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Newborn Screening Program for Cystic Fibrosis in Türkiye: Experiences from False-Negative Tests and Requirement for Optimization

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Background: Since January 2015, the Cystic Fibrosis National Newborn Bloodspot Screening (CF-NBS) program has been implemented in Türkiye with two samples of immune reactive trypsinogen (IRT-1/IRT-2) testing.

Aims: To evaluate the Turkish national CF screening program, which included patients referred to a tertiary pediatric pulmonology center, to ascertain the optimal cut-off values for IRT-1/IRT-2 and to identify alternative strategies for mitigating the number of late-diagnosed false-negative patients (FNPs) who initially exhibited screen negative results but were diagnosed subsequently based on clinical suspicion. The study also compared NBS-positive patients to FNPs to determine the influence of delayed diagnosis.

Study Design: A retrospective cohort study.

Methods: Screening for CF was conducted in accordance with the national CF-NBS program within 48-72 hours of birth by collecting a few drops of heel blood on Guthrie paper. A cut-off value of 90 µg/l was accepted for the first IRT, while 70 µg/l was accepted for the second sample. Infants with elevated IRT values in both samples were referred to the CF centers for a sweat test (ST). Based on the diagnosis, the NBS-positive infants referred to our CF center for ST analysis were divided into three groups: CF; cystic fibrosis-related metabolic syndrome/cystic fibrosis screen positive, inconclusive diagnosis (CRMS/CFSPID); and false-positive NBS. In addition, the study included NBS-negative patients who initially received negative screen results but were subsequently diagnosed with CF based on clinical suspicion.

Results: Of the 227 NBS-positive infants referred within the study period, 53 (23.34%) were diagnosed with CF (true-positive NBS), 11 were classified as CRMS/CFSPID (4.84%), and 163 were classified as false-positive NBS (71.8%). CF was diagnosed in 66 infants, 53 (80.3%) of whom were confirmed using the NBS test, while the 13 (19.7%) patients who were missed on the NBS test were diagnosed based on clinical suspicion (FNP). The study findings indicate that the IRT/IRT approach exhibited a sensitivity of 80.3% and a positive predictive value (PPV) of 23.3%.

Conclusion: The current study is the first to analyze the NBS program for CF using data from the Western Anatolian Region of Türkiye. Due to the low sensitivity and PPV of the IRT/IRT protocol and the high proportion of false-positive infants and FNPs, the current national program is not practicable for Türkiye. False-negative results significantly delay the diagnosis and invalidate the screening objectives. It is essential to establish optimal cut-off values for IRT-1/IRT-2 or revise existing strategies to reduce the number of FNPs missed by the screening program.

INTRODUCTION

Cystic fibrosis (CF) is a global, life-threatening autosomal recessive genetic disorder caused by *cystic fibrosis transmembrane conductance regulator (CFTR)* gene mutations. It is estimated to affect 1 in 2500 to 1 in 3500 live births.[1] The newborn bloodspot screening (NBS) for CF program is a well-established public health approach with universal standards[2] that offers the opportunity to diagnose CF at a younger age, potentially preventing many complications through early intervention and decreasing morbidity and mortality.[3] Improved long-term health outcomes outweigh the risks associated with inaccurate diagnosis due to false-positive results. CF-NBS was incorporated into Türkiye's national NBS program in January 2015 with two repeated immunoreactive trypsinogen (IRT) based screenings (IRT-1/IRT-2).

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Infants with elevated IRT values are classified as positive-NBS and are referred to the CF centers for a sweat test (ST).

The European Cystic Fibrosis Society (ECFS) recommends that an optimal NBS program should strive to achieve a minimum of 95% sensitivity and 30% positive predictive value (PPV). Infants who have been diagnosed using the NBS should be granted early access to CF centers within 35 days of birth, with a maximum of 58 days.[3]

This study aimed to evaluate the Turkish national CF screening program, which included patients referred to a tertiary pediatric pulmonology center, to ascertain the optimal cut-off values for IRT-1/IRT-2 and to identify alternative strategies for mitigating the number of late-diagnosed false-negative patients (FNPs) who initially exhibited screen negative results but were diagnosed subsequently based on clinical suspicion. Additionally, we also aimed to assess the FNPs and determine the impact of delayed diagnosis by comparing NBS-positive patients and FNPs.

MATERIALS AND METHODS

This retrospective cohort study analyzed the Turkish national CF-NBS program and included CF-NBS-positive infants who were referred to our pediatric pulmonology department between January 2015 and January 2023. The study was approved by the Clinical Research Ethics Committee of Ege University Faculty of Medicine (approval number: 22-6.1T/50, date: 28.06.2022).

The NBS-positive infants were divided into three groups: true-positive NBS (diagnosed as CF), NBS-false-positive (healthy infants), and cystic fibrosis-related metabolic syndrome/cystic fibrosis screen positive, inconclusive diagnosis (CRMS/CFSPID). Furthermore, patients who tested negative during the initial screening but were clinically diagnosed during the study were included and referred to as FNPs. The IRT-1 and IRT-2 results of the NBS-negative CF group patients were accessed using their mother's national identification number. Children whose IRT-1 and IRT-2 results could not be accessed were excluded from the study.

The standardized fluorometric enzyme immunoassay method was employed to analyze the IRT values. If the first IRT ≥ 90 µg/l was administered at 72 h of life, the second IRT was performed between the 10th and 21st day.[4] If the IRT-2 value was ≥ 70 µg/l, the screening result was considered positive, and the infant was referred to the CF centers for undergoing the ST.[4] The sweat chloride concentration (SCC) was ascertained using the CFΔ Collection System (UCF 2010 Iontophoresis Unit and UCF 2011 Sweat Analysis Unit), which analyzes the SCC using coulometric endpoint software. Patients exhibiting a SCC ≥ 60 mmol/l were regarded as CF positive and subjected to genetic analysis and clinical assessment. For the intermediate SCC (30-59 mmol/l), a repeat ST is performed. Confirmatory next generation sequencing (NGS) genetic analysis was performed for all children having SCC ≥ 60 mmol/l or intermediate (30-59 mmol/l) to validate screening results.

The NBSPP group was comprised patients with clinical features and laboratory results consistent with CF and SCC ≥ 60 mmol/l and/or genetic analysis results revealing the presence of two CF-causing CFTR mutations in trans. If genetic analysis did not reveal any CF-causing mutation, the patient was diagnosed with CF based on clinical features and a positive ST.[3,4] FNPs were defined as infants who were initially CF-NBS negative but were subsequently diagnosed based on clinical findings or family history consistent with CF.

The CF-Mutation Database-2 (CFTR-2) was searched to identify the clinical and phenotypic spectrum of the variants.[5,6] For mutations not identified using the *CFTR-2* database, the *CFTR-1*[7] and *CFTR*-France[8] databases were searched. We classified CFTR mutations into three genotypes based on the synchronous presence of F508del: homozygous, heterozygous, and others. Additionally, they were classified as severe for the synchronous existence of class I, II, or III variants in the two *CFTR*-gene copies of a patient and mild for the synchronous existence of class IV, V, or VI variants or with severe variants.[9-11] Asymptomatic NBS-positive infants with no symptoms of CF and persistently exhibiting intermediate SCC values (30 to 59 mmol/l) and fewer than two CF-causing CFTR mutations or two CFTR mutations with 0 or 1 accepted as disease-causing, and SCC levels < 30 mmol/l defined as CRMS (CF related metabolic syndrome) in the United States, and CFSPD (CF screen positive inconclusive diagnosis) in other countries.[12]

The indicators of nutritional status, specifically weight-for-age and height-for-age measurements expressed in terms of z-scores, were assessed using World Health Organization (WHO) growth charts.[13] Failure to thrive was diagnosed in children with weight-for-age values below the fifth percentile on standardized WHO growth charts.[14] Respiratory manifestations, as indicated by persistent coughing, recurrent wheezing, tachypnea, and frequent pulmonary infections, were considered to signify pulmonary involvement.[15] CF-related pseudo-Bartter syndrome (PBS) was defined as the presence of hyponatremic, hypokalemic, and hypochloremic metabolic alkalosis without renal tubulopathy, in accordance with the ECFSPR guidelines.[16] The presence of chronic *Pseudomonas aeruginosa* (*P. aeruginosa*) infection was diagnosed when the Leeds criteria were met (i.e., when the respiratory samples were *P. aeruginosa* culture positive for more than 50% of months in the previous 12 months).[17] Since fecal elastase analysis could not be performed at our hospital, the presence of pancreatic insufficiency was diagnosed if the patient required pancreatic enzyme treatment. The demographic and clinical features and laboratory findings of the NBSPPs and FNPs were compared. The sensitivity and PPV of the current program were evaluated. The optimal cut-off values for IRT-1 and IRT-2 were established.

Statistical analysis

The IBM SPSS Statistics software version 22.0 (IBM, Armonk, NY, USA) was employed for statistical analyses. Numerical variables are presented as mean ± standard deviation and median (minimum-maximum), while categorical variables are presented as numbers and percentages. Pearson χ^2 and Fisher's exact χ^2 tests were conducted for categorical data. The Kolmogorov-Smirnov test was applied to determine the normal distribution of the numerical variables. The numerical variables were compared between groups using the Mann-Whitney U test and two independent samples

t-tests. Correlation analysis between continuous parameters was performed using the Pearson's correlation test. The receiver operating characteristic (ROC) curve was employed to determine the diagnostic efficacy of the IRT values for CF. The area under the curve (AUC) for each IRT value was calculated from ROC. A p value < 0.05 was considered statistically significant. The sensitivity and specificity were ascertained for each IRT-1 and IRT-2 cut-off points. The optimal cut-off point was defined based on the highest sensitivity and specificity as determined by Youden's index, which assesses the ability of a diagnostic test to balance sensitivity and specificity.

RESULTS

The study included 300 children who tested positive for CF on the NBS test and were referred between 2015 and 2023. The study excluded seventy-three infants whose IRT values could not be accessed due to the absence of the mother's identification numbers. The study continued with 227 children (132 males) whose median age of presentation was 30 (14-300) days. The median level of IRT-1 was 109.8 (90-293.2) µg/l, while the median level of IRT-2 was 86.75 (70-370.5) µg/l. Among the 227 infants, 23.3% (n = 53) were diagnosed with CF (true-positive NBS), 4.8% (n = 11) with CRMS/CFSPID, and the remaining were classified as false-positive NBS (71.8%, n = 163). The demographic and laboratory characteristics of the NBS-positive infants are presented in Table 1. The CF to CRMS/CFSPID ratio was 4.8:1. No patients were diagnosed with CF during their follow-up. Table 2 illustrates the laboratory and genetic results of the CRMS/CFSID group.

Upon comparing the NBS-positive children with true-positive (CF) and false-positive (healthy infants) children, no significant difference was found among them in terms of sex, gestational age, birth weight, and age at admission. The weight and height-for-age z-score at admission was significantly lower in the true-positive group (-0.68 ± 1.19 vs. 0.30 ± 0.93, $p < 0.01$, and -0.64 ± 1.24 vs. 0.38 ± 0.88, $p < 0.01$). The incidence of failure to thrive in neonates, history

TABLE 1. Demographic and Laboratory Features of Children with Positive NBS (n = 227).

Gender (male/female), n (%)	132 (58.1)/95 (41.9)
Gestational age (week)	38.5 (24/42)
Age of reference (day)	30 (14/300)
Consanguinity, n (%)	42 (18.5)
IRT-1, median (minimum-maximum)	109.8 (90-293.2)
IRT-2, median (minimum-maximum)	86.75 (70-370.5)
Sweat chloride concentrations, median (minimum-maximum)	15 (2-160)
Diagnosis, n (%)	
CF	53 (23.3)
CRMS/CFSPID	11 (4.9)
False-positive NBS	163 (71.8)

CF, cystic fibrosis; CRMS/CFSPID, cystic fibrosis transmembrane conductance regulator-related metabolic syndrome/cystic fibrosis screen positive inconclusive diagnosis; IRT, immunoreactive trypsinogen; NBS, newborn screening.

TABLE 2. Laboratory Characteristics of CRMS/CFSPID Children.

Case No	IRT-1 (µg/l)	IRT-2 (µg/l)	Sweat chloride (mmol/l)	Mutation analysis
Case 1	140	78	52/57	-/-
Case 2	118	70	33/35	-/-
Case 3	92.18	81.93	53/10	L997F
Case 4	129.24	96.23	50/47	-/-
Case 5	107.86	72	31.5	-/-
Case 6	164.73	71	32.60	-/-
Case 7	104.17	80.21	11/9	I807M; 2553A/I148T
Case 8	102.16	78.57	28/14	F508del/E217G
Case 9	124	156.25	41.10/10.42	-/-
Case 10	110	100	31/10	-/-
Case 11	267	76	37/15	-/-

IRT-1, immunoreactive trypsinogen; CRMS/CFSPID, cystic fibrosis-related metabolic syndrome/cystic fibrosis screen positive, inconclusive diagnosis.

of pulmonary infection, recurrent pneumonia, and steatorrhea was significantly more prevalent in the true-positive group. The groups exhibited significant differences in terms of IRT-1 ($p < 0.01$) and IRT-2 values ($p < 0.01$). The comparison of the true-positive and false-positive NBS groups is presented in Table 3.

When NBSPPs (n = 53, 80.3%) and FNPs (n = 13, 19.7%) were compared, no significant difference was detected among the groups in terms of sex, gestational age, birth weight, history of siblings with CF, and consanguinity. NBS-positive CF patients experienced their first clinic visit at a younger age [30 (15-24) days vs. 120 (60-450) days, $p < 0.01$] and were diagnosed at a younger age [2.5 (1-8) months vs. 4 (2-8.5) months, $p < 0.01$]. While there was no significant difference in the clinical and demographic characteristics among the groups, the incidence of failure to thrive, prolonged jaundice, and a history of meconium ileus, PBS, and steatorrhea was higher among the FNPs. Pulmonary involvement, recurrent pneumonia, hospitalization due to pulmonary exacerbation, *P. aeruginosa* colonization, and pancreatic insufficiency were prevalent among the NBSPPs. The frequency of severe/severe mutations (46.8% vs. 15.4%, $p = 0.02$) and median IRT-1 value were higher in the NBSPPs [150 (90-293) (µg/l) vs. 41.77 (24.8-131.20) (µg/l), $p < 0.001$]. There were no differences among the groups based on the SCC, while albumin levels were lower in the NBSPP group (median level, 40.5 vs. 43 mg/dl, $p = 0.03$) (Table 4). A negative correlation was detected between IRT values and age at diagnosis (r = -0.32, $p < 0.001$, and r = -0.26, $p = 0.04$). Furthermore, there was no correlation between IRT values and SCC, serum sodium, chloride, albumin levels, weight, and height-z-scores of children at diagnosis ($p > 0.05$).

A total of 127 alleles and 45 different CFTR mutations were detected in the 66 CF patients. The most common one were F508del (n = 40), G542X (n = 10), and 2184delA (n = 7). One CF patient was not genotyped, and three patients exhibited only one CFTR-mutation (Table 5).

Our study findings indicate that the IRT/IRT approach demonstrates a sensitivity of 80.3% and PPV of 23.3%. The AUC for IRT-1 was 0.764 [95% confidence interval (CI): 0.683-0.845, $p < 0.01$], and the AUC for IRT-2 was 0.834 (95% CI: 0.765-0.904, $p < 0.01$), respectively (Figure 1). The sensitivity (73.6%) and specificity (73%) of IRT-1 were at their highest when the cut-off value was estimated to be 124.25 µg/l. The cut-off for IRT-2 was estimated at 94.3 µg/l, which corresponds to the maximum combined sensitivity (75%) and specificity (75.5%).

TABLE 3. The Comparison of True-positive NBS (Diagnosed with CF) and False-positive NBS (without CF).

	True-positive NBS, (n = 53)	False-positive NBS, (n = 163)	p
Demographic data			
Gender (m), n (%)	28 (52.8)	64 (39.26)	0.05[&]
Birth-weight (gr)	3200 (1500-4140)	3200 (600-4315)	0.25[#]
Gestational age (week)	38 (30-41)	39 (24-42)	0.05[#]
Age at admission (day)	30 (15-124)	30 (14-300)	0.32[#]
Consanguinity, n (%)	16 (30.2)	26 (16)	**0.02[&]**
Weight for age z-score at admission	-0.68 ± 1.19	0.30 ± 0.93	**< 0.001[*]**
Height for age z-score at admission	-0.64 ± 1.24	0.38 ± 0.88	**< 0.001[*]**
Clinical manifestations			
Failure to thrive at neonate, n (%)	32 (60.3)	24 (14.2)	**< 0.001[&]**
Prolonged jaundice, n (%)	5 (9.4)	26 (16)	0.17[&]
Meconium ileus, n (%)	5 (9.4)	-	
Pulmonary infection at least once, n (%)	47 (67.1)	23 (14.3)	**< 0.001[&]**
Recurrent pneumonia	33.9 (64.1)	6 (3.7)	**< 0.001[&]**
Pseudo-Bartter syndrome, n (%)	32 (60.4)	-	
Steatorrhea, n (%)	28 (52.8)	3 (1.8)	**< 0.001[&]**
Laboratory data			
1.IRT (µg/l)	150 (90-293)	103.09 (90-291.6)	**< 0.001[#]**
2.IRT (µg/l)	147.11 (70.6-370.5)	82.36 (70-250.36)	**< 0.001[#]**
Sweat chloride concentrations (mEq/l)	73.5 (14-160)	12 (2-29)	**< 0.001[#]**

P comparison of true-positive NBS (diagnosed with CF) and false-positive NBS (without CF) infants; [&]Chi-square test; [#]Mann-Whitney U test; [*]Two independent sample t-tests; NBS, Newborn screening; CF, cystic fibrosis; IRT-1, immunoreactive trypsinogen.

TABLE 4. The Clinical Features and Laboratory Findings of Cystic Fibrosis Patients.

	CF, (n = 66)	NBS-positive CF, (n = 53) (80.3)	False-negative CF, (n = 13) (19.7)	p
Demographic features				
Gender (m), n (%)	37	28 (52.83)	9 (69.2)	0.22[&]
Birth-weight (gr)	3200 (1500-4140)	3200 (1500-4140)	3300 (2100-4000)	0.34[#]
Gestational age (week) at birth	38 (30-41)	38 (30-41)	38 (34-41)	0.28[#]
Age of reference (day)	44.5 (14-450)	30 (15-124)	120 (60-450)	**<0.001[#]**
Age at diagnosis (month)	2.75 (1-8.5)	2.5 (1-8)	4 (2-8.5)	**<0.001[#]**
History of siblings with CF, n (%)	10 (15.4)	7 (13.2)	3 (23)	0.31[&]
Consanguinity, (%)	21 (31.8)	16 (29.6)	5 (38.5)	0.38[&]
Mutation analyses				
Severe/severe	24 (40)	22 (46.8)	2 (15.4)	**0.02[&]**
Severe/mild	22 (36.7)	16 (34)	6 (46.2)	0.29[&]
Mild/mild	8 (13.3)	6 (12.8)	2 (15.4)	0.54[&]
Others	12 (18)	9 (16.9)	3 (23)	0.48[&]
Clinical manifestations				
Failure to thrive	41 (62.1)	32 (60.4)	9 (69.2)	0.40[&]
The salty taste of sweat	45 (68.2)	37 (69.7)	8 (61.5)	0.39[&]
Prolonged jaundice	7 (10.6)	5 (9.4)	2 (15.4)	0.41[&]
History of meconium ileus	7 (10.6)	5 (9.4)	2 (15.4)	0.41[&]
History of PBS	40 (60.6)	31 (58.4)	9 (69.2)	0.35[&]
History of steatorrhea	35 (53)	28 (52.8)	7 (53.8)	0.59[&]
Weight for age z-score at diagnose	-1.25 ± 1.55	-1.4 ± 1.54	-0.47 ± 1.36	0.05[*]
Height for age z-score at diagnose	-0.75 ± 1.52	-0.75 ± 1.56	-0.75 ± 1.42	0.98[*]
Pulmonary infection at least once	58 (87.9)	47 (88.7%)	11 (84.6)	0.49[&]
History of recurrent pneumonia	40 (60.6)	34 (64.1%)	6 (46.2)	0.19[&]
Pulmonary exacerbation annually	1 (0-6)	1 (0-6)	1 (0-2)	0.17[&]
Hospitalization due to pulmonary exacerbation	26 (40)	22 (42.3)	4 (30.8)	0.33[&]
Pulmonary involvement	40 (60.6)	34 (64.1)	6 (46.2)	0.19[&]
P. aeruginosa colonization, n (%)	6 (9.1)	6 (11.3)	-	
S. aureus colonization, n (%)	11 (16.7)	9 (17)	2 (15.4)	0.62[&]
Pancreatic insufficiency, n (%)	54 (81.8)	44 (83)	10 (76.9)	0.43[&]
Laboratory data				
IRT-1 (µg/l)	141.48 (90-278)	150 (90-293)	41.77 (24.8-131.20)	**<0.001[#]**
IRT-2 (µg/l)		147.11 (70.6-370.5)	-	-
Sweat chloride concentration (mEq/l)	73.18 ± 28.90	74 ± 29.44	64.02 ± 32.28	0.32[*]
Sodium	138 (118-142)	136 (118-142)	137 (125-141)	0.96[#]
Chlorine	101 (57-110)	101 (57-110)	102 (74-107)	0.74[#]
Albumin	41.3 (22.5-53)	40.5 (22.5-53)	43 (39.10-48)	**0.03[#]**

P comparison of NBS-positive and NBS-negative CF patients; [&]Chi-square test; [#]Mann-Whitney U test; [*]Two independent sample t-tests; NBS, newborn screening; CF, cystic fibrosis; PBS, Pseudo-Bartter syndrome; IRT-1, immunoreactive trypsinogen.

TABLE 5. Variant Frequency of Detected CFTR Mutations.

		Variant frequency, n (%)		
CFTR mutations	**Mutation**	**All CF, (n = 66)**	**NBS-positive CF, (n = 53)**	**NBS-negative CF, (n = 13)**
F508del	Disease-causing	40 (31.49%)	33 (32.6%)	7 (26.92%)
G542X	Disease-causing	10 (7.87%)	10 (9.9%)	-
2184del	Disease-causing	7 (5.51%)	6 (5.9%)	1 (3.84%)
D110H	Disease-causing	5 (3.93%)	1 (0.99%)	4 (15.38%)
W1282X	Disease-causing	4 (3.14%)	4 (3.96%)	-
M9521	No database	4 (3.14%)	4 (3.96%)	-
D1152H	Disease-causing	4 (3.14%)	2 (1.98%)	2 (7.69%)
R347H	Disease-causing	3 (2.36%)	2 (1.98%)	1 (3.84%)
N1303K	Disease-causing	3 (2.36%)	3 (2.97%)	-
2789+5G>A	Disease-causing	3 (2.36%)	1 (0.99%)	2 (7.69%)
W1098L	Disease-causing	2 (1.57%)	-	2 (7.69%)
L732X	Disease-causing	2 (1.57%)	2 (1.98%)	-
IVS8+1G>A	Disease-causing	2 (1.57%)	2 (1.98%)	-
G85E	Disease-causing	2 (1.57%)	2 (1.98%)	-
E1044G	No database	2 (1.57%)	2 (1.98%)	-
3120+1G>A	Disease-causing	2 (1.57%)	2 (1.98%)	-
D1152H	Disease-causing	2 (1.57%)	2 (1.98%)	-
2183AA>G	Disease-causing	2 (1.57%)	2 (1.98%)	-
3129del4; 3126del4	Disease-causing	2 (1.57%)	2 (1.98%)	-
W401X	Disease-causing	1 (0.78%)	1 (0.99%)	-
G1349D	Disease-causing	1 (0.78%)	1 (0.99%)	-
S945L	Disease-causing	1 (0.78%)	-	1 (3.84%)
R785X	Disease-causing	1 (0.78%)	1 (0.99%)	-
R117C	Disease-causing	1 (0.78%)	-	1 (3.84%)
R1066L	Disease-causing	1 (0.78%)	1 (0.99%)	-
P5L	Disease-causing	1 (0.78%)	1 (0.99%)	-
IVS 16+5G>A	No database	1 (0.78%)	1 (0.99%)	-
E217G	No database	1 (0.78%)	1 (0.99%)	-
F311L	Disease-causing	1 (0.78%)	1 (0.99%)	-
E92K	Disease-causing	1 (0.78%)	1 (0.99%)	-
E474K	Disease-causing	1 (0.78%)	1 (0.99%)	-
c.2052delA	Disease-causing	1 (0.78%)	1 (0.99%)	-
D1154N	VUS	1 (0.78%)	1 (0.99%)	-
4096-3C>G	Disease-causing	1 (0.78%)	1 (0.99%)	-
1677delTA	Disease-causing	1 (0.78%)	1 (0.99%)	-
E1228G	No database	1 (0.78%)	1 (0.99%)	-
c.482T>G	No database	1 (0.78%)	1 (0.99%)	-
c.3213G>T	No database	1 (0.78%)	-	1 (3.84%)
c.1520_1522del	No database	1 (0.78%)	1 (0.99%)	-
c.4411_delinsGTC	No database	1 (0.78%)	-	1 (3.84%)
c.376G>C	VUS	1 (0.78%)	-	1 (3.84%)
c.1186A>T	VUS	1 (0.78%)	-	1 (3.84%)
c.2339delG	No database	1 (0.78%)	1 (0.99%)	-
p.G780VfsX23	No database	1 (0.78%)	1 (0.99%)	-
c.3659C>T	No database	1 (0.78%)	-	1 (3.84%)

CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; NBS, newborn screening; VUS, Variants of uncertain significance.

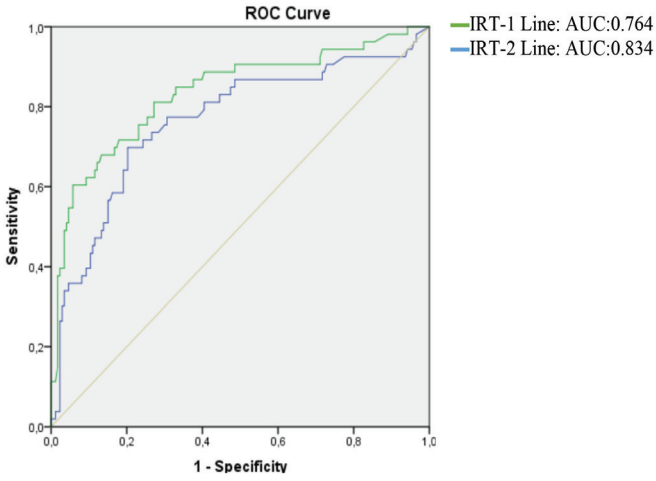

FIG. 1. Receiver operating characteristic curve for IRT-1 and IRT-2 test results.

ROC, Receiver operating characteristic; IRT, immunoreactive trypsinogen; AUC, Area under the curve.

DISCUSSION

This study is the first to evaluate the NBS program for CF with data from the Western Anatolian Region of Türkiye. We aimed to establish the optimal cut-off values for IRT-1/IRT-2, identify potential for improvement, and reduce the rate of FNPs who were initially classified as NBS-negative. Our center is the primary referral center in this region of Türkiye, where CF patients receive specialist CF care. Our study revealed that the sensitivity and PPV of the current program are low, and the number of false-positive infants and FNPs was higher than the documented rate.[3] In the previous studies evaluating the IRT/IRT protocol, the sensitivity ranged from 85% to 95%.[18] The rate of the FNPs in the study was consistent with that of other studies, ranging from 5 to 15%.[18] Maclean et al.[19] reported that CF-NBS may delay the diagnosis by falsely establishing that all CF patients have been identified, which may result in an increased rate of FNPs. Lowering the IRT cut-off values is the simplest method to increase sensitivity,[20] and decrease the FNP rate.[21] Six of the FNPs in the study could have been detected by lowering the IRT-1 cut-off below 42 µg/l. However, this may increase the false-positive referrals, leading to additional follow-up, laboratory testing, and a corresponding increase in cost.[20] According to our findings, the estimated optimum IRT-1 (124.5 µg/l) and IRT-2 (73.67 µg/l) cut-off points with the highest specificity and sensitivity were higher than those implemented today. Nevertheless, the sensitivity and specificity of IRT-1 (73.6%; 73%) and IRT-2 (75%, 75.5%) were below the recommended levels.[3] Our findings were comparable to a previous study conducted in the central Anatolia region of Türkiye, which reported an estimated IRT-1 of 116.7 µg/l and an estimated IRT-2 of 88.7 µg/l.[22] However, increasing the cut-off values will elevate the rate of FNP and delay the diagnosis. De Boeck[21] concluded that IRT/IRT programs having a low PPV of around 10% generally necessitate a second test. As per our findings, the PPV (23.3%) of the current program was lower than the ECFS suggestions.[3] The diagnosis was significantly delayed because of the high rate of FNP in the investigation, indicating the necessity of developing newer strategies. Integrating genetic analysis into a protocol should enhance PPV, lower the rate of infants with false-positive results, and reduce the cost of unnecessary follow-up.[23] Padoan et al.[24] reported that integrating genetic analysis into an IRT/IRT protocol lowers the FNP rate. *CFTR* gene mutations can be determined from the same dried blood sample obtained for performing IRT-1,[20] enabling early diagnosis.[25] The sensitivity of the IRT/DNA test is largely increased by the number of mutations in the *CFTR*-panel. According to a study from Wisconsin, the sensitivity of screening was increased to 99% using the IRT/25 *CFTR*-multi mutation assay.[26] The frequency of *CFTR*-variants differs based on ethnic and geographic origins.[27] Rock et al.[28] recommended the use of NGS with IRT (IRT/NGS) to identify more variants, leading to increased sensitivity and providing more equality among ethnically diverse groups that exhibit a lower incidence of F508del and other prevalent mutations.[29] Due to the ethnic diversity of the Turkish people and the wide distribution of CFTR mutations based on the registry,[4] implementing the IRT/DNA protocol would be challenging and expensive. Dayangaç-Erden et al.[30] suggested that population-specific mutation panels be revised for Turkish people considering their genetic heterogeneity. Unfortunately, employing a more comprehensive genetic analysis as a second-tier test may cause the recognition of the carriers and CRMS/CFSPID[31] and reveal novel variants with unknown phenotypes.[27] Sontag et al.[32] concluded that the IRT/IRT/DNA protocol should be considered to decrease the detection of carriers and CRMS/CFSPID; however, it may delay the diagnosis more than the IRT/DNA protocol.

The study from the Turkish national registry reported that the F508del mutation was the most prevalent in Türkiye, with an allelic frequency of 28%.[4] In the current study, F508del accounted for 31.49% of mutations, followed by G542X (7.8%). D110H (15.38%) was the most prevalent mutation after F508del (26.92%) for FNP. Three NBSPPs with SCC > 60 mEq/l exhibited only one mutation (F508del, E217G, and E1228G, respectively). A female patient with positive NBS demonstrated moderate SCC levels (the patient experienced respiratory infections and failure to thrive during follow-up). Among the 127 variants and 45 varied mutations identified from the CF patients by sequencing, three different variants were determined as variants of unknown clinical consequences (VUS), while 12 variants have not been reported in the *CFTR*-databases (Table 5). Three FNPs exhibited CFTR mutations with no database in trans, and one FNP demonstrated the presence of two concomitant VUS. As a result, the present investigation suggests that the final determination should not be based on genotype and laboratory data. Patients with one or no pathogenic variants should be diagnosed as CF based on their clinical features, even if their SCC is not > 60 mEq/l. The CFTR-2 database has been reported to evaluate over 400 variants; however, approximately 1600 CFTR variants, which are typically uncommon, were unable to be identified.[33] Defining these novel and rare variants detected with sequencing is also essential for administering mutation-based individualized treatments.[30] In a previous European study, it was more feasible to identify

CRMS/CFSPID infants with a CF: CRMS/CFSPID ratio spanning from 1.2:1 (Poland) to 32:1 (Ireland) through strategies that integrated genetic tests.[23] This ratio was 4.8:1 in the current study, and none of the CRMS/CFSPID children developed CF to date during their follow-up. Measuring a second-tier biochemical test for PAP (IRT/PAP) from the same heel prick enables early diagnosis.[34] Screening panel with PAP ensures a lower detection rate of CRMS/CFSPID, thus making it more cost-effective than IRT/DNA protocols.[35] Nevertheless, it appears to reduce sensitivity and PPV.[34]

In the present study, NBSPPs were diagnosed at a younger age than FNPs, with a median age of 2.5 months. However, they were older than had been previously documented. In a study from Türkiye, NBSPPs were diagnosed earlier than FNPs, with a median age of 150 days, similar to our study.[36] A previous study reported that cases diagnosed after 2 months of age result in worse outcomes and require additional therapy.[37] However, according to our study, delay in diagnosing FNP was not associated with more adverse nutritional and growth outcomes at the time of diagnosis. Notably, the weight-for-age z-scores were lower in NBSPPs, even though there was no significant difference ($p = 0.05$). This suggests that poor nutrition may occur as early as two weeks of age.[38,39] The functional classification of the *CFTR* genotype is related to the variations in phenotype and mortality. Patients with at least one class IV or V mutation exhibit milder clinical manifestations, delayed disease onset, and fewer bacterial infections.[10] The current study illustrated that NBSPPs were substantially more susceptible to severe/severe mutations, which resulted in worse nutritional outcomes, hospitalization, bacterial colonization, pancreatic insufficiency, and elevated respiratory symptoms than FNPs.

Clinical characteristics such as a history of failure to thrive, prolonged jaundice, meconium ileus (MI), steatorrhea during neonatal period, and a history of PBS were most frequently observed in the FNPs and should serve as warning signs for specialists. Of the 13 FNPs, three were detected with a family history, two with prolonged jaundice, six with PBS, and two with MI. Pediatric surgeons should be communicated regarding circumstances wherein children with MI may be excluded by screening[40] as these children exhibit lower IRT levels in infancy.[41]

One of the study limitations was the small number of study groups and the low rate of FNPs. More extensive nationwide studies should be designed using the registry to corroborate the findings of the current study. The second limitation was that the definition of pancreatic insufficiency was based on the necessity for pancreatic enzyme treatment, as fecal elastase analysis could not be performed in our hospital.

The IRT/IRT protocol is not feasible for Türkiye because of its low sensitivity and PPV. The rate of false positives from whom CF disease should be excluded and FNPs revealing a delay in the diagnosis were often than accepted. It is essential to define optimal cut-off values for IRT-1/IRT-2 or revise existing strategies to decrease the number of FNPs.

Our findings suggest that the delay in diagnosing FNP was not consistent with adverse nutritional outcomes at the time of diagnosis. However, more extensive studies with a greater number of FNPs, including their follow-up outcomes, are required. The sensitivity of a second-tier test must be enhanced to enhance the PPV of screening, necessitating revised cut-off values for IRT. Applying the IRT/DNA protocol may enhance the sensitivity and enable the early initiation of highly effective modulator therapies before irreversible damage occurs. However, the cost of increased referrals and carrier identification could be challenging for Türkiye. Additional comprehensive nationwide research is warranted to assess the accessibility of screening strategies.

Ethics Committee Approval: The study was approved by the Clinical Research Ethics Committee of Ege University Faculty of Medicine (approval number: 22-6.1T/50, date: 28.06.2022).

Informed Consent: Informed consent was obtained from the patients to participate in the study.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authorship Contributions: Concept- F.Ç.; Design- F.Ç.; Supervision- G.K.Ö., F.G., E.D.; Data Collection or Processing- F.Ç., H.D.Ş., M.B., M.M.Ö., B.G.D., E.O., E.H., Ş.A.Ö.; Analysis and/or Interpretation- F.Ç., G.K.Ö., H.D.Ş., Literature Search- F.Ç., G.K.Ö., Writing- F.Ç., G.K.Ö.; Critical Review- F.Ç., G.K.Ö., F.G., E.D.

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