ORIGINAL ARTICLE

TWO YEARS OF NEWBORN SCREENING FOR CYSTIC FIBROSIS IN NORTH MACEDONIA: FIRST EXPERIENCE

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ABSTRACT

There is a widely accepted consensus on the benefits of newborn screening (NBS) for cystic fibrosis (CF) in terms of reduced disease severity, improved quality of life, lower treatment burden, and reduced costs. More and more countries in the world are introducing NBS for CF as a national preventive health program. Newborn screening for CF was introduced in the Republic of North Macedonia (RNM) in April, 2019, after a pilot study of 6 months in 2018. A two-step immunoreactive trysino-gen (IRT-IRT) algorithm is performed, and then a sweat test for confirmation/exclusion of the CF diagnosis when the IRT values were both over the cutoff (70.0 and 45.0 ng/mL, respectively). In cases with confirmed diagnosis of CF (a sweat chloride concentration ≥60.0 mmol/L) or with intermediate sweat test results (a sweat chloride concentration of between 30.0 and 59.0 mmol/L), CF transmembrane conductance regulator (CFTR) muta-tion analysis is performed. By the end of 2020, over a period of 27 months, including the pilot study period, a total number of 43,139 newborns were screened for CF. Seventeen (0.039%) newborns were diagnosed with CF. In all newly discovered CF cases by screening, the diagnosis was confirmed by determination of the CFTR mutations. The most common CFTR mutation, F508del, was found with an overall incidence of 70.6%. Other more frequent mutations were G542X (11.8%) and N1303K (5.9%). Four mutations were found in one CFTR allele each: G1349D, G126D, 457TAT>G and CFTRdupexon22, with the last one being newly discovered with unknown consequences. An incredibly large difference was found in the incidence of the disease between the Macedonian and Albanian neo-natal population, with almost four time higher prevalence among Albanians (1:4530 *vs.* 1:1284).

Keywords: Cystic fibrosis (CF); cystic fibrosis trans-membrane conductance regulator (*CFTR*) gene mutations; Newborn screening (NBS).

INTRODUCTION

Cystic fibrosis (CF) is the most common, potentially fatal, genetic disorder in Caucasians, with autosomal recessive heredity, affecting around 1 in 3000 live births. The incidence varies in different geographical areas and ethnic groups. The CF diagnosis is classically made on the basis of clinical symptoms consistent with a diagnosis of CF: respiratory (chronic cough and sputum production, airway obstruction manifested by wheezing, recurrent pneumonia or obstructive bronchitis, persistent coloniza-tion/infection with typical CF pathogens, nasal polyps); gastrointestinal and nutritional abnormalities (meconium ileus, loose stools, symptoms of malabsorption, failure to thrive, hypo-proteinemia and edema, distal intestinal obstruction syndrome); salt loss syndrome (salt depletion and metabolic alkalosis) coupled with positive sweat test. The diagnosis of CF is also confirmed with genetic analysis and identification of two cystic fibrosis transmembrane

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conductance regulator (*CFTR*) gene mutations in *trans*. Due to the very diverse and variable clinical expression of the disease as well as the severity of the symptoms, CF diagnosis can often be delayed, when chronic lung disease and malnutrition are already established.

To improve the prognosis and opportunities for a better quality of life for persons with CF, early diagnosis through newborn screening (NBS) and the appropriate preventive and curative care management in specialized CF centers for affected children from the start of life are essential. Today, NBS is considered as important component in the standards of care for CF [1,2].

Newborn screening protocols for CF rely on immunoreactive trypsinogen (IRT) as the primary test and on the sweat test for confirmation or exclusion of the diagnosis of CF [3]. The increase of IRT in the blood of newborns with CF, which can be detected and in a dried drop of blood taken for routine screening, was first described in 1979 [4]. It's the result of complete or partial obstruction of the pancreatic ducts during intrauterine life and the outflow of acinus products into the vascular system. In order to improve the positive predictive value of the NBS program, a second tier test is mandatory. It can be either a second blood sample taken at 3 to 4 weeks of age for IRT-2, or *CFTR* gene mutation analysis using the initial blood spot or more recently a biological dosage of pancreatitis-associated protein (PAP) using the initial blood spot [3]. In the last 2-3 decades, a growing number of countries in Europe, North and South America are introducing NBS for CF, therefore, the number of newborns being screened for CF constantly increases [5]. Newborn screening for CF was introduced as a national program on all newborn population in the Republic of North Macedonia (RNM) from April 2019, after a pilot study in 2018.

MATERIALS AND METHODS

A two-step IRT-IRT algorithm is performed, and then a sweat test for confirmation/exclusion of the CF diagnosis when IRT values were both over the cutoff: 70.0 and 45.0 ng/mL, respectively (Figure 1). Heel prick blood samples are taken by a trained nurse, between 24 and 72 hours of life (when the baby is discharged from the maternity hospital) and spotted on Whatman 903 filter paper (Guthrie card). Premature or sick full-term neonates, who usually have a prolonged stay in neonatal intensive care units, are screened between the first and second week of life. The whole-blood samples are dried at room temperature within 2 hours and transported or mailed to the laboratory for neonatal screening twice per week from each birth center. The whole-blood IRT is measured by the DELFIA® time-resolved fluoroimmunoassay method, using a DELFIA® neo-

natal IRT kit (DELFIA®; PerkinElmer, Wallac Oy, Turku, Finland), and read by a 1420 VICTOR 2D Fluorometer (Wallac Oy, Turku, Finland). The IRT1 cutoff level of 70.0 ng/mL was established as 99.5th percentile of the IRT values obtained in a total of 2.058 newborns with birth weight >3000 g, gestational age >38 weeks, and age >48 hours. The second step in the algorithm is based on request of a second blood specimen from those neonates with elevated IRT-1, 3 weeks after the first screening. For IRT-2, a fixed cutoff level is used at 45.0 ng/mL. If the IRT-2 is over the cutoff value, the positive-screened child is referred for sweat testing. A doctor from the CF team meets the parents/baby before performing the sweat test and provide explanations about the screening results and the sweat test.

The sweat test is performed according to a standardized protocol via quantitative pilocarpine iontophoresis with the Macroduct® collection method. Sweat chloride concentration is measured in a ChloroChek® Chloridometer® (ELITechGroup, Puteaux, France). The sweat test is analyzed immediately and the result is reported to the family on the same day. Based on the recommendations for sweat chloride test results in healthy and CF-affected infants [2,6,7], the following sweat chloride reference ranges are use: ≤29.0 mmol/L, CF rejected or unlikely; 30.0 to 59.0 mmol/L, intermediate; ≥60.0 mmol/L,
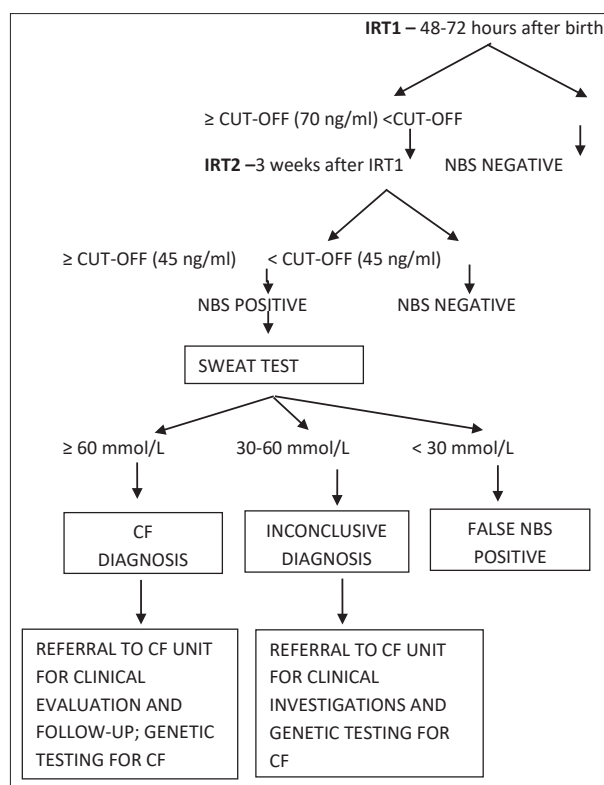

Figure 1. Algorithm for CF NBS in the Republic of North Macedonia.

consistent with a diagnosis of CF. Infants with a positive sweat test result are referred to the specialist CF doctor as soon as possible. A CF doctor informs the family about positive CF diagnosis, gives them information about the disease, the benefit of early diagnosis and treatment for the child's health and arranges admission to the CF Unit for a comprehensive assessment of the child, introduction of therapy and parent training for chest physiotherapy. Parental written informed consent for CFTR mutation analysis is required at the first visit.

DNA is isolated from peripheral blood using the standard phenol-chloroform extraction method. Mutation analysis of the *CFTR* gene is performed initially by SNaPshotreaction [8] for 11 most common regional CFTR mutations (F508del, G542X, N1303K, 621+1G>T, 2184insA, V456F, G126D, G1349D, E822K, R117C, 711+3A>G). For the patients whose genotypes were not determined with the initial molecular screening, several additional methods are applied, including multiplex ligation-dependent probe amplification (MLPA), for detection of deletions/duplications in the *CFTR* gene (SALSA MLPA KIT P091 *CFTR*; MRC-Holland, Amsterdam, The Netherlands); next generation sequencing (NGS), with TruSight Inherited gene panel performed on MiSeq Illumina Personal Sequencer and data analyses on the Illumina Variant Studio (Illumina Inc., San Diego, CA, USA) [8,9].

RESULTS

A total of 43,139 newborns have been screened for CF during the study period of 27 months (6 months pilot study period and 21 months national screening period). Recall rate was 0.44% ($n = 190$). Out of 41 (0.095%) positive screening cases, the diagnosis of CF was confirmed in 17 (0.039%) newborns after the positive sweat test. The average age of diagnosis of infants through NBS was 29 days (range from 23 to 43 days). Fifteen of all diagnosed cases at the end of the first month of life, already had symptoms consistent with the diagnosis of CF: failure to thrive (15), frequent loose stools (15), malnutrition with hypoproteinemia (5), cough, colonization/infection with *Staphylococcus aureus* (9), pneumonia (2) and salt depletion with metabolic alkalosis (1). Two babies, who were pancreatic sufficient, had no symptoms of the disease. During the period of NBS, only one CF case with meconium ileus (genotype F508del/457TAT>G) was missed on screening.

Our first NBS results for CF showed a high incidence of the disease in our geographic region of 1:2538 live newborns. We found a very large difference in the incidence of the disease between the Macedonian and Albanian neonatal population. Twelve of the detected CF cases were ethnic Albanians (70.6%) and five were ethnic Macedonians (29.4%). Moreover, the CF incidence observed among the Albanian neonatal population (1:1284) was almost 4-fold higher than the incidence detected in the Macedonian newborns (1:4530).

The genotype of detected CF patients by NBS is shown in Table 1. The most common CFTR disease causing mutation F508del was found with an overall incidence of 70.6%. Other more frequent mutations were G542X (11.8%) and N1303K (5.9%). Four mutations were found in one CFTR allele each: G1349D, G126D, 457TAT>G and CFTRdupexon22. The latter is a newly discovered mutation with unknown consequences, found in a pancreatic sufficient case in which CF diagnosis was confirmed by two positive sweat tests.

DISCUSSION

Cystic fibrosis is a multisystem genetic disease resulting in complicationsin multiple organs, but especially involving the lungs and pancreas. Since the discovery of the *CFTR* gene associated with CF, there has been a great progress in understanding and in the care of patients with this disease. Cystic fibrosis has been changed from a fatal early childhood disease to a chronic disorder in which most patients with CF are expected to live into adulthood. Early diagnosis by NBS, multidisciplinary care in specialized CF centers, and

Table 1. Genotypes of cystic fibrosis patients diagnosed with newborn screening.

Genotype (legacy name)	Genotype (cDNA name)	*n*
F508del/F508del	c.1521_1523delCTT/c.1521_1523delCTT	10
F508del/G542X	c.1521_1523delCTT/c1624G>T	3
F508del/G1349D	c.1521_1523delCTT/c.4046G>A	1
G542X/N1303K	c.1624G>T/c.3909C>G	1
N1303K/G126D	c.3909C>G/c.377G>A	1
457TAT>G/CFTRdupexon22	c.325_327delTATinsG/CFTRdupexon22[a]	1

[a] HGVS (Human Genome Variation Society) nomenclature: NC_000007.14: g.(117041992_117054830)_ (117054908_117069810) dup.

optimized and preventive treatments are the most important factors that have changed the face of CF [10].

Today, NBS is considered an essential component in the standards of care for CF [2]. The vast majority of newborns in North America, Europe, Australia and New Zealand, and a growing number in South America, are screened for CF [5]. Newborn screening and early appropriate treatment (pancreatic enzyme replacement, fat-soluble vitamins, salt supplementation) has a beneficial effect on growth and nutritional status, and prevent deficiency of fat-soluble vitamins and protein malnutrition [3,11-13]. Cystic fibrosis patients diagnosed with NBS have a lower burden of treatment and fewer hospitalizations for intravenous antibiotic therapy due to exacerbation of lung disease [12,13]. Children diagnosed with CF by NBS are expected to have better lung function and lower incidence of *Pseudomonas aeruginosa* infection, in particular delayed onset of chronic *Pseudomonas aeruginosa* infection [12,14,15]. Newborn screening for CF leads to improved long-term health outcomes and survival for the CF population [12,16]. Screening is a cost-effective public health strategy [17]. In the era of CFTR modulator therapies that correct the basic underlying molecular defect, early diagnosis with NBS will enable the timely introduction of this therapy in the future.

Considering the confirmed benefits from the early diagnosis of CF by NBS, which was introduced in our country as a national program on all newborn poputation from 2019, after a previous pilot study. We performed a two-step IRT-IRT algorithm, and then a sweat test for confirmation/exclusion of the CF diagnosis when IRT values are both above the cutoff values. In cases of positive or borderline sweat tests, mutation analysis of *CFTR* gene was performed.

However, the screening protocols are varied, and there are many different NBS protocols for CF across Europe. Most programs use DNA analysis as a second-tier test, due to the fact that the IRT-1/IRT-2 protocol is not sufficiently sensitive [18]. While five CF screening protocols in Europe (Austria, Portugal, Russia, Slovakia and Turkey) still rely exclusively on biochemical tests, either a repeat IRT measurement at days 14-21 or measurement of PAP in parallel to IRT-1 plus IRT-2 measurement in/after the third week [5,19]. The IRT values decrease in infants without CF over the first 4 weeks of life, but remain high in those with CF. The IRT-1/IRT-2 protocol improves positive predictive value by reducing the number of infants who are referred for a sweat test. Biochemical screening protocols also avoid the issues raised by CFTR mutation analysis as a second step such as carrier detection and limited the number of cases with equivocal diagnosis of CF. Cystic fibrosis screen positive, inconclusive diagnosis (CFSPID), also known as CFTR-related metabolic syndrome (CRMS) in the USA, are infants detected by NBS

with a normal sweat test and two CFTR mutations, at least one of which has unclear phenotypic consequences and infants with intermediate sweat test and one or no CFTR mutations [7,20,21]. Infants with an uncertain diagnosis of CF (CFSPID/ CRMS) require further investigation that should be undertaken with close liaison of the CF Center with the service for molecular genetics. A number of these children will remain free of symptoms throughout their life, but some of them may develop clinical features suggestive of CFTR-related disorder (CFTR-RD) or clinical features of CF later in life [21,22]. Many more CFSPID/CRMS cases are found if DNA analysis is a second-tier test in screening protocol, especially if protocols include large panel of CFTR mutations [22].

During the screening period, one CF case with meconium ileus was missed on screening, that is, the NBS test for CF was false negative. It is well recognized that infants with meconium ileus usually have IRT-1 values below the cutoff and false negative NBS results [23,24]. Therefore, any case of meconium ileus should be considered as CF until proven otherwise.

The incidence of CF in the Republic of North Macedonia, estimated in the short observation period while the NBS program was being implemented, is one of the highest in Europe, on average 1:2500 [11]. North Macedonia is a multiethnic country. In the newborn population, Macedonians of Slavic origin are in the majority, contributing 53.0%, 30.0% are ethnic Albanians, 7.0% are Romas, 5.0% are Turks and 5.0% are of other ethnicities. The NBS for CF revealed a huge difference in disease incidence between the two largest newborn populations in the country, Macedonian (1:4530) and Albanian (1:1284). The closedness of the Albanian population over the centuries, including consanguineous marriages, has contributed to the greater frequency of the pathological *CF* gene in this population. Further years of NBS would give us a more accurate assessment of the incidence of CF in our geographic area and between the different ethnic groups.

CONCLUSIONS

The expansion of CF NBS across Europe and around the world in the last few decades indicates that this screening is considered worthwhile. Newborn screening for CF was introduced for all newborns in the RNM in 2019, included in the preventive program for mothers and children's care of the Ministry of Health. Our first experiences with NBS for CF gave us promising results and objective hope for keeping patients in good health.

Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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