Journal of Cystic Fibrosis 18 (2019) 54 – 63

Journal of Cystic Fibrosis

www.elsevier.com/locate/jcf

Original article

Newborn blood spot screening for cystic fibrosis with a four-step screening strategy in the Netherlands

Jeannette E. Dankert-Roelse [a,*], Marelle J. Bouva [b], Bernadette S. Jakobs [c], Hettie M. Janssens [d], Karin M. de Winter-de Groot [e], Yvonne Schönbeck [f], Johan J.P. Gille [g], Vincent A.M. Gulmans [h], Rendelien K. Verschoof-Puite [i], Peter C.J.I. Schielen [b], Paul H. Verkerk [f]

[a] *School for Public Health and Primary Care, Dept Pediatrics, Maastricht University Medical Centre, Debyelaan 25, 6229 HX Maastricht, the Netherlands*
[b] *Reference Laboratory for Neonatal Screening, Centre for Health Protection, National Institute for Public Health and the Environment (RIVM), Postbus 1, 3720 BA Bilthoven, the Netherlands*
[c] *Department of Clinical Chemistry and Haematology, Elisabeth-Twee Steden (ETZ) Hospital, Hilvarenbeekseweg 60, 5022 GC Tilburg, the Netherlands*
[d] *Department of Pediatric Pulmonology, Erasmus Medical Center, Sophia's Children's Hospital, Doctor Molewaterplein 40, 3015 GD Rotterdam, the Netherlands*
[e] *Department of Pediatric Pulmonology, Wilhelmina Children's Hospital, University Medical Centre Utrecht, Utrecht University, Lundlaan 6, 3584 EA Utrecht, the Netherlands*
[f] *TNO, Dept Child Health, Schipholweg 77, 2316 ZL Leiden, the Netherlands*
[g] *Department of Clinical Genetics, VU University Medical Centre, de Boelelaan 1118, 1081 HV Amsterdam, the Netherlands*
[h] *Dutch Cystic Fibrosis Foundation (NCFS), Doctor Albert Schweitzerweg 3, 3744 MG Baarn, the Netherlands*
[i] *Department of Vaccine Supply and Prevention Programmes, National Institute for Public Health and the Environment (RIVM), Postbus 1, 3720 BA Bilthoven, the Netherlands*

Received 5 February 2018; revised 9 July 2018; accepted 16 July 2018
Available online 23 August 2018

Abstract

Background: Newborn screening for cystic fibrosis (NBSCF) was introduced in the Dutch NBS program in 2011 with a novel strategy.
Methods: Dutch NBSCF consisted of four steps: immuno-reactive trypsin (IRT), Pancreatitis-associated Protein (PAP), DNA analysis by Inno-LiPa (35 mutations), extended gene analysis (EGA) as fourth step and as safety net. Only samples with two CFTR-variants were considered screen-positive, but samples with one disease-causing variant were considered also screen-positive from April 2013. The first 5 years of NBSCF were evaluated during a follow-up ranging from 2 to 6.8 years for sensitivity, specificity, positive predictive value (PPV), ratio of CF/Cystic Fibrosis

Abbreviations: NBSCF, Newborn screening for cystic fibrosis; IRT, immunoreactive trypsin; PAP, Pancreatitis-associated Protein; EGA, extended gene analysis; PPV, positive predictive value; CFSPID, Cystic Fibrosis Screen Positive infants with an Inconclusive Diagnosis; ECFS, European Cystic Fibrosis Society; RIVM, National Institute for Public Health and the Environment; CvB, Centre for Population Screening; NEORAH, Neonatal Registry of positive NBS tests; GP, general practitioner; NCFR, Dutch Cystic Fibrosis register; DPSU, Dutch Paediatric Surveillance Unit; MI, meconium ileus; MLPA, multiplex ligation-dependent probe amplification

* Corresponding author.
E-mail addresses: jeannettedankert@gmail.com, (J.E. Dankert-Roelse), marelle.bouva@rivm.nl, (M.J. Bouva), b.jakobs@etz.nl, (B.S. Jakobs), h.janssens@erasmusmc.nl, (H.M. Janssens), k.m.dewinter@umcutrecht.nl, (K.M. de Winter-de Groot), yvonne.schonbeck@tno.nl, (Y. Schönbeck), jjp.gille@vumc.nl, (J.J.P. Gille), v.gulmans@ncfs.nl, (V.A.M. Gulmans), rendelien.verschoof@rivm.nl, (R.K. Verschoof-Puite), peter.schielen@rivm.nl, (P.C.J.I. Schielen), paul.verkerk@tno.nl. (P.H. Verkerk).

https://doi.org/10.1016/j.jcf.2018.07.008

Screen Positive infants with an Inconclusive Diagnosis (CFSPID) and median age at diagnosis, and were compared to other novel strategies for NBSCF and European Cystic Fibrosis Society (ECFS) Best Practice Standards of Care.
Results: NBSCF achieved a sensitivity of 90% (95% CI 82%–94%), specificity of 99.991% (95% CI 99.989%–99.993%), PPV of 63% (95% CI 55%–69%), CF/CFSPID ratio of 4/1, and median age at diagnosis of 22 days, if samples with two variants as well as samples with one disease-causing variant were considered screen-positive.
Conclusion: The program achieved the goal to minimize the number of false positives and showed a favourable performance but sensitivity and CF/CFSPID ratio did not meet criteria of EFCS Best Standards of Care. Changed cut-off values for PAP and IRT and classification of R117H-7T/9T to non-pathogenic may improve sensitivity to ≥95% and CF/CFSPID ratio to 10/1. PPV is estimated to be around 60%.

Keywords: Newborn screening; Immuno-reactive trypsin; Pancreatitis-associated protein; DNA-analysis; Extended CFTR-gene analysis; Validity

1. Introduction

Newborn screening (NBS) for Cystic Fibrosis (CF), mostly by means of blood spot screening, has been incorporated in many NBS programmes in most western countries in the last decade. NBSCF leads to an earlier diagnosis and improved outcome for children with CF in many countries. With the development of drugs correcting the basic defect, in due time NBSCF will become the essential first step in the management of patients with CF ensuring that treatment starts before irreversible organ damage has occurred [1–3].

NBSCF is performed within the domain of public health and harm of participants in screening programs should be minimal. For most diseases in current NBS programmes the suspected disease is easily confirmed or excluded, but after a positive screening result for CF excluding the diagnosis can be difficult. False positive cases should therefore be avoided. The sweat test is the gold standard confirming or excluding the diagnosis CF. However, in infants below three months of age sweat-tests fail in up to 20% and it may take weeks before a sweat-test is successful, causing a prolonged period of great parental anxiety [4, 5]. Moreover finding carriers is not the aim of NBS as each individual has the right-not-to-know. This right will be violated when carrier status is revealed through NBS [6].

To minimize the number of newborns with false positive screening tests for CF the Health Council of the Netherlands advised to introduce a novel four-step strategy based on the results of a pilot study [7, 8], with a two tier assay for immuno-reactive trypsin (IRT) and Pancreatitis-associated Protein (PAP) followed by DNA analysis (DNA) and extended gene analysis (EGA). This theoretically would lead to a screening method with very high specificity as well as positive predictive value (PPV) and an acceptable sensitivity of 95% [7].

The aim of this study was to assess the performance of this four-step strategy in a routine NBS-program, to evaluate if the pre-set goals were achieved and to compare this approach with the European Cystic Fibrosis Society (ECFS) Best Practice Standards of Care [9] measured as PPV, sensitivity, ratio of CF/Cystic Fibrosis Screen Positive infants with an Inconclusive Diagnosis (CFSPID) [10] and median age at diagnosis, and with other novel strategies for NBSCF.

2. Patients and methods

2.1. The Dutch newborn screening program

In the Netherlands newborn screening is offered free of charge [11]. The Dutch NBS program is centrally organised and coordinated by the Centre for Population Screening of the National Institute for Public Health and the Environment (RIVM-CvB). Future parents get oral and written information about the NBS program. Informative leaflets are provided 2 times, in the third trimester of pregnancy, and when the baby is registered at the municipality. Dutch municipalities report the personal data of each registered newborn in a web-based registry to the RIVM as soon as possible. The medical advisor of RIVM asks local youth health organizations or midwives to take a blood sample between 72 and 168 h after birth. After oral consent of the parents, blood samples are collected mostly at home and sent by regular postal services to one of the five designated national screening laboratories. Screening laboratories report screening results directly in the central database and inform RIVM immediately when a screening result is positive. RIVM registers all positive screening results in a web-based registry (Neonatal Registry of positive NBS tests (NEORAH)). CF-centres enter their diagnostic results into NEORAH. The complete NBS program is annually monitored by an independent institute (TNO, Department Child Health).

2.2. Reporting of positive results of NBSCF to the parents

For each newborn with positive NBS medical advisors of the RIVM arrange a timely referral. The length of time between informing the parents and referral is variable, dependent on the disease and if immediate treatment is urgent or not. After a positive screening for CF, the newborn is referred within a week to a designated CF-centre after consulting the general practitioner (GP). After an appointment has been made for a sweat test the GP informs parents about the positive screening for CF and necessary referral. GP's take care to inform parents not earlier than 48 h before the planned sweat test.

2.3. Study population

NBSCF was offered to all newborns in the Netherlands from May 1st, 2011. All data collected from May 1st, 2011 until January 1st, 2016 were included. The study population consisted of all infants born in this period who got NBS.

2.4. Screening protocol: four-step screening strategy for CF

The screening strategy for CF used in the Dutch routine NBS program consisted of four consecutive steps (Fig. 1). Step 1, IRT, and step 2, PAP, were performed in all five screening laboratories. Step 3, DNA, was performed in two of the five screening laboratories (ETZ Hospital Tilburg, Isala Clinics Zwolle, The Netherlands). For Step 4, EGA, samples were sent to the VU university medical centre.

Concentrations of IRT (AutoDELFIA® or Genetic Screening processor (GSP®) neonatal IRT) were measured in dried blood spots of heel prick cards using a time resolved fluorometric assay (B005–112, PerkinElmer, Turku, Finland, www.perkinelmer.com), according to the manufacturer's protocol. Screening was negative if IRT concentrations were <60 μg/L blood.

In all blood spots with IRT concentrations ≥60 μg/L blood, PAP concentrations were measured by MucoPAP enzyme-linked immunosorbent assay (ELISA) (since 2015 MucoPAP-F) (Dynabio, Marseille, France, www.dynabio.com) following an adapted protocol. When PAP was ≥3.0 μg/L blood, or PAP ≥ 1.6 μg/L blood and IRT ≥ 100 μg/L blood, DNA analysis was performed. Cut-off values for IRT and PAP were based on the earlier study [7,8]. Corresponding percentiles of cut-off values were IRT 60–98.5%, IRT 100–99.7%, PAP 1.6–84%, PAP 3.0–92%.

DNA was extracted from the dried blood spots using the MagNa Pure compact nucleic acid isolation kit and performed on the MagNa Pure Compact (Roche). Mutation analysis of the CFTR gene was performed using Reversed DotBlot analysis (lineblot assay of INNO-LiPa CFTR 19 and INNO-LiPa CFTR17+ Tn, Fujirebio, Belgium). (Appendix A).

If mutation analysis revealed only one variant EGA of all coding exons of the *CFTR* gene (including intron/exon boundaries) was performed by PCR and Sanger sequencing. As safety net, EGA was also carried out in all samples without identified DNA-variants if IRT concentrations were ≥100 μg/L and PAP concentration ≥ 1.6 μg/L blood.

Known CFTR variants were classified as disease causing ("A"), having a variable or uncertain clinical effect ("O") or non-pathogenic ("N"). Database searches for presence of the variant in patients and controls were performed in gnomAD, ClinVar, HGMD, LOVD, CFTR2 (www.cftr2.com)) and in literature searches (PubMed, Google). Not earlier described CFTR variants ("novel" variants) were classified similarly by clinical laboratory geneticists with experience in DNA testing for CF. For classification different tools were used: in silico prediction of the effect of amino acid changes (AlignGVGD, SIFT, Polyphen), and splice prediction algorithms to detect

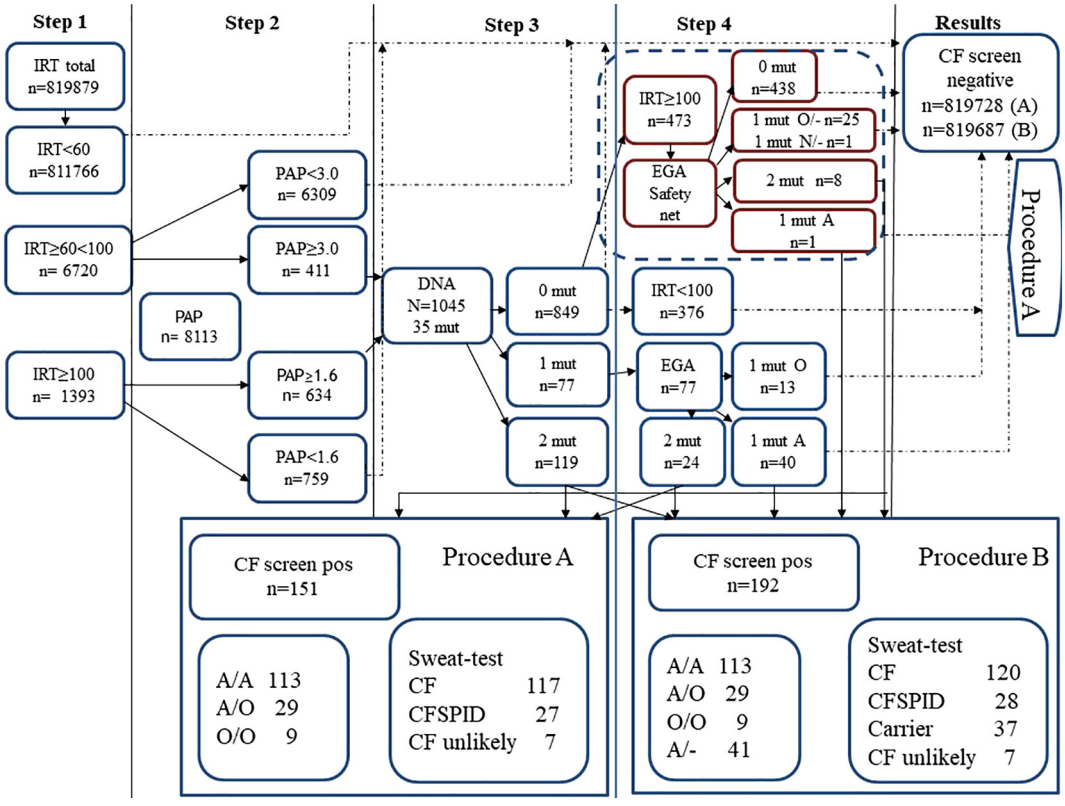

Fig. 1. Flow chart and results of screening program for procedures A and B.

possible splice defects (SpliceSitefinder-like, MaxEntScan GeneSplicer, NNsplice). Variants certainly affecting the proper functioning of the CFTR protein, such as stop and frameshift variants or variants in splice sites were classified "A", variants which might influence the proper functioning of the protein (e.g. missense variants changing only one amino-acid) were classified "O", and variants which based on prediction programs will have no effect on the function of the protein, e.g. variants in introns outside the splice site as "N". CFTR variants known to cause only male infertility were considered non-pathogenic and were ignored.

2.5. Criteria for positive screening tests

From May 1, 2011 until April 9th, 2013 screening was considered positive only if two CFTR variants were found, either both variants were "A", or one "A" variant was combined with an "O" variant, or both variants were classified as "O". Screening was considered negative when DNA- or EGA-analysis revealed only one "O" variant. Detection of one "A" variant was considered as a negative screening result. Parents were informed that their child was a carrier of one CFTR variant and therefore considered as having no CF. They were advised to consult a clinical geneticist for genetic counselling. After two children considered as CF-carriers (as EGA-analysis had failed to detect a second variant) were diagnosed with severe CF at the ages of 1.0 and 1.3 years, the criteria for a positive screening were changed. If only one "A" variant was identified screening was considered positive also. This new criterion was implemented starting April 9th, 2013. Furthermore all infants reported as CF-carriers born between May 1st, 2011 until April 9th, 2013 were recalled for a follow-up visit including a sweat test.

2.6. CF diagnosis

All newborns with a positive screening test were referred to one of seven designated Dutch Cystic Fibrosis centres for a sweat test, performed on the day of referral. Dutch CF centres collected sweat by means of a quantitative pilocarpine ionthophoresis test (QPIT) or the Macroduct collection system and measured [Cl^-] concentrations by a standard colorimetric procedure. For the interpretation of sweat test results we followed recent international guidelines [12], with two exceptions. First, infants with two CFTR variants of variable clinical consequences and a sweat test result of [Cl^-] < 30 mmol/L were considered as CF unlikely and not as CFSPID, eg infants homozygous for the R117H-7T variant belonged to this category. Secondly, because our algorithm includes EGA, children with one "A" variant and a sweat test [Cl^-] < 40 mmol/L sweat were considered healthy carriers. In newborns with two "A" variants CF was confirmed with sweat [Cl^-] ≥ 30 mmol/L. Sweat [Cl^-] ≥ 60 mmol/L confirmed the diagnosis CF in all infants with at least one "A" variant, or two "O" variants. In newborns with two variants of which at least one was disease-causing, CF could not be confirmed nor excluded with sweat [Cl^-] < 60 mmol/L. These infants were first labelled as non-classical CF but in the present analysis considered as CFSPID [10]. CFSPID newborns were followed in the CF centres according to ECFS guidelines [10, 12]. Newborns with CFSPID, healthy carriers and infants unlikely to have CF were considered as having a false positive screening test.

Results of the sweat tests were reported in [Cl^-] concentrations in NEORAH as well as in the Dutch Cystic Fibrosis register (NCFR). In May 2016, a cross check was performed between NEORAH and NCFR evaluating completeness of data of registered patients, CFTR-variants, results of sweat tests and final diagnoses in both registries. Discrepancies were checked and missing data were either enriched from the other registry or added by paediatricians from the CF centres.

2.7. CF patients missed by NBSCF

All patients with a CF diagnosis not identified by NBS were reported directly by the CF-centres to the RIVM. Moreover the Dutch Paediatric Surveillance Unit (DPSU) was used to assess the number of false negative screening results and cases missed for other reasons. All Dutch paediatricians were asked monthly to report all patients missed by the routine NBS program to DPSU.

2.8. Data analysis

Performance was measured in terms of validity (sensitivity and specificity) as well as of PPV, ratio of CF to CFSPID, and median age at diagnosis.

We used data from the five screening laboratories for IRT and PAP-concentrations and for DNA-analyses; data of the EGA-analyses were obtained from the VU medical centre.

TNO provided data of the annual evaluations of the NBSCF, including patients missed by NBS. DPSU also provided data about reported CF patients missed by NBS.

We calculated sensitivity, specificity, PPV and CF/CFSPID ratio for two criteria for screen positive tests, procedures A and B. Procedure A (tests were only screen positive if two CFTR variants were identified) was implemented from May 1, 2011 and calculated as if in use until January 1st 2016. The results of procedure B (tests were also positive as only one disease causing variant was identified) was implemented since April 9th, 2013 and calculated as if in use since May 1st 2011. We compared the results of procedure B to the ECFS standards of care and with recently published values of other novel screening strategies for CF.

3. Results

3.1. Four-step screening strategy for NBSCF

During the study period 819,879 newborns were screened in the Netherlands (participation rate was 99.3% of the total number of newborns during the study period). The results of the

four-step NBS, calculated as procedures A and B, are shown in Fig. 1.

In procedure A 151 newborns were referred for a sweat test which confirmed CF in 117 infants, in 27 the diagnosis could not be confirmed nor excluded (CFSPID), in 7 CF was considered unlikely. Forty-one infants with one A-variant after the fourth step in the screening strategy were considered as healthy carriers and not referred.

In procedure B 192 newborns, 151 with two variants, and 41 with one A variant, were referred for a sweat test. CF was confirmed in 120 infants. Furthermore sweat tests found 37 carriers and 28 CFSPID cases. CF was considered unlikely in seven infants.

In both procedures 16 infants with a positive screening test had meconium ileus (MI).

3.2. Results of IRT and PAP

In total 8113 IRT tests were ≥60 μg/L blood, 0.98% of all screened newborns. In the next step, PAP, 1045 samples (0.13% of all screened newborns) were ≥cut-off values (Fig. 1).

3.3. Results of DNA-analyses

DNA-analysis was performed in 1045 bloodspots. No variants were found in 849 (81.2% of all DNA-analyses), an IRT ≥ 100 μg/L had been found in 473 (45%) of these (Fig. 1), these bloodspots were further analysed in the safety net. One "A" or "O" variant was identified in 77 bloodspots (7.4%), further analysis followed in the fourth step. In 119 (11.4%) two CFTR variants were found (Fig. 1), 96 "A"/"A", 21 "A/O", 2 "O"/"O" (Appendix B). All infants with two CFTR-variants were screen positive in both procedures.

3.4. Extended gene analyses

3.4.1. EGA as fourth step in protocol

Seventy-seven EGA's led to the finding of a second "A" variant in 14 samples and a second "O" variant in 10 (Appendix B). CF-diagnosis was confirmed in 14 (18%) newborns with two "A"-variants, and in three (4%) with "A/O" variants; CF was unlikely in two with "O/O" variants, four with "A/O" variants were labelled as CFSPID. No second variant was identified in 13 infants with one R117H-7T/9T variant (screen negative), nor in 40 samples with one "A"-variant, screen negative in procedure A, but screen positive in procedure B (Fig. 1). One of these had a sweat[Cl^-] of 46 mmol/L, and was considered as CFSPID.

3.4.2. EGA as safety-net procedure

The safety-net procedure ($N = 473$) showed no variants in 438 (93%), in 25 (5%) one "O" variant was found, and one non-pathogenic variant ("N") in one (0.2%), all considered as screen-negative. Eight had two variants and were referred in both procedures A and B, one had one "A" variant and was only screen positive in procedure B (Fig. 1). In three of these CF was confirmed, two were reported as CFSPID, one as a healthy carrier, in three CF was unlikely (Table 1).

In summary, 550 EGA's, 77 as fourth step in the screening protocol and 473 in the safety net, detected in total 31 (6%) "A" variants in 23 newborns. Five variants (1%) in 3 newborns were novel.

An overview of all identified variants is shown in Appendix B.

F508del was the most frequently found "A" variant, and present in 86% of the patients with CF, 57.5% of the CF patients was homozygous for this variant. R117H-7T was the second most frequently found variant and present in 22 of the 28 infants with CFSPID; the diagnosis of CF was made in only one child with R117H-7T/F508del (sweat[Cl^-] 66 mmol/L). The other 6 infants with CFSPID were found by EGA, in three after DNA-analysis revealed one variant, and in three by the safety-net procedure.

3.5. Diagnostic procedures

Diagnostic procedures were started in 87% of all referred newborns without meconium ileus before the age of 30 days, with a median age at referral of 20.6 days. Eight % was referred before the age of 2 weeks, 38% in the third week of life, 32% in the fourth week, and 22% after the age of 28 days. Median age at diagnosis for patients with CF was 21.8 days. In one patient with CF (R117H-7T/F508del) the diagnosis was made at the age of 213 days. For the other 118 patients with CF the range varied from day 0 to day 50, two already had a prenatal diagnosis.

Sweat test results were recorded for most referred newborns; results were missing in 9/120 CF patients (7.4%), all with two

Table 1
Results of safety net.

N of CFTR variants	Variant	Class	Variant	Class	Sweat test	Result
2	c.1679 + 1G > C, p.?	A	c.1679 + 1G > C, p.?	A	104	CF
2	c.1418del, p.Gly473Glufs*54	A	c.1418del, p.Gly473Glufs*54	A	116	CF
2	c.825C > G, p.Tyr275* (Y275X)	A	c.1675G > A, p.Ala559Thr (A559T)	A	123	CF
2	**c.5A > C, p.Gln2Pro (Q2P)**	O	c.3154 T > G, p.Phe1052Val (F1052 V)	O	34	CFSPID
2	**c.1973_1985delinsAGAAA p.(Arg658Lysfs*4)**	A	**c.4056G > C, p.Gln1352His (Q1352H)**	O	12	CFSPID
1	c.680 T > G. p.Leu227Arg (L227R)	A	–	–	31	carrier
2	c.3854C > T, p.Ala1285Val (A1285V)	O	c.4096A > T, p.Ile1366Phe (I1366F)	O	10	healthy
2	c.1001G > A, p.Arg334Gln (R334Q)	O	**c.3964-6C > T, p.?**	O	8	healthy
2	c.91C > T, p.Arg31Cys (R31C)	O	c.3454G > C, p.Asp1152His (D1152H)	O	20	healthy

Novel variants are in bold, legacy names between parentheses.

CF causing "A" variants, in two infants considered as CF unlikely, both homozygous for the R117H-7T variant, and in 22 referred carriers, of which 16 were born before April 9th, 2013. After recall of these infants normal sweat test results were reported for 15, for one child parents refused to have a sweat test performed because prenatal diagnosis already had shown that their child was a carrier.

3.6. False negative screening tests

All infants reported as missed by NBSCF were missed due to a false negative screening test.

Table 2 summarizes test results of 19 CF patients with a false negative screening test. Median age at diagnosis was 0.2 years (73 days). IRT (7/16) or PAP concentrations (8/16) below cut-off value were the most frequent causes of a false negative result. One CF patient was missed because no variants were found in the DNA-analysis. No safety net procedure was performed because IRT concentration was <100 μg/L blood. (Table 2, nr 16). Three infants were missed by EGA. Two infants were diagnosed at the ages of 1.0 and 1.3 years (Table 2, nrs 17 and 18) which led to the change from procedure A to procedure B. From May 1, 2011 until April 9, 2013 the screening program identified in total 19 infants as healthy carriers. All children were recalled for a sweat test. Among these children a third CF patient with false negative screening due to a large deletion not identified by EGA was found at the age of four months (Table 2, nr 19).

3.7. Results and performance

Specificity of both procedures was higher than 99.99%% (Table 3). When patients with MI were excluded sensitivity varied from 86% (procedure A) to 90% (procedure B).

3.8. Prevalence of CF

Prevalence of CF at birth was 1: 6029, in total 136 CF cases were found in a screened population of 819,879 newborns. It is estimated that 10 to 20% of newborns with CFSPID with a

Table 2
Causes of false negative screening results. *

Cause	Case	IRTμg/L	PAPμg/L	DNA test,(legacy names), ¥	EGA test	Sweat test [Cl−]mmol/L#	Age atdiagnosis (yrs)
IRT < cut-off	1	27*	nd (2.7)	nd (F508del/F508del)	nd	86	0
	2	36*	nd (71.8)	nd (F508del/F508del)	nd	na	0
	3	41	nd (4.3)	nd (F508del/F508del)	nd	70	0.1
	4	45	nd	nd (F508del/1303NK)	nd	na	0.2
	5	57	nd (10.6)	nd (F508del/1303NK)	nd	115	0
	6	48	nd (1.56)	nd (F508del/3272-26A > G)	nd	71	0.4
	7	46	nd	nd (F508del/A455E)	nd	62	3.2
PAP < cut-off	8	76	1.5	nd (F508del/F508del)	nd	99	0
	9	78*	1.6	nd (F508del/F508del)	nd	80	0
	10	106*	1.5	nd (F508del/F508del)	nd	91	0.1
	11	122	1.2	nd (F508del/G542X)	nd	na	1.1
	12	123	1.3	nd (F508del/R553X)	nd	91	0.2
	13	200	1.0	nd (F508del/3905insT)	nd	103	2.0
	14	174	1.3	nd (F508del/F508del)	nd	69	0.1
	15	317	0.7	nd (F508del/F508del)	nd	107	0
DNA analysis	16	89	3.6	0 mutations	nd (1259insA/S1159F)	133	0.7
EGA analysis	17**	305	5.6	F508del	No 2nd mutation found(deletion exon 17a/b)	97	1.0
	18**	160	14.3	711 + 1G > T	No 2nd mutation found(deletion exon 11)	99	1.3
	19**	300	10.1	F508del	No 2nd mutation found(deletion exon 19)	90	0.4

nd: not performed in screening program, values in parentheses: values determined in blood spot after confirmed diagnosis of CF.
#: as registered in NCFR, na: no result of sweat test registered.
¥: new nomenclature:
F508del-> c.1521_1523del p.Phe508del (F508del).
N1303 K-> c.3909C > G p.Asn1303Lys (N1303 K)
3272-26A > G -> c.3140-26A > G (3272-26A > G).
G542X -> c.1624G > T; p.Gly542* (G542X).
A455E -> c.1364C > A; p.Ala455Glu.
R553X -> c.1657C > T p.Arg553* (R553X).
3905insT -> c.3773dup p.Leu1258Phefs*7 (3905insT)
711 + 1G > T -> c.579 + 1G > T p.? (711 + 1G > T).
1259insA -> c.1130dup (p.Gln378Alafs*4)
S1159F -> c.3476C > T, p.Ser1159Phe (S1159F).
Deletion exon 17a/b -> c.(2988 + 1_2989−1)_(3367 + 1_3368−1)del (del exon 17a/b).
Deletion exon 11 -> c.(1584 + 1_1585−1)_1679 + 1_1680−1)del (del exon 11).
Deletion exon 19 -> c.(3468 + 1_3469−1)_3717 + 1_3718−1)del (del exon 19).
* Meconium ileus **missed by EGA in Procedure A, referred to CF centre in Procedure B where sweat tests confirmed diagnosis of CF.

Table 3
Performance of screening procedures A and B.

	Procedure A (*N* = 819,879)	Procedure B (N = 819,879)
True-positive (n with MI[a])	117 (16)	120 (16)
False-positive (n with CFSPID)	34 (27)	72 (28)
True-negative	819,709	819,671
False-negative (n with MI)	19 (4)	16 (4)
CF/CFSPID ratio	4/1	4/1
Total CF (n with MI)	136 (20)	136 (20)
Sensitivity, % (CI)	86 (79–91)	88 (81–93)
Sensitivity w/o MI, % (CI)	86 (78–92)	90 (82–94)
Specificity,% (CI)	99.996 (99.994–99.997)	99.991 (99.989–99.993)
PPV, % (CI)	77 (70–84)	63 (55–69)

Procedure A: screen positive if 2 mutations (no referral of infants with only one "A" mutation).
Procedure B: screen positive if 2 mutations or 1 "A" mutation.
[a] MI = meconium ileus. CI = 95% confidence interval.

sweat[Cl^-] ≥ 30 and <60 mmol/L will turn out to have CF [10, 13, 14]. In our population 10 of the 28 newborns with CFSPID showed a sweat[Cl^-] ≥ 30 mmol/L sweat.

3.9. Comparison with other novel NBSCF strategies

We compared the results of procedure B with recently published novel NBSCF approaches (Table 4) [14–18]. Assessing the 4 step strategy with ECFS standards of care we found a considerably higher PPV than recommended (63% versus >30%), and a median age at diagnosis of 22 days which compares quite well with other novel strategies (Table 4) as well as with the ECFS standard <30 days [8]. Sensitivity was below standards of care (90% versus ≥95%), and below the sensitivities observed in other strategies (Table 4).

4. Discussion

The four-step screening strategy for NBSCF introduced since May 2011 appeared to be feasible within the routine Dutch NBS program. Both procedures A and B showed a favourable performance with regard to specificity, PPV, CF/CFSPID ratio, CF/carrier ratio and median age at diagnosis. Sensitivity both for procedures A (86%) and B (90%) was below the value expected before introducing NBSCF in the Dutch NBS program [7] and less than desirable.

Within two years after introducing this screening strategy the criteria for screen negative and screen positive tests had to be adapted. The anticipation that this screening strategy would reliably distinguish healthy CF carriers from CF patients appeared not to be true. Three infants with severe CF were found who NBSCF earlier identified as healthy CF carriers. All three patients had a large deletion that cannot be found by EGA. Although it was known from the NCFR that large deletions occur in the Dutch CF population, the frequency was considered as extremely low. This finding forced us to adapt the program. We reflected if we could add multiplex ligation-dependent probe amplification (MLPA) to the fourth step However this technique requires 50 ng of DNA which cannot be extracted from the limited blood spot material available for CF-testing. Moreover still not all CFTR variants, eg variants hidden within the intron, can be detected. As the estimated PPV of the program (77% in procedure A) was considerably higher than the generally considered acceptable value (PPV > 30%) and the number of identified carriers relatively low we decided to consider all samples with one A variant as screen positive from April 9, 2013 (Procedure B). This change in screening strategy could easily and rapidly be implemented as no other changes were necessary.

The Dutch NBSCF screening strategy differed from other NBSCF programs in several steps.

Main difference was the use of PAP as second tier and EGA as fourth. The use of a second IRT has been in use in many NBSCF programs. It avoids DNA-analysis - which due to legal restrictions cannot be used for screening purposes in some countries - and has an acceptable test validity [6]. A major disadvantage of a second IRT is the fact that a second heel prick is necessary. This can be avoided by the use of PAP analysis as second tier performed in the first blood sample. There are no current screening programs employing solely IRT and PAP as screening strategy, although this screening strategy has the highest estimated cost effectiveness [19]. The low PPV of the IRT-PAP strategy and the considerable risk of failure of sweat tests in newborns led to the decision of adding two more steps in Dutch NBSCF. The choice to add PAP in the screening strategy originates from the observation that substantially less CFSPID and CF carriers were found in an IRT-PAP-DNA-

Table 4
Comparison of Procedure B with results of recently published novel NBSCF approaches and ECFS standards of care.

Study	N screened	Prevalence of CF	Screening method	Median age (days) at diagnosis	Sensitivity (%) (CI) w/o MI	PPV (%)	CF/CFSPID ratio
Kharrazi[14]	2,573,293	1: 6899	IRT/DNA/EGA	34	92	34	0.6
Sontag[16]	1,520,079	1: 5548	IRT/IRT/DNA	32	96.2	19.7	10.8
Sommerburg[17]	328,176	1: 4826	IRT/PAP	nia[a]	96 (0.865–0.989)	9	59
Lundman[15]	181,159	1: 8660	IRT/DNA/EGA	26–33[b]	95	43	1.1
Weidler[18]	410,111	1: 5258	IRTxPAP	nia	97.4	8.2	19.5
Present study (procedure B)	819,879	1: 6029	IRT/PAP/DNA/EGA	22	90 (82–94)	63	4
ECFS standard[9]				≤30	≥95	≥30	≥10

[a] nia: no information available.
[b] Range of median age at first diagnostic follow-up.

EGA approach than in an IRT-DNA strategy [7]. The present study confirms that by adding PAP the number of identified carriers is considerably lower than in an IRT-DNA approach. We found 37 carriers in 1045 DNA-analyses (1 in 28) (Fig. 1), which approximates the expected number, based on a CF-carrier frequency of 1 in 30 in the general population [20]. IRT/DNA based screening identifies about twice as many carriers as expected, about 1 in 15 false positive cases [20].

The other difference is the use of EGA after DNA-analysis. Adding EGA as fourth step showed several advantages. The diagnosis of CF was facilitated in 18% of the infants with one variant in the DNA-analysis due to the finding of a second CF causing variant by EGA. Secondly, generally CF carriers and CFSPID can be better distinguished than in an IRT/DNA approach due to the identification of novel "O" variants by EGA. But because we do not test parents it cannot be excluded that a few CFSPID cases might in fact be carriers with two CFTR variants in *cis* (Appendix B and Table 1). Our approach of categorizing CFTR-variants as probably disease-causing ("A"), variable/uncertain ("O") or non-pathogenic ("N") if known from the literature or databases as well as in not earlier described variants (see Section 2.3) appeared to be correct in all identified infants. Referral of infants with variants of unknown clinical significance could therefore be avoided. EGA led to a CF diagnosis in 4 patients with novel variants (Appendix B). In a strategy using a large panel of rare but earlier identified CF variants as in next generation sequencing [15], instead of DNA-analysis followed by EGA as in our approach, these patients would not have been found. The proportion of nine referrals from the safety-net (5.3% of all referrals in procedure A, 4.7% in procedure B) was considerably lower than the proportion of referrals in a safety-net approach where NBSCF was screen positive if IRT > 99.9% (11.4%) [17]. The safety net led to the identification of three CF patients who would otherwise have been missed, two of them being homozygous for rare variants (Table 1). As our databases do not register ethnicity it is not known if these rare variants were from immigrant populations. In 25 samples in the safety net, EGA identified one "O" CFTR variant (Fig. 1). Because sweat tests will not be helpful in differentiating carriers of deleterious or mild variants and referral might arouse much parental anxiety, we decided to consider these results as screen negative.

Inno-LiPa mutation-panels were chosen as third tier, because of proven validity for small samples from heel prick bloodspots and practical considerations such as availability, costs, necessary equipment and personnel. The panels were not ideal as some of the variants in the panel have never been found in the Netherlands while earlier identified variants (in total 124 were registered in the NCFR) were not part of this panel [22]. Due to the EGA-step in our screening algorithm patients carrying these variants could be identified (non-bold variants identified in EGA step in Appendix B). Moreover, based on the findings in the pilot study and the knowledge that most CFSPID carrying the R117H-7T/9T variant will never develop CF [23] we would have preferred a panel without R117H-7T/9T, as in several other screening programs [14, 16]. This variant was responsible for a large proportion of CFSPID (79%), similarly as in the pilot study (85%) [7].

Despite the four-step strategy the median age at diagnosis (day 22) differed not from other NBS-strategies [21]. Most referrals (79% in Procedure A, 62% in Procedure B) took place after step 3 (Fig. 1).

We found a prevalence of CF at birth of 1: 6029, but the total number of detected CF cases in our program probably is slightly underestimated as some CFSPID cases will later turn out to be CF.

False negative screening results were mostly caused by either IRT- or PAP-values below the cut off level. The sensitivity of IRT as biomarker is estimated to be around 95% [24]. The sensitivity of PAP with current cut-off levels seems to lie in the same range. The addition of PAP as second step therefore almost doubled the number of false negative tests. Remarkably in missed cases, infants with an IRT below cut-off often showed PAP-values considerably above cut-off level, while infants missed due to a low PAP-value often had high IRT-values (Table 2). Theoretically using both biomarkers at the same time instead of in two consecutive steps as in the approach using a product of IRT and PAP has - if combined with DNA-analysis - the potential of a screening strategy with a very high sensitivity and specificity [18]. However, cut-off values should be re-evaluated and it is not clear if the advantage of a lower carrier detection would still be present. The costs of such an approach would probably be considerably higher than our approach.

When comparing the sensitivity of the program with data from the literature it is important to realize that in many NBSCF programs underreporting of missed patients is a problem [16]. The number of unknown CF patients missed in our program is probably low, due to the centralized NBS program, frequent meetings of representatives of all parties involved in the program, including the seven CF-centres and the annual evaluations by an independent institute. Moreover monthly reminders by the DPSU to all Dutch paediatricians to report all patients not identified by the NBS-program will have contributed to a complete report of all missed cases. Up to now no more missed cases were reported born in the period from May, 1, 2011 until January 1, 2016. In the period before NBSCF was introduced a median age at diagnosis of 31.5 weeks (IQR 8.25–106.75) [25] was found. There is still a risk that false negative cases born in this period will be found at a later age but the expected number is likely low.

The low sensitivity and the relatively high number of CFSPID with the R117H-7T-9T variant forced us to investigate how to improve our screening strategy [26]. In July 2016 the following changes were introduced: cut-off values were adjusted: DNA-analysis is performed if IRT ≥ 100 μg/L and PAP ≥ 1.2 μg/L (77th percentile), if IRT ≥ 124 μg/L (99.9th percentile) regardless of PAP value, and as before, if IRT ≥ 60 μg/L and PAP ≥ 3.0 μg/L. The R117H-7T/9T variant is part of the Inno-LiPa panel and cannot be removed from the panel, therefore it is no longer considered as "O" but as "N". The algorithm of four steps was not changed. We calculated that these changes will probably lead to a sensitivity of ≥95%, with a PPV of 60% and a specificity of 99.99% [26].

Changing the Inno-LiPa panels to a next generation sequencing panel that can be adapted to a more specific Dutch mutation panel including large deletions is a future option. In order to identify novel variants the use of a safety net by EGA still will be necessary because of a continuously changing population due to migration.

5. Conclusions

We showed that the Dutch NBSCF program achieves a high specificity, a high PPV, identifies a low number of CFSPID and healthy carriers, and achieves a favourable median age at diagnosis, but the sensitivity of the program needs improvement.

Acknowledgements

We would like to acknowledge the help from the Department of Pediatrics, Maastricht University Medical Centre, especially Tamara Cauberg for providing always promptly all literature I asked for and Edward Dompeling for providing research accommodation. Also we would like to thank all paediatricians and paediatric pulmonologists in the Netherlands for their loyal support and monthly reports in the registries. And finally we acknowledge the Dutch Paediatric Surveillance Unit (DPSU) for their loyal support.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or non-profit sectors.

Conflicts of interest

Dr. Verkerk reports that TNO monitored the newborn blood spot screening (including the CF screening) as a task for RIVM, Bilthoven, The Netherlands, during the conduct of the study. TNO was founded by law in 1932 to enable business and government to apply knowledge. This organisation regulated by public law is independent; it is no part of any government, university or company and provides unbiased information.

Dr. Janssens reports grants from NCFS (Dutch Cystic Fibrosis Foundation), and grants from NIH (R01HL126603) outside the submitted work; grant office Sophia Research B.V. received speakers fee from Vertex. Co-author in studies supported by unrestricted research grants from Chiesi, Gilead and Glaxo Smith Kline.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcf.2018.07.008.

References

[1] Quon BS, Rowe SM. New and emerging targeted therapies for cystic fibrosis. BMJ 2016;352:i859 doi: 10.1136bmj.i859.

[2] Kuk K, Taylor-Cousar JL. Lumafactor and ivafactor in the management of patients with cystic fibrosis: current evidence and future prospects. Ther Adv Respir Dis 2015;9:313–26.

[3] Taylor-Cousar JL, Munck A, Mckone EF, van der Ent CK, Moeller A, Simard C, et al. Tezafactor-Ivafactor in patients with cystic fibrosis homozygous for Phe508del. New Engl J Med 2017 Nov 3. https://doi.org/10.1056/NEJMoa1709846.

[4] Vernooij-van Langen A, Dompeling E, Yntema JB, Arets B, Tiddens H, Loeber G, et al. Clinical evaluation of the Nanoduct sweat test system in the diagnosis of cystic fibrosis after newborn screening. Eur J Pediatr 2015 Aug;174(8):1025–34. https://doi.org/10.1007/s00431-015-2501-0.

[5] Vernooij-van Langen AM, van der Pal SM, Reijntjens AJ, Loeber JG, Dompeling E, Dankert-Roelse JE. Parental knowledge reduces long term anxiety induced by false-positive test results after newborn screening for cystic fibrosis. Mol Genet Metab Rep 2014;1:334–44 eCollection 2014.

[6] Castellani C, Southern KW, Brownlee K, Dankert-Roelse JE, Duff A, Farrell M, et al. European best practice guidelines for cystic fibrosis neonatal screening. J Cyst Fibros 2009;8:153–73.

[7] Vernooij-van Langen AM, Loeber JG, Elvers B, Triepels RH, Gille JJ, van der Ploeg CP, et al. Novel strategies in newborn screening for cystic fibrosis: a prospective controlled study. Thorax 2012;67:289–95.

[8] Health Council of the Netherlands. Neonatal screening for cystic fibrosis. The Hague: Health Council of the Netherlands; 2010 publication no. 2010/01.

[9] Smyth AR, Bell SC, Bojcin S, Bryon M, Duff A, Flame P, et al. European cystic fibrosis society standards of care: best practice guideline. J Cyst Fibros 2014;13(Suppl. 1):S23–42.

[10] Munck A, Mayell SJ, Winters V, Shawcross A, Derichs N, Parad R, et al. Cystic fibrosis screen positive, inconclusive diagnosis (CFSPID): a new designation and management recommendations for infants with an inconclusive diagnosis following newborn screening. J Cyst Fibros 2015;14:706–13.

[11] Verkerk PH, et al. Evaluatie van de hielprikscreening bij kinderen geboren in kalenderjaren 1990–2011. Leiden: TNO; 1992–2012.

[12] Farrell PM, White TB, Howenstein MS, Munck A, Parad RB, Rosenfeld M, et al. Diagnosis of cystic fibrosis in screened populations. J Pediatr 2017;181S:S33–44.

[13] Ooi CY, Castellani C, Keenan K, Avolio J, Volpi S, Boland M, et al. Inconclusive diagnosis of cystic fibrosis after newborn screening. Pediatrics 2015;13596:e1377–8.

[14] Kharrazi M, Yang J, Bishop T, Lessing S, Young S, Graham S, et al. Newborn screening for cystic fibrosis in California. Pediatrics 2015;136:1062–72.

[15] Lundman E, Gaup HJ, Bakkeheim E, Olafsdottir EJ, Rootwelt T, Storrøsten OT, et al. Implementation of newborn screening for cystic fibrosis in Norway. Results from the first three years. J Cyst Fibros 2016;15:318–24.

[16] Sontag MK, Lee R, Wright D, Freedenberg D, Sagel SD. Improving the sensitivity and positive predictive value in a cystic fibrosis newborn screening program using a repeat immunoreactive trypsinogen and genetic analysis. J Pediatr 2016 Aug;175:150–8.

[17] Sommerburg O, Hammermann J, Lindner M, Stahle M, Muckentaler M, Kohlmüller D, et al. Five years of experience with biochemical cystic fibrosis newborn screening based on IRT/PAP in Germany. Pediatr Pulmonol 2015;50:655–64.

[18] Weidler S, Stopsack KH, Hammermann J, Sommerburg O, Mall MA, Hoffmann GF, et al. A product of immunoreactive trypsinogen and pancreatitis-associated protein as second-tier strategy in cystic fibrosis newborn screening. J Cyst Fibros 2016;15:752–8.

[19] van der Ploeg CP, van den Akker-van Marle ME, Vernooij-Van Langen AM, Elvers LH, Gille JJ, Verkerk PH, et al. Cost-effectiveness of newborn screening for cystic fibrosis determined with real-life data. J Cyst Fibros 2015;14:194–202.

[20] Ranieri E, Lewis BD, Gerace RL, Ryall RG, Morris CP, Nelson PV, et al. Neonatal screening for cystic fibrosis using immunoreactive trypsinogen and direct gene analysis: four years' experience. BMJ 1994;308:1469–77.

[21] Barben J, Castellani C, Dankert-Roelse JE, Gartner S, Kashirkaya N, Linnane B, et al. The expansion and performance of national newborn

screening programmes for cystic fibrosis in Europe. J Cyst Fibros 2017; 16:207–13.

[22] Lakeman P, Gille JJ, Dankert-Roelse JE, Heijerman HG, Munck A, Iron A, et al. CFTR mutations in Turkish and North African cystic fibrosis patients in Europe: implications for screening. Genet Test 2008;12:25–35. https://doi.org/10.1089/gte.2007.0046.

[23] Thauvin-Robinet C, Munck A, Huet F, Génin E, Bellis G, Gautier E, et al. The very low penetrance of cystic fibrosis for the R117H mutation: a reappraisal for genetic counselling and newborn screening. J Med Genet 2009;46:752–8. https://doi.org/10.1136/jmg.2009.067215 [Epub 2009 Jun 29].

[24] Munck A, Houssin E, Roussey M. Evaluation of the French nation-wide cystic fibrosis newborn screening program. Arch Pediatr 2008;15:741–3.

[25] Vernooij-Van Langen AM, Gerzon FL, Loeber JG, Dompeling E, Dankert-Roelse JE. Differences in clinical condition and genotype at time of diagnosis of cystic fibrosis by newborn screening of by symptoms. Mol Genet Metab 2014;113:100–4.

[26] Bouva MJ, Dankert-Roelse JE, Verkerk PH, Schönbeck Y, Verschoof-Puite RK, Gulmans VAM, et al. Improvement of the validity of the Dutch combined IRT/PAP/DNA/EGA newborn screening program for cystic fibrosis by re-evaluating mutation classification and optimizing cut-off values. J Cyst Fibros 2016;S37. https://doi.org/10.1016/S1569-1993(13)60170-8.