to medical treatment and underwent amputation. Albisetti (18) reported that the rates of minor and major bleeding complications were 26% and 17%, respectively, in patients treated with tPA. Newall et al. (9) evaluated 26 paediatric patients with arterial or venous thrombosis, who were treated with tPA and reported a major haemorrhage rate of 11.5%. In this study, both the major and minor bleeding complication rate was slightly lower than the literature.

Close follow-up protocols relating to the anticoagulant treatment concomitant to the high- and low-dose systemic thrombolytic therapy and haemorrhage have been previously reported (20). The reported range of dosage of tPA for children is between 0.01 and 0.6 mg/kg/h. Tarango AND Manco-Johnson (10) suggested that the duration of tPA treatment is 6-72 hours for low-dose and 2-6 hours for high-dose tPA. The longer duration of tPA treatment compared to the literature did not lead to a higher complication rate in this study. There are studies showing that low-dose tPA treatment was as effective as high-dose tPA treatment (18-21). In their retrospective study, Gupta et al. (22) evaluated the data of 80 paediatric patients treated with high-dose tPA over 14 years and reported that major complications emerged in 40% of patients and that high-dose tPA treatment had a poor safety profile. Moreover, all patients in this study received unfractionated heparin infusion before tPA treatment. In this study, 52 patients received low-dose, and 5 patients received high-dose tPA treatment. The authors observed complications in two of the high-dose patients (40%) and seven of the low-dose patients (13.5%). Although the patient groups and success rates were comparable, the complication rate was lower in our study. With regard to our findings, the necessity of the high-dose tPA and concomitant treatments may be questioned.

Study Limitations

There were limitations to this study. tPA and concomitant anticoagulant treatment options were used based on the recommendations of the clinicians. A balanced distribution between the groups could not be achieved due to the retrospective study design. It would be better if the data on the duration of thrombosis before tPA treatment was available as it may have affected the success rate of tPA. Statistical analysis could not be performed because of the small number of patients receiving high-dose tPA treatment.

Conclusion

In this study, we detected that low-dose tPA treatment was effective in the treatment of life-, limb- or organ-threatening arterial and venous thromboembolism in children. Howe ver, randomized studies with larger sample sizes and control groups are required.

Ethics

Ethics Committee Approval: The Ethics Committee of Çukurova University Faculty of Medicine approved the study (2019-86).

Informed Consent: Informed consent was not obtained because this was a retrospective study.

Peer-rev iewed: Internally and externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: F.D., S.E., Concept: G.L., D.Y., A.K.B., Design: A.Y., F.D., E.M., Data Collection or Processing: A.Y., G.L., S.E., Analysis or Interpretation: D.Y., A.K.B., Literature Search: A.Y., H.İ.Ş., E.M., Writing: A.Y., G.L., D.Y.,

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Original Article

The Evaluation of Skeletal Manifestations in Patients with Gaucher Disease

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ABSTRACT

Aim: Gaucher disease (GD) is the most prevalent hereditary lysosomal storage disorder, affecting multiple organ systems. It is characterized by a deficiency of the enzyme glucocerebrosidase leading to an accumulation of glucosylceramide in lysosomes. The majority of patients present with hepatosplenomegaly, anemia, thrombocytopenia, bleeding tendencies, skeletal pathologies, growth retardation and in severe cases pulmonary disease. The bone manifestations include bone infarcts, avascular bone necrosis, lytic lesions, osteopenia and osteoporosis. This article gives an overview of the bone manifestations of 20 GD patients and reviews the current literature.

Materials and Methods: The data of 20 patients with GD who were being followed up in Gazi University Hospital Pediatric Metabolism Unit were retrospectively evaluated. Their demographic information including age, gender, clinical findings, enzyme replacement therapy (ERT) status and duration were recorded. Laboratory analyses including serum calcium, phosphorus, vitamin D, the presence of skeletal findings, and bone mineral density (BMD) Z scores were collected from the patient files. The BMD status of patients was compared with their pre-treatment values.

Results: The main symptoms of referral were abdominal distention, cytopenia, bleeding tendency and skeletal findings. All patients had skeletal symptoms. Nineteen patients showed vitamin D deficiency. The medullary involvement of femur and vertebrae was present in 14 (70%), Erlenmeyer flask deformity in 3 (15%), and avascular necrosis in one (5%) patient. Also, one patient (5%) had lytic bone lesions. Ten patients showed osteoporosis (50%), and 8 showed osteopenia (40%) at the time of diagnosis, before the initiation of ERT. The rate of osteoporosis was determined to be 40%, and osteopenia was 35% within the study group after the initiation of ERT.

Conclusion: Physicians, including pediatricians, may be unfamiliar with bone pathophysiology and the complexity of the skeletal manifestations of GD. There is a need to enhance awareness and to improve the diagnosis and treatment of skeletal pathology in patients with GD.

Keywords: Gaucher disease, skeletal involvement, osteoporosis, osteopenia

Introduction

Gaucher disease (GD) is an autosomal recessively inherited inborn error of metabolism (IEM) of glycosphingolipids, caused by loss of function mutations in the *GBA* gene encoding lysosomal glucocerebrosidase, a lysosomal enzyme that is responsible for the breakdown of glucocerebroside. Due to the decreased activity of this enzyme, glucocerebroside accumulates in various tissues, affecting multiple organ systems, and leading to serious complications. GD is subdivided into neuronopathic (types 2 and 3) and non-neuronopathic (type 1) phenotypes. Type 1 is the most prevalent form with an estimated prevalence of 1 in 40,000 to 60,000 cases in the general population (1).

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The skeletal findings associated with GD are osteopenia, osteoporosis, osteonecrosis, osteosclerosis, bone crises, chronic bone pain, Erlenmeyer flask deformity, pathological fractures and vertebral collapse. Vitamin D deficiency is also known to be frequent among patients (2).

Enzyme replacement therapy (ERT) and substrate reducing therapy (SRT) are efficacious in treating the visceral and hematological aspects of the disease, and are also effective in reversing the skeletal findings. Initiating early treatment may prevent skeletal complications which are the most debilitating manifestations of this disease and have a significant impact on the patient's well-being (1,2).

We present the skeletal findings of 20 patients with GD being followed up at Gazi University Hospital, Pediatric Metabolism Unit. Also, an overview of bone manifestations of GD, along with a review of the current literature is given.

Materials and Methods

The data of 20 patients with GD who were being followed up at Gazi University Hospital, Pediatric Metabolism Unit were retrospectively evaluated. All patients had been on ERT for at least six months at the time of the data collection. Their demographic information including age, gender, subtype of GD, age of initial symptoms and diagnosis, clinical findings, age at the initiation of treatment (ERT) and the duration of ERT were recorded. Laboratory analyses including serum calcium (Ca), phosphorus (P), 25-hydroxyvitamin D [25(OH)D], and the presence of skeletal findings were collected from the patient files. The values for vitamin D deficiency and insufficiency were determined according to the criteria of European Society of Paediatric Gastroenterology, Hepatology and Nutrition. Values below 20 ng/mL (50 nmol/L) were accepted as deficiency and values between 20 and 29.9 ng/mL (50-74.9 nmol/L) as vitamin D insufficiency. The cut-off for normal vitamin D levels was ≥30 ng/mL (≥75 nmol/L) (3).

Bone mineral density (BMD) measurements were performed as part of routine annual follow-up, and were assessed by dual-energy X-ray absorptiometry at the lumbar vertebrae, hip, and wrist. Values were reported as Z-scores and T-scores, in relation to the deviation in units of Standard deviation from the median age specific and young adult reference values, respectively (4).

The research was approved by the Ethics Committee of Gazi University Medical Faculty (approval no:2020-444, date: 14.07.2020). Written consent was obtained from all subjects.

Statistical Analysis

Median and ranges were used for descriptive statistics, and paired sample t-test was used to evaluate modifications in mean BMD scores, by using the SPSS 26.0 software package.

Results

The patient group included seventeen patients with GD type 1, and three patients with type 3 (a total of 20 patients). The mean age of patients was 18.72±14.71 years (range: 1-51 years), the mean age of diagnosis was 14.4±11.03 years (range: 1-44 years). The age of initiation of symptoms ranged between 1 to 15 years. The median duration of ERT was 2.52±2.08 (range: 0.5-7) years. Of a total of 20 patients, 8 were male and 12 were female. Ten out of 20 patients were diagnosed after the age of 18 years.

All patients had confirmed low levels of glucocerebrosidase enzyme. Additionally, all patients had genetically confirmed variants of the *GBA* gene. The most common variant detected in GD type 1 patients was the homozygous c.1226A>G variant (10/20 patients), 4 patients had the homozygous c.1448T>C variant. Other detected variants were homozygous c.1214G>A (2/20), compound heterozygous (c.1311-1312insT;c.1226A>G) (1/20) and (c.1226A>G;c.1505G>A) (1/20).

Their main symptoms of referral were abdominal distention (15 cases, 75%), anemia (2 cases, 10%), thrombocytopenia (2 cases, 10%), bleeding tendency (2 cases, 10%) and skeletal findings (1 case 5%). One patient was splenectomized (Table I). All patients had visceral findings at the time of the study (100%).

All patients had skeletal symptoms. Nineteen patients showed vitamin D deficiency. The mean serum concentration of 25(OH)D was 11.22±7.92 (3-40) ng/mL. The mean Ca, P and ALP values were 9.23±0.39 mg/dL (8.3-9.9), 3.91±0.57 mg/dL (3-5) and 124.35±58.58 IU/L (48-272) respectively.

Medullary involvement of femur and vertebrae was present in 14 (70%), Erlenmeyer flask deformity in 3 (15%), and avascular necrosis in one (5%) patient. Also, one patient (5%) had lytic bone lesions. Ten patients showed osteoporosis (50%), and 8 showed osteopenia (40%) at the time of diagnosis, before the initiation of ERT (Table II). Three adult patients claimed to have bone pain (15%). It was observed that the patient with lytic lesions had been diagnosed at the age of 37, and had been receiving ERT for 3 years.

All of the adult patients showed osteopenia before the initiation of treatment. At the time of the study period, while all patients were on ERT, it was seen that 8 patients had osteoporosis, 7 had osteopenia and 5 showed normal BMD findings. Ten patients showed medullary involvement

Table I. Baseline general clinical assessment of patients with Gaucher disease	
Gaucher disease subtype	**Patients (n)**
Type 1 Type 3	17 3
Gender	**Patients (n)**
Male Female	8 12
Mean age ± SD, years (range)	18.72±14.71 (1-51)
Mean age of initation of symptoms, ± SD, years (range)	5.8±4.89 (1-25)
Mean age of diagnosis, ± SD, years (range)	14.4±11.03 years (1-44)
Initital symptoms for referral	**Patients (n)**
Hepatosplenomegaly (Abdominal distension) Anemia Thrombocytopenia Coagulaopathy Bone pain	15 2 2 2 1
Splenectomy	1
Mean ERT duration (years)	2.52±2.08 (0.5-7)
Mean 25(OH)D concentration, ± **SD**, (ng/mL) **(range)**	11.22±7.92 (3-40)
SD: Standard deviation, 25(OH)D: 25-hydroxyvitamin D, ERT: Enzyme replacement therapy	

Table II. Skeletal findings of patients with Gaucher disease		
Skeletal manifestation	**Baseline (n)**	**During ERT (n)**
Medullary involvement of femur and vertebrae	14	10
Osteoporosis	10	8
Osteopenia	8	7
Bone pain	3	0
Avascular necrosis	1	1
Erlenmeyer flask deformity	3	3
Pathologic fractures	0	0
Bone crisis	1	0
Lytic lesions	1	1
ERT: Enzyme replacement therapy		

of bone and vertebrae. None of the patients had complaints of bone pain, bone crisis or pathological fractures during the ERT period.

Discussion

GD, one of the most common lysosomal storage disorders (LSD), is an IEM of the lysosomal enzyme glucocerebrosidase, which induces the deposition of undegraded glycolipid material in organs rich in mononuclear macrophage system including the liver, spleen and bone marrow. There are three clinical sub-types based on age of onset and the presence of neurological manifestations. GD type 1 is the non-neuronopathic type, with primarily skeletal and visceral signs and symptoms which range in severity. The infantile-onset (type 2) and later-onset (type 3) GD involve the central nervous system. The diagnosis is confirmed by analysis of glucocerebrosidase activity in peripheral blood leukocytes or fibroblasts (1,2).

GD is commonly associated with HSM and occasionally other organs, including the kidneys or lungs, are affected. The skeletal manifestations of GD include a variety of bone pathologies prevalent at all ages, due to the progressive glucocerebroside storage, changes of vascularity, and impaired bone remodeling. Bone involvement is broad and can occur in otherwise clinically asymptomatic individuals (5). Furthermore, bone symptoms may present signs in childhood (6). One of the largest cohorts of GD type 1 patients including 2,004 patients evaluated in the International Collaborative Gaucher Group (ICGG) Gaucher registry showed bone manifestations to be prevalent at a rate of between 76% and 94% (7).

The frequently encountered bone manifestations of GD include bone infarcts, bone marrow infiltration, Erlenmeyer flask deformity, avascular necrosis, lytic lesions, osteosclerosis, and fractures due to osteoporosis. The recognition of bone disease related with GD is important since it may lead to serious complications including polyclonal and monoclonal gammopathies and cancer (1-5).

In our study group, the most frequent skeletal manifestation was low BMD including osteopenia and osteoporosis, which was detected in all adult patients before the initiation of ERT. This finding is in accordance with several studies in the literature. Pastores et al. (8) revealed that 61 adult patients with GD had significantly lower BMD than expected for age and sex. The authors stated that the severity of the osteopenia correlated significantly with other clinical indicators, namely disease severity, genotype, prior splenectomy, and hepatomegaly and the severity of skeletal disease as assessed by skeletal radiography. The ICGG Gaucher Registry has indicated that the rate of osteopenia is 55% among all registry patients (8,9). Mistry et al. (10) showed low bone density to be most prevalent in adolescence. Our findings demonstrate that skeletal findings in GD are mainly encountered after childhood, and since ERT is effective in the regression of skeletal pathologies, early diagnosis and treatment may be

an important factor in the prevention of irreversible damage to the skeletal system.

ERT is effective in the treatment of GD and can have a significant impact on skeletal manifestations. De Fost et al. (11) have suggested the response to ERT to be slower for the hematological and visceral symptoms, when compared to skeletal symptoms. The efficacy of ERT in reducing the burden of Gaucher cells in the bone marrow has been demonstrated in several studies (12). It has been shown that the Bone Marrow Burden (BMB) score, a radiological scoring system used to evaluate the severity of bone involvement in GD proposed by Maas et al. (13), improves by 2 or more points after ERT (11). Similar beneficial effects have been observed with SRT (14).

It is important to diagnose focal osteolytic lesions of GD, which typically have a "worm-eaten" appearance, since they are a risk factor for fractures, and they may be mistakenly diagnosed as malignancy related osteolytic lesions (15). In our patient group, only one had focal osteolytic lesions along with bone pain and avascular necrosis. Interestingly, this patient was splenectomised before the diagnosis of GD. It has previously been reported that splenectomised patients have lower bone density (8). Thus, the severe skeletal findings of this patient may be related with the splenectomy.

It is well-known that bone complications respond to ERT later than visceral and hematological manifestations. Robertson et al. (12) reported that the BMB score was improved by 2 or more points in those patients receiving ERT. Also, the fact that bone findings stabilize after 5 years of therapy has been previously reported in the literature, reaching near normal values after 7 years of ERT (2). Our findings are in compliance with the literature, since the skeletal findings including lytic lesions were not completely resolved, when the relatively short duration of ERT was considered.

Although vitamin D deficiency is very common among the general population, GD patients have been reported to have a higher prevalence of low vitamin D levels than the general population. Vitamin D deficiency in GD may result from: low intake, malabsorption, diminished exposure to sunlight or decreased hepatic production of calcidiol (16). In our study group, the rate of vitamin D deficiency was found to be 95%. Unfortunately, we were not able to evaluate the etiologic factors contributing to the vitamin D deficiency in our patient group, due to our research being retrospective. Mikosch and Hughes (5) evaluated 74 GD patients and found a high prevalence of vitamin D deficiency among these patients. Similarly, nineteen out of 20 GD patients in our study group had vitamin D deficiency. Considering the essential role of vitamin D in bone homeostasis and the prevalence of hypovitaminosis D, we suggest evaluating this parameter routinely in all GD patients. In order to optimize the care of bone disease in GD patients, supplementation with vitamin D must be recommended for GD patients with osteopenia or osteoporosis.

Conclusion

BMD normalization was achieved in three patients with ERT. These results suggest that ERT for pediatric and adult patients may be an effective treatment for osteoporosis and osteopenia in these patients and ERT may maintain BMD and lessen the risk of developing osteopenia in patients with near normal BMD scores. We think that since the average duration of ERT was 2.5 years in our study group, the number of patients who benefit from ERT will increase with time, since it is known that bone manifestations stabilize after 5 years of ERT. Also, adequate calcium replacement should be given according to local guidelines.

Physicians, including pediatricians, may be unfamiliar with bone pathophysiology and the complexity of the skeletal manife stations of GD. There is a need to enhance awareness and to improve the diagnosis and treatment of skeletal pathology in patients with GD.

Ethics

Ethics Committee Approval: The research was approved by the Ethics Committee of Gazi University Medical Faculty (approval no: 2020-444, date: 14.07.2020).

Informed Consent: Written consent was obtained from all subjects.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: F.S.E., L.T., Design: Ç.S.K., Data Collection or Processing: A.O., Analysis or Interpretation: İ.O., Literature Search: Ç.S.K., A.O., İ.O., Writing: Ç.S.K., A.O., İ.O., F.S.E., L.T.

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Original Article

Long-term Outcomes of Children with Cow's Milk Protein Allergy in a Pediatric Allergy Clinic

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ABSTRACT

Aim: This study aimed to assess the clinical features, management, and long-term outcomes of pediatric patients with cow's milk protein allergy (CMPA).

Materials and Methods: This is a retrospective study consisting of 246 children with CMPA. Data of the patients were collected from the medical files.

Results: 95.8% of patients experienced the first reactions associated with cow's milk (CM) allergy during infancy. Hen's egg (56%) was the most frequent triggering food coexisting with CMPA, and this was followed by tree nuts (6%), wheat (5%) and lentil (3%). During five years of the follow-up period, tolerance occurred in 78.9% of the patients. The optimal cutoff value for CM sIgE to predict the tolerance status for CMPA was 7.39 kU/L with a sensitivity of 87.3% and a specificity of 58.3%, [95% confidence intervals (CI), 0.655-0.859, p<0.001]. IgE-mediated hypersensitivity reactions [odds ratios (OR) 4,369 (95% CI, 2,298-8,308), p<0.001], family history of atopy [OR 2,943 (95% CI, 1,324-6,541), p: 0.008), CM sIgE>7.39 [OR 9,683 (95% CI, 3,947-23,757), p<0.001], casein sIgE >0.56 [OR 6,909 (95% CI, 2,719-17,557), p<0.001], were the predictors for the persistence.

Conclusion: This study showed that the majority of the CMPA in children gave rise to clinical manifestations in the infancy period, most of them less than six months of age. The prognosis of the disease was favorable with a spontaneous tolerance developed by the age of three in most patients. IgE-mediated hypersensitivity reactions, a family history of atopy and higher specific IgE values were predictive factors for the long-lasting disease.

Keywords: Cow's milk allergy, cow's milk protein allergy, children, skin prick test, tolerance

Introduction

Cow's milk protein allergy (CMPA) is the most common food allergy in children with a prevalence ranging between 1.8%-7.5% (1). CMPA presents with a variety of symptoms involving different systems according to the type of reaction, with a predominance of IgE-mediated reactions. Skin reactions are the most common presentations of CMPA, followed by gastrointestinal and respiratory symptoms. A detailed history, physical examination, skin prick test (SPT) and specific IgE testing may support the diagnosis, but oral food challenge (OFC) is the gold standard for CMPA diagnosis (2). Treatment is the elimination of the dairy products from the infants' diet and if necessary, from the maternal diet in breastfed infants. The majority of patients outgrow their allergy during childhood in the natural course

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of the disease with a favorable prognosis in general (3). Different factors seem to affect the acquisition of this immune tolerance (4). With different rates of resolution and different predictors reported for the tolerance, the natural history of CMPA and strategies for inducing tolerance may change over time (3,5).

In this study, we aimed to assess the natural course of CMPA by investigating its clinical features, management, and the long-term outcomes in pediatric patients with CMPA.

Materials and Methods

Study Population

Ours is a retrospective study of 246 children with CMPA treated between January 2014 and February 2016 at the Department of Pediatric Allergy Immunology in the University of Health Sciences Turkey, Ankara Dr. Sami Ulus Maternity and Child Training and Research Hospital. Data were collected from the medical files. CMPA is classified by the underlying immune mechanism (IgE-mediated, non-IgE-mediated and mixed), the time of presentation and organ system involvement. Reactions within minutes to 2 hours of exposure are considered to be IgE-mediated, while reactions in hours are considered to be non-IgE-mediated or a mix of both (1,2,6). Food allergy diagnosis was based on a combination of clear-cut history, typical clinical presentation and an OFC test. Children were diagnosed with CMPA according to the international guidelines (1,2,7).

Study Measurement

Atopy was evaluated by SPT, prick to prick test (PTP) and sIgE measurements. Initially, a panel of major food [CM, hen's egg (HE), wheat, soy, walnut, hazelnut and peanut] standardized allergen extracts (Stallergens, SA, Antony, France) and fresh milk (FM) were used for skin tests on those patients presenting with suspected food allergy. Individually, the allergen panel was enhanced according to the patient's clinical reactions and diet history. SPTs with inhalant allergens (grass, weed, and tree) (Stallergenes, SA, Antony, France) were performed on children older than two years. A positive SPT was defined as a wheal size of ≥3 mm compared to the negative control. Specific IgE serum levels to food allergens were measured with an enzyme immunoassay system (IMMULITE Siemens, Germany). sIgE levels greater than 0.35 kUA/L were considered as positive.

Oral Food Challenge

Children underwent OFC for diagnostic challenge and determination of tolerance acquisition with CM based on the guidelines' recommendations (8). The age of immunotolerance is defined as the time when FM was tolerated for the first time.

Statistical Analysis

The Statistical Package for Social Sciences (SPSS) version 15 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis of the research data. Odds ratios (OR) with appropriate 95% confidence intervals (CI) were calculated by logistic regression analyses. Receiver operating characteristic (ROC) curve analysis was performed to identify the optimal cut-off CM and casein sIgE value to predict the tolerance status for CMPA. Positive predictive values (PPV), negative predictive values (NPV), sensitivity and specificity values were determined. Statistical significance was defined at $p<0.05$.

Ethical approval was received from the University of Health Sciences Turkey, Ankara Dr. Sami Ulus Maternity and Child Training and Research Hospital local institutional review board (approval number: 2016/2519). Informed consent was not required because the study was conducted retrospectively.

Results

A total of 246 patients with a diagnosis of CMPA were enrolled in this study. The characteristics of the study population are shown in Table I. The diagnoses of CM allergies were as follows: IgE-mediated in 84 (34.1%), non-IgE mediated in 36 (14.6%), and mixed-type in 126 (51.2%) patients. 95.8% of the patients experienced their first reactions associated with CM allergy during infancy (≤12 months). The diagnoses for the 246 cases of CM allergy are shown in Figure 1. Skin symptoms (83.7%) were the most frequently observed clinical manifestation followed by gastrointestinal system (GIS) (17%), respiratory system (6%) and cardiovascular system (1%) involvements. After the ingestion of dairy product by our study group, the most frequently observed symptoms were eczema (49.5%) for skin involvement, blood and/or mucus-streaked stools (11.7%) for the GIS and cough and wheezing (4.8%) for the respiratory system. 12.1% (n=30) of the patients had anaphylaxis with CM, and 4.4% (n=11) of the patients had anaphylaxis with foods other than CM.

The dairy products consumed at the time of the first hypersensitivity reaction were CM-based formula (31.5%), yogurt (23%), breast milk (23), CM (11.9%), cheese (9.4%), butter (0.9%) and condensed CM (0.4%). At the time of the diagnosis, 81.9% of the patients were breastfed (with or without complementary feeding or CM-based formula).

Table I. Characteristics of the study population	
	Study population (N=246)
Gender Male Female	 162 (65.8%) 84 (34.2%)
Current age, median (IQR), months	54 (43-62)
Age of the onset of the allergic symptoms to CM, median (IQR), months	4 (2-6)
Age at the diagnosis of CM allergy, median (IQR), months	6 (4-7)
Food allergy other than CM, no (%)	142 (57.7%)
Presence of allergic diseases, no (%) Atopic dermatitis Allergic rhinitis Asthma	 122 (49.5%) 12. (4.8%) 39 (15.9%)
Accompanying inhalant allergen sensitization, no (%)	12 (4.9%)
Family history of atopy, no (%)	115 (46.7%)
Total IgE, median (IQR), kU/L	40 (19.4-104)
*sIgE CM at the time of diagnosis, median (IQR), kU/L	0.3 (0-2.8)
*sIgE Casein at the time of diagnosis, median (IQR), kU/L	0.1 (0-1.5)
SPT with cow's milk extract at the time of diagnosis, median (IQR), mm	2 (0-5)
PTP with fresh cow's milk at the time of diagnosis, median (IQR), mm	6 (0-10)
Vitamin D level (total 25[OH]D), median (IQR), ng/mL	27 (18.5-39.6)
IQR: Interquartile range, CM: Cow's milk, SPT: Skin prick test, PTP: Prick to prick *Measured at the time of first performance during follow-up	

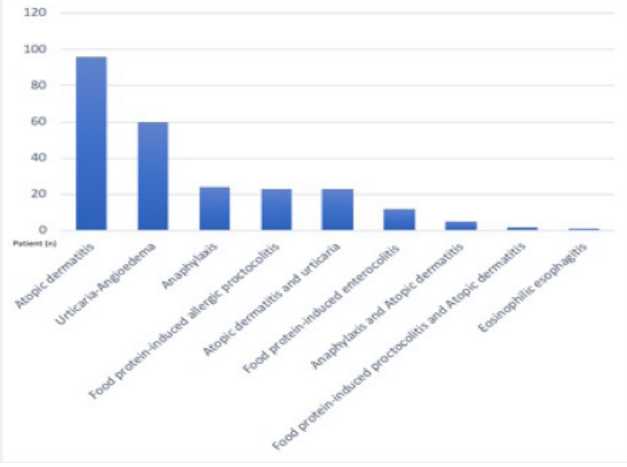

Figure 1. Diagnosis of cow's milk protein allergy

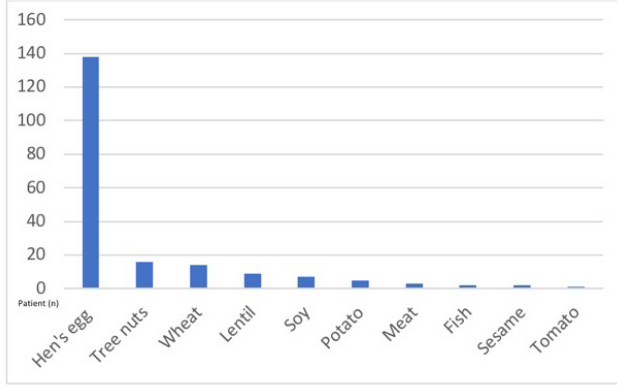

Figure 2. Food allergy other than cow's milk

Those patients who were formula-fed on at least one occasion were 39.2% of the population. The patients were introduced to complementary foods at a mean age of 5 months (range: 1 to 9 months).

Multiple food allergies were determined in 57.7% (n=142) of the patients. The most common food allergy other than CM was HE (n=138). The concomitant food allergies are shown in Figure 2.

At the time that the first SPTs were performed, 70.3% of the patients' PTP performed with FM were found to be positive; however, 46.5% of the patients' SPT with CM were positive. SPT performed with goat's milk was positive in 62.7% of the patients.

During the elimination of CM and its products from the diet, 62.5% of the patients used extensively hydrolyzed infant formula (eHF) or amino acid-based formula (AAF) with a mean (± standard deviation) duration of 11 (±5.9) months. Among those mothers who were on an elimination

diet for CM and its products, 77.3% received supplemental calcium and vitamin D. The vitamin D level of the patients evaluated during the follow-up period was below 20 ng/mL in 30.3% of the patients.

Approximately within five years of follow-up period, tolerance occurred in 78.9% of the patients (61.9% IgE-mediated and 87.6% non-IgE-mediated). 59.3% of the patients developed tolerance by the age of 24 months, and 68.2% by the age of 36 months. ROC curve analysis was performed to identify the optimal cut-off CM and casein sIgE values to predict the tolerance status for CMPA. (Figures 3 and 4). The optimal cut-off value for final CM sIgE was 7.39 kU/L with a sensitivity of 87.3% and a specificity of 58.3%, as well as an area under the curve of 0.757 (95% CI, 0.655-0.859, p<0.001) (Table II). The type of the hypersensitivity reaction, family history of atopy, age of the introduction of complementary feeding, CM sIgE, casein sIgE, wheal size of SPT with CM and wheal size of PTP with FM were associated with tolerance status (p<0.05). The comparison of patients with CMPA according to their status of tolerance or persistence is shown in Table III. IgE-mediated hypersensitivity reactions [OR 4,369 (95% CI, 2,298-8,308), p<0.001], family history of atopy [OR 2,943 (95% CI, 1,324-6,541), p=0.008], CM sIgE>7.39 [OR 9,683

Table II. ROC analysis and diagnostic value of cow's milk and casein spesific IgE for the prediction of the tolerance in patients with cow's milk protein allergy

	Diagnostic scan					ROC curve		p-value
	Cut-off	Sensitivity%	Specificity%	PPV%	NPV%	AUC	95% CI lower-upper	
CM-specific IgE final kU/L	**≤7.39**	87.37	58.33	84.69	63.64	**0.757**	0.655-0.859	**0.001**
Casein spesific IgE final kU/L	**≤0.56**	63.33	80.00	89.06	45.90	**0.758**	0.651-0.865	**0.001**

ROC: Receiver operating characteristic, CM: Cow's milk, PPV: Positive predictive values, NPV: Negative predictive values, AUC: Area under the curve, CI: Confidence interval

Table III. Comparison of the patients with cow's milk protein allergy according to the status of tolerance or persistence

	Tolerant patients N=194	Persistent patients N= 52	p-value
Gender Male, no (%) Female, no (%)	 131 (67.5%) 63 (32.5%)	 31 (59.6%) 21 (40.3%)	0.285
Current age (years), median (IQR), months	55 (46-62)	50 (27-64)	**0.014**
Age at onset of symptoms	6 (3-7)	6 (5-8)	0.182
Age of the introduction of complementary feeding, mean (± SD), months	4.9 (±1.4)	5.4 (±0.8)	**0.022**
Type of hypersensitivity reaction IgE-mediated, no (%) Non-IgE-mediated or mixed type, no (%)	 52 (26.8%) 142 (73.2%)	 32 (61.5%) 20 (38.5%)	**<0.001**
Concomitant food allergy Single food allergy, no (%) Multiple food allergy, no (%)	 84 (43.4%) 110 (56.7%)	 20 (38.5%) 32 (61.5%)	0.531
Family history of atopy, no (%)	80 (41.2%)	35 (67.3%)	**0.007**
CM sIgE initial, median (IQR), kU/L	0.10 (0-1.10)	2.6 (0.1015.65)	**<0.001**
CM sIgE final, median (IQR), kU/L	0.54 (0-4.8)	13.06 (1.64-85.45)	**<0.001**
Casein sIgE initial, median (IQR), kU/L	0 (0-0.69)	0.69 (0-7.6)	0.868
Casein sIgE final, median (IQR), kU/L	0.16 (0-1.8)	4.5 (0.7-35.1)	**<0.001**
SPT with cow's milk extract, median (IQR), mm	0 (0-5)	5 (0-7)	**0.004**
PTP with fresh cow's milk, median (IQR), mm	5 (0-9)	8 (6-11)	**<0.001**
Vitamin D level [total 25(OH)D], median (IQR), ng/mL	26.8 (18.9-38)	28.1 (15.8-43.2)	0.911

IQR: Interquartile range, SD: Standard deviation, sIgE: Specific IgE, CM: Cow's milk, SPT: Skin prick test, PTP: Prick to prick, 25(OH)D: 25-hydroxyvitamin D

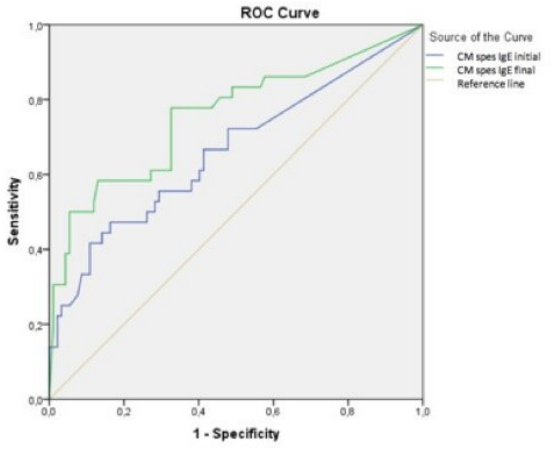

Figure 3. Receiver operating characteristic curve analysis for estimating the optimal cut-off cow's milk sIgE value
ROC: Receiver operating characteristic, CM: Cow's milk, sIgE: Specific IgE

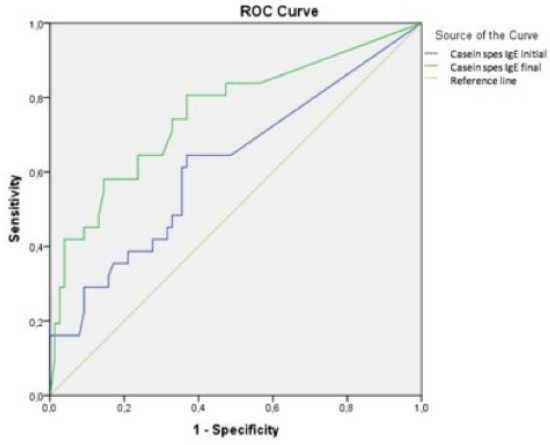

Figure 4. Receiver operating characteristic curve analysis for estimating the optimal cut-off casein sIgE value
ROC: Receiver operating characteristic, sIgE: Specific IgE

(95% CI, 3,947-23,757), p<0.001], and casein sIgE >0.56 [OR 6,909 (95% CI, 2,719-17,557), p<0.001] were associated with longer-lasting disease.

Discussion

This study emphasized the early onset of the symptoms in CMPA, particularly in the first six months of infancy with most of the children presenting with AD. Among the initial diagnostic allergy tests, PTP performed with FM showed higher positivity when compared to SPT with CM. Lentil was one of the prominent triggering foods coexisting with CMPA after HE, tree nuts and wheat. Our study population showed that more than half of the patients developed tolerance by the age of 36 months with a favorable prognosis for CMPA. An earlier introduction of complementary feeding and having non-IgE-mediated or mixed type reaction showed a positive association with the development of tolerance in children with CMPA. Higher CM and casein-specific IgE level, family history of atopy and IgE-mediated reactions showed a negative association with tolerance.

CMPA is more common in infants and peaks in the first year of life with a predominance of the IgE-mediated type of allergy (3). Nearly half of the children with CMPA are estimated to have IgE-mediated reactions (9). In the current study, the median (IQR) age of symptom onset was four months (2-6) which was distinctly earlier than reported in a preliminary analysis of a Turkish national multicenter study of children diagnosed with food allergy (10). In our study population, the mixed group and non-IgE-mediated reactions were more common than IgE-mediated reactions. In a study of pediatric patients with CMPA from Turkey, the authors reported more IgE referenced diagnoses (66.6%) when compared to non-IgE and the mixed group (11). However, Yang et al. (12). reported that the majority of CMPA infants had eczema in China. Approximately one-third of children with AD had a diagnosis of CMPA after OFC, and nearly 40-50% of children less than one year of age with CMPA also had AD (13). We would like to emphasize the main clinic presentation of CMPA in our study population which was AD rather than IgE-mediated reactions like urticaria or angioedema during the first six months of infancy.

The clinical presentation of CMPA involved cutaneous symptoms in up to 90%, GI symptoms in up to 60%, respiratory symptoms in up to 30% and anaphylaxis in 0.8 to 9% of cases (9). Symptoms and signs related to CMPA involved mostly the skin and the GI system in our study group which is consistent with the literature; the anaphylaxis rate was slightly higher than those reported (9).

In studies reported from Turkey, about 30-50% of food allergic children have multiple food allergies (2). In the current study, the rate of multiple food allergies is within the upper limits. HE was the most frequent triggering food coexisting with CMPA similar to the literature, followed by tree nuts and wheat. The third and fourth most common triggering foods differ from one country to another such as peanuts in the USA and Switzerland, wheat in Germany and Japan, sesame in Israel, walnuts in Korea and hazelnuts in Turkey (3). The fourth most common food in our group was lentil, followed by soy, potato and meat. We consider that these differences are related to the frequency of consumption of these foods in specific regions, a variety of the dietary patterns and traditional eating habits.

Skin tests with both FM and CM standardized extract are useful in the diagnostic workup of CMPA, but PTP with fresh food extracts were reported to be more effective in detecting sensitization in comparison to SPT with commercial extracts (14,15). With a cut-off of 3 mm for both allergens tested initially in our group, FM showed more positivity than standardized CM extract similar to previous studies. Mauro et al. (16) reported that PTP with FM showed better sensitivity and NPV than SPT with three milk proteins (a-lactalbumin, casein and b-lactoglobulin), taken singly or all together; however, FM had the least specificity and PPV. Considering the mean age (under six months) of onset in our study group and the wheal size of allergen-induced prick tests which are smaller in infants than in children (due to hyporeactivity), PTP with FM is very useful for the initial diagnostic workup of CMPA, primarily to exclude an IgE-mediated CMPA with high sensitivity and NPV (17).

Providing appropriate nutritional guidance is essential to supply sufficient calorie intake, minerals and elements. Non-exclusive breastfeeding of infants with CMPA requires a substitute-formula with age-appropriate nutritional requirements. The type and severity of the clinical reaction and the availability of the formula affect the selection of the formula (2). More than half of the patients in the current study required eHF or AAF, which are recommended by DRACMA guidelines, with eHF as the first line for uncomplicated cases, and AAF for severe cases (9). Although therapeutic CM elimination was achieved with appropriate diet modification by a dietitian, 30% of the patients in the current study had inadequate vitamin D levels in their blood.

None of the cut-offs for SPT or sIgE proposed in the literature can be used to confirm CMPA. However, many reports suggest possible sIgE and/or SPT cut-off values for CMPA diagnosis in the pediatric population (18). Different values have been recommended in the literature, even when similar statistical methods are used. CM sIgE cut-offs with a 100% PPV varied between 4.18 KUA/L and 50 KUA/L (18). In a group of studies, it was found that casein sIgE was the best predictor but in another study CM sIgE was a better predictor than the specific IgE for its components (19-22). In the current study, we combined IgE-mediated and mixed hypersensitivity reactions for the analyses of the sIgE cut-off for tolerance prediction in patients with CMPA. We found a cut-off level of 7.39 for CM sIgE and a cut-off level of 0.56 for casein sIgE. In a cohort study, it was reported that among children with CMPA, 70% of those with a CM sIgE < 2 kU/L had resolved milk allergy compared with only 23% of those with a CM sIgE >10 kU/L (5). In the current study analyzing CM sIgE level as a categorical variable, subjects with a final CM sIgE less than 7.39 kUA/L had a 9.6-fold increased likelihood of resolving their allergy versus those with levels of greater than 7.39 kUA/L.

No delay in the introduction of complementary feeding in infants with CMPA is recommended for tolerance development in infants and children with CMPA (3). According to our findings, the initiation of complementary feeding was significantly earlier in the tolerant group, which is in accordance with the literature.

The levels of sIgE, SPT wheal sizes, sensitization to multiple foods, and a family history of atopy are reported to be inversely associated with the timing of CMPA resolution (3). The levels of sIgE, SPT wheal sizes and a family history of atopy were inversely associated with tolerance in the current study. However, there was no significant difference in sensitization to multiple foods. The type of immune reaction in CMPA was shown to be associated with the rate and timing of tolerance acquisition, with more frequent and earlier development of tolerance in non-IgE-mediated CMPA than in IgE-mediated CMPA (23). We found in our study group that the immune tolerance developed more frequently in the non-IgE and mixed group than in IgE-mediated CMPA, which is consistent with the literature (4).

The most important limitation of our study is its retrospective design. Selection of the patients for OFC may differ instead of a protocol-defined method. Open OFC was performed for the diagnosis, but it is reported that for the first years of life, open OFC does not seem to cause bias (24). However, the long-term follow-up period and the large number of patients are the strengths of the study reflecting the real-life and clinical course of CMPA in daily practice.

Conclusion

In this study, we evaluated the natural course of CMPA in a group of children followed up in a pediatric allergy clinic. A significant proportion of these children with CMPA presented as AD in early infancy. In addition to HE, tree nuts and wheat, lentil was one of the common coexisting food allergies. The tolerance acquisition rate was more than 50% by the age of 3 years. IgE-mediated hypersensitivity reactions, family history of atopy, higher sIgE values, and the later initiation of complementary feeding were predictors for its persistence.

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Ethics

Ethics Committee Approval: Ethical approval was received from University of Health Sciences Turkey, Ankara Dr. Sami Ulus Maternity and Children Training and Research Hospital local institutional review board (approval number: 2016/2519).

Informed Consent: Informed consents were not required because the study was conducted retrospectively.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: A.E., Z.Ş.E., S.Ö., İ.B., Design: A.E., İ.B., Data Collection or Processing: A.E., Z.Ş.E., İ.B., Analysis or Interpretation: A.E., Z.Ş.E., S.Ö., İ.B., Literature Search: A.E., Z.Ş.E., Writing: A.E., Z.Ş.E., S.Ö., İ.B.

Conflict of Interest: No conflict of interest was declared by the authors.

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Original Article

Maternal Perceptions About Breast-milk Production Predicted the Daily Frequency of Breastfeeding in Infants of Age Up-to Six Months in Gondar Town, Northwest Ethiopia

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ABSTRACT

Aim: The proper quantity and quality of breast-milk availability markedly influenced by the number of breastfeeding (BF) sessions per day. Consistent professional support that is information based may be crucial in improving the frequency of effective BF. Thus, we aimed to provide information on factors predicting maternal BF frequency (BFF).

Materials and Methods: A total of 861 participants were selected by using a cluster sampling method and a community based cross-sectional study design. An Online Data Collection Kit (ODK) technique was applied to collect the face-to-face interviewer administered survey from lactating women. Advanced analyses were carried out. The directly downloaded data from Google Cloud imported to Stata 14. Negative Binomial Regression was employed to model the frequency of BF and its predictors.

Results: Around 77% of mothers breastfed their infants at least 9 times per day, of which 15% of the mother's breastfed more than 12 times per day. The incidence of frequent BF increased among mothers who had postnatal follow-up [Adjusted Incidence Rate Ratio (AIRR): 1.07; 95% confidence interval (CI): 1.01-1.13], who strongly perceived the adequacy of their breast milk production (AIRR: 1.22; 95% CI: 1.04-1.44) and who had preterm births (AIRR: 1.06; 95% CI: 1.02-1.13). Furthermore, a one-centimetre increase in Mid Upper Arm Circumference (MUAC) of the mothers was associated with an increased frequency of BF (AIRR: 1.02; 95% CI: 1.02-1.03).

Conclusion: The ratio of lactating mothers who breastfeed their infants was found to be lower than the Ethiopia Infant and Young Child Feeding Practice guideline. The incidence rate ratio of frequent BF was directly associated with antenatal MUAC, postnatal follow-up, preterm birth, and maternal perception about breast milk production. Though BFF is one of the components of appropriate BF, this issue has received little attention in Ethiopia. Thus, frontline health professional and concerned bodies should give attention to the encouragement of the frequency of BF by giving attention to its identified predictors.

Keywords: Frequency of breastfeeding, infants aged up to six months, Ethiopia, poisson regression

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Introduction

In Ethiopia, the proportion of mothers initiating exclusive breastfeeding (EBF) has increased between the years 2005 and 2019 from 49% to 59% (1). However, EBF is frequently discontinued before 6 months (1,2), despite current recommendations that infants should be exclusively breastfed "on-demand" (according to their appetite) until the age of 6 months of life (1,3). The proportion of exclusively breastfed infants in Ethiopia abruptly drops with age; from 73% to 68% between the ages of 0-1 months and 2-3 months, and further to 40% at the age of 4-5 months (1). The frequently given reasons for this discontinuation is the perceived insufficient milk production (4-6), in other words, a woman perceiving that her supply is inadequate either to satisfy her infant's hunger or to support normal growth (7). The volume of milk consumed at each BF is directly related to the available milk volume of the breast (8). Indeed, the proper quantity and quality of breast-milk availability markedly influenced the number of BF sessions per-day (9).

Thus, exclusive BF and BF duration are correlated issues as artificial feeding during the first few days and weeks of lactation reduces the frequency of breast stimulation required to form breast tissue development and milk production (10). Artificial or formula milk is likely used by mothers who perceive that they have insufficient milk production. BF knowledge is strongly linked with BF confidence and actual lactation duration (5). Several previous studies have testified that BFF falls between the range of eight to twelve times per day (6,8,11-13).

Consequently, evidence-based information can assist efforts to improve the frequency of successful BF by replacing either unskilled or inconsistent professional support. We aimed to provide information that can be used as a guide to clinicians or frontline health workers on those factors affecting BFF.

Materials and Methods

Study Design and Setting

A community-based cross-sectional study was employed to reach lactating mothers who were living in Gondar Town. From the Amhara National Regional State administrative zones. Gondar town is located in the Northern part of it at a distance of 747 km from Addis Ababa (the largest city and capital of Ethiopia). It has 12 counties with a total population of 333,103 (14,15). The town has one governmental referral hospital, more than eight health centres, and more than 15 private medical clinics (16). Lactating women who were living in a randomly selected urban county (clusters) were considered as the study population.

Sample Size

A cluster sampling technique was used to select lactating mothers who had six-month-old infants. At the first stage, a lottery method was applied to find the five urban clusters and at the sec ond stage, a housing census of lactating mothers found in the five selected clusters was conducted. The desired sample size (n) was calculated in Epi-info 7 by the s ingle population proportion formula $n = \frac{\left(z_{\alpha/2}\right)^2}{d^2} pq$ by assuming 95% confidence level of, $z_{a/2} = 1.96$ a margin of error of 5%, design effect of 2, the proportion infants breastfeed more than eight times per 24-hours was 74% (1), and the non-response rate was 10%. The final sample size was 650 but due to a cluster effect, all the participants within the five clusters were included and a total of 861 was finally reached.

Data Collection and the Questionnaire

A face-to-face interviewer administered a structured and pre-tested electronic-based questionnaire to collect the required data from all the participants. The online data collection and management application kit was used (17). A Lenovo-7 tablet with uploaded questionnaires was used by nine trained nurses to collect and send the data through an online Google cloud platform. To confirm consistency, the original English version of the questionnaire was translated to Amharic and back-translated to English before the actual data collection. The investigators of the study supervised the overall process of the data collection activities.

Socio-demographic characteristics such as age, educational status, income, marital status, frequency and early initiation of breastfeeding were collected by using a questionnaire. Maternal and infant nutritional status were collected using the Mid Upper Arm Circumference (MUAC) where an infant with a MUAC ≤110 mm and a mother with maternal MUAC ≤220 mm were considered malnourished (18-20). Food security was assessed using a question, "In the last three months, have you ever worried that your household would not have enough food?" with a response category of "yes" or "no". Information on maternal characteristics including pregnancy intention and perception towards breast-milk production (how satisfied they were with the amount of milk they produced for their baby) were collected. Maternal depression was assessed using the Edinburgh Postnatal Depression scale with a cut-off value of 12 or more indicating depression (21). Social support was

assessed using the Oslo Social Support scale with a cut-off value of 9 and above indicating good support (22). Partner support was assessed by a question "My husband helps me a lot" with response options of "always", "most of the time", "some of the time", and "rarely".

Data Analysis

The data, which was stored online on the Google Cloud Platform, was directly downloaded and imported to the Stata 14 for further analysis. The data completeness was checked and further cleaning was done by running frequencies. Before further analyses were done, the nature of the data was explored through mean, median, proportion/percentage, interquartile range, standard deviations, and exploratory analysis. Preliminary findings were presented using tables. The appropriate assumptions were checked (such as equal mean and variance of the corresponding count data) and the Negative Binomial Regression Model was fitted to model factors associated with the BFF. Adjusted incidence rate ratio (AIRR) with its 95% confidence interval (CI) was used to report factors predicting the BFF.

Statistical Analysis

A statistically significant variable was considered at a p-value $\leq$0.05 in the final model. Akaike information criterion (AIC) and Bayesian information criteria (BIC) were applied to select the model with the best fit for the data. The model with the smallest AIC and BIC was selected as the best fit model for the data and interpreted.

Ethical Consideration

The first approval was obtained from University of Gondar Institutional Review Board ethics committee (approval no: O/V/P/RCS/05/1601/2018, date: 12/06/2018). A supportive letter, which was used in Gondar town health office and respective districts, was given by the University of Gondar Research and Community Service. The purpose of the study and its objectives were explained to each participant including their right to participate or not participate in the study. A personal identifier was not used in order to protect the privacy and confidentiality of the study participants. Before the interview started, written informed consent was secured from the study participants declaring that they were willingly participating in the study. Participants who were seriously ill at the time of data collection were referred to Gondar University Specialized Hospital. Lactating mothers and infants found to be severely malnourished were also counselled about proper nutrition.

Results

Baseline Characteristics of the Respondents

A total of eight hundred and sixty-one (861) mothers were interviewed. The mean [± standard deviation (SD)] age of mothers was 26.5 (±4.53) years. Most of the mothers (80.7%) were orthodox Christian followers and married (96.2%). A substantial proportion of respondents (61.6%) had primary or above educational status, while more than two-thirds of the study participants (71%) were housewives. Around 90% of respondents had low or medium-income with a household mean (± SD) monthly income of 3,509.76 (±2977.61 birr) Ethiopian birrs (Table I). Eight hundred and twenty-five (95.8%) and six hundred and sixty-one (76.8%) mothers had attended Antenatal-care or were attending Postnatal-care services, respectively. Regarding pregnancy needs, seven hundred and thirty-four (85.3%) pregnancies were planned and the majority of women had 3 or less children with a mean (± SD) number of 2 (±1.21) children. Over two-thirds (69%) of BF women strongly agreed that their breast milk production was adequate.

Psychosocial Characteristics of the Respondents

Nearly half (47%) of lactating mothers have constant support from their husband. A significant number of (79.7%) the participants received good social support, and the remaining had poor social support. Regarding their depression status, 6% of the mothers had depression symptoms during pregnancy and 8% of lactating mothers had depression. Daily coffee consumption was reported in 41.2% of lactating mothers.

Baseline Characteristics of the Infant

The majority (68.9%) of the infants included in the current study were above the age of four months. About 61% of infants had initiated breastfeeding within one hour of delivery and a similar proportion (63%) of infants had exclusive BF for up to 6 months. About 15% and 3% of infants were preterm births or low birth weight, respectively. One hundred and twenty-two (14%) of the infants were malnourished. About 22% and 17% of the infants had diarrhea or acute respiratory infection, respectively (Table II).

Predictors of Breastfeeding Frequency Among Lactating Mothers

Among the 861 participating lactating mothers, about 77% reported breastfeeding their infants at least 9 times per day (in 24 hours). Of these, around 15% of mothers frequently breastfed their children more than twelve times per day. An adjusted Poisson regression model output

Table I. Baseline-characteristics of the respondent in Gondar tow, Northwest Ethiopia (n=861)

Variable	Number	%
Age of Respondents		
18-24	280	32.52
25-34	521	60.51
≥=35	60	6.97
Religion of Respondents		
Orthodox	695	80.72
Muslim	166	19.28
Marital Status of Respondents		
Single	33	3.83
Married	828	96.17
Educational Level of Respondents		
No formal education	110	12.78
Grade 1-8	221	25.67
Grade 9-12	321	37.28
Diploma and above	209	24.27
Occupation of Respondents		
Housewife	614	71.31
Student	13	1.51
Government employee	123	14.29
Self-employed	111	12.89
Income of Respondents		
Low	419	48.66
Medium	353	41.00
High	89	10.34
ANC Follow-up		
No	36	4.18
Yes	825	95.82
PNC Follow-up		
No	200	23.23
Yes	661	76.77
Parity		
1	331	38.44
2-3	433	50.29
4-8	97	11.27
Sex of Child Preference		
No	626	72.71
Yes	235	27.29
Pregnancy Planning		
Planned	734	85.25
Unplanned	127	14.75
Breast Milk Production Perception of Respondents		
Don't know	18	2.09
Agree	248	28.80
Strongly agree	595	69.11
Antenatal-MUAC		
<22 cm	123	14.29
>22 cm	738	85.71
Postnatal-MUAC		
<22 cm	71	8.25
>22 cm	790	91.75

MUAC: Mid Upper Arm Circumference, ANC: Antenatal care, PNC: Postnatal care

Table II. Baseline characteristics of infants under the age of six months in Gondar town, Northwest Ethiopia (n=861)

Variables	Number	%
Age of Infant		
1-4 months	268	31.13
5-6 months	593	68.87
Early Initiation of BreastFeeding		
No	332	38.56
Yes	529	61.44
Exclusive BreastFeeding		
No	316	36.70
Yes	545	63.30
Low Birth Weight		
No	835	96.98
Yes	26	3.02
Preterm Birth		
No	733	85.13
Yes	128	14.87
Infant Nutritional Status		
Normal	739	85.83
Malnutrition	22	14.17
Diarrhea		
No	714	82.93
Yes	147	17.07
Acute Respiratory Infection		
No	675	78.40
Yes	186	21.60

revealed that the incidence of frequent breastfeeding increased among lactating mothers who had postnatal follow-up (AIRR: 1.07; 95% CI: 1.01-1.13), who strongly agreed that their breast milk production was adequate (AIRR: 1.22; 95% CI: 1.04-1.44), and who had preterm births (AIRR: 1.06; 95% CI: 1.02-1.13). A one-centimetre increase in MUAC of the mothers was found to be associated with an increase in EBF (AIRR: 1.02; 95% CI: 1.02-1.03) (Table III).

Discussion

Inappropriate breastfeeding practices significantly impair the health, development and survival of infants and improving these practices could save thousands of infant lives each year (2). The first few hours and days of a newborn's life are a critical window for establishing lactation through the early initiation of BF, EBF, and an increased frequency of breastfeeding. The current study aimed to provide information that can be used as a guide to clinicians or frontline health workers when advising mothers about proper breastfeeding.

Most (77%) of the mothers reported breastfeeding their infants at least 9 times per day (in 24 hours), which is similar to a study from Southern Ethiopia with a rate of 74% and Addis Ababa city with a rate of 76% (23,24). The Ethiopia IYCF guideline recommends lactating women breastfeed infants at least 10-12 times per day (25). With respect to this, only 58% achieved this recommendation. In addition, early initiation of breastfeeding and EBF were 61% and 63% respectively, which are also below the recommended IYCF guideline (3). Compared to the current study, lower BFF was reported in Indonesia with a rate of 53.6% (13). These possible differences might be due to socio-demographic differences such as maternal education or employment status. Highly educated mothers are more

Table III. Poisson regression model to identify factors associated with BFF among lactating mothers in XX, 2019 (n=861)

Variables	**Frequency (%)**	**IRR, 95% CI**	**AIRR, 95% CI**
Antenatal MUAC	861 (100%)	1.03 (1.02-1.04)	1.02 (1.002-1.03)*
Postnatal MUAC	861 (100%)	1.02 (1.01-1.04)	1.00 (0.98-1.02)
PNC Follow-up			
No	200 (23.23)	1	1
Yes	661 (76.77)	1.14 (1.08-1.20)	1.07 (1.01-1.13)*
Preterm Birth			
No	733 (85.13)	1	1
Yes	128 (14.87)	1.09 (1.04-1.16)	1.06 (1.02-1.13)*
Breast Milk Production Perception of Mothers			
Don't know	18 (2.09)	1	1
Agree	248 (28.80)	1.09 (0.93-1.29)	1.11 (0.94-1.31)
Strongly agree	595 (69.11)	1.24 (1.06-1.45)	1.22 (1.04-1.44)*
Husband Support			
Rarely	52 (6.04)	1	-
Some of the time	160 (18.58)	1.07 (0.97-1.18)	1.07 (0.97-1.19)
Most of the time	247 (28.69)	1.05 (0.95-1.16)	1.07 (0.97-1.19)
Always	402 (46.69)	1.13 (1.03-1.24)	1.09 (0.99-1.21)
Days With Disability of Mothers			
≤=7days	416 (48.32)	1	-
≥=8days	445 (51.68)	1.07 (1.03 - 1.12)	1.04 (0.89 - 1.09)
Postnatal Depression			
No	69 (8.01)	1	-
Yes	792 (91.99)	0.95 (0.88-1.03)	0.97 (0.89-1.06)

BFF: Breastfeeding frequency, MUAC: Mid Upper Arm Circumference, IRR: Incidence rate ratio, AIRR: Adjusted incidence rate ratio, CI: Confidence interval

likely to be employed and might not have time to frequently breastfeed, unlike those who are less educated and are often housewives. Most of the participants in the current study were housewives with a low educational status. This shows the negative effect of employment on the frequency and duration of breastfeeding (26), and most importantly EBF (25).

In Southeast Ethiopia, the median BFF was found to be 6 times per day (27). This is lower than our finding and this might be due to the age difference of the infants (25,28) included in the study as BFF decreases as the age of the infants' increases (27,28). In the first month, there is frequent breastfeeding with a short duration (lasting for 20 minutes) but, in later months, this frequency gradually decreases while the duration of breastfeeding increases (12,29).

A unit increase in the antenatal MUAC of the mothers resulted in an incremental increase in BFF in this study. Opposing this, a finding from Bangladesh showed that maternal nutritional status was found to be unrelated to the frequency of breastfeeding (6). In general, a previous review (30) and cohort study (31) found an association between maternal weight and the main breastfeeding components. Thus, optimizing maternal BMI during the pre-conception period is essential, which is supported by the current findings indirectly. Unlike our findings where no association between infant nutritional status and the incidence rate ratio of BFF was determined, a study from Bangladesh stated that infant nutritional status was significantly associated with BFF (6).

In this study, mothers who had postnatal follow-up were more likely to frequently breastfeed their infants than their counterparts. This is true as postnatal follow-up helps to provide counselling services about appropriate breastfeeding (3). A major review approach for improving BF rate noted multiple determinants and influences but BF counselling is one of the key interventions to improve the BF rate (32). The incidence rate ratio of BFF was found to be higher among mothers who had preterm birth infants. Better counselling and support about appropriate breastfeeding to mothers of preterm infants might have enhanced their knowledge and practice on the frequency of BF. This evidence implies that the implementation of some breastfeeding counselling, such as about frequency and timing, to the lactating mothers could significantly improve breastfeeding practices.

Similarly, the incidence rate ratio of frequent breastfeeding was higher among mothers who strongly agreed that their breast milk production was enough to feed their infants. Maternal perception about insufficient milk production was found to be one of the reasons for lower BFF and discontinuing BF in another study (6). There is also evidence that, for a mother, the more productive of their breasts was used more frequently than the less productive one, and when the breastfeeding was paired, the more productive breast was offered first more frequently than the less productive one (8). To our knowledge, this investigation is the first to examine BFF in relation to an urban setting. Thus, this finding may serve as baseline information for frontlin e health workers, decision-makers and researchers. However, mothers' recall bias on BF frequency might affect the validity of this study. A prospective follow-up study that is able to observe the duration of BF for each frequent BF with the inclusion of socio-cultural variables would help to determine a more precise estimate.

Conclusion

The ratio of lactating mothers in this study who breastfeed their infants was found to be lower than the Ethiopia IYCF guideline. The incidence rate ratio of frequent breastfeeding was directly associated with antenatal MUAC, postnatal follow-up, preterm birth, and maternal perception about breast milk production. Although BFF is one of the components of appropriate BF, this issue has received little attention in Ethiopia. Thus, frontline health professionals and concerned bodies should give attention to the enhancement of the frequency of BF. In addition, we have also a recommendation to strengthen the public health information and education system in order to promote appropriate breast-feeding.

Ethics

Ethics Committee Approval: The first approval was obtained from University of Gondar Institutional Review Board ethics committee (approval no: O/V/P/RCS/05/1601/2018, date: 12/06/2018).

Informed Consent: Before the interview started, a written informed consent was secured from the study participants to willingly participate in the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: A.F.D., Z.M.N., H.D.D., Design: A.F.D., Z.M.N., H.D.D., Data Collection or Processing: A.F.D., Z.M.N., H.D.D., Analysis or Interpretation: A.F.D., Z.M.N., H.D.D., Literature Search: A.F.D., Z.M.N., H.D.D., Writing: A.F.D., Z.M.N., H.D.D.

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Original Article

Brain Developmental Differences Between Preterm-born Twins and Singletons: A Multi-modal MRI Study

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ABSTRACT

Aim: Twin studies allow for the investigation of genetic and environmental influences on human brain development. The generalizability of their findings depends on the developmental similarity between twins and singletons. This study aimed to evaluate the structural and functional differences in a cohort of preterm-born twins and singletons at term-equivalent age.

Materials and Methods: Eighteen twins and forty-seven singletons were included and scanned at the term-equivalent age. Brain volumes from 3D T1-weighted images, quantitative metrics and structural connectivity from diffusion tensor imaging, and low-frequency brain activity and functional connectivity from resting-state functional MRI (rs-fMRI) were obtained from these neonates.

Results: We found no significant volumetric differences after multiple comparison correction. The diffusivity values in the cingulum cingular part, cingulate gyrus, lateral fronto-orbital gyrus, gyrus rectus, as well as medial fronto-orbital gyrus were significantly higher in the twin group than in the singleton group. Structural connectivity analysis showed higher transitivity in the twin group compared to the singletons, indicating increased local connectivity. For rs-fMRI, the twin group showed greater fractional amplitude of low-frequency fluctuation (fALFF) values in the salience network and several fronto-temporal regions compared with the singleton group. It is worth noting that we found differences both in structural and functional measurements (MD and fALFF) in the prefrontal and cingulate cortex.

Conclusion: The structural and functional differences collectively indicated that preterm-born twins may have delayed brain development compared with gestational age-matched singletons at term-equivalent age, which may be related to perinatal-neonatal problems.

Keywords: Twin-singleton, brain development, preterm-birth, multi-modal MRI, connectivity

Introduction

Knowledge from twin studies allows researchers to understand the contribution of genetic and environmental factors to brain development (1-5). The generalizability of these studies, however, depends on the assumption that brain developmental patterns in twins are comparable to those in singletons. It has been well established that twins have compromised growth in the third trimester starting from about 30 weeks of gestation that may be attributed to certain reasons, such as the different growth

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pattern, placental size, maternal nutrition, and family care after birth (6-8), leading to potential brain developmental differences between twins and singletons.

Neuroimaging, especially magnetic resonance imaging (MRI), has been shown to be a powerful tool to characterize the structural and functional changes in the developing brain. Brain MRI has been employed to investigate twins and singletons, but not to its full potential. Several existing studies (9-11) focused on morphological differences between twin and singleton brains from 10 months to 30 years of age, however, their conclusions were inconsistent. A recent longitudinal study by Sadeghi et al. (12) compared longitudinal diffusion tensor imaging (DTI) metrics between singletons and twins from birth to 2 years old, and showed that the axial diffusivities in the anterior limb of the internal capsule and anterior corona radiata were significantly higher in twins compared with singletons during early development.

Current MRI studies comparing twins and singletons are still limited and their findings are discrepant, and most studies used a single MRI modality (T1-weighted or DTI). Moreover, all the aforementioned studies focused on comparing twins with term-born singletons, while it is known that twins have an increased risk of preterm delivery (13). The unpaired gestational age makes it difficult to separate the effects of twin birth from premature birth in the observed developmental differences compared with singletons (14). Here, we aim to systematically evaluate whether the MRI findings of twins can be generalized to singleton studies by comparing the preterm-born singleton and twin neonates that were born with equivalent gestational ages with a multi-modal MRI approach, including morphological MRI, DTI, and resting-state functional MRI (rs-fMRI) acquired at term-equivalent age.

Materials and Methods

Subjects

Preterm-born infants were enrolled at term-equivalent age for MRI scan. Ethical approval was obtained from the Institutional Review Board at the Children's Hospital of Zhejiang University School of Medicine (2019-11-13). Written informed consent was provided by the parents. Exclusion criteria included 1) congenital malformation or syndrome; 2) acquired brain injury on MRI; 3) visible artifacts on MRI, or a mean frame-wise displacement (FD) exceeding 0.2 mm for rs-fMRI; 4) psychiatric or neurological family history; 5) pregnancy complications; 6) illicit drug or alcohol use during pregnancy.

Image Acquisition

All neonates received 50 mg/kg oral or enema chloral hydrate 30 minutes before scanning from a radiology nurse who was trained and certified to administer sedation. Ear protectors and physiological monitors were used for protection and monitoring. The scans were performed on a Siemens 1.5T Avanto MRI scanner (Siemens Healthcare, Erlangen, Germany) with a 12-channel Siemens head coil. The multi-modal MRI protocol included the following: 1) 3D sagittal T1-weighted imaging using the MPRAGE sequence with repetition time (TR)=1,910 ms, echo time (TE)=3.01 ms, inversion time=1,100 ms, flip angle=15°, resolution=0.82×0.82×1 mm^3, field of view (FOV)=210×210×160 mm^3, and acquisition matrix=256×256×160; 2) DTI using a single-shot echo-planar imaging (EPI) sequence with TR=3,800 ms, TE=86 ms, in-plane resolution=1.64×1.64 mm^2, FOV=210 × 210 mm^2, 25 slices at a slice thickness of 5 mm, 12 gradient directions at a b-value of 750 s/mm^2 and 1 non-weighted image (b0), and 4 repetitions; 3) rs-fMRI using a gradient-echo T2*-weighted EPI sequence with TR=2,000 ms, TE=40 ms, in-plane resolution=3.28×3.28 mm^2, FOV=210×210 mm^2, 24 slices at a slice thickness of 6 mm, bandwidth=200 Hz/pixel, and number of volumes=180.

Image Processing

Image segmentation and structural volumes

The 3D T1-weighted images went through a fully automated segmentation pipeline via the online platform MRICloud (www.mricloud.org) (15), which performed a multi-atlas based segmentation, based on the JHU neonatal multi-atlas (16). Thirty-eight regions of interest (ROIs) were defined, including the gray matter, myelinated/unmyelinated white matter, brain stem, corpus callosum, caudate, putamen, thalamus, and cerebrospinal fluid (CSF), etc. The volumes of the ROIs were obtained and then summed over the two hemispheres (resulting in 18 symmetric ROIs after removing two non-brain-tissue ROIs), assuming negligible laterality in this study.

DTI processing and structural connectivity

a) Pre-processing and tensor reconstruction

All data were manually inspected by a radiologist (T.L.) to exclude diffusion-weighted images (DWI) with

noticeable imaging artifacts, followed by intra-subject registration using a 12-parameter affine transformation (17) to correct for head motion. Then, we employed the standard preprocessing steps with denoising (18), Gibb's ringing removal (19), distortion correction (20), and bias field correction (21). Fractional anisotropy (FA), mean/axial/radial diffusivity (MD/AD/RD) maps were generated from the diffusion tensor using the weighted linear least squares method.

b) Segmentation

The individual DTI data were transformed to the JHU-neonate single brain DTI atlas for image segmentation (22). The individual mean DWI images were aligned to the atlas DWI image with an affine transformation, followed by histogram matching between the atlas and subject images. Following this, the non-linear transformation was performed with large deformation diffeomorphic metric mapping (23,24), utilizing multi-channel contrasts of the mean DWI, FA, and b0 images (25). The DTI images were further inspected for registration failure, and none of the data showed visible registration errors. After transforming to the atlas space, the individual images were automatically segmented into 126 ROIs, as defined in the JHU-neonate atlas. The FA, MD, AD, and RD values were extracted from the ROIs, and an MD threshold of 2×10-3 mm^2/s was used to exclude the CSF voxels. The DTI metrics were then averaged over the hemispheres for statistical analysis.

c) Tractography and structural connectivity

Tractography was performed on the pre-processed DWIs by a tensor-based probabilistic fiber tracking algorithm (26) in MRtrix3 (www.mrtrix.org). The seed voxels were selected randomly within a whole-brain mask, and the following tracking parameters were used: cut-off of 0.06, step size of 0.16 mm, minimum/maximum length of 8/164 mm. Probabilistic tractography was used in this study as it was shown to yield higher connectome reproducibility than the deterministic method (27,28).

To construct the structural network, all deep white matter and cerebellum ROIs were excluded, leaving 62 ROIs as structural network nodes. It is worth noting that the network nodes included not only cortical and subcortical GM but also subcortical WM because the subcortical WM helped to determine the fibers linked to cortical regions (29). The weakest 1% of the connections, which were considered as spurious streamlines, were discarded. Following this, the streamlines were log-transformed to achieve normality (30). Seven network parameters were calculated using the brain connectivity toolbox (31), including the degree, transitivity, local efficiency, global efficiency, modularity, characteristic path length, and small-worldness.

rs-fMRI Processing

a) Pre-processing

The rs-fMRI data were preprocessed using the data processing assistant for resting-state fMRI (DPARSF, Advanced Edition) (32). First, the first ten time points were removed, followed by slice-timing correction and head motion correction, and those subjects with mean FD exceeding 0.2 mm were excluded (33). Following this, spatial normalization was performed via T1-weighted anatomical images that were registered to the JHU-neonate single brain T1 atlas (22), and all fMRI images were resampled to 3 mm isotropic voxels using SPM8. Next, the normalized images were smoothed with the Full Width at Half Maximum set at 6 mm. Finally, linear drift was removed, and the six rigid head motion parameters, as well as sources of physiological artifact extracted from white matter and CSF masks, were regressed out. In addition, bad time points were scrubbed using a threshold of Jenkinson FD>0.2 mm as well as one volume before and two volumes after (33).

b) ALFF, fALFF and functional connectivity

The low-frequency fluctuations were quantified by the amplitude of low-frequency fluctuation (ALFF) and fractional ALFF (fALFF) (34). ALFF was calculated within a specific low-frequency range (0.01-0.1 Hz), then the ratio of the power of the low-frequency band to that of the entire frequency range was calculated as fALFF. Z-transform was performed on both ALLF and fALFF to improve the normality before filtering. Then, the functional connectivity was calculated based on ALFF or fALFF, using the modified atlas as mentioned in 2.3.2 (c), and the correlation coefficient matrices were converted into z map by Fisher's r-to-z transform to improve normality. Correlation coefficients under 0.2, which were considered to be a negligible correlation, were discarded (35). The same network parameters were calculated as those in the structural network.

Statistical Analysis

For demographic information, categorical data were analyzed using the chi-square test. Shapiro-Wilk's test was used to analyze the distribution of measurement data. The Student's t-test was used for normally distributed data, and the Mann-Whitney U test applied to data that did not fulfill the requirements for normality. For the differences in DTI

and network metrics between twins and singletons, analysis of covariance (ANCOVA) was performed with a permutation approach in R-Project (36,37). Then the p-values of DTI metrics extracted from multiple ROIs were adjusted with the Bonferroni method. For ALFF and fALFF, a voxel-based analysis of differences between groups was performed with DPABI (32) using a permutation test method followed by Bonferroni correction for multiple testing using threshold-free cluster enhancement (38). The significance level was set at 0.05 for all tests. For all analyses, gender, birth weight, postmenstrual age (PMA) at scan, and Apgar score at 5 minutes after birth were used as covariates.

Results

Demographic and clinical characteristics

In total, eighteen twins and forty-seven singletons were included in this study. For rs-fMRI analysis, eight singletons were excluded as their mean FD exceeded 0.2 mm. Demographic and basic clinical information of the 65 study participants is provided in Table I, and the information of the 57 subjects used for rs-fMRI analysis is listed in Supplementary Table I. No significant group difference was found in terms of the listed demographic and clinical characteristics.

Table I. Demographic and basic clinical information characteristics of study participants

	Twins	Singletons	p-value
Gender (male/female)	12/6	23/24	0.315[a]
PMA at birth (weeks)	32.06±1.00	32.13±0.99	0.796[b]
PMA at scan (weeks)	40.50±0.99	40.06±1.55	0.185[b]
Birth weight (grams)	1,802.5±254.1	1,850.6±381.0	0.559[b]
Apgar score at 5-min after birth	9.83±0.38	9.74±0.53	0.460[b]

Data are represented as mean ± standard deviation.
[a]Compared by chi-square test, [b]Compared by Mann-Whitney U test

Supplementary Table I. Demographic and basic clinical characteristics of study participants used in rs-fMRI analysis after removing eight singletons with significant head movement (FD>0.2 mm)

	Twin	Singleton	p-value
Gender (male/female)	12/6	18/21	0.168[a]
PMA at birth/weeks	32.06±1.00	32.21±0.73	0.575[b]
PMA at scan/week	40.50±0.99	40.15±1.62	0.324[b]
Birth weight/gram	1802.5±254.1	1835.8±364.8	0.692[b]
Apgar score at 5 min after birth	9.83±0.38	9.74±0.55	0.480[b]

Data was represented as mean ± standard deviation
PMA: Postmenstrual age, FD: Frame-wise displacement, rs-fMRI: Resting-state functional magnetic resonance imaging

Comparison of the structural volumes

Based on the automated segmentation of the 3D T1-weighted images, the structural volumes of the 18 ROIs were compared between the twins and singletons. The myelinated white matter and thalamus in the singleton group demonstrated higher volumes than the twin group (p=0.034 and p=0.012, respectively before correction), but no significant difference was found after multiple comparison correction. The structural volumes and the statistical test results are listed in Supplementary Table II.

Comparison of the DTI metrics

As demonstrated in Figure 1, the MD values of the cingulum cingular part (CGC), cingulate gyrus (CingG), lateral fronto-orbital gyrus (LFOG), gyrus rectus (RG), as well as medial fronto-orbital gyrus in the twin group were significantly higher than the singleton group (adjusted p<0.05). The AD and RD were also increased in these regions (Figure 1), while RD showed differences in three additional ROIs, including the lingual gyrus, fusiform gyrus (Fu), and cingulum hippocampal part. The color maps in Figure 2 demonstrate the spatial distribution of regions with significant differences in MD between the groups, and the colors indicated the percentage of group difference by

Table II. Brain regions that show significant differences in fALFF between the twin and singleton groups

Brain region	Cluster size (number of voxels)	t values	MNI (peak)		
			x	y	z
Ins L	147	4,025	-27	-4	4
STG L					
LFOG L					
PoCG L					
STG R	101	4,268	21	14	-17
Ins R					
LFOG R					
CingG	64	4,123	0	17	-14
RG					

Ins: Insular cortex, CingG: Cingulate cortex, STG: Superior temporal gyrus, LFOG: Lateral fronto-orbital gyrus, RG: Gyrus rectus, POCG_L: Left postcentral gyrus, fALFF: Fractional amplitude of low-frequency fluctuation

Supplementary Table II. Statistical results of structural volume differences between twins and singletons		
ROIs	**p-values**	**Adjusted p-values**
Intracranial volume	0.586	1
CSF	0.836	1
Lateral ventricle	0.646	1
3rd ventricle	0.395	1
4th Ventricle	0.455	1
Cavum septum pellucidum	0.859	1
Gray matter	0.671	1
White matter	0.388	1
Myelinated white matter	**0.034**	0.605
Brain stem	0.114	1
Cerebellum	0.728	1
Corpus callosum	0.210	1
Caudate	0.999	1
Putamen	0.818	1
Globus pallidus	0.837	1
Thalamus	**0.012**	0.223
Hippocampus	0.967	1
Amygdala	0.413	1
P-values before and after the Bonferroni correction were shown. ROI: Region of interest, CSF: Cerebrospinal fluid		

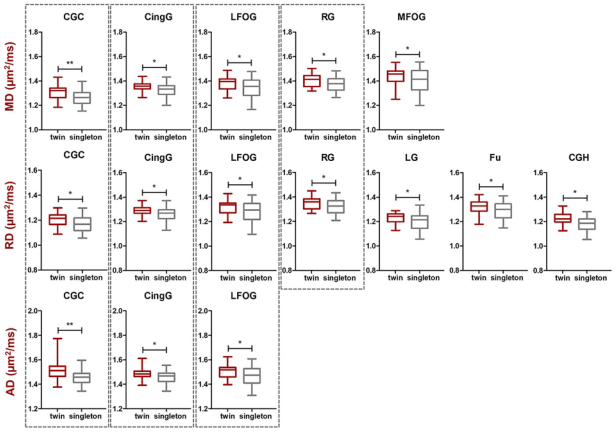


Figure 1. Differences in diffusivity measurements between twin and singleton brains. The MD values in the CGC, CingG, LFOG, RG, and MFOG were higher in the twin group compared with the singletons after multiple comparison correction (adjusted p<0.05). The corresponding RD and AD values were also higher in the twin group
*p<0.05, **p<0.01 by ANCOVA tests followed by Bonferroni correction
CGC: Cingulum cingular part, CingG: Cingulate gyrus, LFOG: Lateral fronto-orbital gyru, RG: Gyrus rectus, MFOG: Medial fronto-orbital gyrus, LG: Lingual gyrus, Fu: Fusiform gyrus, CGH: Cingulum hippocampal part, ANCOVA: Analysis of covariance, AD: Axial diffusivity, RD: Radial diffusivity, MD: Mean diffusivity

calculating the mean MD difference between the groups normalized to the mean MD of the singleton group for each ROI. No significant difference in FA was found between the twin and singleton groups.

Comparison of the structural connectivity

The degree, transitivity, local efficiency, global efficiency, modularity, characteristic path length, and small-worldness of the tractography-based structural network were calculated. Only the transitivity, which reflected local connectivity (39), was found to be significantly higher in the twin group than that in the singleton group (Figure 3), and the result did not change with cut-off thresholds (weakest

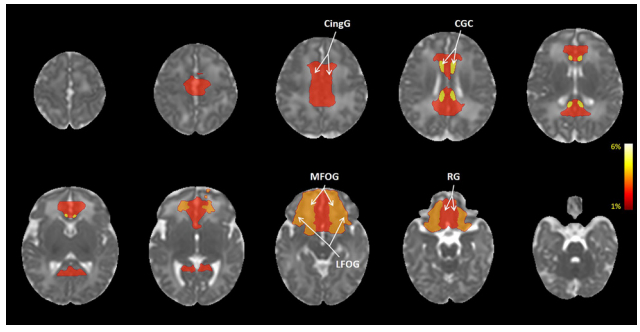


Figure 2. Brain regions showed significantly higher MD in the twin group than in the singleton group. The colors indicate the percentage of group difference by calculating the mean MD difference between the groups normalized to the mean MD of the singleton group for each ROI
CGC: Cingulum cingular part, CingG: Cingulate gyrus, LFOG: Lateral fronto-orbital gyru, RG: Gyrus rectus, MFOG: Medial fronto-orbital gyrus, MD: Mean diffusivity, ROI: Region of interest

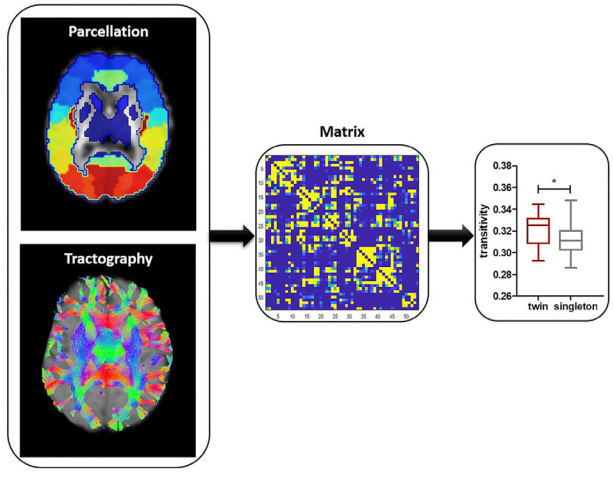


Figure 3. Flow chart of structural network analysis and the statistical result. The DTI data were segmented based on JHU neonatal DTI atlas. Sixty-two ROIs, including cortical GM, deep GM, and subcortical WM, were chosen as the nodes. Tensor-based probabilistic tractography was performed and the fiber accounts between each pair of ROIs were used to obtain the connectivity matrix. Standard network attributes were obtained, and transitivity was found to be significantly higher in the twin group
DTI: Diffusion tensor imaging, ROI: Region of interest

1-5% connections) (Supplementary Table III). Transitivity is a classical variant of the traditional clustering coefficient (40), which is thought to be less biased towards the contribution from low-degree vertices.

Comparison of the rs-fMRI results

As demonstrated in Figure 4, fALFF values in the salience network, which included bilateral insular cortex (Ins) and bilateral cingulate cortex (CingG), and several gyral regions, including the bilateral superior temporal gyrus, bilateral LFOG and bilateral RG, and left postcentral gyrus (POCG_L), were found to be significantly higher in the twin group. Moreover, the fALFF results overlapped with the MD results in several regions, including the CingG, LFOG, and RG (Figure 5). We repeated the analysis with global signal regression, and the results remained largely unchanged (Supplementary Figure 1). No group difference was found in ALFF, and no functional network parameter was found to be different in this study.

Supplementary Table III. Comparison of the tractography-based structural network properties between the singleton group and the twin group

Threshold	1%	2%	3%	4%	5%
Degree	0.067	0.051	0.056	0.083	0.091
Transitivity	**0.014**	**0.024**	**0.012**	**0.024**	**0.024**
Modularity	0.196	0.166	0.141	0.134	0.140
Character path length	0.902	0.902	0.901	0.902	0.902
Global efficiency	0.249	0.098	0.079	0.211	0.251
Local efficiency	0.251	0.138	0.100	0.197	0.312
Small worldness	0.075	0.158	0.132	0.047	0.200

Significance levels (p-values) for seven network properties were listed under a range of cut-off thresholds. The cut-off was set to be the weakest 1-5% connections

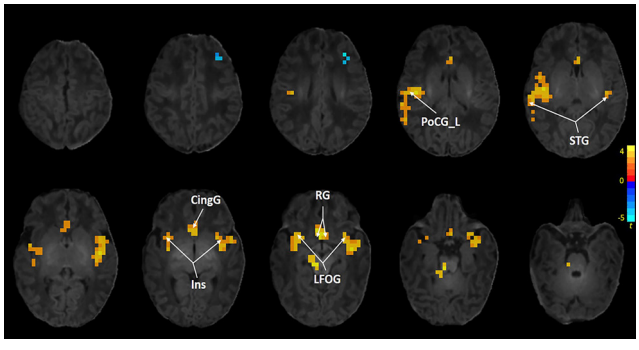
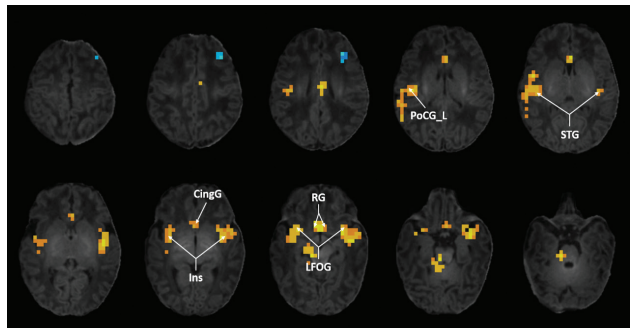


Figure 4. Differences in fALFF between twins and singletons, which were mainly located at the salience network and several fronto-temporal regions. The warm colors indicate higher fALFF in the twins compared with singletons, while the cool colors indicate lower fALFF in the twins compared with singletons. Voxel-based analysis of differences between the groups was performed using Bonferroni correction followed by a threshold-free cluster enhancement method for multiple testing
Ins: Insular cortex, CingG: Cingulate cortex, STG: Superior temporal gyrus, LFOG: Lateral fronto-orbital gyrus, RG: Gyrus rectus, POCG_L: Left postcentral gyrus, fALFF: Fractional amplitude of low-frequency fluctuation



Supplementary Figure 1. Regions that showed significant higher fALFF in the twin group compared with the singleton group after global signal regression. The spatial distribution was similar to that without global signal regression in Figure 5

Ins: Insular cortex, CingG: Cingulate cortex, STG: Superior temporal gyrus, LFOG: Lateral fronto-orbital gyrus, RG: Gyrus rectus, POCG_L: Left postcentral gyrus

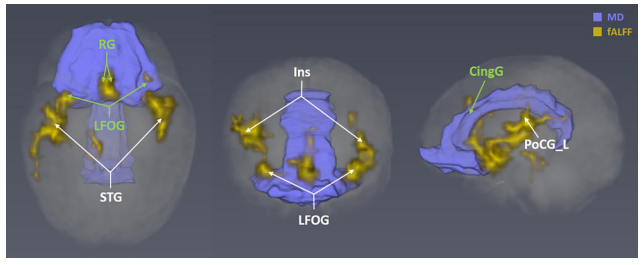


Figure 5. Three-dimensional spatial distribution of the brain regions that show significant group difference in MD (purple) and fALFF (yellow). The fALFF results overlap with the MD results in several regions, including the CingG, LFOG, and RG (green arrow)
Ins: Insular cortex, CingG: Cingulate cortex, STG: Superior temporal gyrus, LFOG: Lateral fronto-orbital gyrus, RG: Gyrus rectus, POCG_L: Left postcentral gyrus, fALFF: Fractional amplitude of low-frequency fluctuation

Discussion

In this study, we performed a comprehensive multi-modal MRI study to examine the structural and functional features in twin and singleton brains at term-equivalent age. Our results revealed considerable developmental differences between the two groups compared to previously reported findings, possibly due to the fact this study recruited gestational age-matched twins and singletons whereas previous studies compared preterm-born twins with term-born singletons. Although the gestational age could be included as a covariate, it is difficult to assess how well the effects of the preterm-birth and twin-birth were separated. Therefore, a direct comparison of preterm-born twins and singletons is necessary.

The volumetric differences were negligible after multiple comparison correction, which was consistent with some of the earlier studies. Knickmeyer et al. (10) compared brain volumes between twins and term-born singletons in the first month of life, and found CSF and frontal white matter volumes were greater in twins than in singletons. Ordaz et al. (11) compared brain volumes between twins and sex-matched unrelated singletons at the pediatric stage, and no significant difference was found. Another morphology study by Hulshoff et al. (9) compared monozygotic twins and dizygotic twins with their siblings in adulthood and found the difference in white matter volume diminished after correction for intracranial volume. Our finding is consistent with the latter two studies but not the first one, which is possibly related to the study population, as well as the use of different brain atlases.

For the DTI measurements, the diffusivities in several cortical gyri, especially the cingulate and front-orbital regions, demonstrated higher values in the twin group than in the singleton group. To the best of our knowledge, only one existing research studied the difference between twins and singletons using diffusion MRI. Sadeghi et al. (12) compared the longitudinal development of white matter between twins and term-born singletons from birth to 2 years of age using a non-linear mixed-effects method, and found the delay parameter of the developmental curve of AD in the anterior limb of the internal capsule and anterior corona radiata was smaller in twins compared to singletons, indicating higher AD in twins during early development. This result is consistent with ours, and their reported regions also showed differences in both FA and MD in our study before multiple comparison correction. However, our data revealed additional regions with higher diffusivities, which is again related to the study population, as well as the use of different brain atlas. Since it is known that the MD value increases with age (41-43), our results indicated a developmental delay in several cortical regions in twins compared with singletons.

For the tractography-based structural connectivity, both groups displayed small-worldness in the whole-brain networks. The transitivity, which indicates local connectivity, was found to be significantly higher in the twins than in singletons. Previous evidence indicated the clustering coefficient of the structural network (similar to transitivity) decreased with brain development from neonate to adult (44,45). However, during perinatal development, some studies found that the preterm-born neonates exhibited an age-dependent increase of clustering coefficient until about term-equivalent age (46,47). These studies indicate the network properties may change in a non-linear pattern during early development, as the brain connectomes experienced ordered strengthening of short-range connectivity followed by growth of long-range connections (48). In the present study, the PMA at scan happened to be the breakpoint of a non-linear trajectory, making it difficult to interpret the developmental difference between twins and singletons. Further longitudinal follow-up studies may be needed to understand the network difference at term-age.

In the rs-fMRI analyses, fALFF values in the salience network and several fronto-temporal regions were found to be significantly higher in the twin group, while no difference in ALFF was found. The discordance of the two parameters may be due to the artifact generated by head motion. Although sedation was used, neonatal subjects still showed more pronounced motion artifacts than adults, which may not be entirely eliminated by motion correction. The fALFF is a normalized version of ALFF, which is thought to be more robust against physiological artifacts and more sensitive to biological difference (34,49). Few studies have investigated the relationship between low-frequency fluctuation and brain development. Bray (50) studied children aged 7-18 years and found that age did not have a significant effect on global fALFF, but the fALFF in the salience network regions demonstrated an age-related decline. Although no neonatal study of fALFF was found, given the above evidence, our results indicate that preterm-born twins with higher fALFF demonstrated a more active spontaneous neuronal activity, which may be associated with delayed neurological development.

It is worth noting that we found differences both in structural and functional measurements (MD and fALFF) in the prefrontal and CingG (indicated with the green arrow in Figure 5), which are involved in several higher-order cognitive functions (51-53). Although the prefrontal and CingG related cognitive functions are immature at an early age (48,54), these regions are known to be rapidly developing during the perinatal stage (55). The agreement between the structural and functional evidence reinforces the differences between twins and singletons at term-equivalent age. These differences in brain development may be associated with the early feeding problems that are more frequent for preterm-born twins, as well as other perinatal-neonatal issues in twins. No difference in the functional network was found, which is slightly different from the result of the structural network. As the structural network is known to develop prior to the functional network (48,56,57), discordant findings between the structural

and functional networks are within expectation (58-60). Moreover, in the current study, both the preterm twins and singletons showed relatively low levels of the functional network due to the immature functional activity in the neonatal brain as well as the use of sedation, so group difference may be difficult to detect.

The current work possesses several limitations. First, this study lacked term-born neonates as a healthy control group. A number of studies have shown that preterm-birth resulted in delayed brain development compared with term-born individuals in terms of DTI metrics (61,62) and brain volumes (63,64), and therefore, we can readily assume that both the preterm-born twins and singletons have altered DTI and volumetric measurements in comparison to healthy controls. Second, information on interventional operations for the preterm-born neonates was absent in this study, which may play a role in interpreting group differences. Although we have excluded those neonates with acquired brain injury on MRI and those with known perinatal diseases, and the Apgar scores at 5-mins after birth were relatively normal (9.83±0.38 and 9.74±0.53 for twins and singlets, p=0.46), this evidence cannot guarantee the healthy condition of the study subjects. Third, we did not investigate the correlation between clinical assessments and MRI in this study. In fact, we followed some of the subjects and performed the Bayley tests at 12 months of age, and no behavioral difference was found between groups. The high drop-out rate and insufficient number of subjects (6 twins and 20 singletons) in this study were not appropriate for statistical analysis. In addition, the number of twins was limited compared with singletons, and therefore, we performed a permutation test for the ANCOVA analysis to overcome imbalanced data in the present study (37). Finally, chloral hydrate was used in this study for sedation. Although sedation is considered safe and is frequently used in infants to reduce head motion in fMRI (65), a previous study has shown that sedation can induce a reduction in brain activity in infants (66). This could have had an impact on the rs-fMRI results. Future studies should consider and mitigate for this factor to improve the reliability of their results. Nevertheless, this was the first attempt to systematically evaluate both the structural and functional differences between twins and gestational-age matched singletons, and the DTI and rs-fMRI results collectively implied a developmental delay in twins at term-equivalent age.

Conclusion

In summary, the multi-modal MRI approach provided comprehensive information about the developmental differences between twins and singletons, compared with the previous single modal approach. Our results demonstrated that the preterm-born twins had higher MD in several cortical regions compared with gestational-age matched singletons at term-equivalent age. In addition, transitivity of tractography-based structural network and fALFF in the salient network were found to be higher in the twin group. These structural and functional differences collectively indicated that preterm-born twins may have delayed brain development compared with gestational age-matched singletons at term-equivalent age, which may be related to perinatal- neonatal problems. Therefore, MRI findings from twin studies on brain development should only be cautiously generalized to singletons, especially at term-equivalent age.

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Ethics

Ethics Committee Approval: Ethical approval was obtained from the Institutional Review Board at the Children's Hospital of Zhejiang University School of Medicine (2019-11-13).

Informed Consent: Written informed consent was provided by the parents.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: D.W., Design: D.W., Data Collection or Processing: H.Z., Y.Y., T.L., X.S., F.T., Analysis or Interpretation: T.L., Literature Search: W.Z., Z.Z., Y.Z., Writing: T.L.

Conflict of Interest: No conflict of interest was declared by the authors.

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Original Article

The Spectrum of *NF1* Gene Variations in Southeastern Turkey

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ABSTRACT

Aim: We aimed to expand the variant spectrum of the *NF1* gene in Southeastern Turkey. Neurofibromatosis type 1 (NF1) disease is an inherited skin disorder with variable severity and heterogeneous systemic involvement. The pathogenic variations of the *NF1* gene are responsible for the *NF1* phenotype.

Materials and Methods: In this study, clinical and molecular manifestations of 92 molecularly confirmed NF1 patients from 86 unrelated families are presented. The next-generation sequencing method (using Ion Torrent PGM™ Platform) was performed to analyze all coding exons of the NF1 gene.

Results: Seventy-six different *NF1* variations were identified with 27 of them being novel. 42.5% of the patients were familial and 57.5% were sporadic. Except for one 20-year-old patient with c.1637dupT variant who presented with pilocytic astrocytoma without cutaneous findings, all the other patients demonstrated several typical clinical criteria of NF1.

Conclusion: Although NF1 diagnostic criteria are the most widely used and proficient clinical diagnostic tool, *NF1* gene analysis can be applied as a definitive diagnostic tool in cases with atypical presentations and in early childhood.

Keywords: NF1, next-generation sequencing, Southeastern Turkey

Introduction

Neurofibromatosis type 1 (NF1; OMIM 162200) is one of the most common hereditary disorders. It is inherited as an autosomal dominant trait. The estimated incidence at birth is 1/3,000 (1,2). Multiple café-au-lait spots, axillary/inguinal freckling, multiple cutaneous neurofibromas, iris Lisch nodules, and choroidal freckling are the characteristic features of NF1 (3). NF1 also manifests as multisystemic disorders with musculoskeletal, vascular, central nervous, and peripheral nervous system involvement such as scoliosis, tibial dysplasia, vasculopathy, glioma, and malignant peripheral nerve sheath tumors (4).

Clinical diagnosis of NF1 is based on the National Institutes of Health (NIH) diagnostic criteria (5). However, without a family history, these criteria may be insufficient in early childhood as most of the clinical features manifest later in life (3).

The NF1 phenotype is caused by germline heterozygous pathogenic variants of the *NF1* gene. NF1 is located at chromosome 17q11.2 and coded neurofibromin 1 protein that acts as a regulator of Ras activity. NF1, one of the largest genes in the human genome, consists of 57 coding exons and 12,362 base pairs transcript length (transcript reference, NM_000267.3).

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In recent years, next-generation sequencing (NGS) technology has enabled many genes, regardless of size, to be analyzed systematically, comprehensively, more easily and more cost-effectively. Most studies demonstrated that NGS with in-solution hybridization-based enrichment

provides a high mutation detection rate comparable to that of conventional direct capillary sequencing methods for the molecular diagnosis of neurofibromatosis. In this study, we aimed to present the variant spectrum of NF1 patients from the region of Southeastern Turkey and investigate if there is a clear genotype-phenotype correlation (6).

Materials and Methods

Profile of the Patients

The clinical and genetic data of the NF1 patients who were referred to the Medical Genetics Clinic, Gaziantep Ersin Arslan Training and Research Hospital between May 2016 and December 2019 were evaluated retrospectively. Peripheral blood samples were obtained after taking informed consent from all participants or the legal guardians of those children under the age of 18. The study was approved by the Ethics Committee of the Gaziantep University Medical Faculty (approval number: 65587614-774.99-291, date: 04/10/2017).

Ninety-two Turkish patients from 86 unrelated families who were both clinically and molecularly diagnosed with NF1 were included in this study. Fifty-four (58.7%) patients were male and thirty-eight (41.3%) were female. Age at diagnosis was in the range of 1-46 years.

The clinical diagnosis was performed based on the presence of two or more of the diagnostic criteria proposed by the NIH Consensus Development Conference (5). The diagnosis of NF1 was established in patients who had two or more of the following NIH criteria: Six or more cafe-au-lait macules (one of them must be greater than 5 mm and 15 mm, prepubertal and post-pubertal respectively); two or more neurofibromas or 1 plexiform neurofibroma; freckling in the axillary or inguinal regions; optic glioma; two or more Lisch nodules; a distinctive osseous lesion (sphenoid dysplasia or tibial pseudarthrosis); and a first-degree relative with neurofibromatosis type 1.

Genetic Analysis

Genomic DNA was extracted from whole blood samples using an automated method (RSC whole blood DNA kit) in the Maxwell® 16 (Promega Corporation, Madison, WI). NF1 (57 coding exons, NM_000267.3) amplicons were designed using the AmpliSeq Designer software (Life Technologies, CA, USA), targeting the complete coding sequence of the *NF1* gene by 120 amplicons. The design target coverage was 99.49%.

Amplicon library was prepared using the Ion AmpliSeq Library Kit Plus, Xpress Barcode Adapters 1-96 Kit (Thermo Fisher Scientific), then pooled together using Qubit 1X dsDNAhs Assay kit and Qubit 4 Fluorometer (Thermo Fisher Scientific). Emulsion PCR, and Ion Sphere Particles enrichment were carried out in the Ion Chef System, then loaded into an Ion 530 chip. NGS was performed via Ion 510 & Ion 520 & Ion 530 kit-Chef (Thermo Fisher Scientific). Data were processed using Ion Torrent Suite Software (Thermo Fisher Scientific) and Ion Reporter Software (Thermo Fisher Scientific).

The Human Gene Mutation Database (HGMD) (7) and Leiden Open Variation Database (LOVD) were used to determine whether a variant was novel or not. Several prediction algorithms, including SIFT (http://sift.jcvi.org), Polyphen2 (http://genetics.bwh.harvard.edu/pph2/), Human Splicing Finder (http://www.umd.be/HSF/) and Mutation taster (http://www.mutationtaster.org) were used to determine any damaging effects on the protein. The Genome Aggregation Database (https://gnomad.broadinstitute.org) was used to estimate the minor allele frequency score.

Nomenclature of the variants was based on the NM_000267.3 (NCBI transcript number). Variants were reviewed using dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/). A minimum 30X coverage for all bases was accepted for a reliable variant calling. Ion Reporter version 5.0 software was used to annotate variants. Integrated Genomics Viewer (http://software.broadinstitute.org/software/igv/) was used for visual assessment of the revealed variants.

Novel variants are reported to the Human Genome Variation Society guidelines and checked by using Mutalyzer tool (https://mutalyzer.nl/about). All variants were classified by using The American College of Medical Genetics and Genomics (ACMG) guidelines criteria (8). Some variants were validated with direct capillary sequencing that was performed by using the BigDyeTerminator kit v3.1 (LifeTechnologies, Darmstadt, Germany) and an automated capillary sequencer (3500xl Genetic Analyzer, Life Technologies). The obtained sequence data were analyzed using the Seq-Scape (Ver. 2.1) program (Applied Biosystems).

Results

Clinical Manifestations of the Patients

We reviewed the clinical data in 73 of the 92 patients. The frequencies of clinical features are sorted by age ranges in Table I. The median age was 8 years. 42.5% of patients were familial and 57.5% were sporadic cases. Seventy-one (97.2%) patients were suffering from café-au-lait spots. Axillary or inguinal freckling was present in 27 patients (36.9%). The other common skin manifestation were cutaneous neurofibromas that accounted for 19.1% (14/73) of cases. Optic glioma was identified in 6.8% (5/73) of the patients. Hamartomas were detected with magnetic resonance imaging in 22 of the patients (30.1%) (Table II).

Characterization of the NF1 Variants

Seventy-six variations of which two were probably somatic were detected in 92 patients from unrelated families. The identified variants were as follows: 36 frameshift variants (41.9%) resulting from small insertions, deletions or indels; 27 non-sense variations (31.4%); 14 missense variants (16.2%); 6 splicing alterations (7%), and 3 in-frame deletions or indels (3.5%) (Figure 1).

Twenty-seven (35%) of the variants were novel and had not been previously reported in HGMD or LOVD. The c.2446C>T, c.3826C>T, c.5839C>T, c.2546dupG showed familial segregation. The c.5107C>T (15%), c.3721C>T (20%) variants were detected at lower fraction percentages; 15% and 20% respectively. The c.2033dupC, c.2446C>T, c.3525_3526delAA, c.3826C>T, c.4084C>T, c.5546G>A, c.6792C>G variations were identified in more than one unrelated family (Table III). Only one of the variations (c.7674G>A) was interpreted as a variant of unknown origin (VUS) based on ACMG criteria. The other three were likely pathogenic and 23 of them were pathogenic. 24 variants produced truncated protein as a result of premature stop codon, which is a significant indication of their pathogenicity. All novel variants were predicted to be deleterious by at least one in-silico analysis.

Discussion

As *NF1* is the one of the most common inherited disorders and *NF1* is one of the largest genes in the human genome, a great number of variations (over 3,000) have been reported in HGMD to date. Even though previously reported pathogenic variation types show diversity, most of them cause severe truncated gene products (9). Most of the pathogenic variants (93%) are small nucleotide alterations (including non-sense, missense, insertion or deletion) and splicing variants. Intragenic deletions/duplications (2%) and microdeletions (5%) are rarely detected (10). In this study, we identified 76 different mutations in 92 families. Most of the variant types are frameshift, non-sense and splice-site, similar to the literature (10-12). The missense variation rate was relatively high (14 variants, 16%) in accordance with similar studies (11-13). There were some conflicting pathogenicity variants such as c.7674G>A and c.2764G>A which were interpreted as VUS based on ACMG criteria. The patient with c.7674G>A had a typical NF1 phenotype, which was a strong indicator showing the variant's pathogenicity. However, the case with c.2764G>A variant did not reach the optimum clinical registry.

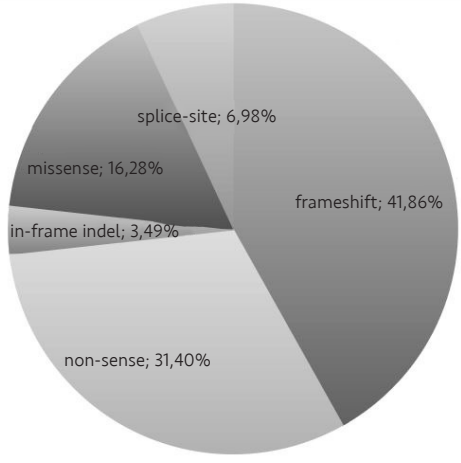

Figure 1. Type of NF1 variations

Table I. Range of the clinical features according to the age groups

Age		6>CALs	Freckling	Neurofibroma	Optic glioma	Lisch nodule	Hamartoma
Year	**n**	**n (%)**					
0-1	15	15 (100)	2 (13.3)	2 (13.3)	2 (13.3)	5 (33.3)	4 (26.6)
2-4	13	13 (100)	4 (30.7)	1 (7.6)	2 (15.3)	4 (30.7)	5 (38.4)
5-18	34	34 (100)	15 (100)	4 (11.7)	1 (12.9)	6 (17.6)	12 (35.2)
19-30	4	3 (75)	3 (44.1)	1 (25)	0 (0)	0 (0)	0 (0)
31-60	7	6 (85.7)	3 (75)	6 (87.5)	0 (0)	2 (28.5)	1 (14.2)

n: Number; CALs: Cafe au lait spots

Table II. Clinical data of the patients

Family	Case	Sex	Age (yr)	Variation (codon number)	Family history	CALs	Neurofibromas	Freckling	Optic glioma	Lisch nodules	Hamartoma	Other clinical findings
F1	C1	M	1	c.1756_1759del	-	+	-	-	-	-	N/R	-
F2	C2	M	13	c.5546G>A	+	+	-	-	-	-	+	-
F3	C3	M	15	c.3525_3526delAA	N/R	N/R	N/R	N/R	N/R	N/R	+	N/R
F4	C4	F	1	c.3739delT	-	+	-	-	+	-	+	-
F5	C5	F	11	c.1019_1020delCT	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
F6	C6	M	2	c.1393-9T>A	+	+	-	N/R	-	-	-	N/R
F7	C7	F	1	c.1428_1431delATTTinsCC	+	+	-	N/R	-	-	-	-
F8	C8	M	7	c.1466A>G	-	N/R	-	-	-	-	-	-
F9	C9	F	13	c.1557dupA	+	+	-	N/R	-	-	N/R	-
F10	C10	M	45	c.1697delC	-	+	+	N/R	N/R	N/R	-	-
F11	C11	M	41	c.1721+3A>C	+	-	+	N/R	N/R	N/R	N/R	-
F12	C12	F	11	c.2446C>T	+	+	+	+	-	-	+	-
F12	C13	M	6	c.2446C>T	+	+	N/R	-	N/R	N/R	-	Short stature
F12	C14	F	28	c.2446C>T	+	+	-	+	N/R	N/R	-	-
F13	C15	F	11	c.2446C>T	-	+	+	+	-	-	N/R	-
F14	C16	F	11	c.2970_2972delAAT	-	+	-	N/R	-	-	-	-
F15	C17	M	1	c.3826C>T	-	+	+	N/R	+	+	+	Seizure
F16	C18	F	2	c.3826C>T	+	+	-	-	-	-	-	-
F16	C19	F	1	c.3826C>T	+	+	-	-	-	+	-	-
F16	C20	M	38	c.3826C>T	+	+	+	+	-	+	N/R	-
F17	C21	F	8	c.3916C>T	-	+	-	+	-	+	+	-
F18	C22	M	10	c.4075_4076delinsAA	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
F19	C23	F	9	c.4084C>T	+	+	+	+	-	+	+	Seizure
F20	C24	M	35	c.4084C>T	-	+	+	-	-	+	-	-
F21	C25	F	9	c.4267A>G	-	+	-	+	+	+	-	Asthma
F22	C26	M	35	c.4537C>T	+	+	-	+	N/R	-	N/R	Arrhythmia
F23	C27	M	9	c.4621delC	-	+	-	+	-	+	+	-
F24	C28	F	2	c.4822_4826delCTGAC	+	+	N/R	+	-	-	-	-
F25	C29	M	5	c.5522T>A	-	+	-	+	-	+	-	N/R
F26	C30	M	1	c.5722G>T	-	+	-	+	N/R	+	N/R	-
F27	C31	M	12	c.5839C>T	+	+	-	+	-	+	+	N/R
F27	C32	F	8	c.5839C>T	+	+	-	+	-	-	N/R	-
F28	C33	M	3	c.6334_6335delCT	-	+	-	-	-	-	-	N/R
F29	C34	M	1	c.6791dupA	-	+	-	-	-	+	+	-
F30	C35	M	1	c.6792C>G	-	+	-	-	-	-	-	N/R
F31	C36	F	13	c.6792C>G	-	+	-	+	-	-	+	-
F32	C37	F	10	c.6792C>G	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R

Table II. continued

F33	C38	F	5	c.7206_7207delCA	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
F34	C39	M	25	c.7237C>T	-	+	-	+	-	-	-	-
F35	C40	M	10	c.7285C>T	-	+	-	-	-	-	-	-
F36	C41	M	29	c.7419G>A	+	+	+	+	-	-	-	-
F37	C42	M	9	c.7486C>T	-	+	N/R	N/R	-	-	-	N/R
F38	C43	F	9	c.953_956delAAAG	-	+	-	N/R	N/R	N/R	-	Scoliosis
F39	C44	M	10	c.1548dupC	+	+	-	yok	-	-	-	-
F40	C45	M	13	-	-	+	-	N/R	-	-	+	-
F41	C46	M	46	c.2867_2868delCCinsA	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
F42	C47	M	3	c.2890dupA	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
F43	C48	F	1	c.3457_3460delCTCA	-	+	-	-	-	-	-	N/R
F44	C49	F	2	c.3525_3526delAA	+	+	-	-	-	-	-	N/R
F45	C50	M	8	c.5844_5845delAA	-	+	-	+	-	-	-	N/R
F46	C51	M	4	c.7674G>A	-	+	-	+	-	+	+	-
F47	C52	F	3	c.2033dupC	+	+	N/R	N/R	-	-	N/R	-
F48	C53	F	7	c.1381C>T	-	+	-	N/R	-	-	+	-
F49	C54	M	4	c.1261-3_1270del TAGTCCGCATTGG	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
F50	C55	F	3	c.1318C>T	-	+	-	+	-	-	+	-
F51	C56	F	16	c.1721G>A	N/R	N/R	N/R	N/R	+	N/R	-	N/R
F52	C57	M	8	c.2546dupG	+	+	-	-	-	+	-	-
F52	C58	M	1	c.2546dupG	+	+	+	+	-	+	+	-
F53	C59	F	2	c.3058delG	+	+	-	+	+	-	+	Skeletal deformity
F54	C60	F	13	c.3114-2A>G	-	+	-	-	-	-	-	-
F55	C61	M	6	c.3610C>G	-	+	-	-	-	-	+	Seizure
F56	C62	M	8	c.5003_5004insGGTA	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
F57	C63	M	3	c.6263delT	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
F58	C64	M	8	c.4288A>G	-	+	-	+	-	-	N/R	N/R
F59	C65	M	36	c.6125delT	+	+	+	+	-	-	+	-
F60	C66	M	12	c.1496T>G	+	+	-	N/R	-	-	-	-
F61	C67	F	4	c.1737_1738delTT	+	+	1 +	-	-	+	-	-
F62	C68	M	21	c.1748A>G	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
F63	C69	F	6	c.1885G>A	+	+	-	N/R	-	-	-	-
F64	C70	F	16	c.2033dupC	+	+	N/R	N/R	N/R	N/R	N/R	N/R
F65	C71	M	13	c.2033dupC	+	+	-	+	-	-	-	-
F66	C72	F	13	c.2097dupC	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
F67	C73	M	9	c.2252-3T>G	+	+	N/R	N/R	N/R	N/R	+	Seizure
F68	C74	M	1	c.2325+3A>G	-	+	-	-	-	-	-	N/R
F69	C75	F	1	c.2604delT	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
F70	C76	M	5	c.2764G>A	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
F71	C77	F	13	c.3470T>A	-	+	N/R	N/R	N/R	N/R	N/R	N/R

Table II. continued

F72	C78	M	2	c.446dupA	-	+	N/R	-	+	+	+	Skeletal deformity
F73	C79	M	10	c.484C>T	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
F74	C80	F	9	c.4817T>A	+	+	-	+	-	-	-	Scoliosis
F75	C81	F	1	c.5719G>T	-	+	-	-	-	-	-	-
F76	C82	M	1	c.7229delT	-	+	-	-	-	-	-	-
F77	C83	M	2	c.7518_7519delGCinsCT	+	+	-	-	-	-	-	-
F78	C84	F	1	c.1404dupT	-	+	-	-	N/R	N/R	-	-
F79	C85	M	20	c.1637dupT	-	-	-	-	-	-	-	Pilositic astrocytoma
F80	C86	F	13	c.1756_1759delACTA	-	+	+ 1	+	-	-	N/R	-
F81	C87	M	21	c.3721C>T	N/R	N/R	N/R	N/R	N/R	N/R	-	N/R
F82	C88	F	8	c.3871delG	N/R	N/R	N/R	N/R	N/R	N/R	N/R	N/R
F83	C89	M	7	c.4816_4817insGG	N/R	+	-	-	-	-	+	N/R
F84	C90	M	1	c.5107C>T	-	+	-	-	-	-	N/R	-
F85	C91	M	4	c.5546G>A	N/R	+	-	N/R	-	+	+	Speech delay
F86	C92	M	38	c.5827delG	-	+	+	-	-	-	-	-

N/R: Not reported, CALs: Cafe au lait spots, M: Male, F: Female
C3-C5-C22-C37-C38-C46-C47-C54-C56-C57-C62-C63-C68-C72-C75-C76-C79-C87-C88 are out of clinical table (Table 1)

There is not a well-recognized hot spot region in the *NF1* gene (14-16). In this study, we found only one recurrent variant (c.6792C>G) in two unrelated families. It did not show significant variant aggregation in any exon (Figure 2). Although we observed relative variant density in exon 13 according to the exon length (Figure 2), it was insufficient to speculate that exon 13 is a hotspot region. Moreover, the variant frequency was lower both in the first and last few exons which code the constitutional amino acids. This can be attributed to the variants on the distal part of the gene being less effective in causing the *NF1* phenotype.

Six functional domains were determined on the *NF1* protein: The cysteine and serine rich domain (CSRD; exons 1-22), the tubulin binging domain (TBD; exons 22-27), the domain responsible for interactions with RAS and GTP hydrolysis (GRD; exons 27-34), the bipartite lipid binging domain (first part) (Sec14; exons 35-36), the bipartite lipid binging domain (second part) (PH; exons 35-36), and the carboxyl-terminal domain (CTD; exons 37-52) (17). The distribution of the variants according to functional domains were CSRD (31/76-40.7%), TBD (9/76-11.8%), GRD (11/76-14.4%) Sec 14 and PH (5/76-6.5%), and CTD (20/76-26.3%). We did not find any variants outside of the functional domains.

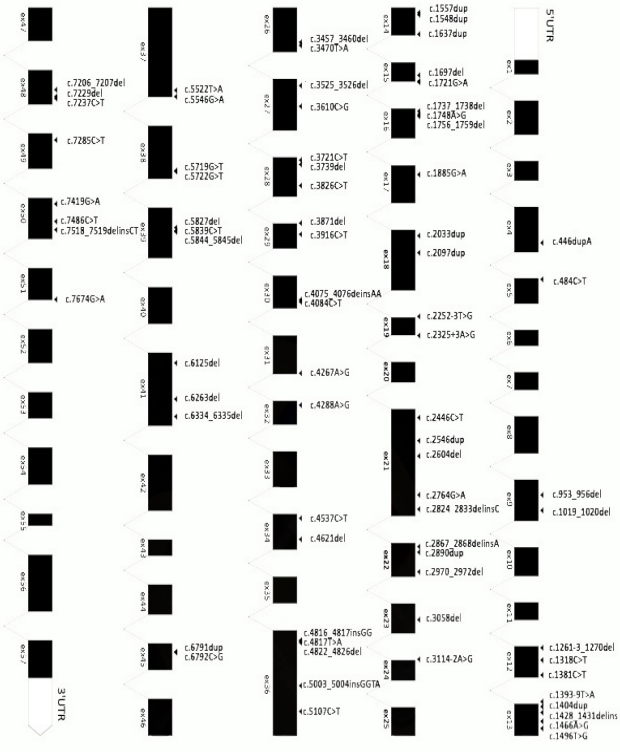

Figure 2. Distribution of the variants according to functional domains

Table III. Distribution of the identified NF1 variants

Family	Variation (codon number)	Protein number	HGMD reference	LOVD	Type	Predicted effect	ACMG criteria	ACMG prediction	Exon
F1, F80	c.1756_1759del	p.Thr586Valfs*18	CD982825	R	Deletion	Frameshift	PVS1, PM1, PM2 PP3, PP5	P	16
F2, F85	c.5546G>A	p.Arg1849Gln	CM1718194	R	Substitution	Missense	PM2, PP2, PP3, PP5	LP	37
F3, F44	c.3525_3526delAA	p.Arg1176Serfs*18	CD000971	R	Deletion	Frameshift	PVS1, PM1, PM2, PP3, PP5	P	27
F4	c.3739delT	p.Phe1247Leufs*19	Novel	N/R	Deletion	Frameshift	PVS1, PM1, PM2, PP3	P	28
F5	c.1019_1020delCT	p.Ser340fs	CD972347	R	Deletion	Frameshift	PVS1, PM1, PM2, PP3, PP5	P	9
F6	c.1393-9T>A	-	CS000058	R(P)	Substitution	Splice site	PM2	VUS	12-13
F7	c.1428_1431delATTTinsCC	p.Lys476Asnfs*14	Novel	N/R	Deletion	Frameshift	PVS1, PM2, PP3	P	13
F8	c.1466A>G	p.Tyr489Cys	CM1111787	R(P)	Substitution	Missense	PM1, PM2, PP2, PP3, PP5	P	13
F9	c.1557dupA	p.Gly520fs	Novel	N/R	Duplicaton	Frameshift	PVS1, PM1, PM2 PP3	P	14
F10	c.1697delC	p.Pro566Leufs*2	CD1815862	N/R	Deletion	Frameshift	PVS1, PM1, PM2, PP3	P	15
F11	c.1721+3A>C	-	CS941514	R(P)	Substitution	Splice site	PM2	VUS	15-16
F12, F13	c.2446C>T	p.Arg816*	CM971040	R(P)	Substitution	Non-sense	PVS1, PM1, PM2, PP3, PP5	P	21
F14	c.2970_2972delAAT	p.Met991del	CD931025	R(P)	Deletion	In frame deletion	PM1, PM2, PM4, PP3, PP5	P	22
F15, F16	c.3826C>T	p.Arg1276*	CM950847	R(P)	Substitution	Non-sense	PVS1, PS3, PM1, PM2 PP3	P	28
F17	c.3916C>T	p.Arg1306*	CM981381	R(P)	Substitution	Non-sense	PVS1, PM1, PM2, PP3, PP5	P	29
F18	c.4075_4076delinsAA	p.Pro1359Asn	Novel	N/R	Indel	In-frame indel	PM1, PM2, PP3, PP5	LP	30
F19, F20	c.4084C>T	p.Arg1362*	CM971046	R(P)	Substitution	Non-sense	PVS1, PM1, PM2, PP3, PP5	P	30
F21	c.4267A>G	p.Lys1423Glu	CM920506	R(P)	Substitution	Missense	PM1, PM2, PM5, PP2, PP3, PP5	P	31
F22	c.4537C>T	p.Arg1513*	CM941093	R(P)	Substitution	Non-sense	PVS1, PM1, PM2, PP3, PP5	P	34
F23	c.4621delC	p.Leu1541fs	Novel	R(P)	Deletion	Frameshift	PVS1, PM1, PM2, PP3, PP5	P	34

Table III. continued

F24	c.4822_4826delCTGAC	p.Leu1608fs	Novel	N/R	Deletion	Frameshift	PVS1, PM1, PM2, PP3	P	36
F25	c.5522T>A	p.Leu1841*	Novel	N/R	Substitution	Non-sense	PVS1, PM1, PM2, PP3	P	37
F26	c.5722G>T	p.Glu1908*	CM143452	R(P)	Substitution	Non-sense	PVS1, PM1, PM2, PP3	P	38
F27	c.5839C>T	p.Arg1947*	CM900173	R(P)	Substitution	Non-sense	PVS1, PM1, PM2, PP3, PP5	P	39
F28	c.6334_6335delCT	p.Leu2112Valfs	CD1415205	R(P)	Deletion	Frameshift	PVS1, PM1, PM2, PP3, PP5	P	41
F29	c.6791dupA	p.Tyr2264*fs	CI962317	R(P)	Duplicaton	Frameshift	PVS1, PM1, PM2, PP3	P	45
F30, F31, F32	c.6792C>G	p.Tyr2264*	CM972796	R(P)	Substitution	Non-sense	PVS1, PM1, PM2, PP3, PP5	P	45
F33	c.7206_7207delCA	p.His2402Glnfs*4	CD031873	R(P)	Deletion	Frameshift	PVS1, PM1, PM2, PP3, PP5	P	48
F34	c.7237C>T	p.Gln2413*	CM000817	N/R	Substitution	Non-sense	PVS1, PM1, PM2, PP3	P	48
F35	c.7285C>T	p.Arg2429*	CM000818	R(P)	Substitution	Non-sense	PVS1, PM1, PM2, PP3, PP5	P	49
F36	c.7419G>A	p.Trp2473*	Novel	N/R	Substitution	Non-sense	PVS1, PM1, PM2, PP3, PP5	P	50
F37	c.7486C>T	p.Arg2496*	CM941096	R(P)	Substitution	Non-sense	PVS1, PM2, PP3, PP5	P	50
F38	c.953_956delAAAG	p.Glu318Valfs*57	CD1512843	R(P)	Deletion	Frameshift	PVS1, PM1, PM2, PP3	P	9
F39	c.1548dupC	p.Glu517Argfs*41	Novel	N/R	Duplicaton	Frameshift	PVS1, PM1, PM2, PP3	P	14
F40	c.2824_2833delAGCAAGTTTTinsC	-	Novel	N/R	Indel	In-frame indel	PM1, PM2, PM4, PP3	LP	21
F41	c.2867_2868delCCinsA	p.Thr956Lysfs	Novel	N/R	Indel	Frameshift	PVS1, PM1, PM2, PP3	P	22
F42	c.2890dupA	p.Thr964Asnfs*11	Novel	N/R	Duplicaton	Frameshift	PVS1, PM1, PM2 PP3	P	
F43	c.3457_3460delCTCA	p.Leu1153Metfs	CD972351	R(P)	Deletion	Frameshift	PVS1, PM1, PM2, PP3, PP5	P	26
F45	c.5844_5845delAA	p.Arg1949Serfs*6	CD941733	R(P)	Deletion	Frameshift	PVS1, PM1, PM2, PP3, PP5	P	39
F46	c.7674G>A	p.Met2558Ile	Novel	N/R	Substitution	Missense	PM2, PP2	VUS	51
F47, F64, F65	c.2033dupC	p.Ile679Aspfs*10	CI951961	R(P)	Duplicaton	Frameshift	PVS1, PM1, PM2, PP3, PP5	P	18
F48	c.1381C>T	p.Arg461*	CM000780	R(P)	Substitution	Non-sense	PVS1, PM2, PP3, PP5	P	12

Table III. continued

F49	c.1261-3_1270del TAGTCCGCATTGG	-	Novel	N/R	Deletion	Splice site	PVS1, PM2, PP3	P	12
F50	c.1318C>T	p.Arg440*	CM950845	R(P)	Substitution	Non-sense	PVS1, PM1, PM2, PP3, PP5	P	12
F51	c.1721G>A	p.Ser574Asn	CM062898	R(P)	Substitution	Missense	PM1, PM2, PM5, PP2, PP3, PP5	P	15
F52	c.2546dupG	p.p.Val850Serfs*15	CI098059	R(P)	Duplicaton	Frameshift	PVS1, PM1, PM2, PP3	P	21
F53	c.3058delG	p.Glu1020Lysfs*2	Novel	N/R	Deletion	Frameshift	PVS1, PM1, PM2, PP3	P	23
F54	c.3114-2A>G	-	CS147208	R(P)	Substitution	Splice site	PVS1, PM2, PP3, PP5	P	23-24
F55	c.3610C>G	p.Arg1204Gly	CM973234	R(P)	Substitution	Missense	PM1, PM2, PM5, PP2, PP3, PP5	P	27
F56	c.5003_5004insGGTA	p.Tyr1668*	Novel	N/R	Insertion	Non-sense	PVS1, PM1, PM2, PP3	P	36
F57	c.6263delT	p.Phe2088Serfs*2	CD1719515	N/R	Deletion	Frameshift	PVS1, PM1, PM2, PP3, PP5	P	41
F58	c.4288A>G	p.Asn1430Asp	CM113590	R(P)	Substitution	Missense	PM1, PM2, PM5, PP2, PP3, PP5	P	32
F59	c.6125delT	p.Leu2042Tyrfs*7	Novel	N/R	Deletion	Frameshift	PVS1, PM2, PP3	P	41
F60	c.1496T>G	p.Leu499Arg	CM1512946	R(P)	Substitution	Missense	PM1, PM2, PP2, PP3, PP5	LP	13
F61	c.1737_1738delTT	p.Phe579Leufs*8	Novel	N/R	Deletion	Frameshift	PVS1, PM1, PM2 PP3	P	16
F62	c.1748A>G	p.Lys583Arg	CM1111788	R(P)	Substitution	Missense	PM1, PM2, PP2, PP5, BP4	LP	16
F63	c.1885G>A	p.Gly629Arg	Novel	R(P)	Substitution	Missense	PS1, PS3, PM1, PM2, PP2, PP3	P	17
F66	c.2097dupC	p.Thr700Hisfs*2	Novel	N/R	Duplicaton	Frameshift	PVS1, PM2, PP3	P	18
F67	c.2252-3T>G	-	CS086414	N/R	Substitution	Splice site	PM2, PP5	VUS	18-19
F68	c.2325+3A>G	-	CS1311513	R(P)	Substitution	Splice site	PM2, PP5	VUS	19-20
F69	c.2604delT	p.Pro869Glnfs*9	CD153889	R(P)	Deletion	Frameshift	PVS1, PM1, PM2	P	21
F70	c.2764G>A	p.Gly922Ser	CM1719434	R(P)	Substitution	Missense	PM2, PP2, PP3	VUS	21
F71	c.3470T>A	p.Val1157Glu	Novel	N/R	Substitution	Missense	PM1, PM2, PP3, PP5	LP	26
F72	c.446dupA	p.Asn149Lysfs*7	Novel	N/R	Duplicaton	Frameshift	PVS1, PM2, PP3	P	4
F73	c.484C>T	p.Gln162*	CM073223	R(P)	Substitution	Non-sense	PVS1, PM1, PM2 PP3, PP5	P	5

Table III. continued

F74	c.4817T>A	p.Val1606Asp	CM1919720	N/R	Substitution	Missense	PM1, PM2, PP3, PP5	LP	36
F75	c.5719G>T	p.Glu1907*	CM043552	N/R	Substitution	Non-sense	PVS1, PM1, PM2 PP3, PP5	P	38
F76	c.7229delT	p.Val2410Glyfs*9	Novel	N/R	Deletion	Frameshift	PVS1, PM1, PM2	P	48
F77	c.7518_7519delGCinsCT	p.Gln2507*	Novel	N/R	Indel	Non-sense	PVS1, PM1, PM2 PP3	P	50
F78	c.1404dupT	p.Lys469*	Novel	N/R	Duplicaton	Non-sense	PVS1, PM1, PM2 PP3	P	13
F79	c.1637dupT	p.Met546Ilefs*12	Novel	N/R	Duplicaton	Frameshift	PVS1, PM2, PP3	P	14
F81	c.3721C>T	p.Arg1241* (%20)	CM000799	R(P)	Substitution	Non-sense	PVS1, PM1, PM2, PP3, PP5	P	28
F82	c.3871delG	p.Val1291Tyrfs*18	Novel	N/R	Deletion	Frameshift	PVS1, PM1, PM2, PP3	P	29
F83	c.4816_4817insGG	p.Val1606Glyfs*4,	Novel	N/R	Insertion	Frameshift	PVS1, PM1, PM2, PP3	P	36
F84	c.5107C>T	p.Gln1703*(%15)	CM143429	R(P)	Substitution	Non-sense	PVS1, PM1, PM2, PP3	P	36
F86	c.5827delG	p.Asp1943Metfs*15	Novel	N/R	Deletion	Frameshift	PVS1, PM1, PM2, PP3	P	39

LP: Likely pathogenic, P: Pathogenic, VUS: Variant of uncertain significance, R: Reported N/R: Not reported

Our results did not reveal any clear relationships between specific NF1 variants and phenotypes. Furthermore, no complete well-known genotype-phenotype correlation has been reported in the literature to date (12,18-20). Only three clear correlations of clinical significance have been identified in particular pathogenic NF1 variants. *NF1* whole-gene deletions are related with early-onset presentation of cutaneous neurofibromas, cognitive abnormalities, somatic overgrowth, and dysmorphic facial features (21,22). The c.2970-2972delAAT variant does not cause cutaneous or surface plexiform neurofibromas (23). Any of the c.5425C>T or c.5425C>A or c.5425C>G variants are related with café-au-lait spots, learning disabilities, short stature, and pulmonic stenosis but not cutaneous neurofibromas (24,25). However, C16 (13 years of age) with the c.2970-2972del variant had neurofibromas, which is a late-onset feature of NF1.

A novel c.1637dupT variation was detected in C85 (20 years of age) who had isolated pilocytic astrocytoma without cutaneous findings. The case of C11 with the c.1721+3A>C variation only had clinical signs of neurofibromas without the accompanying café-au-lait spots. Ocular manifestations such as Lisch nodules and optic nerve glioma were determined in 23% and 6.8% of all cases, which are lower frequencies in comparison with the literature (26,27). Additionally, the frequency of neurofibromas were lower than in the literature.

Mosaic NF1 variants cause mild forms of the NF1 phenotype (28). We detected two different variations with low variation fraction in C87 (c.3721C>T) and C90 (c.5107C>T). Sanger confirmations of these variants were consistent with NGS data. However, a molecular analysis of a second tissue could not be performed in these patients. Both of these patients had classical NF1 symptoms without family history and segmental involvement. We classified both of these patients as mosaic generalized NF1.

Conclusion

In conclusion, *NF1* genetic analysis was a supporting tool for the atypical presentation of NF1 cases especially in the prepubertal period. Additionally, genetic analysis before pregnancy provides preimplantation and prenatal genetic diagnosis for families with *NF1*.

Ethics

Ethics Committee Approval: The study was approved by the Ethical Committee of the Gaziantep University Medical Faculty (approval number: 65587614-774.99-291, date: 04/10/2017).

Informed Consent: Peripheral blood samples were obtained after taking informed consents from all participants or legal guardians of children under the age of 18.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: E.K., Design: E.K., Data Collection or Processing: H.M.A., E.K., Analysis or Interpretation: H.M.A., E.K., Literature Search: H.M.A., E.K., Writing: H.M.A., E.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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Original Article

Spectrum of Clinical Manifestations in Turkish Patients with Williams-Beuren Syndrome: A Monocentric Study

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ABSTRACT

Aim: Williams-Beuren syndrome, which is characterized by dysmorphic facial features, cardiovascular findings, intellectual disability, endocrine abnormalities and a typical cognitive profile, is caused by a microdeletion in the 7q11.23 region. In this study, we aimed to evaluate the dysmorphic and clinical manifestations of patients with Williams-Beuren syndrome.

Materials and Methods: We retrospectively collected data from 27 Turkish patients who had clinically and genetically confirmed Williams-Beuren syndrome. Their multisystemic manifestations, demographic data and dysmorphic facial features were recorded.

Results: All patients had the characteristic facial phenotype. The most frequent dysmorphic facial features were periorbital fullness, short nose, broad nasal tip and wide mouth. Aortic stenosis (59.2%) and pulmonary stenosis (37%) were the most common cardiac findings. Short stature (25.9%), idiopathic central precocious puberty (7.4%), hypothyroidism (congenital, non-congenital or subclinical) (40.7%) and hypercalcemia (3.7%) were the major endocrine manifestations in the patients. Genitourinary abnormalities were detected in 6 patients. All patients had some degree of intellectual disability; most of the patients (62.9%) had mild intellectual disability. Additionally, behavioral problems were frequently detected and the most common abnormality was overfriendliness (77.7%). Renal abnormalities (double collecting system, bladder diverticula and renal calculi) were also detected.

Conclusion: Dysmorphic facial features, which have a crucial role in the diagnosis of Williams-Beuren syndrome, should be assessed in suspected patients with supravalvular aortic stenosis and concomitant intellectual disability in order to make an early diagnosis. It should be kept in mind that endocrine abnormalities, musculoskeletal, neurologic and psychiatric manifestations are also common in patients with Williams-Beuren syndrome, necessitating a multidisciplinary approach.

Keywords: Williams-Beuren syndrome, 7q11.23 deletion, supravalvular aortic stenosis, pulmonary stenosis, intellectual disability

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Introduction

Williams-Beuren syndrome [(WBS), OMIM: 194050] is a congenital disorder that has a prevalence of 1/7,500-1/20,000 in newborns (1). This syndrome is characterized by dysmorphic facial features, cardiovascular disorders [supravalvular aortic stenosis (SVAS), elastin arteriopathy, peripheral pulmonary stenosis], intellectual disability, endocrine abnormalities (hypercalcemia, hypercalciuria, hypothyroidism, and precocious puberty) and a typical cognitive profile with over-friendly behavior (2). The typical facial features include broad forehead, bitemporal narrowing, periorbital fullness, strabismus, short nose, broad nasal tip, malar flattening, thick vermilion of the upper and lower lips, wide mouth, small jaw and large ear lobes. Hypotonicity is frequently detected in infants with WBS who typically have hyperextensible joints and delayed motor development. Connective tissue abnormalities including inguinal/umbilical hernia, bowel/bladder diverticula and rectal prolapse can also be observed. While penetrance is 100%, expression of the phenotypic features is variable in patients with WBS (3).

Williams-Beuren syndrome is caused by the deletion of approximately 1.5-1.8 megabase pairs on chromosome 7q11.23. It encompasses 26-28 genes including elastin (ELN). The *ELN* gene encodes a protein which is one of the two components of elastic fibers. Elastic fibers are important extracellular matrix macromolecules that provide elasticity and resilience to tissues and organs such as the arteries, heart, lungs, skin and ligaments. The atypical deletions are uncommon (2-5%) and range from 200 Kb to 2.5 Mb. The deletion size usually correlates with the phenotype (4-6). Genomic testing methods which determine the copy number of sequences can include chromosomal microarray or targeted deletion analysis by fluorescence *in situ* hybridization (FISH) (3). Most deletions occur de novo; but rare instances of parent-to-child transmission have been reported in patients with WBS (7).

The aim of this monocentric study was to describe the dysmorphic and clinical manifestations in a cohort of Turkish patients with WBS.

Materials and Methods

Twenty-seven patients with clinically and genetically confirmed WBS were included in this study. All patients were examined at the Department of Pediatric Genetics and Medical Genetics of University of Health Sciences Turkey, Dr. Behçet Uz Child Disease and Pediatric Surgery Training and Research Hospital, İzmir, Turkey between June 2012 and July 2020. Clinical data including prenatal signs, demographic features, and age at the time of diagnosis were collected from the clinical records and by parental interviews. The growth parameters of the patients were evaluated in accordance with the specific growth charts for WBS (8). Additionally, the dysmorphic facial features, laboratory test results and imaging results were noted. After diagnosis, all patients were included in a multi-specialist follow-up protocol.

All patients had a typical chromosomal 7q11.23 deletion which was detected by FISH analysis. We excluded those patients who were not genetically diagnosed. The local Ethics Committee approved the study (date: 24.09.2020, number: 2020/13-8), and written informed consent was obtained from all individuals involved. The study was conducted in accordance with the Helsinki Declaration.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 23.0. (IBM Corp. Armonk, NY). Data is presented with descriptive statistics (median with 25^{th}-75^{th} percentiles for continuous variables; frequency and percentage for categorical variables).

Results

The main facial features and clinical findings of the 27 patients (18 males, 9 females) are summarized in Table I. The median age at the time of diagnosis was 4 years (1-8). The median follow-up period of the patients was 5 years (5-8). Ten patients (37%) were diagnosed before the age of 2. The median birth weight of the patients was 2.75 kg (2.25-3.1). All patients had several types of the characteristic facial features of WBS (Table I). The most common facial findings

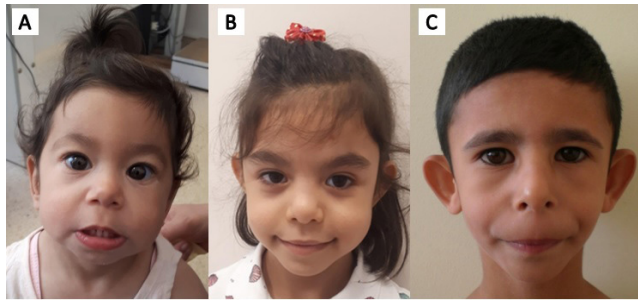

Figure 1. Dysmorphic facial findings of some of the study patients. A: Broad forehead, periorbital fullness, epicanthal fold, short nose, broad nasal tip, full cheeks, wide mouth, long philtrum and small jaw in a 1.5-year-old patient. B: Periorbital fullness, broad forehead, full cheeks, short nose, broad nasal tip and wide mouth in a 7-year-old patient. C: Broad forehead, periorbital fullness, short nose, broad nasal tip, wide mouth, long and smooth philtrum and small jaw in an 8-year-old patient

Table I. Clinical manifestations of the patients with Williams-Beuren syndrome

Clinical findings	n (%)
Sex	
Male Female	18 (66.6) 9 (33.3)
Prematurity	5 (18.5)
Small for gestational age	9 (33.3)
Dysmorphic features Periorbital fullness Short nose Broad nasal tip Wide mouth Long philtrum Full cheeks Small jaw Broad forehead Epicanthal fold Large ear lobes	27 (100) 27 (100) 27 (100) 27 (100) 27 (100) 25 (92.5) 23 (85.1) 22 (81.4) 22 (81.4) 17 (62.9) 13 (48.1)
Short stature	5 (18.5)
Cardiovascular findings	24 (88.8)
Endocrinologic and genitourinary findings Hypothroidism Hypercalcemia Precocious puberty Genital abnormalities Urinary tract abnormalities	19 (70.3) 11 (40.7) 1 (3.7) 2 (7.4) 4 (14.8) 2 (7.4)
Ocular findings	12 (44.4)
Connective tissue and skeletal abnormalities	14 (51.8)
Intellectual disability Mild Moderate Severe Global developmental delay	27 (100) 17 (62.9) 8 (29.6) 1 (3.7) 1 (3.7)
Behavioral traits Overfriendliness Attention deficit disorder Phobia Autistic features Specisific learning disability	25 (92.5) 21 (77.7) 7 (25.9) 7 (25.9) 4 (14.8) 1 (3.7)
Epilepsy	2 (7.4)
Total	27 (100)

were periorbital fullness, long philtrum, short nose, broad nasal tip and wide mouth (Figure 1). The median weight of the patients was 25 kg (7-73) and the median height was 126 cm (73-164) during the final evaluation. Weight over 2 standard deviation score (SDS) was detected in six patients and the other patients were between the normal ranges. Short stature below 2 SDS was observed in 5 individuals and one of them was also small for gestational age (SGA). In two patients with insufficient annual height velocity during follow-up, growth hormone stimulation tests (Clonidine) were performed and adequate growth hormone response (>10 ng/mL) was obtained. Ultimately, growth hormone deficiency could not be demonstrated in any of the patients.

Table II. Echocardiographic results of the patients with Williams-Beuren syndrome

Echocardiographic findings	n (%)
Aortic stenosis Valvular Supravalvular	16 (59.2) 1 (3.7) 15 (55.5)
Pulmonary stenosis Valvular Supravalvular Valvular + Supravalvular Peripheral	10 (37) 3 (11.1) 1 (3.7) 2 (7.4) 4 (14.8)
Aortic stenosis + Pulmonary stenosis	6 (22.2)
Mitral regurgitation (mild)	3 (11.1)
Mitral valve prolapse	3 (11.1)
Aortic regurgitation (mild)	2 (7.4)
Pulmonary hypertension	1 (3.7)
Bicuspid aortic valve	1 (3.7)
Ventricular septal defect	1 (3.7)
No cardiac abnormality	3 (11.1)
Total	27 (100)

Cardiologic evaluation was performed in all patients. Three participants did not have any cardiac abnormalities. In the remaining patients, aortic stenosis and pulmonary stenosis were the most common features. Furthermore, three patients had mild mitral regurgitation; two of them were associated with mitral valve prolapsus. The echocardiographic findings of the patients are given in Table II. More than one cardiac abnormality was detected in 12 patients. Two patients with SVAS and one patient with ventricular septal defect underwent cardiac surgery. Balloon valvuloplasty was performed in two patients due to valvular pulmonary stenosis. Additionally, the severity of peripheral or supravalvular pulmonary stenosis was decreased in two patients during echocardiographic follow-up.

Connective tissue abnormalities and musculoskeletal findings were present in 14 patients (51.8%) (Table I) and 10 of them had uni- or bilateral inguinal hernia. Additionally, 4 patients had a variable degree of thoracolumbar scoliosis, and torticollis was detected in a one-year-old patient.

Hypothyroidism was detected in 11 (40.7%) cases; 2 of them had congenital primary hypothyroidism, 4 of them had non-congenital primary hypothyroidism requiring

L-thyroxine treatment, and 5 of them had subclinical hypothyroidism not requiring any treatment. One of these patients also had vitamin D deficiency. Chronic constipation was present in 5 patients and 4 of these also had hypothyroidism. Additionally, a single patient had asymptomatic hypercalcemia. Idiopathic central precocious puberty was diagnosed (both with Tanner stage 2 and >5.0 IU/L peak Luteinizing-hormone levels after Luteinizing-hormone releasing hormone stimulation) in two patients (8.5-year-old male and 7-year-old female) who were treated with gonadotropin-releasing hormone. The genital abnormalities included unilateral cryptorchidism (n=2), penile hypospadias (n=1) and webbed penis (n=1), which were not attributed to any endocrine/hormonal pathology (Table I). Double collecting system and bladder diverticula were found in one female patient. Additionally, renal calculi were detected in another patient.

Hearing tests were performed in all patients and a mild hearing loss was detected in one male patient. Ocular manifestations were present in approximately half of the patients (Table I). The most common findings were strabismus (n=5, 18.5%) and stellate pattern of the iris (n=3, 11.1%). Myopia (n=1), hyperopia (n=2), astigmatism (n=1) and glaucoma (n=1) were also detected.

All patients had some degree of intellectual disability and one of them also had global developmental delay (Table I). Almost all of the patients had several types of behavioral problems (Table I). One patient, who had mild intellectual disability, also had a specific learning disability. Echolalia was remarkable in one patient with global developmental delay and autistic features. Epilepsy was detected in two patients and Chiari malformation type 1 was found in two other patients.

Discussion

In this monocentric study, we evaluated the dysmorphic features and clinical abnormalities in a cohort of 27 Turkish patients with WBS, which is a congenital genetic disorder. The microdeletion at 7q11.23 involves the gene coding for elastin, which is an important protein in the composition of elastic fibers of connective tissue. This gene may explain some phenotypic characteristics and disorders present in WBS such as connective tissue abnormalities and cardiovascular diseases.

The majority (~80%) of patients with WBS have structural cardiovascular abnormalities typically related to arterial stenosis (9). These cardiovascular disorders are usually associated with increased morbidity and mortality (10). Patients with combined SVAS and pulmonary stenosis or coronary artery stenosis may develop biventricular hypertrophy and hypertension, which increases the risk of myocardial ischemia, arrhythmias and sudden death (10,11). The incidence of sudden death in one cohort of 293 patients with WBS was 1/1,000 patient years, which is 25 to 100 times higher than the age-matched population (12). Bruno et al. (13) reported that SVAS was the most frequent malformation representing 71% of cases, and the incidence of pulmonary stenosis at the valvular level was 11%. Pulmonary artery stenosis was the other common finding and its incidence was reported to be approximately 40% in patients. Of those patients with cardiac anomalies, 34.5% had a single defect and 65.5% had multiple defects (14,15). In the current study, cardiovascular anomalies were evident in most of our patients (88%). Consistent with the literature, the most common findings were aortic and pulmonary stenosis. In 6 patients, SVAS was accompanied by pulmonary stenosis. A single cardiac abnormality was detected in half of the patients with established cardiac findings. Previously, it has been reported that peripheral pulmonary stenosis is associated with a good long-term prognosis (14). In our study, 10 patients had pulmonary stenosis and 2 of them (one peripheral, one supravalvular) showed improvement during follow-up. Pulmonary balloon valvuloplasty was performed in two other patients with valvular pulmonary stenosis.

The phenotypical traits in WBS patients are well characterized, but become more evident with increasing age; therefore, the probability of finding a 7q11.23 deletion is greater in older individuals (16,17). Additionally, patients with mild or insignificant cardiac involvement are older at the time of diagnosis compared to patients with SVAS or severe cardiac symptoms (15). In our study, all of the patients had typical facial characteristics, but only 10 patients (37%) had been diagnosed before the age of 2. Eight of these patients had more than one cardiac finding and 6 of them had both aortic and pulmonary stenosis. In the remaining 17 patients who were diagnosed after 2 years of age, aortic stenosis and pulmonary stenosis were detected in 9 and 2 patients, respectively. Additionally, 2 patients had mild mitral regurgitation due to mitral valve prolapsus and 1 patient had pulmonary hypertension. Three patients (2.5, 5 and 10 years of age at the time of diagnosis) did not have any cardiac abnormalities. On the basis of these findings, we may suggest that although SVAS is the most common cardiac abnormality in patients with WBS, SVAS accompanied by pulmonary stenosis may be a stronger indicator for this disease. Furthermore, in patients with suspected SVAS and intellectual disability, assessment of characteristic facial features may enable the early detection of the disease.

Recently, endocrine abnormalities have been reported in detail in patients with WBS. Although pre- and postnatal growth retardation is common in this syndrome, growth hormone deficiency has not been considered as a major cause. This may be attributed to restricted prenatal growth, failure to thrive in infancy or restricted growth in childhood. In a previous study, the frequency of short stature was reported to be 33% at preschool ages and 67% at school ages in patients with WBS. About 50% to 60% of the patients with WBS reach a final adult height below their target height range (18,19). The other endocrine abnormality, congenital hypothyroidism, is rare, but subclinical hypothyroidism occurs in 31% of patients, and occurs more frequently in children than in adults (20). Additionally, precocious puberty is common in WBS and true precocious puberty has been reported in 3-18% of the patients (21). Hypercalcemia was also reported in 5-50% of individuals with WBS. It is usually mild but it may present with vomiting, constipation, irritability and muscle cramps (22). In the present study, short stature was detected in 5 patients and one of them had SGA. Hypothyroidism (congenital/non-congenital primary or subclinical hypothyroidism) and central precocious puberty were detected in 11 (40.7%) and 2 (7.4%) patients, respectively. Furthermore, hypercalcemia was detected in a single asymptomatic patient. Endocrine abnormalities are not uncommon causes of morbidity in patients with WBS; therefore, close monitoring and follow-up are needed for the management and the prediction of the prognosis of these endocrine dysfunctions.

The risk of external genital abnormalities, renal and urinary tract structural abnormalities (renal ectopia, agenesis, hypoplasia, duplication, horseshoe kidney, hydronephrosis, and vesicoureteral reflux) in WBS cases were demonstrated to be more common (up to 73%) than the normal population (23). In this study, 4 (14.8%) cases had external genital abnormalities and 2 (7.4%) cases had renal and urinary tract structural abnormalities. However, none of them contributed to any endocrine/hormonal pathology. The relatively low frequency of urogenital anomalies in our study may be explained by the low number of patients and the absence of further investigations such as voiding cystourethrogram or dimercaptosuccinic acid scintigraphy.

Musculoskeletal manifestations can be commonly observed in WBS. Atypical posture and exaggerated or abnormal spinal curvatures are also a frequent finding in patients with WBS. Lordosis was described in 38% of infants and 90% of adolescents and young adults. Additionally, while kyphosis was reported in 10-21% of patients, scoliosis was detected in 12% to 20% of patients (24). In a recent study, Damasceno et al. (25) identified scoliosis in 34% of their patients. In the present study, thoracolumbar scoliosis was detected in 4 patients (14.8%), whereas neither lordosis nor kyphosis was observed in any cases. Additionally; torticollis, which is a rare musculoskeletal abnormality in WBS, was detected in a one-year-old male patient. Physical therapy was performed in all of these patients. These musculoskeletal features are not specific to WBS, but they should be kept in mind and assessed when necessary to prevent disease progression.

Seventy-five percent of patients with WBS have intellectual disability, which is usually mild. The cognitive profile of these patients is distinctive. It consists of good verbal short-term memory and language while visuospatial constructive cognition is highly impaired (26). Furthermore, patients with WBS have a typical personality profile which includes overfriendliness, excessive empathy, attention problems and social disinhibition. Emotional regulation, perseveration, and specific phobias can also be observed. Some patients have overlapping symptoms with the autism spectrum disorder (27,28). In the present study, all patients had intellectual disability, but most of them to a mild degree (62.9%). Overfriendliness (77.7%) was the most common personality profile which is consistent with the current literature. Additionally, 4 patients had autistic features. While 3 of these patients also had moderate or severe intellectual disability, the other one had global developmental delay. Patients with WBS usually have mild intellectual disability and are usually socially motivated. However, it should be emphasized that WBS has been found to be a risk factor for autism.

Conclusion

In conclusion, WBS is a complex, genetic disorder characterized by a highly variable phenotype. The most common and significant findings in patients with WBS are dysmorphic facial features and cardiac abnormalities. Furthermore, WBS should be considered in patients with SVAS and concomitant intellectual disability. Additionally, it should be kept in mind that endocrine abnormalities, musculoskeletal, neurologic and psychiatric problems are also frequent in WBS, necessitating a multidisciplinary approach. Early diagnosis is crucial for the management of the syndrome and the prediction of its prognosis.

Ethics

Ethics Committee Approval: University of Health Sciences Turkey, İzmir Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital, Clinical Research Ethics Committee approved the study (date: 24.09.2020, number: 2020/13-8).

Informed Consent: Written informed consent was obtained from all individuals involved.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: S.G., F.H., Design: B.Ö., S.A., C.Z., Data Collection or Processing: S.G., F.H., C.Z., S.A., Analysis or Interpretation: S.G., F.H., M.M.Y., T.M., Literature Search: B.Ö., S.A., C.Z., Writing: S.G., M.M.Y., T.M.

Conflict of Interest: No conflict of interest was declared by the authors.

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Original Article

The Effect of Diabetes Camp on Glycemic Variability in Children and Adolescents with Type 1 Diabetes Mellitus

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ABSTRACT

Aim: Glycemic variability can be affected in diabetes camps as a result of sports, social activities and nutrition. Close glucose monitoring is necessary to reduce glycemic variability, especially hypoglycemia. The aim assessment of glycemic variability and time in range by use of the flash glucose monitoring system (FGMS) in children and adolescents with type 1 diabetes.

Materials and Methods: Thirty-three children and adolescents between 10-18 years of age who participated in the 2018 diabetes camp of Ege University were included. Their glycemic variability indexes were recorded.

Results: The mean age and duration of diabetes mellitus in the study group was 13.3±0.5 and 4.9±0.7 years respectively. Twelve (43%) of the participants were boys and 16 (57%) were girls. Ten (35.7%) of the participants used continuous subcutaneous insulin infusion (CSII) pump therapy while 18 (64.3%) used multiple dose insulin therapy. When the participants were evaluated according to time in range (TIR), the duration of TIR increased, and level 1 and level 2 hyperglycemia decreased during the camp. Participants using CSII had spent more time in level 2 hypoglycemia before camp, but during and after the camp, similar values were reached for both groups. Before the camp, participants with good metabolic control had a longer duration of hypoglycemia than those participants with poor metabolic control. During and after the camp, level 1 and level 2 hypoglycemia periods were similar between the two groups.

Conclusion: In diabetes camp, healthy diet, regular exercise, and close glycemic control improve glycemic variability. By using FGMS, normoglycemia periods can be increased without increasing hypoglycemic attacks. As a result, using FGMS had a positive effect on diabetes management and the control of hypoglycemia periods during the diabetes camp.

Keywords: Diabetes camp, glycemic variability, flash glucose monitoring system

Introduction

In children and adolescents, glycemic variability, hypoglycemia and glycemic excursions are seen more than in adults due to their unpredictable activity, eating habits and hormonal changes (1). Either by using multiple dose insulin (MDI) or continuous subcutaneous insulin infusion (CSII) treatments, persons with type 1 diabetes mellitus have to monitor their blood glucose frequently in order to improve metabolic control. The burdens of self-monitoring of blood glucose (SMBG) include pain, disturbance of sleep

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due to night-time tests, inconvenience and embarrassment (2). It also has many limitations including insufficient identification of glycemic variability and hyperglycemic or hypoglycemic episodes due to intermittent monitoring, the unreliability of individual recorded data, and inadequate compliance (3). With the help of FGMS Abbott FreeStyle Libre, glucose levels are checked by scanning the sensor with a reader, thus eliminating the need for SMBG testing (4). It reports the current glucose concentration, glucose trends, and displays the previous 8 hours as a trend (5,6). The aim of this study was to evaluate the duration of level 1 and level 2 hyperglycemia, time in range (TIR), and level 1 and level 2 hypoglycemia percentages in type 1 diabetic children and adolescents, by means of Abbott FreeStyle Libre FGMS, during a summer camp.

Materials and Methods

A retrospective study with type 1 diabetic children and adolescents aged 10-18 participating in a summer camp was conducted.

All participants gave written informed consent. This study was registered and approved by the ethics committee of Ege University with approval number 20-11.1T/45 and conducted in accordance with the Declaration of Helsinki.

The inclusion criteria were being between 10-18 years, a diagnosis of type 1 diabetes mellitus at least 1 year prior to the camp, the absence of any disease that could impair exercise capacity and a willingness to participate in the study. The medical staff, who supported the children during the camp, consisted of nutritionists, nurses of diabetology, pediatric assistant doctors, students, and specialists in pediatric endocrinology and diabetes.

All camp participants who were using Abbott FreeStyle Libre sensor were included in the study. However, routine glucose control measurements for therapeutic decisions were made by Accu Check Performa glucometer. Participants at the camp performed activities such as swimming, cycling, running and dancing for a minimum of 2 hours per day. The duration of the camp was 5 days. After camp was completed, data from FGMS was downloaded to a computer by specially designed software that created a series of reports of the glycemic picture 5 days before, 5 days during and 5 days after the camp. TIR was defined as the percentage of glucose readings and the time between 70-180 mg/dL (3.9-10 mmol/L). Level 1 hyperglycemia was defined as the percentage of readings and time between 181-250 mg/dL (10-13.9 mmol/L). Level 2 hyperglycemia was defined as the percentage of readings and time greater than 250 mg/dl (13.9 mmol/L). Level 1 and 2 hypoglycemia were defined as the percentage of readings and time between 69-54 mg/dL (3.9-3 mmol/L) and the time less than 54 mg/dL (3 mmol/L) respectively (6). All raw data was classified according to TIR, hypo- or hyperglycemia.

In year 2020, standard deviation (SD) and coefficient of variation (CV) calculation program was added to Libre infrastructure. Unfortunately in 2018 in the Turkish version of software of Libre, the personal SD and CV was not calculated. For this reason, each participants' intrapersonal mean and SD of all recorded glycemic measurements (approximately 1,500) were calculated by researchers and for the coefficient of variance, the mean/SD formula was used (7).

Statistical Analysis

Analysis was carried out using SPSS for Windows 25.0, descriptive statistics are reported using mean ± SD for normally distributed variables, and median for skewed data. Since the sample size was smaller than 30, it does not meet the normality assumption of a t-test. Thus, groups in this study were compared by Mann-Whitney U test, which is a non-parametric equivalent of the 2-sample t-test. Trends across more than 2 groups were ANOVA and Freidman analysis. P-values of less than 0.05 were considered statistically significant.

Results

Of the 33 participants, 5 were not included in the study due to early detachment of the sensor. Mean age and duration of diabetes mellitus were 13.3±0.5 and 4.9±0.7 years respectively. Twelve (43%) of the participants were male and 16 (57%) were female. Forty percent (n=12) of the participants' diabetes duration was greater than 5 years. Ten (35.7%) of the participants were using a CSII pump while 18 (64.3%) were using MDI therapy. The mean glucose of all the participants before, during, and after camp were 199±52.5 mg/dL (11±2.9 mmol/L), 171±32.1 mg/dL (9.5±1.8 mmol/L), and 194±45.2 mg/dL (10.8±2.5 mmol/L) respectively (Table I).

The percentage of readings within TIR was 60.3±15.3% during the camp, 47.4±17.7% before the camp and 44.1±17.5% after the camp (p=0.005). Participants with poor metabolic control had an increased percentage of TIR in comparison to their pre-camp values (p=0.003, Table II). As was expected, pre-camp TIR values were higher in the good metabolic control group in comparison to the poor metabolic control group (p=0.037). However, no differences were found during or after the camp. In the poor metabolic control group, level 2 hyperglycemia values were lower during the camp than their pre- and post-camp values (p=0.039). Those

participants with good metabolic control had longer periods of level 2 hypoglycemia compared with the poor metabolic control group before the camp (p=0.033) whereas the duration of level 2 hypoglycemia was similar during and after the camp (Table II).

The CSII and MDI groups had a similar percentage of TIR, level 1 and level 2 hyperglycemia, and level 1 hypoglycemia values before, during and after the camp. In the CSII group, the duration of level 2 hypoglycemia was more than the MDI group before the camp (p=0.03). Similar values were reached during and after the camp between the groups (Table III). The mean HbA1c of the last year before summer camp was 7.7% (61 mmol/mol) in the CSII group and 8.4% (68 mmol/mol) in the MDI group.

The total daily insulin dosage before and during the camp was not significantly different (0.81±0.23 and 0.79±0.24 u/kg respectively). During the camp, basal insulin dosage was significantly lower than the pre-camp and post-camp values (0.36±0.15, 0.31±0.14 and 0.38±0.14 u/kg respectively, p<0.001).

SD was 74.1±36.48 before the camp, it was reduced to 67.4±31.6 during the camp and increased to 74.95±30.10 after the camp but this is statistically insignificant. CV before, during, and after the camp was 40.61%, 41.2%, and 39.2%, respectively.

Discussion

There have been significant changes in the management and treatment of type 1 DM with breakthroughs in technology. One of the challenges in diabetes, especially in children, is the measurement of capillary blood glucose. Many children do not measure blood glucose due to pain, loss of time and shame, and do not adequately manage

Table I. Duration of hypoglycemia, normoglycemia and hyperglycemia periods before, during and after summer camp

	All participants n=28			
	Before camp	**During camp**	**After camp**	**p-value**
Mean glucose readings mg/dL (mmol/L)	199±52.5 (11±2.9)	171±32.1 (9.5±1.8)	194±45.2 (10.8±2.5)	0.002
Level 1 hypoglycemia (%)	2.07±2.85	1.41±3.07	0.7±1.31	0.085
Level 2 hypoglycemia (%)	3.06±3.19	3.36±2.41	2.18±1.73	0.280
Normoglycemia (%)	47.48±17.79	60.35±15.35	44.18±17.5	0.002
Level 1 hyperglycemia (%)	24.38±6.86	20.54±7.21	27.63±9.42	0.008
Level 2 hyperglycemia (%)	22.99±19.3	14.23±11.52	25.29±13.80	0.032
Daily scan count (n)	15.6±12.1	51.89±33.61	27.53±14.76	<0.001

Data are mean ± SD, Time in range (TIR): 70-180 mg/dl (3.9-10 mmol/L), Level 1 hyperglycemia: 181-250 mg/dL (10-13.9 mmol/L), Level 2 hyperglycemia: >250 mg/dL (13.9 mmol/L), Level 1 hypogl ycemia: 69-54 mg/dL (3.9-3 mmol/L), Level 2 hypoglycemia: <54 mg/dL (3 mmol/L)
SD: Standard deviation

Table II. Duration of hypoglycemia, normoglycemia and hyperglycemia periods before, during and after summer camp according to metabolic control

	HbA1c≤7.5 %				**HbA1c>7.5 %**			
	Before camp	**During camp**	**After camp**	**p-value**	**Before camp**	**During camp**	**After camp**	**p-value**
Level 2 hypoglycemia %	3 (0-11.9)	0.2 (0-13.1)	0.1 (0-5.1)	0.013	0.4 (0-5.5)	3.5 (0.2-7)	2.3 (0-5.5)	0.460
Level 1 hypoglycemia %	3.7 (0.4-11.9)	2.6 (0.4-7)	1.8 (0-4.9)	0.165	1.6 (0-7.9)	0.1 (0-6.9)	0 (0-2.8)	0.404
TIR %	51.1 (43.9-71.7)	58 (29.9-93)	42.1 (22.2-87.9)	0.27	41.1 (9.5-81.1)	59.9 (41.5-89)	43.6 (18.3-73.4)	0.003
Level 1 hyperglycemia %	23.5 (15.2-31.6)	20.7 (4.2-30.2)	30.3 (7-51.1)	0.053	26.7 (10.8-36.3)	19.8 (4.1-31.2)	28 (15.8-38.4)	0.076
Level 2 hyperglycemia %	9.4 (2.8-20.5)	10.1 (0-43.2)	23.2 (0-31.3)	0.303	29.9 (3.3-77.6)	8.8 (0.9-33.1)	24 (5.8-63.1)	0.039

Data are median (min-max), Time in range (TIR): 70-180 mg/dL (3.9-10 mmol/L), Level 1 hyperglycemia: 181-250 mg/dL (10-13.9 mmol/L), Level 2 hyperglycemia: >250 mg/dL (13.9 mmol/L), Level 1 hypoglycemia: 69-54 mg/dL (3.9-3 mmol/L), Level 2 hypoglycemia: <54 mg/dL (3 mmol/L)
Min: Minimum, max: Maximum

Table III. Duration of hypoglycemia, normoglycemia and hyperglycemia periods before, during and after summer camp according to treatment method

	CSII (n=10)				MDI (n=18)			
	Before camp	**During camp**	**After camp**	**p-value**	**Before camp**	**During camp**	**After camp**	**p-value**
Level 2 hypoglycemia %	2.2 (0.8-11.9)	1.4 (0-13.1)	0.5 (0-5.1)	0.0242	0.1(0-5.5)	0 (0-6.9)	0 (0.8-2.8)	0.71
Level 1 hypoglycemia %	3 (1.3-11.9)	5.4 (0.4-6.2)	2.5 (0.2-5.5)	0.432	1.4 (0-8.8)	2.1 (0.2-7)	2.1 (0-5.5)	0.5
Normoglycemia %	45.2 (24-51.8)	56.5 (41.5-66.7)	40.4 (24.2-47.2)	0.016	50.6 (9.5-81.1)	60.3 (29.9-93)	44.5 (18.7-87.9)	0.024
Level 1 hyperglycemia %	29.5 (19.6-31.6)	20.6 (17.7-29.1)	31.3 (17.1-33.7)	0.011	24.1 (10.1-31.1)	19.4 (4.1-31.2)	28 (7-51.1)	0.032
Level 2 hyperglycemia %	19.3 (9.4-43.2)	11.9 (4.7-39.5)	30.6 (16.2-38.8)	0.056	15.9 (2.8-77.6)	9.4 (0-43.2)	23.5 (0-63.1)	0.091

Data are median (min-max), Time in range (TIR): 70-180 mg/dL (3.9-10 mmol/L), Level 1 hyperglycemia: 181-250 mg/dL (10-13.9 mmol/L), Level 2 hyperglycemia: >250 mg/dL (13.9 mmol/L), Level 1 hypoglycemia: 69-54 mg/dL (3.9-3 mmol/L), Level 2 hypoglycemia: <54 mg/dL (3 mmol/L).
Min: Minimum, max: Maximum, CSII: Continuous subcutaneous insulin infusion, MDI: Multiple dose insulin

diabetes, especially when they are away from parental control, such as at school (8). FGMS was developed to replace capillary blood glucose measurement and contributes to the management of diabetes by recording glucose values every 15 minutes, showing the trend of the previous 8 hours and creating graphs of glycemic variability (9).

The importance of exercise in the treatment of diabetes is indisputably known, but each individual's glycemic variability with exercise is different and the effect on blood glucose depends on the duration and type of exercise (10,11). Although exercise rules are determined by many associations, there are individual differences in practice (12,13). Many persons with diabetes avoid sports because of the fear of hypoglycemia, the difficulty in follow-up of strict SMBG, and a lack of knowledge about exercise management (14). Diabetes camps are one of the most intensive experiences for exercise and contribute to exercise training (15,16).

Although insulin dosage is decreased in diabetes camps, hypoglycemia is seen frequently with the effects of strict exercise (17-19). We observed that increasing the number of scans by Abbott FreeStyle Libre and promoting strict exercise increased the duration of normoglycemia during the camp. When the participants returned to their natural routine, similar results to pre-camp values were observed; positive motivation was not permanent, and it seems that permanent lifestyle changes are obligatory to improve metabolic control. Parallel to our results, various studies show that those persons with the poorest metabolic control had the greatest metabolic improvements during the camp, but this is not sustained after the camp (20).

In the CSII group, the duration of level 1 hypoglycemia was longer before the camp. This was due to the fact that the pumps are not reinforced by the sensor and lower HbA1c values are present in CSII group. However, there was no difference in the duration of level 1 hypoglycemia according to the treatment model during the camp, suggesting that the lack of knowledge about hypoglycemia awareness/management in this group might be the reason.

Glycemic variability is a favorite target of scientific research in diabetology. It was found to be related to microvascular complications (21). In non-diabetic persons, after intense exercise, despite no changes in mean blood glucose levels, there is increased glycemic variability and increased periods of hypoglycemia (10). Although light exercise and glycemic variability in type 2 diabetes mellitus has been studied, the information about the effect of exercise on glycemic variability in children with type 1 diabetes was inadequate (11,22). It is hoped that treatment approaches that will reduce glycemic variability during exercise will be found, but it needs to be clarified according to exercise type and treatment modalities. As we investigate the literature, our study was the first of its kind using FGMS during a summer camp in a pediatric sub-population evaluating glycemic variability calculated as CV. There are two other studies which evaluated the accuracy and satisfaction of Abbott FreeStyle Libre which concluded that the FSL is accurate in children. However, its accuracy depends on the glucose trend and Abbott FreeStyle Libre user's satisfaction survey revealed that most of the respondents rated satisfaction with Abbott FreeStyle Libre positively (23,24). In a summer camp conducted in Slovenia, children with type 1

diabetes using CSII were investigated. They found that CGM was as safe and effective as SMBG, and reduced the time spent in hyperglycemia in a sub-population of children with suboptimal glycemic control.

Study Limitations

In this study, the number of capillary blood glucose measurements were not recorded, a quality of life and the Abbott FreeStyle Libre satisfaction questionnaire were not applied, and unexpected events were not recorded.

Conclusion

Considering all the data, due to its easy usability, guidance with diabetes management, lack of requirement for calibration and good participant satisfaction, Abbott FreeStyle Libre can be used during summer camp. With its contribution, the duration of normoglycemia can be increased without increasing the duration of hypoglycemia and glycemic variability.

Ethics

Ethics Committee Approval: The study was registered and approved by the ethics committee of Ege University with approval number 20-11.1T/45 and conducted in accordance with the Declaration of Helsinki.

Informed Consent: All participants gave written informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: A.A., Ş.D., Design: G.A., G.D., Data Collection or Processing: H.I., D.G., Analysis or Interpretation: Ş.D., Literature Search: B.E., Y.A.A., Writing: A.A., S.Ö.

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Original Article

The Frequency of Ketoacidosis and Associated Factors at the Diagnosis of Type 1 Diabetes in Turkish Children: A Single-center Experience and Literature Review

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ABSTRACT

Aim: We aimed to investigate the frequency of diabetic ketoacidosis (DKA) and the associated factors at the time of diagnosis of type 1 diabetes (T1D) in children in a tertiary health center in Turkey, and to review previous studies conducted in Turkey.

Materials and Methods: Data of 180 children with T1D (98 boys) aged 1 to 18 years were analyzed retrospectively. All children were consecutively diagnosed as having T1D at our pediatric endocrinology clinic between April 2016 and December 2019. To conduct a literature review, we screened PubMed, Google Scholar, Web of Science, and article reference lists as well as the proceedings of the national conferences organized by the Turkish Pediatric Endocrinology and Diabetes Society for the period until January 1st, 2020.

Results: DKA was detected in 81 (45.0%) children with T1D at the time of diagnosis in this cohort. An association between DKA and high glycated hemoglobin (HbA1c) levels at the time of diagnosis was determined (p=0.038). Furthermore, a relationship was also detected between severe DKA (pH<7.1 or serum bicarbonate <5 mmol/L) and children residing in rural areas, as well as mothers with education less than high school (p=0.003 and p=0.022, respectively). This study, together with a literature review of 49 other studies, identified that 4,037 (45.6%) of 8,837 children with newly diagnosed T1D presented with DKA at diagnosis between 1981 and 2019.

Conclusion: In this cohort, presentation with DKA at the time of diagnosis of T1D in children was associated with high levels of HbA1c, and presentation with severe DKA was associated with rural life as well as low education levels of mothers. Almost half of all children with T1D presented with DKA in Turkey. There should be greater effort to increase awareness among society and health professionals for the early detection of T1D in children.

Keywords: Children, frequency, ketoacidosis, type 1 diabetes

Introduction

Type 1 diabetes (T1D) is a common chronic disease with significant morbidity and notable mortality rates in children (1,2). There is an increasing trend in the incidence of T1D in children and adolescents globally, including Turkey (3,4). Diabetic ketoacidosis (DKA) is a major acute complication of T1D and has a serious risk of mortality and morbidity. DKA develops as a result of severe insulinopenia and presents symptoms of hyperglycemia, ketosis, and acidosis. DKA is commonly detected at the time of T1D diagnosis in children and it is also detected in children previously diagnosed with T1D, usually following the interruption of insulin treatment due to an intervening disease, deliberate or unintentional omission of insulin, or when the prescribed insulin dose is no longer sufficient (5).

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A systematic review investigating 46 studies from 31 countries examining more than 24,000 children with T1D reported the frequency of DKA to have six-fold variability ranging between 12.8% and 80.0% (6). Sweden, Canada, and Finland had the lowest rates, and the highest DKA frequencies were reported in the United Arab Emirates, Romania, and Saudi Arabia (6). The frequency of DKA in children and adolescents was previously reported to be 41.9% and 64.9% at a tertiary health center in Elazığ province in Turkey at two different times (7,8). This study aimed to determine the current DKA frequency and associated factors at the time of diagnosis of children with T1D at the Elazığ province and to review literature data on DKA frequency in Turkish children with T1D at the time of diagnosis.

Materials and Methods

Data of 180 children with T1D (98 boys) aged 1 to 18 years were analyzed retrospectively. All children were consecutively diagnosed as having T1D at our pediatric endocrinology clinic in Elazığ province in Turkey between April 2016 and December 2019.

The study protocol was performed according to the Declaration of Helsinki and approved by the Ethics Committee of Non-Interventional Research of Fırat University (decision number: 0010, date: 28.11.2019). The requirement for informed consent was waived due to the retrospective nature of the study.

The hospital where the study was conducted provides healthcare services especially to residents within the provincial borders of Elazığ. Additionally, this hospital also accepts patient applications from neighboring provinces. For this study, the following demographic, clinical, and laboratory data of those children with T1D were collected and evaluated: sex, age at diagnosis, the season of diagnosis, the pre-diagnostic diabetes symptoms (polyuria and polydipsia), T1D presence in the family, parental education status, place of residence, blood glucose concentration at admission, venous blood pH and bicarbonate concentration, serum C-peptide levels, and glycated hemoglobin (HbA1c) concentrations. Children and adolescents diagnosed as having diabetes other than T1D, such as type 2 diabetes, monogenic diabetes, and diabetes developing due to secondary causes, were excluded from the study. Additionally, patients with no or insufficient laboratory data (such as those diagnosed at an external center) and whose type of diabetes was not fully defined were also excluded from the study.

DKA is defined as the presence of the following laboratory test results: Blood glucose>200 mg/dL, venous pH<7.3 or serum bicarbonate <15 mmol/L, and ketonemia (blood β-hydroxybutyrate ≥3 mmol/L) or ≥ moderate ketonuria. The severity of DKA is described in three categories: Severe DKA (pH <7.1 or serum bicarbonate <5 mmol/L), moderate DKA (pH 7.1-7.2 or serum bicarbonate 5-10 mmol/L), and mild DKA (pH 7.2-7.3 or serum bicarbonate 10-15 mmol/L) (5). Those children who presented with DKA were compared with the children who presented with non-DKA at the time of diagnosis according to their residential area type (urban or rural), age groups (<5 years, 5-9 years, and >10 years), duration of diabetes symptoms (≤2 weeks or >2 weeks), and education level of parents (less than high school or high school and above). An analysis was also performed for those children who presented with severe DKA at the time of diagnosis.

A compilation from a literature review on DKA prevalence at diagnosis was made via the following sources: PubMed, Google Scholar, and Web of Science, which were screened using the date criteria December 2019 and with the keywords DKA, T1D, and Turkey in children and adolescents. Additionally, the abstract books of national meetings organized between 1996-2019 by the Turkish Pediatric Endocrinology and Diabetes Society were examined for reports on T1D-related studies. Google Scholar and Web of Science were also screened for all case series with data on the frequency of DKA at diagnosis together with study references and studies cited in them. Congress papers of the studies published as articles were excluded from the study. The following data were obtained from studies that were included in the review: (a) Year of study, (b) diagnosis period of children with T1D, (c) number of children with T1D, (d) frequency of DKA and severe DKA at diagnosis, and (e) factors associated with DKA.

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS 22.0, SPSS Inc., Chicago, U.S.A.) program for Windows was used for the statistical analysis of the data. Demographic, clinical characteristics and laboratory results were evaluated using descriptive and frequency statistics. Student's t-test or the Mann-Whitney U test was used in the comparison of the averages, and the chi-square test was used to compare frequencies. Logistic regression analysis was performed for parameters with significant differences between groups. Statistical values of $p<0.05$ were considered significant. The odds ratio (OR) was calculated with 95% confidence intervals (CI) for nominal values that were found to be significant through regression analysis.

Results

In this study, we analyzed data obtained from 180 children with T1D, of whom 98 (54.4%) were boys, with a mean age of 10.1±4.2 (range: 1.1-17.9) years. DKA at diagnosis was detected in 81 (45.0%) of these children. When the patients were classified by the severity of DKA, 26 (32.1%) patients had severe DKA, 37 (45.7%) had moderate DKA, and 18 (22.2%) had mild DKA. Of the patients with DKA at diagnosis, 41 (50.6%) were male with a mean age at diagnosis of 9.7±4.2 years. The mean age was not significantly different from that of patients with no DKA at diagnosis. When compared by age groups, the number of patients in both groups was similar. Furthermore, there was no statistical difference between those patients with and those without DKA when compared according to their duration of diabetes symptoms before the diagnosis, the season in which diabetes was diagnosed, the presence of T1D in first-degree relatives, and parental education status.

When the groups were compared as per their residence area, the frequency of DKA at diagnosis was significantly higher for those living in rural areas (p=0.030). A comparison of the laboratory data at diagnosis revealed that children with DKA had higher HbA1c and lower C-peptide levels (p=0.017 and p=0.001, respectively). Those children with severe DKA had lower average C-peptide levels, a higher occurrence of rural residency, and inadequate maternal education levels (below high school) (p=0.024, p=0.003, and p=0.022, respectively). A regression analysis of children with severe DKA indicated a relationship between children living in rural areas [OR 3.95; 95% CI: (1.69-8.72); p=0.003] and having mothers with lower education levels [OR 1.34; 95% CI: (1.12-1.60); p=0.022]. High levels of HbA1c at diagnosis was found to be a risk factor for all patients with DKA (p=0.017). The comparison of clinical features by the presence of DKA and severe DKA at diagnosis in children with T1D is shown in Table I.

Table I. Comparison of clinical features by presence of DKA and severe DKA at diagnosis in children with type 1 diabetes

	DKA present	No DKA	p-value	Severe DKA present	No severe DKA	p-value
Number (%)	81 (45.0%)	99 (55.0%)		26 (14.4)	154 (85.6)	
Sex (M/F)	41/40	57/42	0.370	10/16	88/66	0.091
Age at diagnosis (years)	9.7±4.2	10.4±4.2	0.262	9.4±4.3	10.2±4.2	0.448
Diagnosis age groups (%) <5 years 5-9 years >10 years	 12 (14.8%) 33 (40.7%) 36 (44.4%)	 14 (14.1%) 32 (32.3%) 53 (53.5%)	0.442	 5 (19.2%) 10 (38.5%) 11 (42.3%)	 21 (13.6%) 55 (35.7%) 78 (50.6%)	0.657
Diabetes symptoms period (≤2 weeks/>2 weeks)	54/27	54/45	0.126	15/11	93/61	0.831
Diagnosis Season (%) Spring Summer Fall Winter	 21 (25.9%) 19 (23.5%) 18 (22.2%) 23 (28.4%)	 22 (22.2%) 24 (24.2%) 25 (25.3%) 28 (28.3%)	0.932	 8 (30.8%) 4 (15.4%) 6 (23.1%) 8 (30.8%)	 35 (22.7%) 39 (25.3%) 37 (24.0%) 43 (27.9%)	0.661
Residence Area (rural/urban)	14/67	6/93	**0.030**	8/18	12/142	**0.003***
T1D in Family (%)	4 (4.9%)	5 (5.1%)	1.000	1/25	8/146	1.000
Mother's education level (%) Lower than high school High school and above	 58 (71.6%) 23 (28.4%)	 67 (67.7%) 32 (32.3%)	0.627	 23 (88.5%) 3 (11.5%)	 102 (66.2%) 52 (33.8%)	**0.022****
Father's education level (%) Lower than high school High school and above	 47 (58.0%) 34 (42.0%)	 57 (57.6%) 44 (42.4%)	1.000	 19 (73.1%) 7 (26.9%)	 85 (55.2%) 69 (44.8%)	0.132
Glucose (mg/dL)	471±158	435±194	0.171	491±116	444±187	0.085
C-peptide (ng/mL)	0.409±0.468	0.726±1.198	**0.001**	0.324±0.217	0.629±1.023	**0.024**
HbA1c (%)	12.2±2.5	11.3±2.4	**0.017*****	12.5±2.9	11.6±2.4	0.173

Parameters with significant difference between the groups, and a significant relationship with logistic regression analysis; *p=0.001, **p=0.023, ***p=0.038
DKA: Diabetic ketoacidosis, M: Male, F: Female

The literature search performed for this study revealed 24 research articles, 18 national and seven international meeting proceedings for studies with data on the frequency of DKA at diagnosis in Turkey. These studies all together showed that 4,032 (45.6%) out of 8,837 children with T1D had DKA at the time of diagnosis between 1981 and 2019 in 24 provinces. In 18 studies evaluating severe DKA, the frequency of DKA at diagnosis was reported to be 6.0-41.5%, and the frequency of severe DKA among patients with DKA was reported to range between 12.7% and 63.0% (Table II) (7-55). Changes in the frequency of DKA and severe DKA at diagnosis according to the time intervals in which the children were diagnosed as having T1D are shown in Figure 1.

Discussion

Diagnosis of T1D in children at the stage of hyperglycemia and ketonemia before the development of DKA is critical for reducing diabetes-related mortality and morbidity levels. This study reports on the experience of a tertiary health center and data in the literature about the prevalence of DKA and DKA-associated factors at the time of diagnosis for children and adolescents with T1D in Turkey.

The initial data from our clinic on the frequency of DKA at diagnosis was reported to be 64.9% from a total of 74 children and adolescents between June 2004 and June 2007 (7). A second study conducted between June 2013 and February 2016 reported a frequency of 41.9% for DKA in a group of 93 patients (8). The results of this work indicate a significant decrease in DKA frequency compared with the first study, and a comparison with the current study on the topic indicates no change.

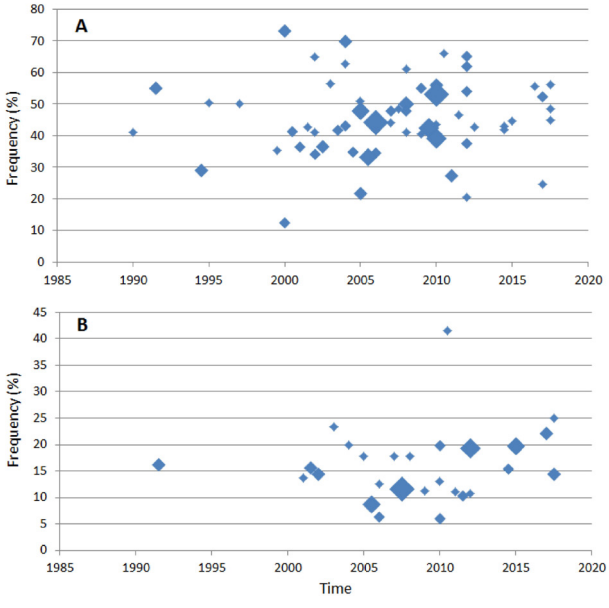

Figure 1. Reported frequencies of DKA (A) and severe DKA (B) at diagnosis of children with type 1 diabetes in Turkey. Symbols were scaled according to the sizes of case series and indicated the middle of the time periods for these case series
DKA: Diabetic ketoacidosis

A decrease in the frequency of DKA at diagnosis was reported in studies conducted at some centers in Turkey (10,15,28). However, there are also publications reporting no change (11) or an increase in its frequency (21,29,33). Fifty studies, including this one, reported DKA findings at diagnosis in approximately half of 8,837 children and adolescents with T1D in Turkey. There is an inverse correlation between T1D incidence of communities and the frequency of DKA at diagnosis (56). This can be explained by an increased awareness of T1D and its symptoms. The increase in T1D incidence in a 20-year period in Northern Finland and the decrease in DKA risk in diagnosis support this proposition (57). The fact that Turkey is listed as a country with moderate T1D incidence may be related to the relatively high incidence of DKA at diagnosis (4,38,58,59). However, despite the increase in T1D incidence in some countries that are well-organized and have good access to health systems, it has been reported that there has been no decrease in the frequency of DKA at diagnosis. For example, in a study conducted in a center in Australia, it was observed that the risk of DKA did not change in children and adolescents with newly diagnosed T1D in the period from 1998 to 2010 (60). Similarly, in Auckland, the largest city in New Zealand, the frequency of DKA at diagnosis remained unchanged between 1999 and 2013, at around 27% (61). The symptoms of diabetes in children with newly diagnosed T1D are usually noticed within a few days to a few weeks before their presentation to hospital. In the EURODIAB study conducted in 24 centers across Europe, 75% of children younger than 15 years with newly diagnosed T1D were found to have had symptoms of diabetes for at least two weeks (56). There has been no significant reduction in the frequency of DKA and severe DKA at diagnosis in children with T1D in our country for years. This strongly implies a failure in the recognition of early T1D symptoms by families and healthcare providers. In our country, a program called "Diabetes Program at School" has been carried out since 2010 to raise awareness of T1D via schools and teachers, enabling the early diagnoses of T1D and decreasing the frequency of DKA in school children. Within the scope of this program, posters about diabetes were hung in schools, a short film was broadcast on national channels, meetings were held at schools and a website was launched (62).

Table II. Studies reporting frequency of DKA at T1D diagnosis in children in Turkey

Order	Reference	Province	Time period	T1D n	DKA n	DKA frequency	Severe DKA frequency
1	Kendirci et al. (9)	Kayseri	1981-1999	190	78	41.0	
2	Demir et al. (10)	İstanbul	1985-2004	395	196	48.5	15.9
			1985-1998	NA	NA	55.0	16.2
			1999-2004	NA	NA	42.6	15.6
3	Ardicli et al. (11)	Ankara	1990-2010	354	180	50.8	
			1990-2000	105	53	50.5	
			2000-2010	249	127	51.0	
4	Demir et al. (12)	Ankara	1990-2017	621	259	41.7	
5	Bober et al. (13)	İzmir	1991-1998	62	18	29.0	
6	Şen et al. (14)	Afyonkarahisar	1993-2007	52	38	73.1	
7	Bideci et al. (15)	Ankara	1995-2004	73	30	41.0	
			1995-1999	32	16	50.0	
			2000-2004	41	14	34.1	
8	Darcan et al. (16)	İzmir	1995-2005	128	16	12.4	
9	Arı Yuca et al. (17)	Van	1995-2009	166	68	41.0	14.4
10	Kocabaş et al. (18)	Antalya	1996-2013	89	31	34.8	
11	Aktaş et al. (19)	Şanlıurfa	1998-2001	17	6	35.3	
12	Şimşek et al. (20)	Düzce	1998-2003	46	19	41.3	
13	Acar et al. (21)	İzmir	1999-2014	282	122	43.2	9.5
			1999-2003	44	16	36.4	13.7
			2004-2008	96	40	41.7	6.3
			2009-2014	142	66	46.5	10.3
14	Haliloglu et al. (22)	İstanbul	1999-2016	517	251	48.4	11.6
15	Taskin et al. (7)	Elazığ	2000-2004	74	48	64.9	
16	Karaguzel et al. (23)	Antalya	2000-2005	115	42	36.5	
17	Candemir et al. (24)	Denizli	2001-2007	53	37	69.8	
18	Karadağ et al. (25)	İstanbul	2002-2006	51	22	43.1	
19	Çayır et al. (26)	Erzurum	2002-2010	129	57	44.2	
20	Sağlam et al. (27)	Bursa	2003-2008	490	163	33.2	8.7
21	Ucar et al. (28)	İstanbul	2003-2012	401	177	44.2	
			2003	NA	17	56.5	23.3
			2004	NA	22	62.8	20.0
			2005	NA	22	47.8	17.8
			2006	NA	18	45.0	12.5
			2007	NA	22	47.8	17.8
			2008	NA	22	47.8	17.8
			2009	NA	17	40.4	11.2
			2010	NA	18	43.4	13.1

Table II. continued

			2011	NA	10	27.3	11.1
			2012	NA	9	20.4	10.7
22	Cizmecioglu et al. (29)	Kocaeli	2005-2008	95	37	38.9	11.6
			2005	23	5	21.7	
			2006	29	10	34.5	
			2007	25	11	44.0	
			2008	18	11	61.1	
23	Arıcan et al. (30)	Kayseri	2006-2013	453	192	42.4	
24	Aydın et al. (31)	Ankara	2007-2013	92	36	39.1	
25	Demir et al. (32)	İzmir and Manisa	2008	139	57	41.0	
26	Özsu et al. (33)	Kocaeli	2008-2010	124	66	53.2	16.5
			2008	NA	NA	50.0	
			2009	NA	NA	55.0	
			2010	NA	NA	56.0	
27	Dilek et al. (34)	Edirne	2006-2018	315	195	61.9	19.3
28	Esen et al. (35)	Ankara	2009-2011	111	59	53.1	19.8
29	Karamık et al. (36)	Ankara	2009-2015	115	62	53.9	
30	Demir et al. (37)	İzmir and Manisa	2010	84	34	40.5	6.0
31	Demirbilek et al. (38)	Diyarbakır	2010-2011	41	27	65.9	41.5
32	Evliyaoglu et al. (39)	İstanbul	2010-2014	184	69	37.5	
33	Demiral et al. (40)	Eskişehir	2010-2015	103	44	42.7	
34	Baran et al. (41)	Diyarbakır	2011-2013	83	54	65.1	
35	Kara et al. (42)	Ankara	2011-2013	50	27	54.0	20.0
36	Cicek et al. (43)	Kayseri	2011-2019	323	144	44.6	19.7
37	Aras et al. (44)	Diyarbakır	2013-2016	142	61	42.9	15.4
38	Esen (8)	Elazığ	2013-2016	93	39	41.9	15.1
39	Kara (45)	Bursa	2015-2018	144	80	55.6	
40	Araslı Yılmaz et al. (46)	Ankara	2016-2018	149	78	52.3	22.1
41	İşleyen and Bolu (47)	Adıyaman	2016-2018	45	11	24.5	
42	Esen and Okdemir*	Elazığ	2016-2019	180	81	45.0	14.4
43	Ersoy et al. (48)	Manisa	2017-2018	64	31	48.4	25.0
44	Yazkı et al. (49)	Adana	2017-2018	66	37	56.1	
45	Ökten et al. (50)	Trabzon	NA	33	20	60.6	
46	Şimşek et al. (51)	Ankara	NA	67	21	31.3	
47	Hatun et al. (52)	33 centers	NA	498	227	45.6	
48	Karagüzel et al. (53)	Trabzon	NA	100	54	54.0	
59	Bala et al. (54)	Van	NA	101	52	51.4	
50	Ozbek et al. (55)	Diyarbakır	NA	538	279	51.9	
				8837	4032	45.6	

*This study, NA: Not available
DKA: Diabetic ketoacidosis, T1D: Type 1 diabetes

Although a possible positive effect of this program on the frequency of DKA at the time of diagnosis was reported in one local study (28), this effect was not demonstrated by studies designed throughout the country.

Due to various factors, it can be expected that the risk of DKA at diagnosis is greater in younger children owing to the difficulty in recognizing the classic symptoms of T1D, especially in children aged <2 years, as the possibility of a diagnosis of T1D is considered less likely by physicians, and also the faster development of dehydration and acidosis in young children. Furthermore, beta-cell destruction in the T1D development process in young children may be more aggressive (6). Usher-Smith et al. (63) conducted a meta-analysis study investigating 31 studies and reported that children aged under 2 years had a 3-times greater risk of being diagnosed as having DKA than children older than 2 years (OR 3.41; 95% CI: 2.54-4.59). This risk is present for children aged up to five years, albeit to a lesser extent (OR 1.59; 95% CI: 1.38-1.84) (63). In our patient series, this age group was not evaluated in terms of DKA risk due to the small number of children aged <2 years. However, when children with and children without DKA were compared according to their average age and age groups (<5, 5-10, and 10-18 years), there was no statistically significant difference. Similarly, in some studies conducted in our country, it was reported that the risk of DKA at diagnosis was not different in children aged <5 years (10,27,37,39). On the other hand, some studies found that the incidence of DKA was higher in children aged <5 years (21,28,32,38,46). However, in only two of these studies, the relationship between DKA risk and causality of being aged under 5 years was shown through regression analysis (28,32).

Lower risk of DKA at diagnosis has been reported to be associated with having parents with high education levels and first-degree relatives with T1D (6). This reduced risk can be explained by higher awareness of T1D and familiarity with the signs and symptoms of hyperglycemia. A low frequency of DKA at diagnosis in children was associated with their mothers' high school or higher education levels in a study from Lithuania, and another study from Finland also reported a decreased frequency of DKA for children with at least one parent with an academic degree (64,65). In contrast, no relationship was detected between the level of parental education and frequency of DKA at the time of diagnosis of children with T1D in a German study. However, the same study also reported a higher risk of severe DKA (pH ≤7.2) in children with parents that had <9 years of education in comparison to parents with ≥12 years of education (OR 3.54; 95% CI: 1.10-11.35) (66). Ucar et al. (28) reported no association between parental education and the risk of DKA at the time of diagnosis in Turkish children with T1D. There was no association between the education level of the parents and the risk of DKA at diagnosis in our patient series; however, having a mother with less than high school education was associated with severe DKA.

In a meta-analysis, Usher-Smith et al. (63) evaluated five studies investigating the relationship between having a relative with diabetes and the risk of DKA at T1D diagnosis in children and concluded that having a relative with diabetes was a risk-reducing feature for DKA. In this study, it was found that having a first-degree relative with T1D made no difference in terms of either DKA risk or severe DKA risk. Ucar et al. (28) found that the frequency of DKA was lower (p=0.042) in applicants who had first and/or second-degree relatives with T1D, but no causal relationship was detected (p=0.21). Although a family history of a parent or sibling with T1D was found to be a risk factor in the first of two different studies conducted by Demir et al. (37) in İzmir and Manisa in 2008 and 2010, the second study reported that it was not a risk factor (32). Apart from these studies, two studies from our country reported that a T1D history in the family made no difference in terms of DKA risk at diagnosis (38,52). However, Ozbek et al. (55) reported that children with a family history of diabetes had a significantly lower frequency of DKA (p=0.04), although at a statistically borderline value. These different results were thought to be related to the low incidence of childhood T1D throughout the community.

This study detected that the DKA diagnosis rate was higher for those living in rural areas. Three studies, including one from our country, found that living in rural or urban areas did not affect the risk of DKA at diagnosis. There was no difference in DKA diagnosis rates between those living in villages or the countryside in comparison with those living in the city center, towns or suburbs in two different studies conducted in Finland, Sweden, and Lithuania (64,65). According to a study by Demirbilek et al. (38) conducted in the province of Diyarbakır, Turkey, there was also no difference in DKA diagnosis rates between those living in the city center, suburbs or villages. This study found that there was a causality relationship between living in villages and the frequency of severe DKA at diagnosis. This may be due to limited access to health services due to rural isolation and may be an indirect factor in the presence of DKA during diagnosis.

This study detected a weak causality relationship between the presence of DKA at diagnosis and HbA1c levels,

but no similar relationship was found with the frequency of severe DKA. In two related studies conducted in Turkey, it was reported that children with DKA had higher HbA1c levels (32-37). However, in another study, no difference was found between the groups (38). The lack of a relationship between severe DKA and high HbA1c levels in this study could be because there was a small number of children with severe DKA and the possibility of the rapid development of T1D in some children. Compared with older children, the lower C-peptide level of children aged <2 years in the diagnosis of T1D suggests that beta cell damage may be more aggressive in younger children (65). In this study, we found low levels of C-peptide in children with DKA and with severe DKA groups compared with the no DKA or non-severe DKA groups but no causal relationship was detected. Ucar et al. (28) reported that serum C-peptide ≥0.6 ng/mL was associated with a reduced risk of DKA at diagnosis (OR 0.55; 95% CI: 0.33-0.92). Furthermore, three other studies conducted in Turkey investigated the differences in C-peptide levels in children with DKA and without DKA at diagnosis of T1D; two reported that children who presented with DKA had lower levels of C-peptide than those without DKA (37,39). However, in another study, no difference was found between the groups (38).

In a meta-analysis of 21 studies, Usher-Smith et al. (63) examined the effect of sex on the frequency of DKA in the diagnosis of T1D, and 20 of these studies showed no effect of sex. A study evaluating 2,121 children aged under 15 years from Germany reported that the frequency of DKA at T1D diagnosis was higher for girls (OR 1.30; 95% CI: 1.07-1.58; p=0.008) (67). When nine studies with sufficient data for meta-analysis were evaluated together, the OR for boys was 0.93 [95% CI: (0.76-1.14); p=0.472] (63). Another study reported that female sex was not associated with an increased risk of severe DKA (pH≤7.2) [OR 0.68; 95% CI: (0.26-1.83); p=0.450] (66). In our patient series, neither risk of DKA nor risk of severe DKA at diagnosis was associated with the sex of the child with T1D. In seven studies conducted in Turkey, the effect of sex on the frequency of DKA at diagnosis was investigated, and two of these studies reported that the frequency of DKA was higher for girls. The first of these studies by Ardicli et al. (11) investigated the clinical features of 354 children with T1D diagnosed over 40 years and they found that the frequency of DKA at diagnosis was higher for girls (55.6% vs. 44.0%, p=0.008). A second study by Demir et al. (10) reported that the frequency of DKA at diagnosis was higher for girls (55.1% vs. 41.7%, p=0.042). On the other hand, five other studies reported that sex was not associated with the frequency of DKA at the time of diagnosis of T1D (27,28,32,39,55).

Difficulty in accessing health services due to a lack of health insurance, low socio-economic status, and being a minority have been reported as other factors associated with the risk of DKA presence at diagnosis (6). A relationship of the above factors with the presence of DKA in our country is probably unlikely because general health insurance provides diagnostic, treatment, and rehabilitation services for all children aged less than 18 years in Turkey. This study does not discuss the ethnic and socioeconomic characteristics of the patients and, as such, these features were not evaluated.

Study Limitations

The high number of patients in this study provides strong data on the frequency of DKA at the time of T1D diagnosis, which makes this study powerful. However, as the designs of the studies examined in this review were heterogeneous and only the abstracts of some studies were evaluated, estimating the factors associated with the presence of DKA at T1D diagnosis was limited.

Conclusion

This study researched several databases rigorously and systematically and detected that approximately half of 8,837 children with T1D presented with DKA at diagnosis, and there was no decrease in the frequency of DKA in Turkey between 1981 and 2019. Our findings indicate that for an earlier childhood T1D diagnosis across Turkey, awareness must be raised among members of society and health professionals. Also, there is a need for investigations to determine the reasons for the persistence of the high frequency of DKA at the time of T1D diagnosis in Turkey.

Ethics

Ethics Committee Approval: The study protocol was performed according to the Declaration of Helsinki and approved by the Ethics Committee of Non-Interventional Research of Fırat University (decision number: 0010/28.11.2019).

Informed Consent: The requirement for informed consent was waived due to the retrospective nature of the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Medical Practices: İ.E., D.Ö., Concept: İ.E., D.Ö., Design: İ.E., D.Ö., Data Collection or Processing: İ.E., D.Ö., Analysis or Interpretation: İ.E., D.Ö., Literature Search: İ.E., D.Ö., Writing: İ.E.

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Original Article

Clinical and Electrophysiological Prognostic Factors of Childhood Absence Epilepsy

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ABSTRACT

Aim: Childhood absence epilepsy is common idiopathic epilepsy in childhood. This epilepsy, which has been shown to impair cognition, needs to be treated promptly and correctly. Therefore, determining its prognostic factors before treatment can provide prediction on the duration of treatment, drug selection, and drug dosage.

Materials and Methods: The electroencephalography (EEG) and clinical findings of patients diagnosed with childhood absence epilepsy who were monitored for at least 12 months in the pediatric neurology clinics of two university hospitals between 2016 and 2020 were reviewed retrospectively. The patients were divided into two groups as responsive and unresponsive, according to seizures, EEG findings, and recurrent seizures after treatment. The epidemiological and clinical features of the two groups were compared.

Results: Sixty-three patients who were diagnosed with childhood absence epilepsy according to the Panayiotopoulos criteria participated in this study. Thirty-nine (62%) of the patients were responsive to treatment (group 1), the remaining 24 patients (38%) (group 2) were unresponsive to treatment. Fifteen patients were valproate resistant, and nine patients relapsed after drug treatment withdrawal in group 2. The mean age of the patients was 7.87±1.68. The mean follow-up period was 29.1±13.6 (13-72 months) months. The mean age was lower in the responsive group of patients. The time between the onset of seizures and treatment was significantly longer in group 2. The number of patients with occipital intermittent rhythmic delta activity (OIRDA) in the responsive group was higher. A significant difference was found in the number of spike-slow wave complex and the amplitude of discharges between the two groups.

Conclusion: In this study, it was seen that young age was an advantage for treatment response. Early initiation of treatment and OIRDA were good prognostic factors, while high amplitude and numerous discharges were among the poor prognostic factors.

Keywords: Absence, prognostic factors, amplitude, EEG, response

Introduction

Childhood absence epilepsy (CAE) constitutes 10-17% of all childhood epilepsies (1). Seizures usually begin between the ages of 4-10 years, and the peak age is between 6-7 years. It is characterized by seizures, most of which last 4-8 seconds, accompanied by brief staring spells and occasional automatism or blinking, which may repeatedly occur during one day. There is a unique pattern in electroencephalography (EEG) in which 3Hz spike-slow waves are bilateral, symmetrical, and simultaneous (1-3).

Although the success rate of treatment in only absence-type without generalized motor seizures has been observed to be 60-95%, 25 to 40% of patients are resistant to antiepileptic treatment in CAE (4-8).

In general clinical practice, if the patient is seizure-free, and two years of treatment completed, discontinuation of

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the drug is preferred (9,10). EEG findings are important in this decision process. Some pediatric neurology clinics perform check-up EEGs after drug discontinuation if the patient is close to the driving license age or in cases where families hardly notice absence seizures. The drug discontinuation process continues if there are single brief focal discharges during sleep, but treatment usually continues for 1-2 years if generalized discharges are seen in control EEG. In addition, some clinics perform EEG without any conditions, and some do not repeat EEG if there is no complaint with the patient (11,12).

Different prognostic factors were found in studies with recurrent seizures and EEG findings after treatment cessation in CAE (4,13,14). Determining these prognostic factors without discontinuing the medication of the patients may provide the appropriate duration of drug usage and then complete remission.

Although there are many studies on juvenile absence epilepsy (JAE) and juvenile myoclonic epilepsy (JME) with recurrent seizures after treatment discontinuation, very few studies have evaluated the recurrent clinical findings and prognostic factors associated with CAE patients (6,14). There are few studies showing the relationships between resistant epilepsy or unresponsiveness to treatment with epidemiological features, clinical course, EEG features (4,8).

In almost all studies, patients with generalized motor seizures have been associated with poor prognosis. Different rates of remission, seizure-free time, and EEG recovery times were found in different studies with different drugs (15,16). However, there are many variables in these studies that could affect the conclusion. This study aims to show the prognostic factors in selected CAE patients who did not have motor seizures and who used the same drug in the same dose range.

Materials and Methods

The medical and EEG records of 63 cases diagnosed with CAE and who were followed up for at least 12 months between 2016 and 2020 in two different pediatric neurology outpatient clinics of two tertiary treatment centres were reviewed retrospectively. The local ethics committee approved this study (118/88 11.12.2020). CAE was defined according to the Panayiotopoulos criteria (17). The patients' gender, age, age of seizure onset, history of febrile convulsions, family history of epilepsy, time between the onset of seizures and diagnosis, time until the control of seizures with antiepileptic treatment, EEG findings, follow-up period, antiepileptic drugs used and their doses were noted. The authors evaluated the maximum duration of generalized discharges, the maximum amplitude of ictal spike discharge, the effect of hyperventilation (HPV) and photic stimulation (PS) on EEG, focal abnormality, and the presence of occipital intermittent rhythmic delta activity (OIRDA) in interictal EEG.

Definitions

Hyperventilation sensitivity (HPV): EEG background rhythm change during HPV or ictal discharge with HPV.

Photoparoxysmal response (PS): Change in EEG background rhythm or emergence of ictal discharges with PS.

The number of slow spike-wave complexes: Each slow spike-wave observed between 4-20 sec was counted as one complex regardless of the duration of the complex block. The slow spike-wave complexes and properties during HPV were not used in the study data.

Slow spike-wave complex duration: The longest duration (sec) of slow spike-wave complex in one-hour EEG.

A typical absence seizure on electroencephalogram, characterized by 3 Hz (2,7-3,5 Hz) generalized slow spike-wave complexes, with an abrupt onset and offset, lasting 4-20 seconds.

Time for EEG recovery time: The first EEG which does not have slow spike-wave complexes observed in 1-hour EEG after the treatment is started, but abnormal findings may be found in later EEGs. This time period does not mean that the discharges do not occur again.

Time until control of seizures with antiepileptic treatment: When the seizure disappears after treatment, seizures may occur later. This time period does not mean that the seizures do not recur.

Participants Groups

Group 1: Responsive group

1. Those cases whose seizures were controlled for one year with valproate treatment and their seizures did not recur during the follow-up, and generalized slow spike-wave complexes were not observed in EEG.

2. Those patients whose seizures did not recur after treatment was discontinued, and no generalized slow spike-wave complexes were detected in their EEGs after treatment.

Group 2: Non-responsive group

3. Those cases whose seizures continued despite an effective dose of valproate treatment (25-40 mg/kg/day, 2-3 divided doses, orally) or whose generalized slow spike-wave complexes on EEG persisted despite being seizure-free for one year.

4. Those patients with recurrent seizures or generalized spike-wave complexes in their EEGs after the withdrawal of antiepileptic drug treatment.

Inclusion and Exclusion Criteria

Clinical criteria

Inclusion

• Frequent (many per day), brief (4-20 sec) typical absences with abrupt and severe consciousness impairment.

• Age of onset between 4-10 years.

Exclusion

• Absences with marked eyelid or perioral myoclonus, single or rhythmic limb, and myoclonic trunk jerks.

• Absences with mild or not clinically detectable consciousness impairment.

• Other types of epileptic seizures

EEG criteria

Inclusion

• Generalized, spike or double-spike and slow spike-wave regular complexes at 3 Hz (2.7-3.5 Hz)

Exclusion

• Discharge fragmentation and multiple spikes

• Discharges are characterized by multiple irregular spikes or slow waves followed by multiple spikes.

• Predominantly brief discharges of less than four seconds

Statistical Analysis

Measurements such as mean, median, frequency, and standard deviation (SD) were made using the Statistical Package for Social Sciences software for Windows, version 23.0, and the results are given as mean ± SD. Variables were evaluated with chi-square and Student's t-test. Student's t-test was used to compare the means between the two groups. The normality of the distribution between the two groups was evaluated with the Kolmogorov-Smirnov test. A value of $p<0.05$ was considered statistically significant.

Results

A total of 63 cases (40 female, 23 male) were included in this study. Thirty-nine (62%) of the patients were treatment-responsive (group 1), 15 of the remaining 24 (38%) were valproate-resistant, and 9 were patients with relapse after drug treatment withdrawal (group 2). The mean age of the patients was 7.87±1.68 years. The mean follow-up period was 29.1±13.6 (13-72) months. There was no significant difference between the follow-up periods of the drug treatment withdrawal patients from group 1 and group 2. Gender distribution among the groups was normal, and there is no significant difference between these two groups ($p>0.05$). The mean age was lower in the responsive group of patients ($p<0.05$). The family history of epilepsy of the patients was 32%, having a febrile convulsion was 21%, and there was no difference between the groups ($p>0.05$) (Table I).

The time between the onset of seizures and treatment was significantly longer in group 2. However, there was no difference between the two groups in terms of the number of seizures before treatment (Table II).

There was no difference in photic effect, hyperventilation effect, and focal discharge between the two groups. The number of patients with OIRDA was higher in the responsive group ($p<0.05$).

The first EEG recording time after the initiation of treatment was 2.84±1.94 months, the clinical evaluation of the patient after the initiation of treatment was 2.50±1.61 months. There was no difference in the evaluation times of the patients between the groups ($p>0.05$).

A significant difference was found in the number of slow spike-wave complexes and the amplitude of discharges during 1 hour of EEG between the two groups (Table II) ($p<0.05$).

The duration between first seizure-free and first normal EEG with the initiation of treatment was significantly shorter in the responsive group (Table I,II) ($p<0.05$).

Discussion

In our study, 62% of the patients responded to treatment according to the study criteria. There are similar results in the literature (4,14). Kim et al. (14) found the effect of 3 different antiepileptics and combined therapy, and the

Table I. Clinical features of patients with childhood absence epilepsy

Variables	AED* responsive patients (n=39)	AED non-responsive patients (n=24)	p-value
Sex ratio (female:male)	24/15	16/8	0.68
Age at diagnosis	7.48±1.62	8.50±1.64	0.02
Family history of epilepsy	13	7	0.73
History of febrile convulsion	11	6	0.78
Seizure duration after the diagnosis (months)	4.25±3.25	8.41±6.48	0.01
The daily mean number of absence seizures before the admission	15.58±15.5	16.5±10.58	0.78
Seizure free time after treatment (months)	3.28±2.91	5.00±3.71	0.04

*AED: Antiepileptic drug

Table II. EEG findings in patients with epilepsy

EEG finding	AED responsive patients (n=39)	AED non-responsive patients (n=24)	p-value
Number of 2.7-3.5 Hz generalized spike-wave complexes	5.71±4.01	11.16±10.56	0.02
OIRDA	17	4	0.02
Photoparoxysmal response	4	2	0.23
Maximum amplitude of epileptic discharges (μV)	307.69±55.94	370.83±80.64	0.01
Hyperventilation sensitivity	32	13	0.57
Focal epileptic activity	8	9	0.17
The maximum duration of generalized spike-wave complex (sec)	7.53±3.20	8.04±3.38	0.14
Time to first normal EEG after treatment (months)	4.72±3.57	8.45±6.05	0.01

EEG: Electroencephalography, AED: Antiepileptic drug, OIRDA: Occipital intermittent rhythmic delta activity

highest effect with a single drug was reported with valproate treatment, with a rate of 70%. Absence epilepsy is the most cognitive disruptive type of idiopathic generalized epilepsy (18-20). Therefore, prognostic studies might be important for the early detection of risk factors for poor prognosis, how often electrophysiological and clinical follow-up needs to be performed, and the timing to the withdrawal of the drug treatment.

The wide range of response rates among studies in CAE may be due to the definition of CAE, the criteria for non-response, and treatment with different doses and different drugs.

Grosso et al. (5) evaluated relapse and remission rates using the ILAE 1989 and Panayiotopoulos diagnostic criteria (17,21). Since the Panayiotopoulos criteria are much more stringent both clinically and electrophysiologically (excluding many other diagnoses such as motor seizures, atypical absence, and possible juvenile syndromes), recurrence and non-response rates were found to be relatively low (17). The success rate of our study was 62%, and the relapse rate after withdrawal of treatment was 32%.

Wirrell et al. (4) conducted the first study investigating the prognostic factors of absence epilepsy. Sixty percent of 86 patients, most of whom had CAE and some of whom had JAE, responded to the first antiepileptic. However, different antiepileptics were used as the first antiepileptic. There are different results in the comparative superiority studies of these different antiepileptics in the literature (15,16).

Although ethosuximide is recommended as the first option in CAE in meta-analysis studies, the difficulty of obtaining it in our country has led us to employ valproate treatment, which has comparable efficacy (22). Another feature of our study is that valproic acid was initiated as the first antiepileptic in all patients in the same dosage range; thus, variability due to different drug activities was eliminated.

Myoclonus or generalized motor seizures occurring after the onset of absence seizures indicate that JME and JAE have converted to juvenile idiopathic epilepsy (JIE) or they already have JIE and have a poor prognosis compared to CAE (23). Patients with seizures other than absence were not included in our study. Late-onset age, absence status, mental retardation, multiple spike waves on EEG and slow background activity, and discharge shorter than 3Hz are other factors associated with poor prognosis in various studies (5,6,24). In our study, patients with background rhythm abnormalities on EEG were excluded. The fact that most of the known poor prognostic factors were excluded from our study group is valuable for us to determine the previously unassessed characteristics of the patients independently from other factors.

There are studies in the literature indicating that discharge below 3 Hz is a poor prognostic factor (5). However, there was no difference between the groups in terms of the frequency (Hz) of spike-slow wave complex on EEG in our study. This data also could be associated with using the Panayiotopoulos criteria for the definition of CAE.

OIRDA is characterized by rhythmic bursts at 2.5-4 Hz over the occipital regions, which was defined as a good prognostic factor in most studies and was significantly associated with good prognosis in our study (14,25).

Female predominance is present in CAE, and 63% of our patients were female. However, no prognostic significance of gender was found in our patients. Although there is a study showing that the male gender is a poor prognostic factor, most studies showed gender is not a prognostic factor for CAE (4,6,13,26).

Family history of epilepsy or absence epilepsy did not show prognostic features in our patient group. In most literature, the presence of seizures in the family is a risk factor for the development of seizures, while it is often not

significant in terms of prognosis (4,5,14).

In our study, the average age of the patients in Group 1 was significantly lower. It is known that absence seizures below the age of 4 may be of genetic origin as in GLUT-1 deficiency and are one of the poor prognostic factors (27-29). Epilepsies that begin after puberty or after the age of 10 are likely to evolve into JAE and JME and are associated with poor prognosis (5,24). Wirrell et al. (24) showed 65% remission with a mean age of 5.8 years, and 44% of patients who did not respond to antiepileptics converted to JME in a long observation period of 20 years. The age range in our study was 4 to 10 years. Under four and over ten years of age, which are associated with poor prognosis, were not included in this study, although age was found to be significant between the two groups. In group 2, the average age was higher ($p<0.05$).

In CAE, the occurrence of focal discharges is around 20-50%.28,29 It was present in 27% of our patients, but it did not have any prognostic significance.

We also had patients with photosensitivity but this had no prognostic value. However, Incecik et al. (13) found this to be a poor prognostic factor in their study. The relation of PS, especially to myoclonic seizures, is known. PS response was observed in a small number of our patients. This may be related to the exclusion of any seizure other than absence in this study. In addition, since the age limit was 10 in this study, patients with photosensitivity who could evolve to JME were eliminated.

As expected, the presence of ictal discharges with hyperventilation was 80%, and this EEG feature, which was seen at a high rate in both groups, was not prognostic. Since the duration and number of discharges that occur during HPV can cause confusion, they were not used in this study. However, discharges with HPV were recorded as HPV sensitivity.

There has been no study evaluating the amplitude of generalized ictal discharges as a prognostic value to date. Non-responders had a higher amplitude of generalized ictal discharges in EEG at the time of diagnosis in our study. We could not determine a cut-off value due to the small number of patients, but this is an important finding. Prospective randomized future studies need to determine amplitude values for the prediction of prognosis.

Valproate was started immediately after diagnosis, and the first normal EEG and first seizure-free detection time afterward were significantly shorter in the responsive group than the non-responsive group. Callenbach et al. (30) showed that failure to be seizure-free in the first six months was a significant risk factor for relapses. In this study, the duration between diagnosis and first normal EEG of the responsive group was 4.7 months. While this is consistent with the literature, seizure-free time after treatment was longer than in the literature with 3.2 months (14). One of the reasons for this might be that we started valproate as an initial treatment (10 mg/kg/day) with a low dose and increased it slowly, when compared to many other studies.

While the number of daily seizures in both groups before treatment was similar, a short duration between diagnosis and treatment initiation for absence seizures was a positive prognostic factor. The mean time between the recognition of absence seizures and the initiation of treatment in the non-responsive group was eight months. As a result of this long period of time without treatment in group 2, an epileptic network may have developed, as in many epilepsies.

Another reason for not admitting to the hospital for such a long time may be low socioeconomic status, which is an independent risk factor for resistant epilepsies (31). However, we did not evaluate this aspect of the patients in this study.

Although there was a significant difference between the groups in term of the number of discharges on EEG at the time of diagnosis, there was no difference between their maximum duration. This is due to definitions of duration time and slow spike-wave complexes. The number of spike-wave complexes was calculated by defining all discharges that distinctly appeared for a certain time as "one" complex. Duration of discharges shows the time that this one longest complex lasts. In Sadleir et al.'s (28) study, ictal discharges were on average 9.4 seconds, whereas, in our patients, it was 7.5 seconds and 8 seconds in Groups 1 and 2 respectively. Kessler and McGinnis (11) determined that patients whose shortest seizure lasted longer than 7.5 seconds were more likely to respond to initial treatment than those whose shortest seizure lasted less than 7.5 seconds. Future studies are needed to examine patients in this respect and to find cut-off values.

The short follow-up period, the low number of patients, and the retrospective nature of the study are the limitations of this study.

The advantages of the study are the scarcity of studies on this subject, the fact that some of the evaluated prognostic parameters have not been found in any studies to date, all patients receiving the same drug as a first antiepileptic in the same dose range, and EEG and patients being evaluated by the same clinician.

Conclusion

Young age, OIRDA, and early treatment were determined as good prognostic factors. High amplitude and high frequency of spike slow-wave discharge as poor prognostic factors in CAE. Family history, febrile convulsion,

photoparoxysmal response, focal epileptic activity, and HPV were not related to prognosis.

Ethics

Ethics Committee Approval: The local ethics committee approved this study (118/88 11.12.2020).

Informed Consent: Retrospective study.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: H.G.T., Design: H.G.T., Data Collection or Processing: T.K., Analysis or Interpretation: H.G.T., P.E., Writing: P.K., P.E.

Conflict of Interest: There is no conflict of interest is declared by the authors.

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Original Article

Attention-deficit Hyperactivity Disorder and Gluten Sensitivity in Children

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ABSTRACT

Aim: Dietary factors are considered one of the possible environmental risk factors for attention-deficit hyperactivity disorder (ADHD). The aim of this study was to demonstrate the relationship between ADHD and celiac disease (CD) and non-celiac gluten sensitivity (NCGS) in children.

Materials and Methods: In this prospective study, children with ADHD, aged 6-18 years, were asked about the presence of gastrointestinal symptoms and their relationship with gluten intake with a previously prepared questionnaire form. Also, they were screened for CD [anti-tissue transglutaminase immunoglobulin (Ig) A and total IgA] and NCGS (anti-gliadin IgA/IgG antibodies).

Results: Of the 117 children (76% male), the mean age was 9.2±2.4 years. Ninety-six patients (82%) had no gastrointestinal complaints. There was no relationship between gluten intake and complaints in the patients who had constipation (12.8%), recurrent abdominal pain (2.5%), dyspeptic symptoms (1.7%), and irritable bowel syndrome (0.8%). None of the patients had anti-tissue transglutaminase IgA or IgG positivity. Only 1 (0.8%) patient had anti-gliadin IgA, and 6 (5.1%) patients had anti-gliadin IgG positivity. There was no relation between the presence of symptoms and anti-gliadin IgG positivity (p=0.08).

Conclusion: There was no increase in the frequency of CD and NCGS in children with ADHD.

Keywords: Attention-deficit hyperactivity disorder, celiac disease, children, non-celiac gluten sensitivity

Introduction

Wheat allergy (WA), celiac disease (CD) and non-celiac gluten sensitivity (NCGS) are the most common gluten-related disorders (1-3). In recent years, the relationship between neuropsychiatric disorders and gluten ingestion such as CD and NCGS has been reported (3-6).

Attention-deficit hyperactivity disorder (ADHD) is characterized by hyperactive, inattentive and impulsive behavior and it is a common neuropsychiatric disorder in childhood. There is no consensus on the etiology of ADHD, but an interaction of genetic factors and environmental causes such as dietary factors have been considered as the main trigger factors. Also, the effect of elimination diets on the treatment of ADHD has been studied. The benefit of elimination of food additives from the diet has been reported in a minority of children with ADHD, but the role

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of other elimination diets such as gluten or casein-free diets and oligoantigenic diets on the improvement of ADHD was inconclusive in children (7,8).

Depending on the hypothesis of the effect of diet on neurodevelopmental disorders, we aimed to evaluate the association of ADHD and the gluten-related disorders of both CD and NCGS in children.

Materials and Methods

The children who had been diagnosed as ADHD according to the criteria of "Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition" in our child and adolescent psychiatry department were evaluated for CD and NCGS at our pediatric gastroenterology outpatient clinic between 07.12.2015 and 01.03.2016, prospectively. First of all, the patients and their parents were informed about the possible relationship between ADHD and gluten-related disorders and then they were asked if they would like to participate in the study. Subsequently, a previously designed informed consent form was given to the parents and their children (>12 years old). Children aged between 6-18 years old, diagnosed as ADHD and willing to participate in the study with a signed informed consent form were recruited into this study. Those children who had mental retardation, previously known CD, or refusing to give blood samples were excluded from the study.

The demographic findings of the patients, their medical history, their physical examination findings and anthropometric measurements were recorded. According to the body mass index (BMI) of the patients, overweight (≥85%-<95%), obesity (≥95%) and underweight (<5%) were defined. Those children who had height-for-age Z-score <-2 standard deviation were considered short stature (9,10).

In order to determine NCGS, their gastrointestinal complaints were investigated with a previously prepared questionnaire form which consists of the following issues; abdominal pain, bloating, diarrhea, constipation, and abdominal distension. In the presence of any of these complaints in daily life, the relation to gluten intake was investigated. If they had a gluten-related symptoms, the following steps were planned; to give a gluten-free diet, to follow the patient for the presence of symptoms and, if there was an improvement within a month, to re-introduce gluten into the diet. If the symptoms occurred after re-introducing gluten, NCGS would be considered (11). Regardless of the presence of symptoms, anti-gliadin (AGA), immunoglobulin (Ig) A and IgG levels were studied in all patients.

All patients were screened for CD via anti-tissue transglutaminase IgA antibody and total IgA levels. In cases of low IgA levels for age, anti-tissue transglutaminase IgG antibody tests were studied. If the anti-tissue transglutaminase IgA or IgG antibody were positive, the following procedures were planned; to perform an upper gastrointestinal endoscopy, to take multiple biopsies from the small bowel; 4 biopsies from the second part of the duodenum and 2 biopsies from the duodenal bulb for histopathological examination in order to establish a biopsy-proven CD (12).

According to our laboratory data, the cut-off values of anti-tissue transglutaminase IgA and IgG, AGA IgA and IgG were lower than 18 U/mL. Those values >18 U/mL were accepted as a positive test.

Statistical Analysis

The data analysis was performed using the Statistical Package for Social Sciences software 17.0. Descriptive statistics, independent samples t-test, and the non-parametric Mann-Whitney U test were used as appropriate, p-values <0.05 were considered statistically significant. The study was approved by the local ethics committee of our hospital (protocol number: 2015/073).

Results

A total of 117 children diagnosed as ADHD, with a mean age of 9.2±2.4 years (age range of 6-17 years) were recruited into this study. Of these children, 89 (76%) were male.

Ninety-six patients (82%) had no gastrointestinal complaints. Fifteen (12.8%) patients had constipation, 3 (2.5%) had recurrent abdominal pain, 2 (1.7%) had dyspeptic symptoms, and 1 (0.8%) had irritable bowel syndrome. Of the 15 patients with constipation, 3 (2.5%) had fecal incontinence. None of these symptoms were related to gluten intake (p>0.05).

The patients did not differ in terms of age and gender according to the presence of symptoms (p=0.93, p=0.98 respectively).

The anthropometric measurements revealed obesity in 5 (4.2%) patients and underweight in 1 (0.8%) patient according to age- and gender-appropriate BMI. Five (4.2%) patients were stunted and two of these were also underweight.

None of the patients had anti-tissue transglutaminase IgA positivity. Seven (5.9%) patients had IgA deficiency. Of these patients, none of them had anti-tissue transglutaminase IgG positivity.

Only 1 (0.8%) patient had AGA IgA positivity (in titer 37.3 U/mL), 6 (5.1%) patients had AGA IgG positivity but all in low titers (<50 U/mL).

Among those patients who had gastrointestinal symptoms, only 14.2% (3/21 patients) had AGA IgG positivity. Of these, 2 patients had functional constipation, and one patient had dyspeptic symptoms unrelated to gluten intake. There was no relation between the presence of these symptoms and AGA IgG positivity (p=0.08).

Discussion

CD is an immune-mediated inflammatory systemic disorder, triggered by gluten intake in genetically susceptible individuals. CD may present with classical form such as diarrhea, growth failure, but more frequently with non-classical signs (1,12). The neuropsychiatric problems such as depression, anxiety, behavioral and personality disorders, ataxia, migraine, epilepsy, and ADHD have been observed in 6.8% to 33.3% of children with CD at presentation (3-6). Additionally, children with CD were found to have an increased risk (1.4-fold greater) for psychiatric disorders in the future (3). Recently, Kristensen et al. (13) reported high depression and anxiety scores in newly diagnosed adult CD patients and improvement after a minimum 1-year gluten-free diet.

There are limited studies that observe the relationship between ADHD and CD in children. The diagnose of ADHD had been found 1.2-fold higher in children with CD than in the normal population (3). Niederhofer and Pittschieler (14) reported a high ADHD symptomatology score after the diagnosis of CD and a significant decrease in ADHD score after 6 months gluten-free diet. Additionally, Niederhofer (15) reported a high frequency of CD (14.9%) among children and adults with ADHD. On the other hand, Gungor et al. (16) reported 0.27% of the children with ADHD had biopsy-proven CD which is lower than the prevalence in healthy children (0.47%) in our country (17). In our study, none of the patients had positive anti-tissue transglutaminase antibody and therefore CD. This may be due to the small size of our study population.

NCGS is a clinical condition, characterized with intestinal and extraintestinal symptoms which are triggered by gluten intake and resolve with gluten removal, in the absence of CD and WA (1,2,18). The most common gastrointestinal symptoms are abdominal distension, bloating, diarrhea, and abdominal pain, followed by constipation, nausea, vomiting, and heartburn. The most common extra-intestinal symptoms are anxiety, lack of well-being, dizziness, and trouble in focusing (1,2,18,19). In our study group, 17.9% of the children had gastrointestinal complaints, but none of these were related to gluten intake. The most common complaint was functional constipation (12.8%) with a similar prevalence to healthy children in our country (20). We did not investigate whether they had confusion, anxiety, or trouble in focusing because of their primary diagnosis.

Unfortunately, there is no consensus on a screening test for NCGS. In patients with NCGS, positivity of AGA IgG antibodies were found more frequently than in the general population but it still unclear that this is helpful in diagnosing NCGS (11). In healthy people, the frequency of AGA IgG antibody positivity is 2% to 8% (18). AGA IgA and IgG antibody were found in 7.7% and 56.4% of adults with NCGS, respectively and after gluten withdrawal, a disappearance of AGA IgG antibody was reported (1,21). In our study, 0.8% of the patients had positive AGA IgA and 5.1% had positive AGA IgG antibody, which is not more frequent than in the general population.

ADHD is a common neuropsychiatric disorder in childhood, characterized by hyperactive, inattentive and impulsive behavior. There is no consensus on the etiology of ADHD, but interactions of genetic and environmental factors are thought to be the main triggering factors. Diet is thought to be related to ADHD symptoms (7,8). In previous studies, it was reported that a minority of children with ADHD benefit from the exclusion of food additives, but the role of elimination diets such as a gluten-free diet, a casein-free diet, or oligoantigenic diets was inconclusive (8). It was reported that approximately 33% of children with ADHD may respond to dietary intervention in a meta-analysis (22). The children with ADHD who ate a well-balanced diet had lower inattention score, than those who prefer the sweetened desserts and junk food (23). In the meta-analysis which reviewed the randomized controlled trials for exclusion and challenge of colorants, improvement of hyperactivity in 8% of patients with ADHD was observed (22). Additionally, a minority of children with ADHD benefit from the exclusion of food additives (7). The oligoantigenic diet, which consists of milk and dairy products, egg, soy, peanuts, wheat and seafood elimination, demonstrated a significant decrease in hyperactive behaviors in 64% of children in randomized controlled trials (24). In light of these findings that demonstrated the effect of dietary gluten intake on ADHD, we investigated the relationship between ADHD and gluten-related disorders, however we could not find any relationship.

Study Limitations

The major limitation of our study is the age range (only children) and the small number in the study population. Though wheat and wheat-based foods play an important role in our traditional eating pattern, the intense ingestion of gluten in daily life leads to difficulty in revealing the exact relationship between symptoms and gluten intake,

especially in children with ADHD.

Conclusion

Although this study demonstrates that CD and NCGS are not common in children with ADHD, larger sized studies are needed.

Ethics

Ethics Committee Approval: The study was approved by the local ethics committee of our hospital (protocol number: 2015/073).

Informed Consent: Children aged between 6-18 years old, diagnosed as ADHD and willing to participate in the study with a signed informed consent form were recruited into this study.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: S.S., C.T.K., G.D., Design: C.T.K., G.D., G.H., Ö.Ü., Data Collection or Processing: C.T.K., G.D., G.H., S.S., S.T.G., Analysis or Interpretation: C.T.K., G.D., S.T.G., Literature Search: C.T.K., S.S., S.T.G., Writing: C.T.K., G.H., G.D., Ö.Ü.

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Original Article

Assessment of Knowledge and Opinions of Family Medicine Residents About the Diagnosis and Treatment of Enuresis in Children

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ABSTRACT

Aim: Enuresis (EN) is a common problem in childhood. Family physicians have an important role in revealing children with EN. The aim of this study was to assess the knowledge and attitude of family medicine (FM) residents regarding the diagnosis and treatment of enuresis in children.

Materials and Methods: Family medicine residents of the University of Health Sciences, İzmir Tepecik Training and Research Hospital were invited to complete a questionnaire concerning enuresis in children. Those who had completed their paediatrics rotation training were defined as group 1, and those who had not completed their paediatrics rotation as group 2. The responses were compared between the groups.

Results: Sixty (88%) of the FM residents agreed to complete the survey. The mean age of the participants was 28.7 (25-35) years, 38 (63%) had completed their paediatrics rotation (group 1). The question about the age of night-time bedwetting was more often answered correctly in group 1 (19/38) (p=0.025). In the question on the symptoms of non-monosymptomatic enuresis; the constipation option was marked as a symptom by only 15 of the participants (25%), with 13 (87%) being in group 1 (p=0.03). The correct response rates to the questions about other symptoms of non-monosymptomatic enuresis, the causes of secondary enuresis, the need for treatment, treatment options and the follow-up of patients with enuresis were similar between groups 1 and 2.

Conclusion: As a result of this study, more correct answers, which revealed adequate knowledge and experience regarding enuresis, were obtained from those who had concluded their paediatrics rotation.

Keywords: Bedwetting, child, enuresis, family medicine, paediatrics, primary care

Introduction

Enuresis (EN) is a common problem in childhood (1-3). EN Awareness is not widespread enough which may lead to a lack of help for children in need. Therefore, family medicine and primary care physicians should have sufficient knowledge regarding EN. We aimed to assess the knowledge and attitude of family medicine residents of our hospital about the diagnosis and treatment of nocturnal enuresis in children.

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Materials and Methods

This study was conducted among family medicine residents of the University of Health Sciences, İzmir Tepecik Training and Research Hospital. In June 2019, multiple-choice questions were sent to the residents via the internet. Participants in the study were asked to answer demographic questions such as their age, gender, status of their paediatrics rotation training, and eight multiple-choice questions (regarding the definition, frequency, etiology, differential diagnosis, treatment, etc) about bedwetting in children. The responses of all the participants were evaluated. Those who had completed their paediatrics rotation were defined as group 1, and those who had not completed or were currently in the process of performing their paediatrics rotation as group 2, and the responses were compared between the two groups.

Ethical approval was taken from the local Ethical Committee (2019/9-23). This study was conducted in accordance with the Helsinki Declaration.

Statistical Analysis

IBM SPSS V.22.0 software was used for statistical analysis. The data that is normally distributed are given as mean + standard deviation, and asymmetrical distributed data as median (maximum, minimum). Statistical significance was checked with parametric and non-parametric tests. A p-value less than 0.05 was considered statistically significant. The responses given by family residents who had completed (group 1) and those who had not (group 2) completed their paediatrics training were compared.

Results

Of the 68 individuals who were family medicine residents, 60 (88%) agreed to fill out the questionnaire form. The mean age of the participants was 28.7 (25-35) years, 45 (75%) were female. There were 38 (64%) family medicine residents in group 1.

Age at Diagnosis

The results of the answers to the question "After which age should night-time bedwetting be evaluated?" were as follows: Sixteen participants (27%) opted for 4 years, 23 participants (38%) opted for 5 years, 8 participants (13%) opted for 7 years, and 13 participants (22%) opted for the choice of "I have no idea". When the age of 5 was considered as the correct answer, the number of participants who gave this answer was 23 (38%) and the number of participants who gave the wrong answers was 37 (62%). The proportion of those who answered correctly was higher among those in group 1 (19/38) than in group 2 (4/22), and the difference was statistically significant (p=0.025) (Table I).

Prevalence

The correct answer for question concerning "the incidence of bedwetting" was 1/10. Other choices such as 1/100, 1/1,000, 1/10,000 were considered incorrect. Twenty-one participants (35%) answered this correctly. Of those who gave the correct answer, 16 had completed their paediatric rotation training and 5 had not, however the difference was not significant (p=0.129).

Non-monosymptomatic Enuresis

A question for which multiple options could be selected was asked concerning the symptoms that suggest daytime urination disorders. For this question, the rates of marking each choice were evaluated separately. The rates of correctly defining the symptoms of bladder and bowel dysfunction (BBD) are shown in Table II. The correct answer rate for the symptoms was 55-81%. Only 15 (25%) participants chose constipation as a BBD symptom. 13 of these 15 (87%) were in group 1 and there was a statistically significant difference with group 2 (p=0.03).

Causes of Primary Enuresis

The choices offered and the answers given for "the etiology of primary enuresis" question are shown in Table II. Psychological causes are predominant in the etiology of secondary enuresis and 62% opted for this choice.

Causes of Secondary Enuresis

The answers given to the question of "secondary enuresis etiology" are shown in Table II. Although urinary tract infection (51%) and psychogenic causes (42%) were chosen more commonly, the percentage of participants who thought that neurogenic bladder might be a cause of secondary enuresis was 42%. There was no statistically significant difference between the answers in groups 1 and 2.

Table I. Answers to the questions that have only one correct choice

	All participants (n=60)	Group 1 (n=38)	Group 2 (n=22)	p-value
Urinary incontinence diagnosis age	23	19	4	0.025
Urinary incontinence frequency	21	16	5	0.129
Treatment requirement	43	25	18	0.542

Table II. Answers to the questions that have more than one correct choice

Non-monosymptomatic enuresis	All (%) (n=60)	Group 1 (n=38)	Group 2 (n=22)	p-value
Urgency	42 (70)	24	18	0.108
İntermittent urination	43 (72)	25	18	0.151
Daytime incontinence	49 (82)	32	17	0.367
Difficulty starting urination	33 (55)	21	12	0.584
Constipation	15 (25)	13	2	**0.028**
Urine retention	45 (75)	27	18	0.272
No idea	-	-	-	-
Urination for 3-7 times a day	51 (85)	4	5	0.183
Causes of primary enuresis				
Genetic	38 (63)	24	14	0.597
Sleeping disorders	33 (55)	26	7	**0.006**
Psychological	37 (62)	25	12	0.277
Nocturnal polyuria	43 (72)	30	13	0.090
No idea	4 (7)	2	2	0.468
Causes of secondary enuresis				
Psychological	49 (82)	30	19	0.363
Diabetes mellitus	36 (60)	23	13	0.563
Urinary tract infections	51 (85)	34	17	0.183
Diabetes insipidus	35 (58)	21	14	0.360
Renal diseases	29 (48)	20	9	0.272
Neurogenic bladder	25 (41)	13	12	0.103
No idea	3 (5)	1	2	0.302

Need for Treatment

A single answer was required for the question "Is nocturnal enuresis a condition that should be treated in children?". Four (7%) participants answered this question as "treatment is unnecessary because it can resolve spontaneously", 9 (15%) as "if the child has low self-esteem, it should be treated", 2 (3%) as "the decision of treatment is up to the family", 42 (72%) as "it should be definitely treated"; and 2 (3%) as "I have no idea". Of the 43 residents who thought that nocturnal enuresis in children should definitely be treated, 25 (58%) were in group 1 but the difference between two groups was not statistically significant (p=0.542).

Table III. Answers to treatment options

Treatment options	All (%) (n=60)	Group 1 (n=38)	Group 2 (n=22)
BMT	6	4	2
BMT + alarm	9	6	3
BMT + desmopressin	2	2	0
BMT + alarm + desmopressin	8	7	1
BMT + oxybutynin	1	0	1
BMT + alarm + oxybutynin	4	2	2
Alarm + oxybutynin	1	1	0
BMT + alarm + desmopressin + oxybutynin	21	14	7
No idea	8	2	6

BMT: Behavioural and motivational therapies

Treatment Options

It was stated that more than one answer could be given to the question "which is/are the treatment option(s) for nocturnal enuresis in children?". The choices were "behaviour therapies", "oxybutynin", "an alarm device", "desmopressin", and "I have no idea". Behavioural and motivational therapy, an alarm and/or desmopressin are the recommended treatment options of primary monosymptomatic enuresis. Fifty-one participants (85%) opted for behavioural therapy as one of the choices and 28 (47%) opted for oxybutynin. Only 8 (13%) participants marked all three choices of behaviour, alarm and desmopressin therapy, whereas 8 (13%) had no idea. The answers given for the treatment options are shown in Table III.

Follow-up of a Patient With Enuresis

The choices for the question "which specialities should follow and treat a child with enuresis?" were family medicine, paediatrics, paediatric nephrology, paediatric urology, and paediatric nephrology/urology specialist for unresponsive or complicated cases.

Thirty-six participants (24 from group 1 and 12 from group 2), stated that enuresis could be managed by family medicine specialists, while 50 (group 1: 31, group 2: 19) thought it should be managed by a paediatrician. Only 43 participants (group 1: 28, group 2: 17) stated that cases unresponsive to treatment should be treated by paediatric nephrologists and urologists. There was no statistical difference between groups 1 and 2 regarding the choice of speciality in the follow-up (p=0.354).

When all questions were evaluated, the rate of correct answers in those questions relating to the age at diagnosis

of enuresis, sleep disorders in the etiology of primary enuresis and constipation symptoms as a bladder/bowel dysfunction was significantly higher in group 1. As for the other questions, there were no significant differences between the two groups.

Discussion

Enuresis, also called bedwetting, is defined as urinary incontinence during sleep in children over the age of five years (3). According to ICD-10 and DSM-V diagnostic criteria, urinary incontinence lasting for at least 3 months and occurring at least once a month is needed for diagnosis (2). When asked about the age at diagnosis, almost half of the participants gave the correct answer with the majority of the correct answers being in group 1. Having the correct information about which age to evaluate enuresis is important for health workers so that patients can be treated and monitored without delay.

Among five-year-old children, the prevalence of EN is 10-25%. This rate regresses to 10-15% by the age of 7, to 5% by the age of 10 and to 1% in adulthood (1,3). Only one third of participants stated that they thought that enuresis is as common as to be seen in one in every 10 children. Actually, this reflects that the high number of children who suffer from enuresis is not adequately known even by physicians.

Primary enuresis occurs due to three major pathogenic mechanisms; nocturnal polyuria, detrusor hyperactivity and sleep pattern disturbance with difficulty in waking up (2,4,5). In addition, genetic causes, reduced functional bladder capacity, and delayed maturation are also considered to be factors included in its etiology. Nocturnal polyuria was the most frequently selected etiology of primary enuresis. Those who evaluated sleep disorder as an etiologic factor were markedly more in the group that had completed their paediatrics rotation. This was thought to be due to the fact that children with enuresis have difficulty in waking up at night. More than half of the participants thought that psychological causes are related with primary enuresis. Two to three decades ago, enuresis was believed to be caused by psychological problems. However, currently it is thought that psychological symptoms are more the result of enuresis rather than its cause (4,6).

It is known that there is a genetic predisposition for primary enuresis (7). The risk increases by 44% when either the mother or father had a history of enuresis and by up to 77% when both parents had a history of enuresis (7). Two out of three participants knew that genetics is involved in the etiology.

Secondary enuresis, which is the resumption of bedwetting after at least 6 months of bladder control, may develop in a child following a stressful period (separation of parents, history of birth of a sibling and so on) (1,8). Furthermore, organic causes such as urinary tract infections, diabetes mellitus, diabetes insipidus, renal diseases and sometimes obstructive sleep apnoea, hypothyroidism, or medications (valproate) can also be involved (9). Neurogenic bladder, on the other hand, usually develops due to congenital spinal dysraphism and is among the causes of continuous incontinence. Although urinary tract infection and psychological issues are well-known causes of secondary enuresis, organic causes were found to be less known among the participants.

If a child has additional symptoms to enuresis, he/she should be investigated for signs of bladder dysfunction (2). This is because the determination of BBD is important in the treatment of these patients. The rates of correctly defining BBD symptoms ranged from 55% to 85%, and the least selected choice was 'straining to urinate'. It is important to know all the symptoms of bladder dysfunction in order to fully and correctly evaluate patients. As the rectum has the same embryonic origin, bowel dysfunction can accompany bladder disorders or can aggravate symptoms of bladder dysfunction (8,10). Even constipation is a part of bladder dysfunction and even a factor unfavourably affecting monosymptomatic enuresis should be investigated and treated (6,11). In cases where constipation is not treated, the response rate expected from urination disorder treatment will be low. The rate of considering constipation as a symptom was rather low (25%).

Many children with a bed-wetting problem have low self-esteem compared with their peers. Studies have shown that their school performance and quality of life scores are also low (8,12). However, this is overlooked in many families, and therefore, they do not present to healthcare centres. In cases like these, every child has the right to be treated regardless of family decision or the psychological trauma on the child. The goal is to increase awareness and treatment approaches via "world bedwetting day" (www.worldbedwettingday.com). The majority (72%) of family medicine residents thought that EN should be treated.

Behavioural and motivational therapy is the first-line treatment in primary monosymptomatic enuresis (10). These children should be a part of their treatment as motivation is important for treatment success (13). The family should be comforted by explaining that enuresis is not a condition that needs to be hidden and may resolve with treatment

or spontaneously with time (4). Recommendations like stopping fluid intake 3-4 hours before sleeping in the evening and voiding the bladder before going to bed are included in behavioural therapy (14). Knowledge regarding behavioural approaches is especially important in order to inform these patients in their primary care.

Alarm treatment and desmopressin are recommended in evidence A at level 1 for PEN (9,15,16). Alarm treatment is a non-invasive treatment method that can be selected especially in children with sleep disorders, low bladder capacity or nocturnal bladder detrusor hyperactivity (15). It is known to be as effective as desmopressin (80%) by the end of the first month. Desmopressin, which decreases urine production through increasing water reabsorption from the renal tubules, has high efficacy starting from the first dose (16). Likewise, oxybutynin, an anticholinergic drug, can be recommended for children with non-monosymptomatic EN with bladder dysfunction. It acts by inhibiting detrusor muscle contractions; therefore, it should be given to BBD patients who need frequent voiding or who have low bladder capacity (3). It is important to use drugs with different mechanisms for appropriate indications in the eligible patients. Therefore, doctors who follow EN are expected to be fully competent in the indications, follow-up and adverse effects of any treatments to be applied. The treatment procedure to be followed should be planned together with the child and family.

Children who applied to a primary health care centre with incontinence and who had no symptoms of bladder dysfunction as a result of the initial evaluation can be treated by paediatricians as primary monosymptomatic enuresis (1). Maintenance treatment of these cases can be carried out by family physicians (6). Patients unresponsive to treatment or with BBD need to be referred to paediatric nephrologists or urologists for further examination and treatment. Although a high rate of participants thought that complicated cases should be referred to these specialities, they also concluded that this information should be further emphasized. For cases with PEN, those who develop psychological symptoms, such as low self-esteem because of enuresis, should be referred to receive psychological help. Similarly, for cases with secondary enuresis, those without an organic cause or thought to be accompanied with a psychiatric disorder should be evaluated by a paediatric psychiatrist. In this study, only 60% of family physicians thought that they could follow patients with EN. This suggests that family physicians should be supported with postgraduate medical training programs.

Conclusion

Primary care physicians have many opportunities to recognise and/or follow-up children with enuresis such as at routine visits or periodic health screening. Since enuresis is a serious problem that needs to be recognized and managed as early as possible, we can say that children with enuresis can have a better quality of life and their families experience less psychosocial problems when their primary care physicians have more awareness of this issue.

Ethics

Ethics Committee Approval: Ethical approval was taken from the local ethical comittee (2019/9-23) conducted in accordance with the Declaration of Helsinki.

Informed Consent: Written informed consent was obtained from the participants who volunteered to participate in the study.

Peer-review: Internally peer-reviewed.

Authorship Contributions

Concept: S.A.Ç., Design: H.P., B.K.D., Data Collection or Processing: E.U., D.A., Analysis or Interpretation: G.E., F.M., Literature Search: E.S., S.A.Ç., Writing: S.A.Ç.

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Original Article

Phenol Administration in Adolescents with Pilonidal Sinus is Effective and Related to High Patient Satisfaction Rates

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ABSTRACT

Aim: Surgical methods are the most commonly used methods in the treatment of pilonidal sinus (PS) in the world but their recovery durations and return to work/school periods are long and the complication rates are high. Among minimally invasive methods, phenol therapy is frequently used in adults and successful results are reported. However, studies on phenol treatment in adolescents with PS are limited and their number of patients is small. Therefore, our study aims to determine whether 80% liquid phenol treatment can be used as first-line therapy in this age group.

Materials and Methods: Primary and 9 between 14 and 19 years who were treated with 80% liquid phenol were included in this study. Patient data were obtained from their files retrospectively.

Results: Of the patients, 69 (69.7%) were male and 30 (30.3%) were female. The mean age of the patients was 17.4±1.3 (minimum-maximum 14-19). The total recurrence and complication rates after phenol administration were 10.7% and 16.1%, respectively. According to the Likert-type questionnaire, the mean satisfaction rate was 8.7±1.7 (minimum-maximum 5-10).

Conclusion: In our study, it has been shown that 80% liquid phenol treatment can be successfully applied in adolescents with PS with low recurrence and low major complication rates. In addition, we think that 80% liquid phenol treatment should be recommended as the first-line treatment option for both primary and postoperative recurrent adolescent patients with PS, as it ensures a quicker return to work/school and shows high satisfaction rates.

Keywords: Adolescence, phenol, pilonidal sinus

Introduction

Pilonidal sinus (PS) disease is a chronic inflammatory disease that usually affects young men in their twenties. The disease usually manifests itself with chronic inflammatory discharges of the sinuses or acute developing abscesses and can be asymptomatic as well. Although there is no advantage in treating asymptomatic disease, patients with symptomatic disease should be treated (1). In spite of the fact that many methods have been described for the treatment of PS, optimal treatment is still controversial. The ideal PS treatment should be a method that can be applied with minimal tissue excision, offers good cosmetic

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results, does not require hospitalization, has a low-cost, is easy to use, allows a rapid return to work/school, and has low complication and recurrence rates (2,3).

The main goal of surgical treatment methods is to excise PS and then leaving this area to secondary healing or to close this area with flap or flapless methods (1,4-6). However, none of these surgical methods provide 100% improvement, so to perform the operation, hospitalization is required, patients cannot return to work for a long time and recovery periods are defined in weeks (1,4-7). In addition, postoperative complication rates are found to be 8.9-49% (4,5). In spite of this, surgery is still the most common treatment in PS (1).

Besides surgical treatments, phenol application has been used for the treatment of adult PS patients for a long time as a simple and easy to use method with high success rates (3,8,9). In the literature, the number of studies evaluating the adolescent patient group with phenol was limited and the published works focus on the use of phenol in a crystallized state (10-12). As far as we can see, there are no papers with liquid phenol in the adolescent PS literature. Our study aims to discuss the efficacy and satisfaction rate of 80% liquid phenol application in patients with adolescent PS in the light of the literature and to determine whether liquid phenol treatment is a usable method in this age group.

Materials and Methods

Local ethics committee approval (26.09.2018-252) was obtained for this study. Between January 2013 and January 2018, adolescent patients aged between 14 and 19 years who applied to a 3rd step reference hospital for primary or recurrent PS and who accepted to be treated by the phenol method were included. The patients' data were collected by one physician's personal log and analyzed retrospectively. Demographic data, comorbidities, body mass index (BMI), complications and PS recurrences were evaluated.

None of the patients underwent preoperative prophylactic antibiotic or laboratory tests. The differential diagnosis of anal fistula in the patients with sinus located near the anal canal was ruled out by magnetic resonance imaging. 80% liquid phenol was applied by the same physician to all patients. After phenol administration, patients were called for twice in the first week, then weekly until the wound was closed, and at 2nd, 6th and 12th months after the wound was closed. After that, patients were called for check-up only once a year. The closure of the wound within 2 months or the failure to reopen within this period was defined as recovery. Patients with recurrent or non-healing wounds were treated with phenol for a second and/or third time. By applying a Likert-type satisfaction questionnaire to patients who came to the check-up postoperatively, the patients were asked to evaluate their outcomes in terms of procedure tolerance, postoperative pain, complication, return to daily work and cosmetic appearance (0-2; poor, 3-4; fair, 5-6; average, 7-8; good; 9-10 excellent). Finally, the patients were invited to the outpatient clinic with the purpose of evaluating their recurrences and complaints. In the situation of those who could not come to the polyclinic, patients were interviewed by telephone.

Patients who had acute inflammation or abscess at the beginning of the treatment, those who did not come to the follow-up check-ups, and those who had no contact information or could not be reached were not included in this study. In addition, patients who had developed recurrence after phenol treatment and then preferred another treatment instead of phenol for the 2nd and/or 3rd time were excluded from the study.

Statistical Analysis

Statistical analyzes were performed using SPSS version 17.0 software. The conformity of the variables to normal distribution was examined using analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk tests). Descriptive analyzes were given as mean ± standard deviation for variables that were not normally distributed. Descriptive statistics were made by giving demographic characteristics, frequency and percentage values. In continuous data, independent groups t-test was used to compare binary groups such as "yes" or "no". Pearson's chi-square or Fisher's Exact chi-square test was used in the analysis of categorical data. The cases where the p-value was below 0.05 were considered statistically significant.

PS Treatment Technique with Phenol

All patients were treated in a day surgery unit. Written informed consent was obtained from all patients before the procedure. After positioning the patient in the Jackknife position, hair was removed from the presacral area and local anesthesia was applied. During the phenol application, petroleum jelly (Vaseline; Unilever, London, UK) and dry dressing were used to protect the circumjacent healthy tissue and anal region. Those with narrow sinus mouths were either dilated with a small incision (0.5-1 cm) to remove hair from these openings, and then the cavity was debrided. Bleeding from these procedures was stopped by

electrocautery or pressure. After 80% liquid phenol was impregnated into the swab cotton, it was gently pressed onto the cavity and held for approximately 2 minutes. This procedure was repeated 3 or 4 times. After the procedure was completed, by gently pressing on top of the cavities, the remaining phenol and debris were removed. This area was covered with dry dressing. Patients were given painkillers to use when necessary. None of the patients were prescribed oral antibiotics or pomade with local antibiotics. During the first follow-up on postoperative day 3, a gentle debridement was performed and the debris remaining inside was removed by entering from the sinus using clamps without any local anesthesia. No physical restraint was recommended to the patients in the postoperative period, and they could return to their work/school and daily activities immediately or when they felt ready.

Results

Of the patients, 69 (69.7%) were male and 30 (30.3%) were female. The mean age of the patients was 17.4±1.3 years (miimumn-maximum 14-19). While 77 patients (77.8%) had primary PS, 22 (22.2%) patients had recurrent PS disease after surgical intervention. Co-morbidities were found in 11 patients (coeliac disease, leukemia, myasthenia gravis, insulin resistance, familial Mediterranean fever and asthma). The mean BMI was found to be 25±4.1 (16-37) (Table I).

The rate of recurrence after the first phenol administration was 26.2% (99/26). Four of the patients who incurred recurrence after the first application of phenol were discharged from the study because they wanted surgery and two patients were excluded because they did not come to follow-up. Among the remaining 93 patients, recurrence rates were found to be 14% (93:13) and 10.7% (93:10), respectively, when second and third phenol were administered to those patients with recurrence. A total of 15 (16.1%) patients developed complications following the procedure. Eleven (11.8%) patients with minor complications who developed maceration and superficial burns were treated with routine dressings. Oral antibiotics were given to 2 (2.1%) patients who developed cellulitis. Two (2.1%) patients developed abscesses, which were drained, and they used oral antibiotic. According to the Likert-type questionnaire, the mean satisfaction rate was 8.7±1.7 (minimum-maximum 5-10) (Table II).

Recurrence and complications were compared in terms of age, gender, BMI, time from the onset of complaints to admission to the hospital, natal cleft depth, sinus number and location, smoking, abscess drainage before phenol administration, how many baths the patient takes a week, surgery before phenol administration and whether relatives have PS (Table III). There was only a significant relationship between smoking and total complication rates ($p<0.05$).

Table I. Patient and PS characteristics

Average age, n (%)	min.-max. 14-19 mean 17.48±1.3
Male/female ratio %	69/30
BMI, n (median)	min.-max. 16-37
	Mean 25±4.1
Additional morbidity, n (%)	11 (11.1%)
Primary PS, n (%)	77 (77.8%)
Recurrent PS, n (%)	22 (22.2%)
(Before phenol application, all surgical operations)	

PS: Pilonidal sinus, BMI: Body mass index, min: Minimum, max: Maximum

Discussion

Our study emphasizes that phenol therapy can be used successfully in adolescent patients with PS and highlights that it may be an alternative to surgical treatment. Although there are many factors that determine the success of PS therapy, the superiority of treatment modalities in the PS literature is usually analyzed by measuring patient satisfaction, recurrence and complication rates. In the current study, recurrence and complication rates were found to be similar to surgical treatments and adult phenol applications. This finding makes the current study

Table II. Data after phenol application

Observation, mo (median)	31.0 (8-68)
Need for analgesics, d (median)	2.2±2.3 (0-10)
Return to work/school time, d (median)	0-7 (3.9±2.2)
Recovery time, d (median)	14-53 (24.4±6.7)
Recurrence rate after one phenol application, n (%)	99/26 (26.2%)
Recurrence rate after two phenol applications, n (%)	93/13 (14.0%)
Recurrence rate after three phenol applications, n (%)	93/10 (10.7%)
Total complications, n (%)	15 (%16.1)
Minor complications, n (%) (maceration, superficial burn on the skin)	11 (11.81%)
Major complications (pus and cellulitis), n (%)	4 (4.3%)
Follow-up period in months	32.2±24 (8-62 month)
Total satisfaction points (average)	8.7±1.7 (3-10)

Table III. Factors affecting first recurrence and complications after phenol administration

	Initial recurrence after phenol administration			**Complication after phenol administration**		
Variables	**No**	**Yes**	**p-value**	**No**	**Yes**	**p-value**
	n=73	**n=26**		**n=83**	**n=16**	
	(mean ± SD)	**(mean ± SD)**		**(mean ± SD)**	**(mean ± SD)**	
Age	17.6±1.3	17.0±1.19	0.066	17.4±1.3	17.2±1.5	0.328
BMI	25.0±4.3	25.0±3.5	0.987	25.0±4.1	25.2±4.3	0.844
Duration of complaints (months)	8.5±9.4	6.5±4.8	0.166	8.1±8.2	7.5±10.3	0.788
Natal cleft depth (mm)	29.9±11.4	30.7±11.2	0.756	30.7±11.0	27.3±13.3	0.271
Number of sinuses	2.2±1.2	2.5±1.3	0.280	2.3±1.3	2.5±1.4	0.598
Smoking per day	3.8±7.3	1.4±4.4	0.054	3.7±7.2	0.6±2.5	0.003
Number of pre-treatment abscess drainage	0.1±0.3	0.3±0.6	0.127	0.2±0.5	0.2±0.4	0.959
Number of baths per week	3.5±1.7	2.8±1.0	0.114	3.4±1.7	3.3±1.5	0.870
Categorical variables	**n (%)**	**n (%)**	***p-value**	**n (%)**	**n (%)**	***p-value**
Gender						
Male	52 (71.2)	17 (65.4)	0.577	59 (71.1)	10 (62.5)	0.556
Female	21 (28.8)	9 (34.6)		24 (28.9)	6 (37.5)	
Location of sinus						
Midline	54 (74.0)	20 (76.9)	0.766	63 (75.9)	11 (68.8)	0.542
Midline and lateral	19 (26.0)	6 (23.1)		20 (24.1)	5 (31.3)	
Previous operation before phenol administration						
No	58 (79.5)	19 (73.1)	0.502	65 (78.3)	12 (75.0)	0.750
Yes	15 (20.5)	7 (26.9)		18 (21.7)	4 (25.0)	
Relative has PS						
No	53 (72.6)	19 (73.1)	0.963	63 (75.9)	9 (56.3)	0.129
Yes	20 (27.4)	7 (26.9)		20 (24.1)	7 (43.8)	

Independent t-test and *Pearson's or test Fisher's test exact chi-square were used.
P<0.05 was considered significant. BMI: Body mass index, PS: Pilonidal sinus, SD: Standard deviation

worthwhile because one of the most important factors in determining the methods to be used in the treatment of PS is the recurrence and complication rates. In this current study, the recurrence rate was 10.7%. Our complication rate was 16.1% in total and the major complication rate was 4.3%. In a study examining patients in the adolescent age group who were treated with crystallized phenol, both recurrence and complication rates were reported to be 2.5% with a single application (10). However, recurrences after phenol administration are usually seen after the first year, and the recurrence rates may be low since the mean follow-up period was only 8.1 months in the study mentioned (8,13). In addition, it is stated in the mentioned study that a good hair cleaning by opening the sinus mouths sufficiently leads to low recurrence rates. Although we applied a similar technique and applied more than one phenol treatment, we could not achieve similar recurrence rates. It is striking that there is only a difference in the form of phenol between the mentioned study and the technique applied in our study. For this reason, we wanted to compare our data with a group of similar age treated with liquid phenol. However, we could not find a study in the literature where liquid phenol was applied to adolescent PS patients. However, we think that the form of phenol used in the treatment (crystallized or liquid) cannot explain this important difference in recurrence rate as crystallized phenol melts rapidly at body temperature and turns into liquid phenol form.

In the adult literature, the recurrence rates of phenol and its complication rates are reported to be 0-18.6% (1,3,14) and 0-15.2% (3,12,14), respectively. As can be seen, the rates of recurrence and complication due to phenol application can be quite different in the literature. It is known that this difference in recurrences varies inversely with the number of phenol applications (3) and in direct proportion to the follow-up period (13). Considering the median follow-up period of 31.0 (8-68) months in the current study, our recurrence rate is consistent with the adult phenol literature. Our complication rate was higher than the adult literature. It is notable that our study showed higher complication rates compared to both adolescent and adult age groups. We have continued our experience in adolescents as we use liquid phenol in adult PS patients and because liquid phenol can be easily directed to the desired area by absorbing it in swab cotton. However, since we make multiple applications in the same session with phenol-impregnated swab wipes, the substances protecting the skin can be removed during repetitive procedures. These findings indicate that more attention should be paid to the protection of surrounding tissues, especially during multiple liquid phenol applications.

In order to compare the recurrence and complication rates in the current study with surgical methods in the literature, we preferred the surgical methods that are frequently used in the treatment of PS in the world. While the most commonly used are excision and open wound healing or primary closure in the midline, the Karydakis flap and Limberg flap are commonly used for off-midline procedures (1). The recurrence and complication rates of the above mentioned surgical treatments vary between 1.4-45% and 8.9-49% in the literature (5,9,10,15-19). We think that recurrence rates in our study provide acceptable rates when compared with the surgery groups used in the treatment of PS in the literature. In addition, because the anatomy is not impaired after the application of phenol, the application can be easily repeated at an out-patient hospital. However, one disadvantage of phenol is that in order to achieve high success rates, it can be said that it usually requires multiple applications as in our study (3). Although the complication rates in our study are not lower than for surgical treatments in the literature, our major complications are usually easily treated complications such as abscess and cellulitis. After surgical treatment, complete wound separation due to abscess, seroma and hematoma may be seen (20) and sometimes complications resulting from surgery result in more morbidity than the disease itself (21). In addition, the disadvantages of the aforementioned surgical treatments include the need for patients to be admitted to hospital (5,7,21,22) and the difficulty of completing the learning curve of flap methods (1). The phenol method is a technique that is easy to apply, does not require hospitalization and whose learning curve is shorter.

When we evaluated the patients in our study in terms of recurrence and complications, a correlation was found only between smoking and the development of complications. However, in studies evaluating recurrence and complications after PS surgery in the literature, the onset of symptoms, sinus number, and BMI were found to be risk factors (23,24). Recurrence and complications were not affected by the characteristics of the patient in general, probably because the anatomy did not change in our treatment with phenol and only the diseased area was intervened.

The second important issue we investigated in our study was the satisfaction rates of the patients. Total satisfaction rates are determined by patient tolerability, complication rates, postoperative pain, quick return to daily work/school and cosmetic results. The satisfaction rates of the adolescents in our study were found to show some differences in terms of patient satisfaction when compared with the adult phenol applications in literature and the frequently used surgical applications in the treatment of PS. In a study comparing the 40-80% liquid phenol administration in adult PS patients (25), the recovery times were similar to our study, while return to work/school and use of painkillers lasted for a shorter period than for our patients. These differences may be explained by the use of prolonged pain killer because of the increased pain due to the more active life of the adolescent age group. In addition, adolescents who are already in school life may not feel the necessity to start school, while adults who have a business life are obliged to start working as soon as possible. This may explain why adolescents with similar wound healing times return to work/school after longer periods of time. Compared with the surgical techniques commonly used in the treatment of PS in the literature, patient satisfaction rates (5,26,27) are comparable with wound healing times (6,28,29), however, it can be seen that the current study is more advantageous than the surgical treatments in terms of return to work (6,7,30-33) and use of painkillers (7,21).

Considering the advantages of phenol therapy, the satisfaction rates in the current study were found to be similar to the satisfaction rates of the surgical groups. This finding is consistent with the study conducted by Doll et al. (26). Doll et al. (26) stated that patients did not care about a long hospital stay or a long wound healing

period if there was no recurrence and they did not find a relationship between different surgical PS treatment methods and satisfaction. In addition, they reported that the age of the patient at the time of surgery did not affect patient satisfaction, but the average satisfaction rate of patients under 20 years of age was lower than the total average, in addition, patients without recurrence had higher satisfaction rates than those with recurrence. Since the patients in our study were in the adolescent age group and the rates of recurrence were found to be comparable with the surgery groups, we thought that we obtained similar satisfaction rates to the surgical groups.

Finally, another point worth mentioning is the use of surgical prophylactic antibiotics (SP) to which though we have not paid due attention while comparing phenol treatment with surgical methods. While SP is usually applied to surgical methods (1,21,34), during the administration of phenol, there is no administration of SP if the patient does not have diabetes mellitus or immunosuppression (3,25,35). This situation is important in terms of antibiotic resistance that threatens the world and also provides an advantage in terms of patient cost.

Study Limitations

The study was retrospective. In addition, the fact that a small number of patients did not accept invitation to the polyclinic, the case if there were recurrence or not were defined according to their own declaration. This may have led to the omission of asymptomatic recurrences.

Conclusion

This study is important in terms of showing that 80% liquid phenol application is a method that can be used in the treatment of adolescent PS in comparison with the commonly used surgical methods and phenol applications in adults in the literature. Although 22.2% of the patients in our study were previously surgically treated recurrent patients, they provided similar recurrence rates to surgical treatments and adult phenol applications in the literature. In addition, low rates of major complications, less anatomical changes compared to surgical methods, and rapid return to work/school are among the important advantages of the method. For all these reasons, we think that phenol application should be recommended as the first treatment method in patients with primary and postoperative recurrent PS. For stronger evidence, prospective randomized reports with high volume and long-term follow-up are recommended to compare with surgical methods in this age group.

Ethics

Ethics Committee Approval: Local ethics committee approval (26.09.2018-252) was obtained for this study.

Informed Consent: Written informed consent was obtained from all patients before the procedure.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: C.K., Design: C.K., Data Collection or Processing: A.S., M.M., Analysis or Interpretation: İ.S., M.E., Literature Search: C.K., A.S., M.E., M.M., H.B.D., İ.S., G.K., C.A., Writing: C.K., M.M.

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Original Article

Bilirubin Levels at 1st and 3rd Postoperative Months are Significant in Determining the Success of the Kasai Portoenterostomy

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ABSTRACT

Aim: The aim of this retrospective study was to determine the indicators of survival with native liver (NLS) of patients operated for biliary atresia (BA).

Materials and Methods: This review included 53 BA patients in a 13-year period. There were two groups: (1) NLS and (2) necessitating transplantation. Age at operation, and bilirubin levels on the 7th day, 1st and 3rd months postoperatively were recorded. Mann-Whitney U and logistic regression analysis were used for statistical analysis for NLS and liver transplantation (LTx).

Results: Kasai portoenterostomy (KPE) was performed on 38 patients, and 15 were directed to LTx due to cirrhotic liver at presentation. Twenty-three of 38 patients with KPE survived with native liver, and 15/38 patients required LTx during follow-up. Mean age at portoenterostomy for NLS and necessitating LTx was 54.43±24.64 and 68.33±24.35 days respectively ($p>0.05$). The 1st and 3rd month bilirubin levels were lower in the NLS group ($p<0.01$). The 1st month and 3rd month bilirubin levels after KP were significant predictors for survival with NLS. A cut-off value of 5.7 mg/dL bilirubin level at the 1st month predicted the necessity of transplantation after KPE with a sensitivity of 83.3% and specificity of 78.9%.

Conclusion: Bilirubin levels of the 1st and 3rd months are reliable predictors for the success of portoenterostomy.

Keywords: Biliary atresia, liver transplantation, biliary cirrhosis, cholestasis

Introduction

Biliary atresia (BA) is a progressive, destructive, and inflammatory disease of the extrahepatic and intrahepatic bile ducts (1,2). The first-line surgical treatment for BA is Kasai portoenterostomy (KPE) introduced by Morio Kasai in 1955 (3). The reported 5-year survival rate with native liver after KPE varies between 35-55% (4,5). Nevertheless, the majority of cases require liver transplantation (LTx) in long-term follow-up. Indications of LTx for BA include biliary cirrhosis, liver failure, portal hypertension, gastrointestinal bleeding, growth retardation, pruritis and hepatopulmonary syndrome.

Several studies have looked into the potential indicators for native liver survival after KPE including age at portoenterostomy, biochemical parameters, histopathological findings and postoperative complications (4-7). Age at portoenterostomy is widely accepted as a prognostic factor. However, there are some counterviews in the literature. For instance, Pakarinen et al. (4) reported that age at KPE is a prognostic factor for clearance of jaundice in

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postoperative follow-up. On the other hand, Ramachandran et al. (8) advocated that age is not a criterion for success of KPE (8). Another popular issue is "prognostic indicator" for success of KPE in the postoperative follow-up period. Ramos Gonzales et al. (6) have shown that total bilirubin >2 mg/dL at 3 months after surgery was an independent predictor for the need for LTx. Similarly, Hukkinen et al. (7) have calculated that conjugated bilirubin levels ≥2.5 µmol/L at postoperative 6th month was a significant predictor, increasing the risk of cirrhosis 35-fold. In this study, we aimed to determine the indicators and predictors of NLS of our patient series operated on for BA in our center.

Materials and Methods

This study was approved by the Institutional Review Board and Research Ethical Committee in accordance with international ethical standards and the World Health Organization Helsinki Declaration (approval no: 20-8T/13). All admissions and surgical procedures were performed after informed consent of the family/parents/caregivers was given.

Study Design and Data Collection

In our clinical protocol, all patients presenting with prolonged jaundice and light-colored stools are initially evaluated with biochemical analysis and detailed hepatobiliary ultrasonography done after starvation of minimum 6 hours. Triangular cord thickness, abnormal gall bladder morphology (starvation and after feeding) as well as findings of subcapsular arterialization, parenchymal heterogeneity, and findings of "BA splenic malformation (BASM)" are considered suggestive for BA. Patients are listed for laparoscopic exploration and intraoperative cholangiogram the day after they are admitted; we generally need to follow this fast-track diagnostic approach since the majority of patients are referred relatively late (Table I), and we usually do not have the liberty of losing another day of valuable time with work-up. In cases where BA is detected during laparoscopy (and cholangiogram), the surgery is completed with KPE if found rational, considering the status of the liver. Extended portal hilar dissection as described in the literature (9) is the preferred method for KPE. Liver biopsy is taken from patients with evident cirrhotic liver (presence of distinctive nodules on the surface with granular appearance, stiffness of the liver) and is sent to frozen section pathological examination during laparoscopy, and those who are reported to display histopathological findings of evident cirrhosis who would not benefit from the KPE are considered for primary LTx, and the procedure is terminated.

Patients are followed in close collaboration with pediatric gastroenterology. Direct/indirect bilirubin levels, gamma-glutamyl transpeptidase (GGT) and liver function tests are checked weekly for the first post-KPE month, and monthly after the first month or as required depending on the clinical progress. Patients who develop liver failure, persistent ascites, portal hypertension, gastrointestinal bleeding, hepatopulmonary syndrome, failure to thrive or pruritis during the follow-up are referred to the LTx program, of which the senior author is also part of the transplant team.

For this study, the hospital records of those patients who were operated for BA between January 2005 and December 2018 were reviewed retrospectively. Demographics, days of admission, age at KPE and surgical findings were detailed. The patients were then divided into two groups; the first group consisted of patients surviving with their native liver (KPE-NLS) after KPE and the second group included those necessitating transplantation (KPE-nLTx) during the follow-up. The two groups were compared according to age at KPE, bilirubin and GGT levels at postoperative 7th day, 1st and 3rd month to determine and compare the success of KPE, as suggested in other studies (10,11).

Statistical Analysis

All statistical analyses were performed using Statistical Package for Social Sciences version 21.0 software for Windows (IBM SPSS Statistics for Windows, Version 21.0.

Table I. Age (days), bilirubin and GGT levels at diagnostic laparoscopy/cholangiography for the two groups

	KPE (38 patients)	Primary LTx (15 patients)	p-value
Age at laparoscopy/cholangiography (days)	53.36±26.49	94.2±26.41	<0.00001
Total bilirubin (mg/dL)	8.60 (7.02-10.05)	8.60 (7.55-12.57)	0.354
Conjugated bilirubin (mg/dL)	6.70 (4.60-8.25)	7.25 (6.17-9.0)	0.424
GGT (U/L)	444.0 (232.5-913.0)	524.5 (212.25-1033.25)	0.990

GGT: Gamma-glutamyl transpeptidase, KPE: Kasai portoenterostomy, LTx: Liver transplantation

Armonk, NY: IBM Corp., USA). The patients' characteristics and clinical parameters were assessed for normality with "Kolmogorov-Smirnov" and "Shapiro-Wilk" tests. Independent "t-test" was used for samples with normal distribution and samples following non-normal distribution were analyzed with "Mann-Whitney U" test. Logistic regression analysis was used to determine predictive factors for NLS. As a result of logistic regression analysis, "receiving operating characteristic" (ROC) curve analysis was used to determine whether the variables had a diagnostic value. Survival curves and tables were constructed for the survival analysis using the "Kaplan-Meier" method. The effect of variables on survival included in the study was examined by "Cox regression" analysis; a "p" value <0.05 was considered significant.

Results

Fifty-three patients operated with for BA were included in this study. Mean age at presentation was 64.9±32.1 (1-176) days. Diagnosis of BA was confirmed with laparoscopy and intraoperative cholangiography for all patients. Fifteen patients with a mean age of 94.2±26.41 (64-176) days at operation who had histopathologically confirmed evident cirrhotic liver during diagnostic laparoscopy did not receive KPE and they were referred to the LTx program for primary LTx. Thirty-eight patients with a mean age of 53.36±26.49 days at operation underwent KPE during the same session. Those patients who were not suitable for KPE had a significantly later presentation when compared to those who received KPE (p<0.00001). Preoperative total/conjugated bilirubin, and GGT levels were statistically similar between the two groups (Table I).

Twenty-three of the 38 patients with portoenterostomy survived with their native livers (KPE-NLS) and the remaining 15 patients required LTx (KPE-nLTx) during their follow-up. Time to LTx following non-functioning KPE was 13.5 (3.9-43.5) months. Three-year NLS was 65.8% and >5-year survival rate was 60.5%. None of the remaining 23 patients with NLS required LTx to date of writing (Figure 1).

In order to determine the effect of native liver survival, portoenterostomy days were assessed for the two groups. Mean age at operation was 54.43±24.64 days for the KPE-NLS patients, and 68.33±24.35 days for the KPE-nLTx patients. Although the mean age at operation was higher

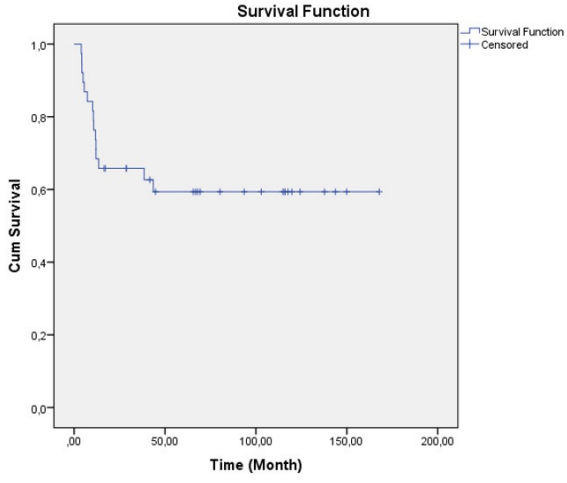

Figure 1. Native liver survival function after Kasai portoenterostomy

Table II. Total bilirubin, conjugated bilirubin and GGT levels of the two groups after Kasai portoenterostomy

	KPE-NL	KPE-nLTx	p-value
7th day			
Total bilirubin (mg/dL)	7.60±2.53	9.28±2.75	0.086
Conjugated bilirubin (mg/dL)	5.90 (4.70-6.90)	7.35 (4.52-9.72)	0.213
GGT (U/L)	635.0 (314.75-1171.5)	1099 (213.75-1695.75)	0.660
1st month			
Total bilirubin (mg/dL)	3.86±3.25	8.39±5.41	0.007
Conjugated bilirubin (mg/dL)	2.0 (0.57-3.62)	5.40 (3.22-9.12)	0.032
GGT (U/L)	530.5 (291.25-1050.5)	1029.0 (380.0-2126.25)	0.182
3rd month			
Total bilirubin (mg/dL)	0.65 (0.40-4.95)	8.35 (2.05-22.85)	0.003
Conjugated bilirubin (mg/dL)	0.55 (0.20-4.05)	6.45 (1.20-16.72)	0.001
GGT (U/L)	288.5 (184.75-492.5)	157.0 (57.0-551.25)	0.305

KPE: Kasai portoenterostomy, NL: Native liver, GGT: Gamma-glutamyl transpeptidase

Table III. Univariate logistic regression analysis to predict the need for liver transplantation after Kasai portoenterostomy		
	Odds ratio (95 CI)	**p-value**
Age at operation	1.025 (0.995-1.056)	0.105
Total bilirubin (preoperative)	0.917 (0.686-1.226)	0.559
Total bilirubin (7th day)	1.305 (0.956-1.782)	0.093
Total bilirubin (1st month)	**1.299 (1.038-1.626)**	**0.022**
Total bilirubin (3rd month)	**1.185 (1.021-1.375)**	**0.026**
Conjugated bilirubin (preoperatively)	0.881 (0.672-1.156)	0.362
Conjugated bilirubin (7th day)	1.343 (0.934-1.932)	0.112
Conjugated bilirubin (1st month)	**1.377(1.024-1.852)**	**0.034**
Conjugated bilirubin (3rd month)	**1.202 (1.031-1.403)**	**0.019**
CI: Confidence interval		

in the KPE-nLTx group, there was no statistically significant difference between the two groups. When the groups were compared according to bilirubin levels in the postoperative period, "total/conjugated bilirubin" levels were similar at postoperative 7th day; however, 1st and 3rd month levels were found to be significantly higher in those patients who required LTx after KPE (Table II). There were no statistically significant differences for postoperative GGT levels in both groups.

Predictive factors for the need for LTx after KPE were evaluated by univariate logistic regression analysis. Total/conjugated bilirubin levels in postoperative 1st and 3rd months were determined to be the most important predictive factors for LTx after KPE (Table III). It was also found that a "1-unit increase" in total bilirubin level at postoperative 1st month increased the risk of LTx by 1.299-fold [odds ratio (OR); 1.299, confidence interval (CI): 1.038-1.626, p<0.05], and also a "1-unit increase" in total bilirubin level in the postoperative 3rd month increased this risk by 1.185-fold (OR: 1.185, CI: 1.021-1.375, p<0.05) (Table III). Age at KPE and bilirubin levels on the 7th postoperative day were not predictive factors for determining the necessity of LTx. As a result of logistic regression analysis, postoperative 1st and 3rd month total and conjugated bilirubin levels were reliable predictive factors for LTx after portoenterostomy.

ROC curve analysis was carried out using these variables to define a cut-off value to determine the requirement for LTx after KPE. ROC curve analysis revealed that "total bilirubin level with a cut-off value of more than 5.70 mg/dL at the 1st postoperative month" was an important indicator (AUC: 0.787, %CI: 0.610-0.965, p<0.01) for future LTx with a sensitivity of 83.3% and specificity of 78.9% respectively (Figure 2). Cox regression analysis has also shown that a one-unit increase in total bilirubin levels at the 1st postoperative month increased the risk of LTx 2.172-fold (95% CI: 1.033-4.566, p<0.05).

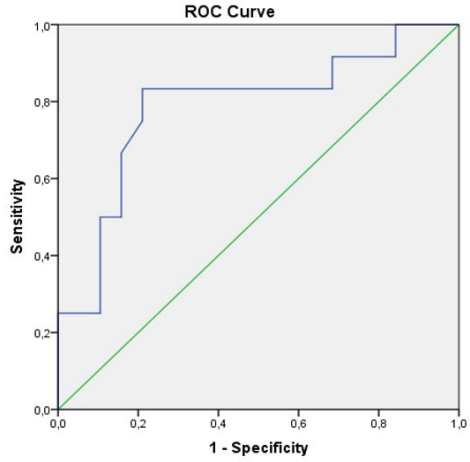

Figure 2. ROC curve analysis to define the cut-off value of 1st month bilirubin for determining the possibility of liver transplantation following Kasai portoenterostomy
ROC: Receiving operating characteristic

Discussion

BA in the 21st century still remains "full-of-unknowns". Although significant progress has been achieved in the effort to elucidate the pathogenesis of this anomaly, KPE is the first surgical option for patients born with BA ever since its introduction in 1955 (3,9,12). On the other hand, BA is the most common indication for LTx in childhood as the success of KPE is limited and depends on many factors. Age at presentation and surgery are considered and have been shown to be two of the most important determinants (4,13). Similarly, the phenotype of the anomaly (i.e., the type of atresia), BASM, an association of Cytomegalovirus infection

and the experience of the surgeon all play an important role in native liver survival after KPE (5,9). Nevertheless, aiming to achieve and prolong native liver survival, KPE remains a bridging operation to LTx for the majority of BA patients. Despite a successful KPE, at least two-thirds of patients, if not all, will require LTx at some point (5,14,15). This has led the researchers try to identify factors that may determine and answer the question of which patient will deteriorate to liver failure and consequently LTx and which will actually achieve native liver survival.

Age at KPE is an important factor thought to determine the success of surgery in the literature (5,13). Schreiber et al. (13) divided their patients into three groups according to KPE age as follows; operated before 30 days, between 31-90 days and after 90 days, and they showed that the 10-year native liver survival rates were 49%, 25% and 15% respectively suggesting that the earlier the KPE, the better the success of surgery (13). In the series by Ferreira et al. (16) analyzing their 117 patients, they have found that the only variable significantly associated with failed biliary drainage was surgery beyond 90 days of age. When they looked at the survival analysis of their patients, absence of biliary flow ($p<0.0001$), age at surgery >90 days ($p=0.035$), and the presence of BASM ($p<0.0001$) alone could predict death or the need for LTx (16).

On the other hand, there are also counterviews in the literature stating that age at KPE is not an indicator for native liver survival (8,17). Quithsi et al. (17) were not able to show any correlation between age at portoenterostomy and survival in their series of 29 BA patients. Ramachandran et al. (8), in their series of 62 infants with BA, operated on 17 patients presenting later than 90 days of age, and showed that one-third of those patients benefitted from KPE concluding that age was not a criterion for the success of the Kasai procedure. Ihn et al. (18), in their large series of 214 patients over 29 years, analyzed their patients in two eras (before and after 2006), and came to the conclusion that the impact of age at the time of KPE on operative outcomes became less significant over time with the increase in the single surgeon's experience and improvement in medical treatment for BA (18).

The findings in our series were also similar to those concluding that age did not matter. However, we believe that the issue of age should be carefully interpreted. The fact that patients presenting relatively late (>90 days) may also benefit from hepatic portoenterostomy does not necessarily mean that all those patients, regardless of the condition of their liver, should receive KPE. The condition of the liver may vary widely; the damage to liver is not the same for the same given age in all patients. Therefore, a patient with a liver that is lobulated, nodular and quite stiff at laparoscopic exploration with confirmed evident cirrhosis on histopathology is hardly likely to benefit from KPE. Nevertheless, a liver that is relatively soft and less nodular in the absence of cirrhosis should definitely be considered for KPE even if the patient is older than 90 days of age. In our series, 38 patients underwent KPE regardless of their ages and 15 patients were directly programmed for primary LTx without portoenterostomy due to evident cirrhosis on histopathological findings during laparoscopic exploration.

The aim of the portoenterostomy is to ensure the biliary drainage and to delay/prevent biliary cirrhosis. Therefore, clearance of jaundice is an important indicator for the success of surgery.

Correspondingly, in our series, those who had a failed KPE and clearance of jaundice required LTx within a period of 13.5 months, and those who had successful clearance of jaundice had a >5-year survival rate of 60.5%. When we further aimed to identify which parameter would be an indicator of NLS or the need for LTx in the future, total and conjugated bilirubin levels in postoperative 1st and 3rd months were determined to be reliable indicators suggesting a clear association between the clearance of jaundice and NLS. Conjugated bilirubin level of 2.0 mg/dL at the 1st month, and normal bilirubin values attained at the 3rd month ensured a 60.5% rate of >5-year NLS. On the other hand, each "1-unit rise" (in mg/dL) in total bilirubin levels increased the risk of LTx by 1.2-fold. A cut-off value of 5.70 mg/dL in total bilirubin at the 1st month of surgery was calculated to be an important indicator for future LTx with a sensitivity of 83.3% and specificity of 78.9% respectively. Huang et al. (11) emphasized that total bilirubin level <4.85 mg/dL at postoperative 1st week was an important predictive factor for NLS. In another study by Khanna et al. (19), the ratio of postoperative 7th day total bilirubin to its preoperative value was found to be reliable predictor for biliary atresia outcome. In our series, 7th day bilirubin levels did not seem to have a predictive effect on outcome. Similarly, Noor et al. (20) also evaluated bilirubin, GGT, and alanine aminotransferase levels at postoperative 7th day of KPE, and no clear association was found between these parameters and survival (20). Rodeck et al. (10) reported that a cut-off bilirubin concentration of 57 µmol/L (3.33 mg/dL) at postoperative 6th week was a predictor for event free survival with a sensitivity of 80% and specificity of 78.6% (10), and this finding was quite similar to ours suggesting that bilirubin levels in postoperative follow-

up, especially at the 1st month, is a consistent parameter to predict native liver survival. Some recent studies have also looked into other biochemical parameters, such as liver function tests and GGT etc., as predictive parameters (20-22). However, in our patient series, we were not able to demonstrate a correlation between serum GGT levels and long-term outcome.

Conclusion

KPE still remains the one and only surgical option in the treatment of patients born with BA. However, controversy still exists as to how patients are best managed and the predictors of its outcome. Although the total number of patients in our series seems to be a limitation, we attained conclusive significant results concerning these debatable issues including age at KPE, postoperative prognostic factors determining the native liver survival after KPE and LTx in the management of BA. Our series has shown that bilirubin levels at postoperative 1st and 3rd months can predict native liver survival after KPE or the need for LTx regardless of age at surgery. We believe these parameters can be used as reliable predictors for the outcomes of patients after KPE.

Ethics

Ethics Committee Approval: It was approved by the Institutional Review Board and Research Ethical Committee in accordance with international ethical standards and the World Health Organization Helsinki Declaration (approval no: 20-8T/13).

Informed Consent: All admissions, surgical procedures were performed after informed consent of the family/parents/caregivers.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Data Collection or Processing: Ü.Ç., G.S., M.K., Analysis or Interpretation: Ü.Ç., Critical Review: M.O.E., Writing: M.O.E.

Conflict of Interest: No conflict of interest was declared by the authors.

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Original Article

Endoscopic Management of Complicated High-grade Vesicoureteral Reflux in the First Year of Life

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ABSTRACT

Aim: The treatment of vesicoureteral reflux (VUR) in infants is controversial. Subureteric injection is considered by some to be a popular alternative to long-term antibiotic prophylaxis. In this study, we reviewed our experience in endoscopic subureteric injection to correct high-grade reflux in infants with documented indications for antireflux surgery.

Materials and Methods: The hospital records of patients with grade 4-5 VUR and breakthrough urinary tract infections who had undergone endoscopic subureteric injection in the first year of life between 2009 and 2016 were reviewed retrospectively. Radiologic success was defined as complete resolution of reflux determined via voiding cystourethrogram obtained at least three months after the injection, and clinical success was defined as the downgrading of reflux grade to below three and the absence of urinary infection.

Results: A total of 23 patients (5 girls, 18 boys) with 34 high-grade refluxing units were included in this study. The mean age at first injection was 6.3±1.8 months (1-11 months). The radiologic success rate with initial injection was 61.7%, and it was 85.2% after repeated injections. The overall clinical success rate after first injection was 70.6% and 97.1% after repeated injections. The mean injected material volume was 0.34±0.27 (0.1-1) mL per ureter.

Conclusion: The management of high-grade infantile reflux is still controversial with insufficient data. Published studies comparing endoscopic treatment and antibiotic prophylaxis have inconclusive results due to their wide range of success rates. Although it needs to be supported by prospective studies, endoscopic treatment is a successful alternative in high-grade VUR infants with breakthrough infection.

Keywords: Vesicoureteral reflux, infant, endoscopic reflux treatment, subureteric injection, paediatric

Introduction

The management of vesicoureteral reflux (VUR) in infants, whether diagnosed with antenatal hydronephrosis or urinary infections, remains controversial. In this specific group, there are factors that complicate decision making, such as the demanding features of antireflux surgery in small babies or the possibility of spontaneous resolution even in high-grade reflux (1-3). Subureteric injection for VUR gained worldwide popularity for its easy application and short hospital stay with superior patient comfort in children, including infants (4-6). In this study, we reviewed our experience in order to evaluate the efficacy and safety of endoscopic subureteric injection to correct high-grade reflux in infants with documented indications for antireflux surgery.

Materials and Methods

The hospital records of those patients with grade 4-5 VUR and breakthrough urinary tract infections (UTIs) who had undergone endoscopic subureteric injection in the

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first year of life between 2009 and 2016 were reviewed retrospectively. We defined breakthrough UTI as a UTI with high fever (>38 °C) and with documented catheter collected urine culture positivity that occurred during a course of antibiotic prophylaxis. Data including patient demographics, injected material volumes, VUR grades according to their preoperative and postoperative voiding cystourethrograms (VCUG), the circumcision status of the boys, and the success rates of the treatment were recorded. Reflux was classified according to the International Reflux Study Committee's Classification System. The procedure was performed under general anesthesia using a 9.5 Fr 6° cystoscope (Storz, Tuttlingen Germany). Polyacrylate polyalcohol copolymer (PPC) (Vantris®, Promedon, Argentina) was administered submucosally at the 6 o'clock position of the ureteral orifice, until the creation of a prominent bulge, by use of a Williams cystoscopic injection needle (Cook Medical®, Bloomington, USA). Radiologic success was defined as complete resolution of reflux determined via VCUG obtained at least three months after the injection, and clinical success was defined as the downgrading of reflux degree to below three and absence of urinary infection. The injection was repeated if persistent reflux above grade 2 was documented. Ultrasonography was performed at the postoperative first, third, and sixth months, and then annually for the follow-up of obstructive findings such as new onset or increased hydroureteronephrosis. This study was approved by the Ege University Medical Research Ethics Committee under the number 20-1T/47. Written informed consent was obtained from all parents of the patients.

Statistical Analysis

The chi-square test and t-test were used for statistical analyses with IBM SPSS 23.0.

Results

A total of 23 patients (5 girls, 18 boys) with 34 high-grade refluxing units were included in this study. The mean age at first injection was 6.3±1.8 months (1-11 months). Four patients had contralateral low or moderate grade (1-3) reflux. There was grade 5 reflux in 27 units and grade 4 reflux in 7 units. Eleven patients had bilateral high-grade refluxing units, and 9 of them were bilateral grade 5. The mean duration of follow-up was 69.53±24.65 months. Continuous antibiotic prophylaxis (CAP) was started with amoxicillin for babies under three months of age, and with co-trimoxazole after this period. Ten patients who were under prophylaxis had confirmed breakthrough UTI and underwent endoscopic injection after their first breakthrough infection. In 13 patients, prophylaxis was changed to co-trimoxazole or cefixime and they were kept under observation due to the questionable compliance of their families to CAP regimens. The mean time interval from surgery to first postoperative VCUG was 5.63±0.90 months. The radiologic success rate with initial injection was 61.7%, and it was 85.2% after repeated injections. Three refluxing units at first injection and one refluxing unit at second injection were downgraded to grade 2 and grade 1. Including these patients, the overall clinical success rate at first injection was 70.6% and 97.1% after repeated injections. The mean injected material volume was 0.34±0.27 (0.1-1) mL

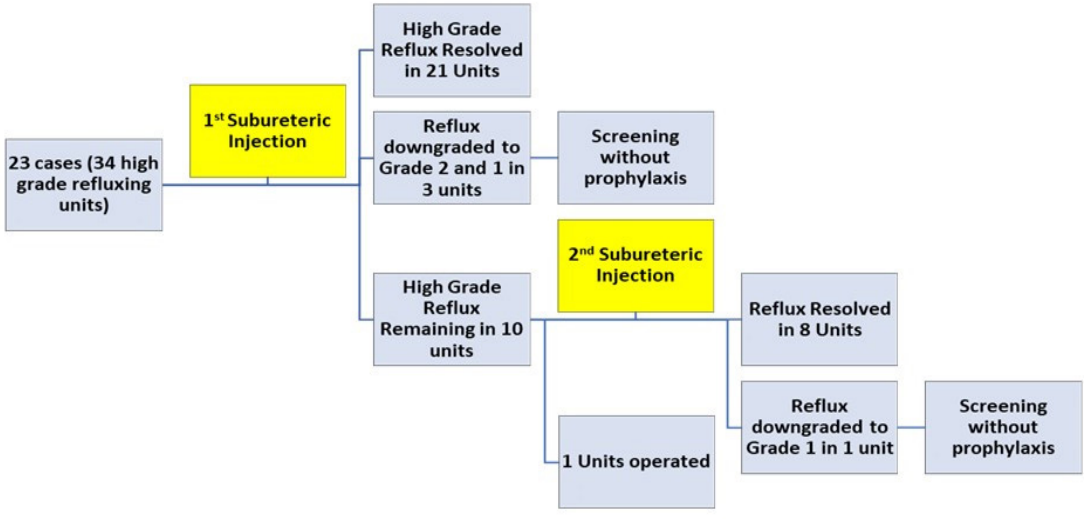

Figure 1. Flow chart of the overall management

per ureter. There were no significant correlations between the volume of injected material and the degree of reflux, the clinical success rate or the radiological success rate (t-test, p>0.05). A flowchart depicting the steps of overall management for the study group is summarized in Figure 1.

Eleven refluxing units of 9 infants with bilateral grade 5 reflux were successfully treated at the first injection, while 6 of them required a second attempt. The overall radiologic success rate for bilateral grade 5 refluxing units was 83.3%, and the clinical success rate was 100%. In one patient whose reflux downgraded from 5 to 2, antibiotic prophylaxis was terminated with no subsequent UTI.

Most of the boys (16/18) were uncircumcised, and 10 of them were circumcised during the general anesthesia given for endoscopic injection treatment. Those children of parents who did not accept circumcision and one patient with megameatus were not circumcised. When the patients were divided into two groups according to their circumcision status, there was no statistical difference in the clinical or radiological success rates between the groups (chi-square, p>0.05).

Eventually, only one patient required surgical correction for persistent VUR. In this case, ureteroneocystostomy was performed in line with the family's choice due to successive febrile urinary tract infections. During the open surgery, a fibrous capsule surrounding the substance was noted and successfully removed. This did not complicate ureteric dissection. No sign of obstruction, such as increased hydroureteronephrosis, was observed in any patients in a mean follow-up period of five years.

Discussion

Controversy regarding the management of VUR in the first year of life continues. The majority of VUR diagnosed in this group belong to higher grades (7). Increased risk of new scar formation in dysplastic kidneys and breakthrough infections during follow-up are strongly related to higher reflux grades (8). However, the rate of spontaneous resolution in this age group, even in high-grade reflux cases is impressive (9). Ureteroneocystostomy is a demanding surgery in infancy with an increased risk of complications due to small anatomy and fragile mucosa of the bladder. However, for a small unique group of infants with breakthrough infections as in our study group, antireflux procedures may be indicated in certain conditions like urosepsis attacks, breakthrough infections, and severe kidney damage (10). The reported series of endoscopic treatments were not promising except for a few studies (5,11). We found that the success rate in our study is higher than those of most studies that published their endoscopic injection experience in infant high-grade reflux. This promising result was the major factor that motivated us to reassess the place of endoscopic treatment in this difficult group of patients.

Several studies draw attention to a higher risk of recurrent and complicated UTI with resistant microorganisms under prophylaxis (12-14). Garin et al. (12) presented resistant bacteria in all cases of recurrent pyelonephritis with VUR under prophylactic antibiotic treatment. In a randomized controlled study, Hari et al. (13) showed similar results. In 2008, Pennesi et al. (14) reported that prophylaxis had no effect on infection and renal damage but caused recurrent UTI by resistant microorganisms in a case-control study. In a Swedish reflux trial, the trimethoprim resistant infection rate in girls under prophylaxis was higher than the endoscopic treatment and surveillance groups (15). Although the RIVUR study has shown that prophylactic antibiotics reduce the risk of UTI recurrence, the probability of resistant UTI has increased significantly (16). Following successful endoscopic treatment, we did not observe any febrile UTI except one case who eventually required ureteroneocystostomy, and resistant bacterial infection was not documented.

In 2010, a prospective study in children between 1 and 2 years of age who had grade 3 or 4 VUR was conducted by the Swedish reflux group (17). Three groups including endoscopic treatment, antibiotic prophylaxis, and follow-up without treatment were compared in terms of resolution of reflux, UTI, and renal scar development. The resolution rate was higher in the endoscopic treatment group than the two other groups (18). Their study showed a success rate of 71% with endoscopic treatment, excluding grade 5 cases. Another branch of their study showed no difference in UTI frequency after endoscopic treatment; however, the difference between successful and unsuccessful cases was not stated (15). The same group published another prospective study in 2016, including infants with grade 4 and 5 refluxes (19). Endoscopic treatment and prophylaxis groups were compared, and reflux resolution was 59% in endoscopic treatment, and 21% in the prophylaxis group after 1-year follow-up. The success rate after endoscopic treatment was 31% in bilateral grade 5 reflux cases. They revealed no statistical difference between the endoscopic treatment and antibiotic prophylaxis groups regarding recurrent UTI and new renal scar formation (19). They also found that multiple recurrent infections were only seen in patients with persistent dilating reflux in follow-up. Our reflux resolution rates, including grade 5 reflux cases

after first and repeat injections, were 63.1% and 89.4% respectively, which were higher than similar studies in the literature. In addition, we achieved an unexpectedly high success rate of 85% in bilateral grade 5 refluxes in a group with previously documented poor results with endoscopic treatment (6). None of the cases in our series had UTI in the follow-up period.

Another study which published similar results to our study in infants with moderate and severe VUR, but with a smaller group of Grade 5 reflux (3.6%), showed a minor recurrent infection rate of 1% (11). We may speculate that the relatively low success rates of endoscopic treatment in two Swedish studies were the reason why they could not find significant difference between their prophylaxis and endoscopic treatment groups regarding urinary infection and new scar formation rates (6,18). Thus, with higher success rates, endoscopic treatment could be superior to prophylaxis not only for reflux resolution but also for the prevention of recurrent UTI and new scar formation.

Long-term reflux recurrence is another issue to be considered following endoscopic treatment, which is reported to be between 13.4% and 19% within 2 years. Since our current protocol does not include routine VCUG in long term follow-up except for cases with febrile UTI, we cannot give an overall long-term recurrence rate for this study. However, a recent review of our protocol revealed a 0.9% long-term VUR recurrence in cases with recurrent febrile UTI that we obtained a VCUG for (20).

Our previous series of endoscopic VUR treatment in children of all ages showed a ureteric obstruction rate of 2.8% (21). Despite being a smaller group, this study, with similar technique and material, revealed no obstruction in a mean follow-up period of 5.7 years.

PPC is a relatively new material with particle size larger than most other materials (22-24). Our successful result is probably related to the material, which has a non-biodegradable feature.

Study Limitations

The limitations of our study are its retrospective nature, the lack of a control group and the low number of cases. However, the high success rate in this special age group with severe reflux is noteworthy. Another issue to mention is that although the parents were informed and aware of febrile urinary tract infections, there may have been infections treated elsewhere which were not reported to us. Prospective randomized studies are required to confirm our results.

Another limitation of our study was the difficulty of excluding the possible protective effect of circumcision due to the small number of patients. Elective circumcision "might be", but so far has not proven to be a better first-line treatment in this specific, high-risk patient group. In the study by Alsaywid et al. (25), the rate of infection after the circumcision was lower than before circumcision. However, a multivariate analysis evaluating other factors that could contribute to this, such as age and the usage of prophylaxis, was missing in that study. Another study by Braga et al. (26) also states that uncircumcised status is a risk factor for febrile UTI in babies with antenatal hydronephrosis. However, they do not make any comment on circumcision as a first-line treatment for breakthrough UTI. On the other hand, in the study of Herz et al. (27), circumcision status did not affect UTI incidence in infants with antenatal hydronephrosis. Also, circumcision did not have any effect on reflux resolution.

Conclusion

The management of high-grade infantile reflux is still controversial with insufficient data. Published studies comparing endoscopic treatment and antibiotic prophylaxis have inconclusive results due to their wide range of success rates. Endoscopic treatment is a successful alternative in infants with high-grade VUR suffering breakthrough infections. Prospective randomized studies with larger numbers may support our findings.

Ethics

Ethics Committee Approval: This study was approved by the Ege University Medical Research Ethics Committee under the number 20-1T/47.

Informed Consent: Written informed consent was obtained from all parents of the patients.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Design: A.T., S.T., A.A., İ.U., Data Collection or Processing: A.T., İ.Y., Ö.K., Analysis or Interpretation: S.T., A.A., İ.U., Writing: A.T., S.T., İ.Y., Ö.K., A.A., İ.U.

Conflict of Interest: No conflict of interest was declared by the authors.

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Case Report

Imaging Diagnosis of Anomalous Total Coronary Artery From the Pulmonary Artery: Case Report

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ABSTRACT

Anomalous total coronary artery from the pulmonary artery is an extremely rare congenital coronary artery malformation. Only a few cases have been reported over the years, but with no comprehensive medical imaging data. We present the imaging findings of this case with trans-thoracic echocardiography, trans-esophageal echocardiography, computed tomography angiography (CTA) and cardiac catheterization.

Keywords: Anomalous total coronary artery from the pulmonary artery, echocardiography, CTA, cardiac catheterization

Introduction

Anomalous total coronary artery from the pulmonary artery (ATCAPA) is an extremely rare but lethal congenital coronary artery malformation (1,2). The patient's right coronary artery (RCA) and left coronary artery (LCA) have a common trunk that originated from the pulmonary artery. This could lead to myocardial ischemia and eventually death due to heart failure. Since the disease is rarely reported, the pathogenesis is unclear to date. This paper reports a detailed imaging diagnosis of ATCAPA as well as surgical treatment verification.

Case Report

A 6-month-old boy was hospitalized with symptoms of polypnea and cough. He had been treated at a local hospital 5 months previously for the neonatal pneumonia, and underwent trans-thoracic echocardiography (TTE) at the local hospital before being admitted to our clinic. Tertiary systolic murmur could be heard in the precardiac region on physical examination. The electrocardiogram showed right deviation of the electric axis, enl argement of the left ventricular and atrium, and abnormality of ST-segment (Figure 1). The BNP was up to 20,971.5 pg/m.

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TTE showed spherical enlargement of the left ventricular, enlarged left atrium, and diffused incrassation of the endocardium (4.3 mm). The mitral valve chordae tendineae and papillary muscle had become hyperechoic with severe mitral regurgitation and 40.3 percent-ejection fraction. The foramen oval was patent. There was no coronary artery arising from the right coronary sinus. A vessel originated from the right lateral wall of the main pulmonary artery trunk and tracked backward and downwards to the side of the left coronary sinus with no distinct boundary (Figure 2). It seemed the LCA and RCA arose from here. Three branches originated from proximal LCA. The RCA tracked between the aortic and pulmonary artery trunk. The direction of the coronary arteries flow was from proximal to distal. Intraoperative transesophageal echocardiography (TEE) revealed a vessel originating from the right posterior lateral wall and was tracked downwards to the side of the left coronary sinus with an intumescentia, then branched LCA and RCA (Figure 2). The direction of flow was shown to be consistent with the TTE.

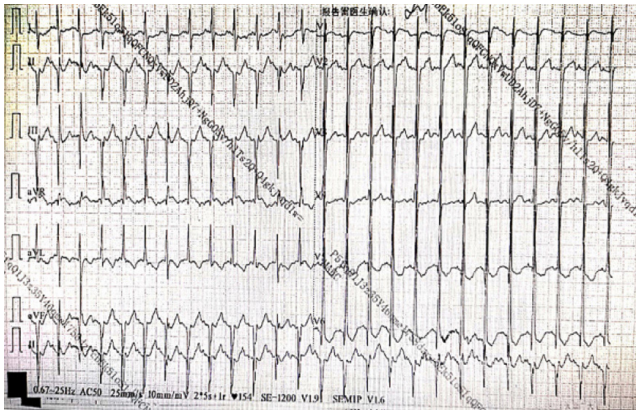

Figure 1. The preoperative ECG showing the right deviation of the electric axis, enlargement of the left ventricular and atrium, and abnormality of ST-segment
ECG: Electrocardiogram

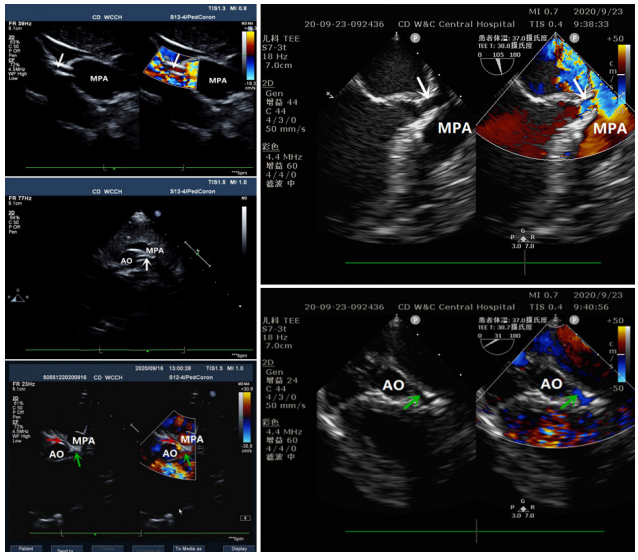

Figure 2. TTE and TEE showing a vessel (white arrow) originated from the right lateral wall of the main pulmonary artery (MPA) trunk, and tracked downwards close to the aorta (AO), then branched LCA (green arrow) and RCA (red arrow)
TTE: Trans-thoracic echocardiography, TEE: Transesophageal echocardiography, RCA: Right coronary artery, LCA: Left coronary artery

The cardiac computed tomography angiography (CTA) showed the RCA and LCA with a common trunk originating from the pulmonary artery, and three branches of the LCA were observed (Figure 3). Cardiac catheterization was also performed for accurate diagnosis and to detect existing collateral circulation or not. Ascending aortography showed no coronary arteries originating from the aorta. The pulmonary arteriography revealed the same as CTA (Figure 3), and the length of the common trunk of the RCA and LCA was 3.2 millimeters. No definite collateral circulation was detected by the previously mentioned TTE, TEE, CTA, or cardiac catheterization.

The above mentioned imaging diagnoses were verified by surgical operation (Figure 4). The abnormality of the left ventricular endocardium and mitral apparatus coincided with the TTE findings. Nevertheless, the mural coronary arteries of both the proximal LCA and RCA had not been observed by the imaging examinations, but only by surgical operation. The common trunk of the RCA and LCA was holistically transferred to the aorta.

Discussion

ATCAPA, with no definite pathogenesis and incidence, is an extremely rare pathogeny of infantile myocardial ischemia. It is rarer than anomalous left coronary artery from the pulmonary artery (ALCAPA) with an incidence rate

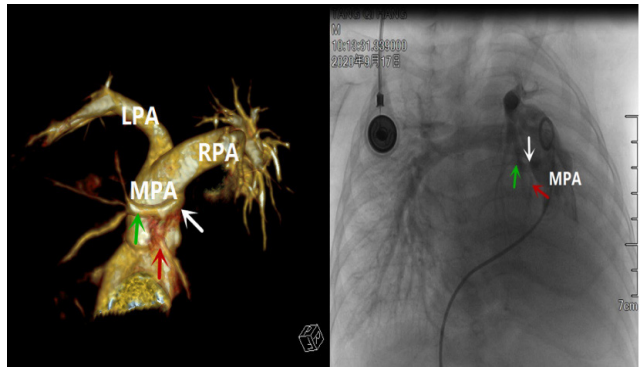

Figure 3. CTA and cardiac catheterization comforming the RCA (red arrow) and LCA (green arrow) with a common trunk (white arrow) originated from the main pulmonary artery (MPA)
CTA: Computed tomography angiography, RCA: Right coronary artery, LCA: Left coronary artery

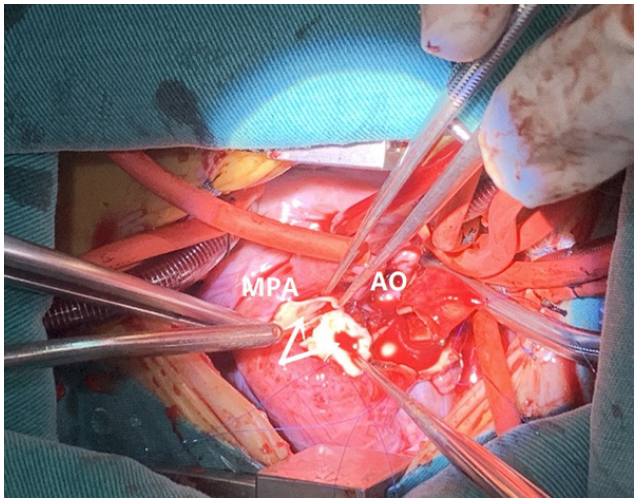

Figure 4. Tansfering the common trunk (white arrow) of RCA and LCA to the aorta by surgical operation
RCA: Right coronary artery, LCA: Left coronary artery

of 0.008% (3) and anomalous right coronary artery from the pulmonary artery (ARCAPA) with an incidence rate of 0.002% in the general population (2).

Those patients with ATCAPA have the co-pathogenic mechanism of ALCAPA and ARCAPA. The clinical symptoms of patients with ATCAPA are related to changes of relative pressure between systemic and pulmonary circulation and the level of coronary collateral circulation (4,5). The main manifestations of the child in this case study are similar to the clinical symptoms in ALCAPA patients. Myocardial ischemia is progressively aggravated with increasing age (6). Incredibly, this patient survived to 6 months of age with hypoxic coronary flow from the pulmonary artery and no certain coronary collateral circulation. There are three possible explanations for this: (a) This patient has some coronary collateral circulations that could not be detected by imaging examinations and cardiac catheterization, (b) the long-term myocardial ischemia and left ventricular diastolic dysfunction with severe mitral regurgitation led to elevated left atrial filling pressure and, additionally, the patient, with repeated pulmonary infection, may have had increased pulmonary vascular resistance, which could enable the flow of pulmonary artery pump into the coronary artery during the diastolic period, (c) the patient's immature myocardium was able to better tolerate hypoxia. Due to long-term myocardial ischemia, the echocardiographic findings of this patient show spherical enlargement of the left ventricular and diffused incrassated endocardium. It should be carefully distinguished from endocardial fibroelastosis and dilated cardiomyopathy, which usually have normal coronary artery and no obvious incrassated endocardium in patients with dilated cardiomyopathy.

The gold standard for imaging diagnosis of anomalous origin of the coronary artery from the pulmonary artery is cardiac catheterization, which can clearly show the location of the origin of the coronary artery and judges the coronary flow direction. The CTA scan is comparable to cardiac catheterization except that it cannot indicate flow direction. Echocardiography is the first choice for imaging examinations for infant heart disease. In this case, both coronary arteries originated from the right back of pulmonary artery, and went close to left coronary sinus. Additionally, it was found in the operation that both proximal of the left and RCAs are partially mural coronary arteries. Such a situation could easily lead to the misdiagnosis that the coronary artery originates from the aortic sinus, which should be distinguished from our case carefully. The coronary artery should be carefully examined when an obvious growth of left ventricular and myocardial ischemia are detected by echocardiography in infants. CTA and cardiac catheterization should be integrated in the diagnostic process if necessary. The integration of multiple imaging techniques is of great value in the prompt detection and diagnosis of coronary artery origin diseases, the selection of operation time, as well as an improvement in prognosis.

Ethics

Informed Consent: The patient's parents approved to use this case for publication.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: J.M., Design: R.M., J.M., Data Collection or Processing: R.M., Z.L., S.Y., Y.D., Y.Y., H.L., M.J., Analysis or Interpretation: R.M., Writing: R.M.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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Case Report

A Rare Cause of Reversible Splenial Lesion Syndrome (RESLES): Benign Convulsions with Mild Gastroenteritis

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ABSTRACT

Transient lesions involving the splenium of corpus callosum is defined as reversible splenial lesion syndrome (RESLES). Benign convulsions with mild gastroenteritis is a rare condition which may be associated with RESLES. Since the prognosis is excellent, the awareness of this association is important to prevent unnecessary investigations and anti-epileptic drug therapy.

Keywords: Splenium, corpus callosum, gastroenteritis, seizure, RESLES

Introduction

Reversible splenial lesion syndrome (RESLES) is a rare clinico-radiological entity characterized by a transient lesion in the splenium of corpus callosum (1). RESLES may result from various causes such as infections, metabolic derangements, high-altitude cerebral edema, seizures or antiepileptic drug withdrawal. In the paediatric population, the most common form of RESLES is mild encephalitis/encephalopathy with a reversible splenial lesion (MERS) which may occur during infectious diseases (2). In MERS, encephalopathy lasts longer than 12 hours and no evidence of inflammation is found in the cerebrospinal fluid. Disturbance of consciousness lasting less than 12 hours or an absence of altered mental status during the clinical course is defined as non-MERS form of RESLES (1).

Benign convulsions with mild gastroenteritis (CwG) was first described by Mooroka in 1982. It is characterized by; (1) occurrence in previously healthy infants or young children aged between 6 months and 3 years; (2) afebrile generalized convulsions sometimes seen in clusters related to gastroenteritis without moderate to severe dehydration; (3) normal laboratory examination including electrolytes, blood glucose and cerebrospinal fluid; (4) normal interictal electroencephalography (EEG); and (5) excellent seizure and developmental outcomes (3). Rotavirus, norovirus and other round-shaped viruses are the most common causative agents related to this entity (4).

Case Report

A previously healthy 42-month-old girl with diarrhoea and vomiting for two days was admitted to our hospital

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due to a brief afebrile generalized tonic-clonic seizure. Her past medical history and family history were unremarkable. On admission, she was alert and had normal physical and neurological examination. During the emergency room follow-up, she had another afebrile generalized tonic-clonic seizure with a duration of 3 minutes and rapidly regained consciousness. Laboratory studies including hemogram, liver and kidney function tests, uric acid, serum electrolytes and acute phase reactants were normal. Stool sample analysis revealed no pathogens. EEG was found to be normal. Cranial magnetic resonance imaging (MRI) revealed increased T2-signal intensity in the splenium of corpus callosum and hypo-intense signal on apparent diffusion coefficient images (Figure 1). She had no further seizures and was discharged on the third day of hospitalization with no antiepileptic drug treatment. The follow-up cranial MRI of the patient performed after 4 weeks showed complete resolution of the lesion (Figure 2). The patient is now seizure free for 6 months with a normal neurological examination and developmental milestones.

Discussion

In 2007, Natsume et al. (5) first described the association of RESLES and CwG in two paediatric cases. After this initial description, a few cases of different ethnic origins, mainly from Asia, were published (6-8) (Table I). In a multicentre study conducted by the Tokai Pediatric Neurology Society, the frequency of RESLES in CwG was presented as being 22% (9). Kato et al. (8) reported nine Japanese cases with a transient splenial lesion of corpus callosum occurring during rotavirus gastroenteritis. While eight of these cases presented with encephalopathy, and were classified as MERS, only one case had clinical features consistent with the non-MERS form of RESLES due to CwG. In another study among 233 patients with RESLES, five patients with no clinical manifestations of encephalitis such as delirium or an altered level of consciousness were diagnosed with RESLES secondary to CwG. Acute seizure treatment with anticonvulsive drugs such as diazepam or phenobarbital, anti-viral agents and rehydration was administrated if necessary in the acute period, but no long-term antiepileptic treatment was given (7). Similar to these cases in the literature, the present case had only two brief generalized seizures without any encephalopathic features during a gastrointestinal infection. Although there is no specific laboratory finding for the diagnosis of CwG, Yoo et al. (10) found that serum uric acid levels are significantly higher in CwG patients than in patients with acute gastroenteritis and febrile seizures. However, the uric acid level of the present case was found to be within the normal range. The presence of a splenial lesion detected on the cranial MRI performed for the recurrent afebrile seizures led to the diagnosis of non-MERS form of RESLES secondary to CwG. No long-term antiepileptic therapy was given. The complete resolution of the splenial lesion as seen in the follow-up MRI, normal neurological development and the absence of recurrent afebrile seizures confirmed the diagnosis.

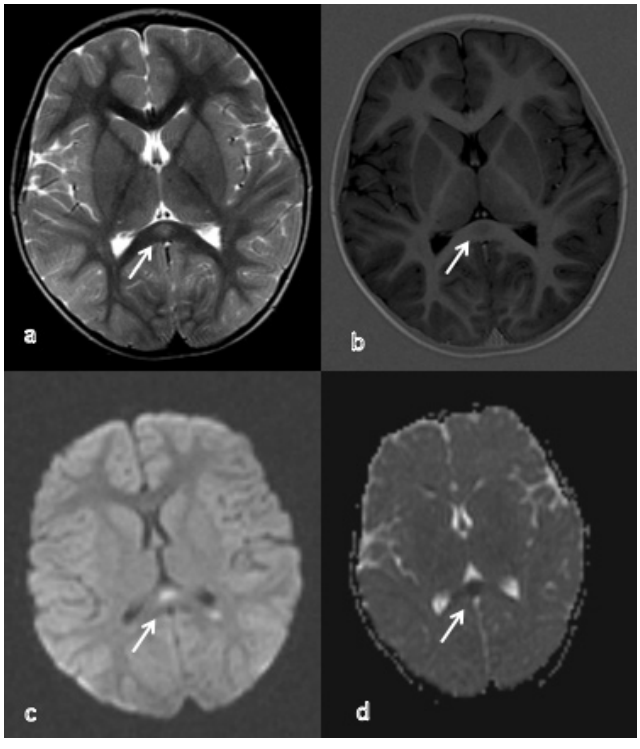

Figure 1. Cranial magnetic resonance images on admission demonstrating a splenial lesion of the corpus callosum. Axial T1-weighted (a), axial T2-weighted (b), diffusion-weighted (c), and the corresponding apparent diffusion coefficient map (d)

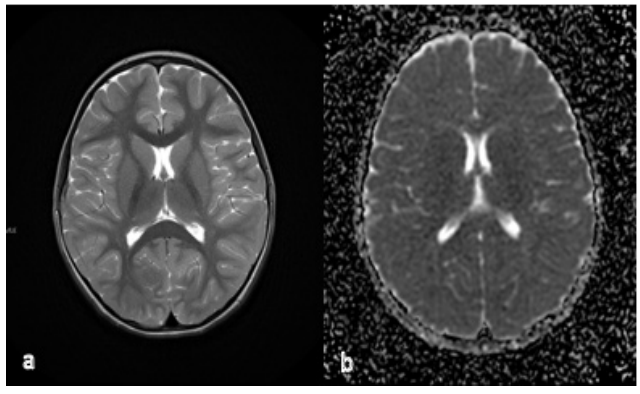

Figure 2. Follow-up cranial magnetic resonance images demonstrating the resolution of the splenial lesion. Axial T2-weighted (a) and apparent diffusion coefficient map (b)

Table I. Data of the published cases with non-MERS form of RESLES secondary to CwG

Publication	Number of patients	Age-months	Symptoms	Etiology	EEG	Treatment	Day of normal follow-up MRI/Outcome
Natsume et al. (5) 2007	2	24 36	5 episodes of focal seizures 2 episodes of generalized seizures	Rotavirus -	Normal	Diazepam, phenobarbital -	9/good 21/good
Kato et al. (8) 2009	1	12	3 episodes of tonic seizures	Rotavirus	Intermittent spikes, occipital slow wave	Diazepam	6/good
Jang and Lee (6)2010	1	30	2 episodes of generalized tonic-clonic seizures	Rotavirus	Normal	Rehydration, empiric antibiotics and acyclovir	6/good
Jiang et al. (7)2014	5	36 14 28 30 24	4 episodes of generalized tonic-clonic seizures 3 episodes of generalized tonic-clonic seizures 5 episodes of generalized tonic-clonic seizures 2 episodes of generalized tonic-clonic seizures 4 episodes of generalized tonic-clonic seizures	- - - - Rotavirus	Normal Normal Normal Normal Occipital slow wave	Rehydration, ribavirin, diazepam Rehydration, ribavirin, phenobarbital Rehydration, ribavirin, phenobarbital Rehydration, ribavirin, diazepam Rehydration, ribavirin, diazepam, phenobarbital	11/good 10/good 15/good 10/good 12/good
Presented case	1	42	2 episodes of generalized tonic-clonic seizures	-	Normal	Rehydration	30/good

MERS: Mild encephalitis/encephalopathy with a reversible splenial lesion, CwG: Convulsions with mild gastroenteritis, RESLES: Reversible splenial lesion syndrome, EEG: Electroencephalography, MRI: Magnetic resonance imaging

Conclusion

In young children with recurrent afebrile seizures and gastroenteritis without fever, dehydration and ion imbalance, the association of RESLES and CwG should be kept in mind. Since the prognosis is excellent with no permanent neurological sequelae, awareness of this rare clinico-radiological entity is important in order to avoid unnecessary investigations and long term anti-epileptic drug therapy.

Ethics

Informed Consent: The consent form was received from the parents of the patient.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: N.U., A.D., S.K., S.Y., Design: N.U., A.D., S.K., S.Y., Data Collection or Processing: N.U., A.D., S.K., S.Y., Analysis or Interpretation: N.U., A.D., S.K., S.Y., Literature Search: N.U., A.D., S.K., S.Y., Writing: N.U., A.D., S.K., S.Y.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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Case Report

Pediatric COVID-19 Associated Rhabdomyolysis

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ABSTRACT

The incidence of rhabdomyolysis secondary to various causes has been reported, especially for viral infections such as influenzas. It is well established that Coronavirus disease-2019 (COVID-19) can include a large array of symptoms during the disease course, but there have been few reports of COVID-19-related rhabdomyolysis. We report a 10-year-old boy who presented with fever, some dry cough, myalgias, and painful walking. His COVID-19 polymerase chain reaction test performed through the nasopharyngeal swab was positive. His first creatinine kinase level was 8,000 U/L. He was treated with isotonic intravenous fluids because of rhabdomyolysis. Muscle weakness and pain are common symptoms of the 2019 novel COVID, but physicians should be aware of the possibility of rhabdomyolysis, especially when patients complain of local pain and weakness in their muscles. Pediatric clinicians should be aware of this complication related to the novel 2019 coronavirus. Timely diagnosis and proper treatment improve the patient's prognosis.

Keywords: COVID-19, rhabdomyolysis, pediatric

Introduction

Coronavirus disease-2019 (COVID-19) is a worldwide health concern affecting people of all ages. The prevalent symptoms of COVID-19 are fever, dry cough, tiredness, and muscle pain; while headache, conjunctivitis, and diarrhea are less common. There have been few reports of COVID-19-related rhabdomyolysis in adults (0.2% of 1,099 patients in China) (1) and fewer in children (2). Rhabdomyolysis is defined as muscle necrosis that presents with muscle weakness, myalgia, and sometimes dark urine. Herein, we report a case of rhabdomyolysis in a patient diagnosed with COVID-19.

Case Report

A 10-year-old boy, previously healthy, initially presented to the pediatric urgent care unit with 2 days of fever and some dry cough and acute muscle weakness and pain of both lower limbs. He was unable to stand due to weakness and pain in both legs. He had no symptoms of diarrhea, vomiting, rhinorrhea, or decreased sense of smell or taste. His medications include azithromycin and acetaminophen.

On the initial examination, he had a fever of 38.5 °C and a respiratory rate of 18. Oxygen saturation was 95 percent in room air. On clinical examination, mild crackles were heard at the base of both lungs. His chest computerized tomography scan revealed small ground-glass nodules, indicating viral pneumonia, scattered across the left lower lung.

His lab testing revealed marked lymphopenia (absolute lymphocyte count: 900), elevated C-reactive protein (CRP) (20 mg/L) (neg <3 mg/L), and marked elevation of creatine phosphokinase (CPK) at 8,000 units per liter (U/L) (range: 24-170 U/L). Lactate dehydrogenase was 500 U/L (normal range: 120-250 U/L). Liver function tests were normal

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Table I. Patient's Laboratory Data

Lab tests	WBC (/mm³)	PMN (/mm³)	LYM (/mm³)	Hb (g/dL)	Plt (/mm3)	CRP (mg/l)	CPK (U/L)	Na (mEq/L)	K (mEq/L)	Bun (mg/dL)	Cr (mg/dL)	Ca (mg/dL)	Glucose (mg/dL)	Blood culture	AST (U/L)	ALT (U/L)	Urine PH
First	2,800	1,800	900	13	150,000	20	8,000	137	4/5	19	0.8	9	90	Neg	117	40	6
Last	5,200	1,900	2,900	12/8	284,000	2	170	135	3.8	12	0.5	9	100	Neg	35	32	7/5

WBC: White blood cell, PMN: Polymorphonuclear neutrophils, CRP: C-reactive protein, CPK: Creatine phosphokinase, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, Lab: Laboratory

except for aspartate aminotransferase which was 117 U/L (normal range: <37 U/L).

His creatinine was 0.8 milligrams per deciliter (mg/dL), which was in the normal range. His urine test demonstrated the presence of blood on a urine dipstick test without any red blood cells (testing of the unspun urine was positive for "heme" on the dipstick, but the visual and microscopic examination of the sediment from the fresh urine specimen was negative for red blood cells (RBC) which suggested myoglobinuria). Renal Doppler and abdominal ultrasound scans were normal. His electrocardiogram was normal and no arrhythmia was observed. The toxicology screen was negative. The patient's COVID-19 polymerase chain reaction test performed via nasopharyngeal swab was positive. His flu test was negative. He was given a 20 cc/kg normal saline bolus and started on isotonic intravenous fluids (at two times the maintenance rate) containing sodium bicarbonate. Fluids were titrated to achieve alkalinization of the urine with a goal urine pH of 8.0. The patient was prescribed hydroxychloroquine and azithromycin for five days. On hospital day 3, his fever stopped and myalgia improved relatively. On hospital day 4, his CPK was 2,000 U/L and his urine was negative for blood, so the fluid rate was decreased (Table I). Two days later, the patient was discharged as his clinical and laboratory symptoms had improved.

Discussion

Rhabdomyolysis is a life-threatening syndrome that is caused by the destruction of muscle cells for various reasons such as viral myositis, trauma, connective tissue disorders, pharmacological, and metabolic disorders (3,4). Viral-associated rhabdomyolysis especially due to influenza is a common cause of rhabdomyolysis in pediatric patients (5).

Several hypotheses explain the mechanism of rhabdomyolysis due to viral causes. First of all, a direct attack of the virus on muscle cells can lead to rhabdomyolysis (6). The second reason is due to the immune system's response to the viruses, which leads to a cytokine storm and eventually muscle destruction. The third factor is the circulating toxins of viruses that directly affect muscle cell membranes (4). However, the mechanism of COVID-19-induced rhabdomyolysis is not yet known.

Herein, we present a pediatric patient with severe rhabdomyolysis without renal involvement associated with COVID-19 infection. To our knowledge, only a few cases of COVID-19-associated rhabdomyolysis have been previously reported in children (2,7). Gilpin et al. (8) presented a case of a 16-year-old adolescent with rhabdomyolysis as an initial presentation of COVID-19, Anwar and Al Lawati (9) presented a 16-year-old boy with COVID-19-induced rhabdomyolysis who eventually died, and Ashley M. Gefen also presented a 16-year-old boy with severe rhabdomyolysis without renal involvement which was related to COVID-19 infection (similar to our case) (2) (Table II).

Another cause of rhabdomyolysis is metabolic diseases. In the above patient, due to the negative family history, unrelated parents, and no history of recurrent cramps or exercise intolerance, the possibility of associated metabolic disease was ruled out (10). Muscle pain and weakness are common symptoms of COVID-19 (11). Myalgias have been observed in more than half of patients with COVID-19 infection. Elevated serum CPK levels indicate the severity of the disease ranging from mild to frank rhabdomyolysis (11). CPK levels should be measured in suspected patients (12). The first step in treating rhabdomyolysis is intense fluid therapy to maintain urine volume and prevent acute renal failure, an early correction of electrolyte disorders, and a correction of

Table II. Summary of previous case reports

Characteristic / Author	Age (year)	Sex	Coexisting conditions	Fever (>38°C)	Cough & shortness of breath	Muscle pain	CT findings	COVID-19 RT-PCR test	CPK (U/L)	Corticosteroids or antiviral therapy	Survived
Gilpin et al. (8)	16	Male	Asthma	38/3	No	Shoulders and Thighs	Negative	Positive	116,640	No	Yes
Gefen et al. (2)	16	Male	Autism, Morbid Obesity	38/9	Yes	Arms, Legs, and Back	Not available	Positive	427,656	No	Yes
Anwar and Al Lawati (9)	16	Male	Negative	38/5	Yes	Myalgia and Leg Weakness	Not Available	Positive	Not Available	No	Died

CT: Computed tomography, COVID-19: Coronavirus disease-2019, CPK: Creatine phosphokinase

metabolic acidosis. Normal saline should be administered as soon as possible at the onset of muscle injury and continued until recovery. However, there is little clinical evidence for its effectiveness. Forced alkaline diuresis may be performed with bicarbonate administration to reduce the renal toxicity of heme (4). The best treatment and the amount and speed of bicarbonate administration are not well known (13). A large cohort study in adults showed the presence of rhabdomyolysis was associated with increased mortality (14). Therefore, the prompt diagnosis and timely treatment of rhabdomyolysis help to reduce complications of those patients with COVID-19.

Conclusion

Rhabdomyolysis can be the first symptom of COVID-19 or it can occur at any time during the course of the disease. Pediatric physicians should be aware of this complication. Timely diagnosis and proper treatment improve the patient's prognosis.

Ethics

Informed Consent: All participants gave written informed consent.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Pari.A., P.A., Design: Pari.A., P.A., Data Collection or Processing: Pari.A., P.A., Analysis or Interpretation: Pari.A., P.A., Literature Search: Pari.A., P.A., Writing: Pari.A., P.A.

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Case Report

Probable Vertical Transmission of SARS-CoV-2 Infection in the Countryside of São Paulo State, Brazil: Case Report

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ABSTRACT

We reported a probable vertical transmission of severe acute respiratory syndrome-Coronavirus-2 (SARS-CoV-2) infection in Presidente Prudente, a city of the western region of São Paulo. Furthermore, regionally updated data on SARS-Coronavirus diseases-2019 pregnant women pinpointed the vulnerability of the region with higher levels of infected and dead mothers compared to nationwide data.

Keywords: SARS-CoV-2, vertical transmission, pregnant women

Introduction

Worldwide, Brazil ranks third for the number of individuals infected with severe acute respiratory syndrome-Coronavirus-2 (SARS-CoV-2) and second with regard to deaths. São Paulo, the richest and most populous state, is the epicenter and ranks first for the number of infected patients and deaths. Considered one of the poorest and most undeveloped regions, western São Paulo comprises 45 municipalities with an estimated population of 753,344 inhabitants. In May 2020, pregnant and postpartum women were included in the group most susceptible to the effects of Coronavirus diseases-2019 (COVID-19) in Brazil. Transient immunosuppression for maintaining the tolerance of fetal semi allograft makes them more vulnerable to viral infections. Furthermore, changes in the respiratory and circulatory systems predispose pregnant women to worse clinical outcomes when infected with viruses such as the H1N1 virus (1). In December 2020, the nationwide profile of pregnant women with SARS who were hospitalized and deaths confirmed by COVID-19 were updated. From February to December 2020, 4,467 of 9,581 (44.7%) pregnant women were diagnosed with COVID-19 and 233 (5.2%) died (2). However, although mothers and their newborns should be considered a high-risk population in the development of prevention and management strategies for COVID-19, in Brazil, little public data are available on vertical transmission, and cases are restricted to case reports (3-7).

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Case Report

A 35-year-old gravida 2, para 1 was admitted to a private hospital in Presidente Prudente, in the countryside of São Paulo, Brazil, on July 15, 2020, at 35+5 weeks of gestation, and up to that time, the pregnancy had been uneventful. She was referred with rhinorrhea and body pain and presented premature labor with no cause and clear amniotic liquid. She had controlled insulin-dependent type I diabetes mellitus and was reported to have been in contact with her sister who had been diagnosed with COVID-19. Despite epidemiological data, the obstetrician and the patient did not consider the possibility of COVID-19 infection and the viral load of the amniotic fluid and umbilical cord were not collected. She had had no other symptoms and was not treated for COVID-19. A cesarean delivery was performed with rupture of the amniotic membranes, with clear amniotic liquid, without lumps or meconium.

A male neonate was delivered; the birth weight was 3,100 g, Apgar scores were 9 and 10 at 1 and 5 min, respectively. Gestational age according to the New Ballard score was 35 weeks and 5 days. He was taken to a bath in running water immediately after delivery, without contact with the mother after birth. Still in the delivery room, he developed tachypnea and moaning (Silverman Andersen Bulletin=6), and was taken to the neonatal intensive care unit (NICU). He was placed in an incubator with fasting, probe open orogastric tubes, maintenance serum, and continuous positive airway pressure with an inspired oxygen fraction (FIO_2) of 30% and aerosol precautions. Chest radiography was performed, showing alterations compatible with pulmonary interstitial edema. His initial diagnoses were preterm newborn, suitable for age pregnancy, premature labor, and early respiratory distress. A real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) test for nasopharyngeal SARS-CoV-2 RNA was performed with indeterminate results on day 2 (July 17, 2020) and positive results on day 7 (July 21, 2020). Nasopharyngeal samples were collected from the mother for COVID-19-rRT-PCR and the result was positive. The newborn remained in the NICU until day 7, under specific precautions. No contact with his mother or family, and no breastfeeding were allowed. On day 9, chest tomography was performed, showing consolidation in the upper segment of the left lower lobe associated with discrete bilateral ground-glass opacities (Figure 1). Laboratory values were at normal levels on day 1 and in the following segment. The newborn showed progressive improvement and was discharged on day 11 weighing 2,940 g, and anti-COVID-19 IgG and total antibodies were positive. Written informed consent was obtained to publish this paper. The project was approved by the Ethical Committee of Oeste Paulista University, Brazil. Protocol number 4.469.118; December 16, 2020.

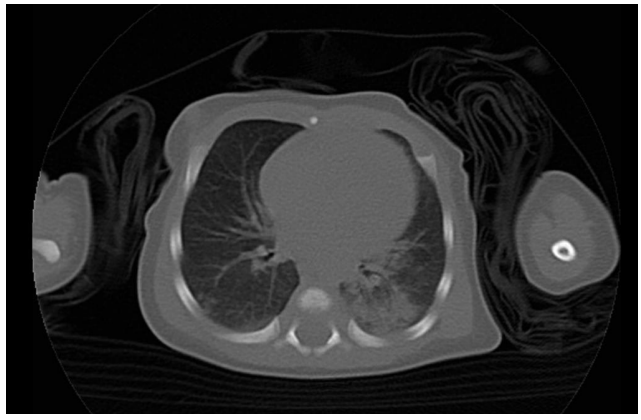

Figure 1. Chest tomography: Axial section with window for viewing parenchyma performed on the 9th day of hospitalization

Discussion

It is possible to consider vertical transmission of COVID-19 in the newborn in this case. According to the criteria of the São Paulo Pediatric Society, suspected and confirmed cases in pregnant women and newborns are defined when the newborn has a positive result for COVID-19 by RT-PCR in respiratory tract samples with swab collection. (https://www.spsp.org.br/2020/03/30/coronavirus-e-recem-nascido-o-que-se-sabe-ate-o-momento-30-03-2020/). Following the delivery, strict measures were taken to reduce the risk of in fection, and the newborn did not have contact with his mother or other family members, a fact that strongly supports the diagnosis. Although congenital SARS-CoV-2 infection can be confirmed by PCR of placental tissue and the umbilical cord, few reports are available using this methodology in Brazil (4), and in most cases, the results were obtained from nasal/oropharynx swabs. In São Paulo, in 3 cases reports of vertical transmission of COVID-19, positive results for SARS-CoV-2 by RT-PCR were obtained from oropharynx swabs (5). In São Luis, Maranhão state, a positive result in RT-PCR4 for SARS-COV-2 was obtained from nasal swab at 6 hours after birth and a probable vertical transmission was described (6). In Rio de Janeiro, nasal and oropharynx swabs were also collected, and RT-PCR tests for SARS-CoV-2 were positive in a maternal SARS-CoV-2 infection associated with systemic inflammatory response and pericardial effusion in the newborn (7). Symptoms of groaning and tachypnea started immediately after birth and the newborn was sent to the NICU for ventilatory support. Chest tomography

demonstrated alterations compatible with pulmonary interstitial edema and consolidation associated with ground-glass opacities. Respiratory manifestations are among the main clinical symptoms reported in newborns infected with COVID-19, and about 30% require admission to the NICU. Furthermore, chest imaging examination should be actively performed in newborns suspected of COVID-19 infection (8). In a review of newborns infected with COVID-19, non-specific radiographic findings were described in 32% of cases, with ground-glass opacity in 8% and linear opacities in 4% (9).

The delivery and neonatal support in this case occurred in a tertiary private hospital with an available NICU in the countryside of São Paulo state. However, such facilities are not available in most regions of Brazil, including the municipalities of western São Paulo, which only has 5 NICUS, all of them being in the city of Presidente Prudente. In 2020, in the western region, 29 pregnant women were diagnosed with SARS, and 15 (51.7%) were diagnosed with COVID-19; two deaths occurred (13.3%), and one infected newborn died. These rates are higher than the countrywide rates, not only for infections (51.7% vs 44.7%), but also for deaths of pregnant women (13.3% vs 5.2%). All the cases of vertical transmission of COVID-19 occurred in large cities in different regions of Brazil, including São Paulo and Rio de Janeiro in the southeast, Curitiba in the south, and São Luis in the northeast (3-7).

Conclusion

This case report focuses on a newborn in a city of São Paulo State. Furthermore, regionally updated data on pregnant women with SARS-COVID-19 demonstrates the asymmetry and vulnerability of regions with higher levels of infected mothers and deaths compared with the nationwide data.

Ethics

Informed Consent: Written informed consent has been obtained to publish this paper.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Medical Practices: P.R.N.S., Concept: P.R.N.S., L.E.P.C., Design: P.R.N.S., Data Collection or Processing: B.M.S., J.A.C.G., V.M.S., L.A.C.G., Analysis or Interpretation: P.R.N.S., L.E.P.C., Literature Search: P.R.N.S., B.M.S., J.A.C.G., V.M.S., L.A.C.G., L.E.P.C., Writing: L.E.P.C., P.R.N.S.

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