Bilateral sweat tests with two different methods as a part of cystic fibrosis newborn screening (CF NBS) protocol and additional quality control

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Abstract: Infants with positive CF newborn screening (NBS) results are called to a CF Centre for verification. Those, in whom the sweat test is elevated, undergo further medical procedures. The aim of our study was to evaluate the applicability of Nanoduct – a new system measuring sweat conductivity and giving immediate results in a CF NBS protocol. Measurements with Nanoduct were compared with the classic pilocarpine method. During 3 years 487 infants from CF NBS had both sweat tests performed on the same day, at the same CF centre. CF infants had a mean conductivity of 99.8±18.8 mmol/L and a mean chloride concentration of 74.0±18.4 mmol/L. Non-CF infants values were 29.8 ±7.7mmol/L and 19.2±6.6 mmol/L respectively. A good correlation between both tests was found (95% confidence level (CI); r=0.87). The optimal cut off, based on follow up experience of screened children, for conductivity tests was 50 mmol/L and for chloride concentration was 34 mmol/L (no lost CF, 11 false positive) with 100% sensitivity and 97.5 % specificity. In conclusion Nanoduct is a very useful and reliable tool in CF NBS protocol, allowing more time efficient organization of the diagnostic and training procedures. Simultaneous bilateral sweat testing with two different methods (concentration and conductivity) provides an extra quality control system.

Key words: sweat test, conductivity, newborns, cystic fibrosis, screening

Introduction

National CF NBS started in Poland in September 2006. The programme was introduced and coordinated by the Screening Department and CF Centre in the Institute of Mother and Child and was rolled out across the country. Screening for CF was based on the 3 stage IRT/DNA/IRT protocol. Infants with positive neonatal CF screening results were called to a CF Centre for verification. The same day two sweat tests were performed: classical Gibson-Cook and Nanoduct based on conductivity. Thanks to quick conductivity test results diagnosed infants could pass all the trainings and return home on the same day [1,2].

Measurement of sweat chloride concentration by the quantitative pilocarpine iontophoresis test (QPIT), as described by Gibson and Cook [3,4], is considered to be the most accurate method to diagnose CF and has been accepted as the standard for sweat testing [5]. However, this method involves multiple steps for collection and analysis of the sweat sample and involves the risk of volumetric, gravimetric, condensate and evaporation errors in the procedure. In this context, the measurement of sweat conductivity is an increasingly used method because it is easier to perform and requires a smaller sample than QPIT [6].

Sweat contains a variety of ions which contribute towards measurements of conductivity and osmolarity. Conductivity represents the nonspecific measurements of the total anion activity in a solution and therefore has a higher concentration in sweat than chloride. The reading is displayed in equivalent NaCl molarity units (mmol/L) [7]. The determination of conductivity in

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Abbreviations: CF – cystic fibrosis; NBS – newborn screening; CFTR – Cystic Fibrosis Transmembrane Conductance Regulator; IRT – immunoreactive trypsin; QPIT – quantitative pilocarpine iontophoresis test; PS – pancreatic sufficient; PI – pancreatic insufficiency.

sweat, as a test for approximation to electrolytes in a non selective manner, has been shown to be as effective as the quantitative determination of sweat chloride or sodium concentration [8,9].

Sweat collected in Macroduct coils and transferred to the Sweat-Chek conductivity analyzer (Wescor Inc., Logan, Utah) has been used in some settings. Recently, a new conductivity analyzer, Nanoduct, has been developed for use especially in the neonatal population.

Nanoduct is a new diagnostic system that induces, collects and analyzes sweat in one step while the required electrodes and sensors are attached to the patient [10]. Compared to the sweat volumes between 75 to 100 µl required by other systems, this system needs only 3 µl of sweat and the results are available within half an hour.

The aim of our study was to evaluate the applicability of Nanoduct, a new system measuring conductivity and giving immediate results in a CF NBS protocol. Nanoduct measurements were compared with classic pilocarpine sweat chloride concentration as reference to assess its accuracy and precision as a diagnostic analytical procedure.

Materials and methods

Patients. 1057 (528 conductivity and 529 chloride concentration) sweat tests were performed between 01.09.2006 and 01.06.2009 at the CF Centre of the Institute of Mother and Child in Warsaw.

487 infants aged 4-6 weeks with positive results of screening tests (IRT-I over 99.4 percentile and one or two *CFTR* mutations or IRT-II over cut-off) were called for further evaluation in our CF Centre (sweat tests and specialist consultation). Control included 45 healthy infants 4-8 weeks old, who did not need a recall within CF NBS programme (negative screening result after the first step).

Sweat tests. In the infants two kinds of sweat tests were performed (quantitative pilocarpine iontophoresis and conductometry). Both tests were performed on the same day, by the same qualified personnel. Quantitative pilocarpine iontophoresis chloride was tested according to the Gibson and Cook method [3], i.e. quantitative analyses of sweat chloride by a manual titration using the Schales and Schales mercuric nitrate procedure. This method is well established as the gold standard but is time consuming and open to many risks of error.

In the Nanoduct system continuous flow analysis eliminates a separate collection phase and analyzes samples within minutes [7]. The possibility of human error is significantly reduced. The system provides automatic display of initial sweating rate and automatic averaging of conductivity. With shorter stimulation time and smaller sample requirement (3 microliters of sweat), reliable results are available in a few minutes. Nanoduct's small components fit easily on neonates. It is an integrated diagnostic system that simplifies the procedure for all patients. Nanoduct incorporates the classic method of inducing sweat by pilocarpine iontophoresis. The pilocarpine is carried into the dermis of the patient from Pilogel® Iontophoretic discs via a controlled electrical current supplied by the Nanoduct Inducer/Analyzer. This is followed by continuous flow analysis of sweat electrolyte concentration using the conductivity sensor.

DNA analysis, based mainly on the sequencing of eight *CFTR* regions, covered 505 *CFTR* variants including the 16 most common *CFTR* mutations in the Polish population.

Depending on DNA and sweat tests analysis results, infants were divided into 3 main groups: CF, non-CF and observational group. CF group included infants with confirmed diagnosis and known *CFTR* mutations causing CF, non-CF consisted of the following subgroups: control (healthy infants with negative screening test), healthy infants from CF NBS recall (false positives) and *CFTR* mutation carriers. Observational group consisted of infants without established diagnosis, who needed further clinical observation, repeated sweat tests and more detailed salt transport investigations, also nasal potential difference (NPD). The observational group consisted of 6 infants. Four of them had the same genotype: F508del/R117H,IVS8-7T/IVS8-9T, one R553X/ R117H,IVS8-7T/IVS8-7T and one G542X/ R117H, IVS8-7T/IVS8-9T.

The infants had pancreatic function assessed clinically, based on weight gain and stool features with laboratory confirmation by faecal elastase-1. Depending on the results they were described as: pancreatic sufficient (CF/PS) and pancreatic insufficient (CF/PI). Infants from all the other groups were pancreatic sufficient.

Statistical analysis. The results of both sweat tests were assessed statistically using descriptive measures for one variant, centiles, mean, SD, and median. The association of both methods was assessed through the correlation coefficient. The Mann-Whitney U test was applied for pair wise group comparison. Limits of agreement were calculated with 95% confidence intervals. The capability of the conductivity and QPIT to discriminate between CF and non-CF was assessed by constructing a receiver operating characteristic (ROC) curve and overall accuracy of the best cut-off values was assessed through calculation of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and kappa coefficient.

Results

Among 487 infants from CF NBS, in 481 cases both sweat tests were performed. Due to technical reasons 3 infants had no Nanoduct and 3 had no iontophoresis (not enough sweat). A summary of the tests is presented in Table 1 and 2.

There were 45 CF diagnosed children, 6 staying under observation. In the control group of 45 infants, one child had no Nanoduct result. CF infants had a mean conductivity of 99.8±18.8 mmol/L (mean 852±24.2 mmol/L if pancreatic sufficient-PS versus 106.1±11.7 mmol/L if pancreatic insufficient-PI) and a mean chloride concentration of 74.0±18.4 mmol/L (64.3±19.0 mmol/L in PS vs. 78.0±16.8 mmol/L in PI) (Table 1 and 2). CF children who were pancreatic sufficient had lower (nearer to normal range) values in both tests versus higher values of pancreatic insufficient infants. Non-CF infants values were 29.8±7.7 and 19.2±6.6 mmol/L respectively. Similar means, medians characterized all non-CF infants whatever was their origin (Table 2). In Mann-Whitney U test p>0.02 confirmed no differences between the groups. Nanoduct mean value +3SD in control group was 50.2 mmol/L and mean +3SD in healthy infants recalled for sweat tests was 53.4 mmol/L. Chloride concentration mean value +3SD was 44.1 mmol/L in control infants, mean +3SD was 39.7 mmol/L in healthy infants originating from recalls.

Table 1. Sweat conductivity (Nanoduct) and chloride titration (quantitative pilocarpine iontophoresis) in 3 main groups

Group	Conductivity NaCl [mmol/L]						Chloride titration Cl⁻ [mmol/L]					
	N	Mean	SD	Min	Max	Med.	N	Mean	SD	Min	Max	Med.
CF	42	99.6	19.0	57.0	126.0	105.5	45	74.0	18.4	38.8	121.3	75.6
Non-CF	480	29.8	7.7	11.0	60.0	28.0	478	19.2	6.6	6.8	54.0	17.6
observation	6	46.2	14.9	32.0	66.0	41.0	6	33.6	13.8	16.2	49.4	36.6

N – number of tests, Med. – median

Table 2. Sweat conductivity (Nanoduct) and chloride titration (quantitative pilocarpine iontophoresis) in subgroups

Group	Conductivity NaCl [mmol/L]						Chloride titration Cl⁻ [mmol/L]					
	N	Mean	SD	Min	Max	Med	N	Mean	SD	Min	Max	Med
CF/PS	13	85.2	24.2	57	126	91	13	64.3	19.0	38.8	92.1	70.6
CF/PI	29	106.1	11.7	67	126	109	32	78.0	16.8	53.3	121.3	77.7
observation	6	46.2	14.9	32.0	66.0	41.0	6	33.6	13.8	16.2	49.4	36.6
Carrier	142	29.5	7.7	17	57	28	141	19.3	5.7	10.9	39.9	18.0
Healthy	294	30.1	7.8	12	60	28	292	19.1	6.8	6.8	46.1	17.5
Control	44	28.6	7.2	11	46	27	45	19.2	8.3	11.5	54	16.2
Total	528	35.5	21.0	11	126	29	529	23.9	18.5	6.8	121.3	18.3

N – number of tests; Med. – median; CF – children with pancreatic sufficiency (CF/PS) and pancreatic insufficiency (CF/PI); Healthy – healthy infants from CF NBS recall (false positives); Control – healthy infants with negative screening test

Optimal level of cut-off values were calculated from receiver operating characteristics (ROC) (Fig. 1 and Fig. 2). Receiver operating characteristics (ROC) curve for conductivity sweat test (Fig. 1) showed the capacity to predict cystic fibrosis diagnosis based on molecular analysis and clinical examination. Optimal cut off based on screened children follow up experience for the conductivity test was 50 mmol/L (no lost CF, 11 false positive) with 100% sensitivity, 97.5% specificity and PPV 79.25 (Table 3). No false positives were found in values over 60 mmol/L. The conductivity values between 50 and 60 mmol/L were assumed borderline.

Optimal cut off for chloride concentration was established at 34 mmol/L (no lost CF, 11 false positive) with 100% sensitivity and 97.5% specificity (Fig. 2 and Table 4). No false positives were found in the range of values over 46 mmol/L. The chloride levels between 34 and 46 mmol/L were assumed borderline (Tab. 5).

A good correlation between both tests was found (CI 95%, r=0.87) (Fig. 3). There were distinctive clusters of positive and negative results.

Discussion

Conductivity

Conductivity method provides a very high capacity to identify CF and non-CF [6]. There are two systems for sweat conductivity measurements.

The Macroduct system, which collects sweat into capillary tubing, developed to limit sweat volume loss from evaporation. Allied to conductivity measurements, sweat analysis can be performed on aliquots as small as 5 µl, on a minimum acceptable sample of 15 µl collected over 30 minutes. The collection of pure liquid sweat facilitates the measurements of conductivity, although it is also possible to measure chloride on the sample collected through a capillary system [11]. Studies proving good correlation between chloride concentration and conductivity on the same sample of sweat have been performed [6].

The Macroduct system has been further developed to perform sweat collection and analysis in infants; Nanoduct provides measurements of conductivity on samples as small as 3 µl. However, unique continuous flow analysis eliminates a separate collection
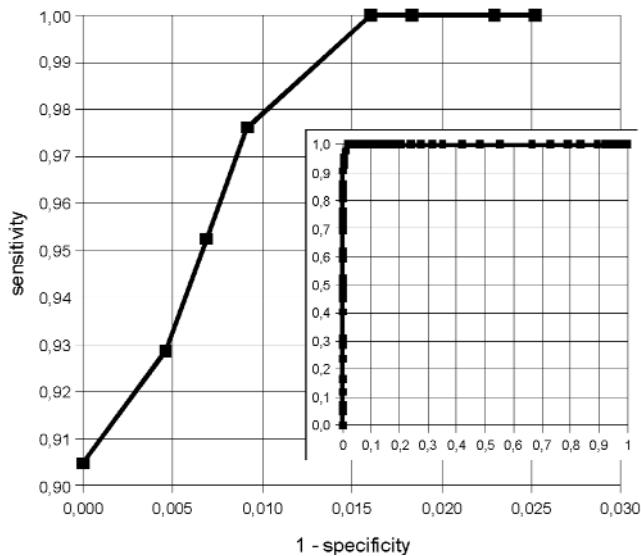
Fig. 1. Receiver operating characteristics curve for conductivity sweat test showing the capacity to predict cystic fibrosis diagnosis (based on molecular analysis and clinical examination). Each filled square represents the cut-off value starting from 50 (lower left corner) to 61 (upper right corner).
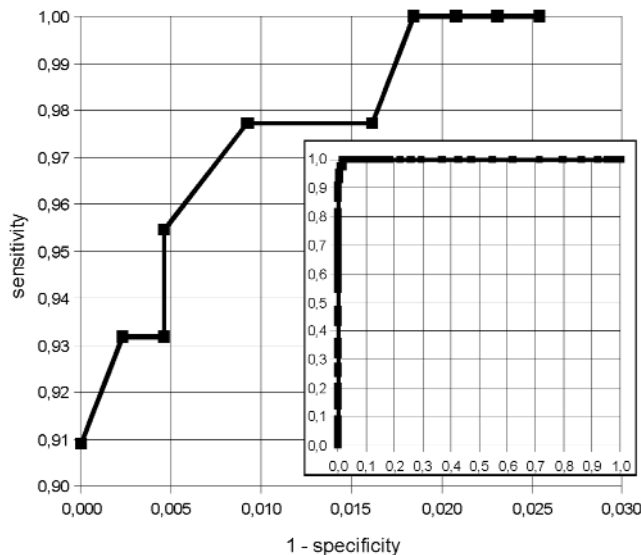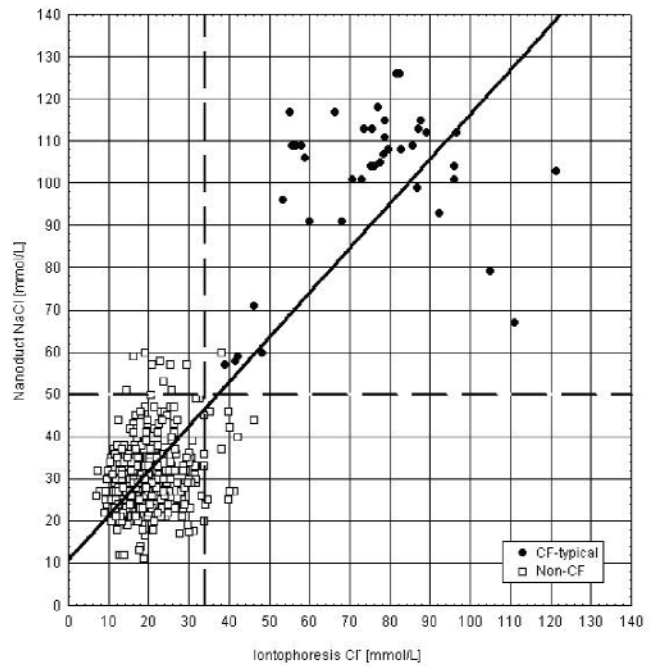

Fig. 2. Receiver operating characteristics curve for chloride titration sweat test showing the capacity to predict cystic fibrosis diagnosis (based on molecular analysis and clinical examination). Each filled square represents the cut-off value starting from 34 (lower left corner) to 47 (upper right corner).

Fig. 3. Correlation between sweat tests results obtained with the conductivity method (Nanoduct) and chloride titration (Iontophoresis); N=522, r=0.87, CI 95%. N – number of tests. r – correlation coefficient. CI – confidence interval.

phase and the performance of two different methods on the same sample of sweat is no more possible.

Indeed, the Nanoduct system is more adapted for infants which is why we use it in our screening procedure, although in our pilot CF NBS in the years 1999-2003 we were working on Macroduct [1].

Although the producer recommends the same reference values for both systems (Macroduct and Nanoduct: 0 to 60 mmol/L are in the normal range; 60 to 80

mmol/L are borderline; and readings above 80 mmol/L should be considered abnormal) discussing the results of different studies the system used is always mentioned [7].

In Lezana study (Macroduct) on mixed age group, results were as follow: conductivity ≥90 mmol/L confirmatory, value <75 mmol/l negative, values 75-89 mmol/L corresponded to the equivocal range [6]. According to our data it could not be applied for infants.

Barben`s group [12] working on Nanoduct in the mixed age group found 59 mmol/L as an upper limit of normal conductivity, so also higher than our infants data (50 mmol/L). He reported median conductivity for CF 115 mmol/l, non-CF 37 mmol/L, while in our infant population the values were slightly lower: CF 105,5 mmol/L, non-CF 28 mmol/L.

Median value of 24 mmol/L in healthy infants for the Macroduct test was found by the Liverpool group [13]. They performed both tests in 5-6 week old healthy infants. They reported excellent limits of agreement between the two methods. They showed that conductivity is as valid as QPIT in the age group 5-6 wk and that chloride greater than 30 mmo/L should be assessed in a specialist CF Centre.

According to the Cystic Fibrosis Foundation (CFF) a patient having a conductivity result ≥50 mmol/L should be referred for QPIT sweat chloride analysis [10]. The manufacturer of the Sweat-Chek (Wescor)

Table 3. Data for receiver operating characteristics curve for conductivity sweat test (Cut-off = the concentration of NaCl in mmol/L. TP = the number of true positive. FN = the number of false negative. FP = the number of false positive. TN = the number of true negative. PPV=positive predictive value. NPV=negative predictive value and kappa coefficient)

Cut-off	TP	FN	FP	TN	Specificity	Sensitivity	PPV	NPV	Kappa
50	42	0	11	425	0.975	1	79.25	100	0.872
51	42	0	10	426	0.977	1	80.77	100	0.882
52	42	0	8	428	0.982	1	84.00	100	0.904
53	42	0	8	428	0.982	1	84.00	100	0.904
54	42	0	7	429	0.984	1	85.71	100	0.915
55	42	0	7	429	0.984	1	85.71	100	0.915
56	42	0	7	429	0.984	1	85.71	100	0.915
57	42	0	7	429	0.984	1	85.71	100	0.915
58	41	1	4	432	0.991	0.976	91.11	99.77	0.937
59	40	2	3	433	0.993	0.952	93.02	99.54	0.935
60	39	3	2	434	0.995	0.929	95.12	99.31	0.934
61	38	4	0	436	1	0.905	100	99.09	0.945

Table 4. Data for receiver operating characteristics curve for chloride titration sweat (Cut-off = the concentration of Cl- in mmol/L. TP = the number of true positive. FN = the number of false negative. FP = the number of false positive. TN = the number of true negative. PPV=positive predictive value. NPV=negative predictive value and kappa coefficient)

Cut-off	TP	FN	FP	TN	Specificity	Sensitivity	PPV	NPV	Kappa
34	44	0	11	422	0.975	1	80.00	100	0.876
35	44	0	10	423	0.977	1	81.48	100	0.886
36	44	0	9	424	0.979	1	83.02	100	0.897
37	44	0	9	424	0.979	1	83.02	100	0.897
38	44	0	8	425	0.982	1	84.62	100	0.907
39	43	1	7	426	0.984	0.977	86.00	99.77	0.906
40	43	1	4	429	0.991	0.977	91.49	99.77	0.939
41	43	1	4	429	0.991	0.977	91.49	99.77	0.939
42	42	2	2	431	0.995	0.955	95.45	99.54	0.950
43	41	3	2	431	0.995	0.932	95.35	99.31	0.937
44	41	3	1	432	0.998	0.932	97.62	99.31	0.949
45	41	3	1	432	0.998	0.932	97.62	99.31	0.949
46	41	3	1	432	0.998	0.932	97.62	99.31	0.949
47	40	4	0	433	1	0.909	100	99.08	0.961

Table 5. Suggested normal values for sweat tests

Group	Sweat conductivity NaCl [mmol/L]	Chloride titration Cl⁻ [mmol/L]
Normal	< 50	< 34
Borderline	50 - 60	34 - 46
CF	> 60	> 46

recommends that conductivity results <60 mmol/L are normal, >80 mmol/L are positive for CF, 60 to 80 mmol/L are borderline [7]. Lezana suggested even narrower values for the conductivity method to diagnose or exclude CF (90,75) [6].

Our infants values (Nanoduct) were: >60 mmol/L confirmatory, value <50 mmol/l negative, values 50-60 mmol/L corresponded to the equivocal range (Tab. 5). It proves, that as in the case of chloride concentration values, conductivity values are also different in the infant population.

Chloride concentration

Cut off values. A critical issue with regard to the sweat test in infants is the choice of cut-off values. The CFF consensus paper on the diagnosis of CF from 1998 supported the original Gibson and Cooke values, with a sweat chloride of >60 mmol/L to be diagnostic, >40 mmol/L in infants up to the age of 3 months as possible CF [10].

Those values, however, were found in CF NBS programmes not to be accurate in infants. Data from the Victorian NBS [14] found infants with sweat chloride in the 35-39 mmol/L range who had CF. An Australasian consensus statement recommended the following values for sweat chloride in infants following NBS: Cl ≥60 mmol/L – cystic fibrosis, Cl 30-59 mmol/L – borderline, Cl ≤29 – normal [15]. Lowering borderline cut-off to 30 mmol/L in infants following screening was also supported by Farrell et al. [16]. They found the mean sweat chloride in healthy infants to be 10.6±5.3 mmol/L. (95% CI, 9.9, 11.3) and in F508del carriers 14.9±8.3 mmol/L (95% CI, 13.4, 16.4)

Sweat chloride of 30 mmol/L was 4 standard deviations above healthy control infants and 2 standard deviations above F508del carriers. There is not such a difference between healthy controls and carriers in our data. We did not find statistically significant differences between healthy infants with no mutations and one mutation carriers.

Taking into consideration new data on sweat chloride tests results in healthy and CF-affected infants emerging from NBS programmes, the CFF consensus

from 2008 also updated sweat reference ranges for infants up to 6 months: ≤29mmol/L, CF unlikely; 30-59 mmol/L, intermediate; ≥60 mmol/L, indicative of CF [17].

In Mackay's data, the variation between the chloride concentration obtained from the two different sides would suggest that, had only one side been tested, that up to 13 patients (4.4% of total group, 48% of those with at least one intermediate value) could be classified differently [18]. We agree that comparable results from sweat collected from two sites simultaneously permits improved confidence and is a useful internal quality control procedure, even more so, when two different methods are applied.

Observational group. An example of the complexity of mutation analysis is found in the evolving picture of individuals who are compound heterozygotes for CF-causing mutation and the R117H mutation in the *CFTR* gene. The likelihood of CF in this group is driven by the length of a polythymidine tract in intron 8 of the R117H allele. The presence of the 5 T tract in the R117H background is usually associated with CF, whereas R117H(7T) is more often associated with isolated male infertility or pancreatitis [19]. But individuals from both groups may display sweat chloride values in the normal, intermediate or diagnostic range and some individuals with R117H(7T) may present with CF lung disease. Thus R117H(7T) is a mutation that when present in trans with a CF-causing mutation can cause a variable phenotype, ranging from normal to CF. Still unknown diagnosis was the reason to assess our analysed infants with R117H(7T) as an observational group. There are ongoing discussions whether the R117H variant should be included in CFTR mutation panels [20].

Borderline sweat tests. Infants with borderline sweat tests (Cl 34-46 mmol/L and Nanoduct 50-60 mmol/L) after CF NBS pose a difficult problem as not all will turn out to have CF. These children should be seen by a physician experienced with CF, the sweat test should be repeated at least twice on separate days. An extended mutation analysis should be performed.

Some *CFTR* mutations which are clearly "CF causing" (in particular, 3849+10kb C>T) are associated with normal or equivocal sweat electrolytes values. Close liaison with local molecular genetics is needed to determine these infants and to eventually include these mutations in CF NBS mutation panel [21,22].

Chest radiography, stool examination (faecal elastase), blood for electrolytes, albumin, liver function, fat-soluble vitamins may add to the clinical picture. We also recommend repeat assessment at 3-6 months of age (including another sweat test). We recognize that it is possible that some of these infants may not

have clinical evidence of CF for many years. "Missing" these infants on NBS is an acceptable limitation of the programme that is by nature a screening programme

Conductivity versus chloride concentration

Our study confirms the results from previous studies, that conductivity is reliable diagnostic analytical procedure for CF [2,6].

Until now the conductivity method has not been accepted by the CFF or the National Committee for Clinical Laboratory Standards (NCCLS) as a definitive method for the disease [5], in spite of good agreement between conductivity and chloride titration found in several studies [6,8,13]. However, the methods used to assess conductivity as diagnostic tool (ROC curve, sensitivity, specificity, predictive values, kappa) clearly indicated also in our study that the conductivity method has an excellent capacity (certainly not worse than QPI) to discriminate between CF and non-CF subjects. We agree with the other studies, that conductivity might be as reliable as Quantitative chloride analysis to diagnose or exclude CF [6,12]. Patients with equivocal values of conductivity had also equivocal QPIT values.

To our knowledge our work is the first to assess bilateral use of different sweat tests as a part of routine CF NBS protocol.

Conclusions

Because of the excellent capacity to discriminate between CF and non-CF subjects Nanoduct is a very useful tool in CF NBS protocol, allowing more time-efficient organization of the diagnostic and training procedures.

The new analyzing system is quick, simple and reliable and could be used as a diagnostic test in addition to screening.

It has a failure rate comparable to other sweat tests and could be used as a simple bedside test to confirm, exclude or suspect CF. In cases with borderline values other additional methods are indicated. The positive result should be always confirmed with another sweat test.

Simultaneous bilateral sweat testing with two different methods (concentration and conductivity) provides an extra quality control system.

We hope that our study provides some evidence based answer to the usefulness of conductivity relative to chloride results in real patients, coupled to *CFTR* mutation analysis in the context of developing world wide screening programmes.

Conflict of interest: The authors declare that there are no conflicts of interest.

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