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Reclassifying inconclusive diagnosis after newborn screening for cystic fibrosis. Moving forward

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A B S T R A C T

Background: Newborn screening for Cystic Fibrosis (CF) is associated with situations where the diagnosis of CF or CFTR related disorders (CFTR-RD) cannot be clearly ruled out.

Materials/patients and methods: We report a case series of 23 children with inconclusive diagnosis after newborn screening for CF and a mean follow-up of 7.7 years (4–13). Comprehensive investigations including whole CFTR gene sequencing, *in vivo* intestinal current measurement (ICM), nasal potential difference (NPD), and *in vitro* functional studies of variants of unknown significance, helped to reclassify the patients.

Results: Extensive genetic testing identified, *in trans* with a CF causing mutation, variants with varying clinical consequences and 3 variants of unknown significance (VUS). Eighteen deep intronic variants were identified by deep resequencing of the whole CFTR gene in 13 patients and were finally considered as non-pathogenic. All patients had normal CFTR dependent chloride transport in ICM. NPD differentiated 3 different profiles: CF-like tracings qualifying the patients as CF, such as F508del/D1152H patients; normal responses, suggesting an extremely low likelihood of developing a CFTR-RD such as F508del/TG11T5 patients; partial CFTR dysfunction above 20% of the normal, highlighting a remaining risk of developing CFTR-RD such as F508del/F1052V patients. The 3 VUS were reclassified as variant with defective maturation (D537N), defective expression (T582I) or with no clinical consequence (M952T).

Conclusion: This study demonstrates the usefulness of combining genetic and functional investigations to assess the possibility of evolving to CF or CFTR-RD in babies with inconclusive diagnosis at neonatal screening.

Keywords:

Cystic fibrosis

CFTR

Neonatal screening

Functional tests

1. Introduction

Newborn screening (NBS) for Cystic Fibrosis (CF) is associated with situations where the diagnosis of CF cannot be clearly ruled out [1,2]. The prevalence of these unclassified situations, also

called Cystic Fibrosis Screen Positive Inconclusive Diagnosis (CF-SPID) is variable according to the population and the screening algorithm, ranging from 1 to 6% in Australia and Canada to 11% in France [3] and 21% in California [4].

A number of studies have followed up CFSPID babies and evaluated the advent of CF [3–13]. This paper focuses on the evolution of CFSPID to CFTR-related disorder (CFTR-RD) later in life, in babies carrying compatible genotypes. This diagnostic dilemma and the uncertainty of evolution make the management of these chil-

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dren challenging. This also causes parents to face a great deal of anxiety about their infants' prognosis and uncertain future [14].

Usually, those patients have an intermediate sweat test between 30 and 60 mmol/L. In a number of cases, genetic testing identifies variants of unknown clinical significance (VUS) and variants with varying clinical consequence (VVCC), which, in *trans* with a CF-causing mutation, may cause either CF or a CFTR-RD [15,16]. To increase interpretation complexity, a number of these variants show an incomplete penetrance for the different phenotypes, which makes their clinical relevance challenging [17]. This is the case of R117H [18] or the 5T allele of the poly-T tract in intron 8 whose severity is related to the associated TG repeat [19]. Thus, there is an urgent need to implement a strategy aiming to detect infants who might convert to CF or a CFTR-RD in order to propose appropriate follow up and possible access to CFTR modulators.

We report a case series of 23 children with inconclusive diagnosis after NBS for CF. All carried a CF-causing variant in *trans* of a VVCC or a VUS. Comprehensive investigations including whole CFTR gene sequencing, *in vivo* functional experiments by intestinal current measurement (ICM) and nasal potential difference (NPD), along with *in vitro* functional studies of VUS, led to reclassify the patients. This classification was related to clinical follow-up.

2. Methods

2.1. Population

Patients were referred for confirmation of diagnosis for CF after detection, at neonatal screening of an IRT above the 99.4 percentile and two CFTR variants identified within the 3-tier-screening Polish program (IRT/DNA/DNA sequencing) [20,21]. The first sequencing step includes sequencing of exons:7, 10, 11, 13, 17B, 18, 20, 21, fragment of intron 19 to identify 3849+10kbC>T and search for dele 2,3(21 kb). The second step in case a variant is identified includes sequencing of exon 1, 2, 3, 4, 5, 6, 8, 9, 12, 14A, 14B, 15, 16, 17A, 19, 22, 23 and 24, search for 5T and TG repeat.

3 524 660 newborns were screened between 2006 and 2016 in the newborn screening catchment area of Warsaw. 165 were diagnosed as CF; 89 were diagnosed as CRMS/CFSPID (including IVS8-TG11T5, TG11T5 thereafter) or 52 (without TG11T5). These 23 cases were from the 89 who accepted the investigation. Parents gave the consent to participate in that study.

Patients were enrolled if they presented an intermediate sweat test and carried 2 variants with at least one VVCC or if their genotype included a VUS. They all underwent regular clinical and microbiological follow-up every 3 months according to existing standard of care including NPD and ICM [22]. The mean clinical follow-up was 7.7 years (4–13). The patients were informed about functional tests and data collection was made following the regulations described by the Declaration of Helsinki and the Huriet-Sérusclat law on biomedical research ethics. This study was approved by the Bioethics Committee at the Institute of Mother and Child, Warsaw.

2.2. Whole CFTR gene sequencing, bioinformatics analysis

A whole CFTR gene analysis was carried out in all patients in order to find other potential pathogenic variants undetected by DNA sequencing included in the NBS program. Libraries of single stranded DNA fragments were prepared using a Nextera Rapid Capture custom design (Illumina, San Diego, CA, USA), covering the whole CFTR locus on chromosome 7q32, and sequencing of the libraries was carried out on a MiSeq instrument (Illumina) according to the manufacturer's protocol (detailed protocol in Supplemental materials). Secondary analysis and annotation of the variants was performed using our in-house pipeline Nenufaar [23].

We classified CFTR variants regarding clinical, genetics and functional data reported in CFTR1 [24], CFTR2 and CFTR-France [25] databases. CFTR2 categorizes variants as CF causing, VVCC or non-CF-causing. CFTR-France helps differentiate among VVCC (i) those with an increased penetrance for CF, (ii) those with a low penetrance for CF and a high penetrance for CFTR-RD, and (iii) those with a low penetrance for CFTR-RD, when they are frequently observed in healthy individuals in *trans* with a CF-causing variant. Missense VUS were analyzed by CYSMA [26] to predict their pathogenic impact on the protein and by data aggregator MobiDetails [27]. CFTR deep-intronic variants of potential interest were prioritized by Nenufaar and analyzed using three different splicing predictors (details in Supplemental materials) and were entered in MobiDetails to assess their Variant Allele Frequency (V.A.F.) frequency in the general population (gnomAD) and find potential reports in general databases (e.g., dbSNP, ClinVar).

2.3. In vitro studies to assess synthesis and maturation of the CFTR protein

We used expression vector pTracer-CMV containing the CFTR-wild-type (WT) cDNA, provided by Pr. Fanen, to assess the impact of VUS [28]. pTracer-CFTR constructs (WT and mutants) were transfected into HeLa cells ($n = 4$ independent transfections) to quantify CFTR protein expression and maturation by western blot experiments (mature form, 180 kD-C band and immature non glycosylated form, 150 kD-B band) (detailed protocol in Supplemental materials).

2.4. In vitro studies to assess the function of the CFTR protein

CFTR activity was measured in transiently transfected HEK293 cells (with pTracer-CFTR WT and mutant constructs) using halide-sensitive yellow fluorescent protein YFP-H148Q/I152L [29]. This reflects CFTR activity as the Iodide (I^-) anion transported by CFTR quenches the fluorescent probe (e.g.; decrease in fluorescence in response to cAMP is indicative of CFTR activity) (detailed protocols in Supplementary materials).

2.5. Intestinal current measurement (ICM) and nasal potential difference (NPD)

ICM was performed to investigate CFTR Cl^- transport. NPD was additionally performed in patients who did not accept ICM and to better characterize patients carrying VUS or VVCC, particularly those with unknown penetrance for CFTR-RD. ICM and NPD were performed according to the European Cystic Fibrosis Society Clinical Trials Network (ECFS-CTN) standard operating procedures [30,31]. All NPD and ICM tests were interpreted by two investigators, blinded to the clinical characteristics of the patients.

Briefly, CFTR activity was assessed in the rectal epithelium by the IsC change upon Forskolin (Δ Forskolin) which evaluates the cAMP dependent active transport, and by the ICM Derichs score Δ Forskolin + Δ Carbachol + Δ Histamine. CFTR function in the nasal epithelium was determined based on the NPD variation in response to Amiloride perfusion in high Chloride (Cl^-) solution (Δ Amiloride); and the sum of the changes in NPD after perfusion with the low Cl^- solution and isoproterenol (Δ Total Cl^- Response Δ TCR). Normal values were derived from previous values in healthy controls (Δ Forskolin = 16.6(14) μ A/cm²; Δ TCR = -18(11) mV and Δ Amiloride = 7.2(5) mV) [30,31].

Table 1

Summary of clinical, genetic and functional data at the moment of the test of 23 patients presenting with inconclusive diagnosis at neonatal screening for CF.

	Genotypes		
	CF / VVCC or VVCC / VVCC (CF or CFTR-RD)	CF / VVCC CFTR-RD-low penetrance	CF / VUS
Number of patients	12	7	4
Age, Mean (min-max), years	5.1 (1–10)	4 (1.5–10)	4.8 (3–8)
IRT ng/ml, Mean (min-max)	215 (105–370)	169 (145–239)	151 (89–225)
Sweat chloride concentration (mmol/L) Mean (min-max)	38 (30–58)	34 (26–45)	22 (16–36)
Number of patients with sputum production	6	1	0
Number of patients with PI*	0	0	0
Lung imaging	Wall thickening ($n = 4$), mucus plugging ($n = 2$)	Wall thickening $n = 2$	Not reported
Bronchial colonization	<i>S.aureus</i> ($n = 4$)	$n = 0$	$n = 1$
Number of patients with respiratory symptoms suggestive of CF 3 years later	$n = 2$	$n = 0$	$n = 0$
Δ Forskolin, (μ A/cm ²)	61 (39–87)	55 (30–95)	51 (46–59)
Mean (min-max)	$n = 9$	$n = 6$	$n = 4$
Number of patients			
Derichs score, Median (min-max)	261 (181–434)	257(134–345)	240 (208–269)
Detailed genotypes (number of patients if $n > 1$)	F508del/D1152H ($n = 3$) F508del/R117H;T7 ($n = 2$) R553X/TG12T5 F508del/F1052V F508del/R1162L ($n = 2$) N1303K/R1162L F508del/L997F TG12T5/TG13T5	R553X/TG11T5 ($n = 2$) F508del/ TG11T5 ($n = 4$) F508del/R31C	F508del/D537N 3849+10kbC>T/T582I F508del/M952T ($n = 2$)

CFTR-RD: CFTR-related disorders; *PI: Pancreatic Insufficiency, based on pancreatic elastase < 200 μ g/g of stool; VVCC: Varying Clinical Significance; VUS: variants of unknown significance.

3. Results

3.1. Patients

Clinical, genetic and functional data of the cohort of infants are summarized in Table 1. Mean follow up of the patients was 7.7 years (4–13). IRT varied from 89 to 370 ng/ml. Nineteen patients had a sweat test between 30 and 60 mmol/L (mean sweat test: 37 mmol/L (range: 30–58 mmol/L), while 4, carrying a VUS (D537N, T582I, and M952T) or a TG11T5 variant in *trans* of a CF-causing variant had a normal sweat test below 30 mmol/L. At the time of investigation, mean age of the patients was 4.7 years (range: 1–10 years). All patients were pancreatic sufficient, based on elastase > 200 μ g/g of stools. *S.aureus* was found in sputum of 5 patients. Three years later, the three patients carrying F508del/D1152H (P7, P8, P9) demonstrated sputum production, wall thickening and bronchial wall thickening with bronchiectasis for P8. Sweat test was assessed in 15 out of the 23 patients at the 3 year follow up. Two patients (P1, F508del/R117H;T7 and P5, TG12T5/TG13T5) displayed conversion of the sweat chloride above 60 mmol/L and were categorized as CF. Three additional patients increased significantly their sweat chloride (P2, R553X/TG12T5; P7 and P9 (both F508del/D1152H)) while 4 patients decreased the chloride sweat value below 30 mmol/l (P4 and P10, both F508del/R1162L; P16 and P23, both F508del/TG11T5). The remaining patients displayed no significant changes (Supplementary Table S1)

3.2. Genetic testing

One patient was TG12T5/TG13T5 (VVCC/VVCC) and 22 carried a CF-causing variant (F508del $n = 17$, R553X $n = 3$; N1303K $n = 1$; 3849+10kbC>T $n = 1$) combined with a non CF-causing variant on the other parental allele. Final classification of this 2nd variant was based on CFTR2 and CFTR-France as explained in Supplementary Table S2. Three patients carried the D1152H VCC variant

which displays an increased penetrance for CF; 4 carried a VCC variant with increased penetrance for CFTR-RD (TG12T5, TG13T5, R117H;T7, F1052V); 3 patients carried variants categorized as non CF-causing (L997F, R1162L) in CFTR2 but considered as CFTR-RD causing in the CFTR-France database; 7 patients carried TG11T5 ($n = 6$) and R31C ($n = 1$). Those variants are associated with low penetrance for CFTR-RD, according to their prevalence in the general population [13]; 4 patients carried a VUS (D537N ($n = 1$), T582I ($n = 1$) or M952T ($n = 2$)).

Whole CFTR gene sequencing identified on average 200 variants per patient. After secondary analysis and prioritization by Nenufaa, we focused on 18 deep intronic variants identified in 13 different patients (Supplementary Table S3) either because they had a V.A.F. lower than 0.01 suggestive of a potential pathogenic effect ($n = 17$) and/or *in silico* predictions suggestive of a possible impact on splicing ($n = 5$). The c.1209+425C>T variant was very unlikely to be pathogenic because of its high frequency in the general population (>0.01). Several variants were probably in complex allele (*i.e.*, on the same chromosome) with exonic variants, as they displayed the same frequency in the general population. The variants c.54–10943G>A and c.1393–1489_1393–1488del were probably in *cis* with R117H, and c.2620–3342A>G in *cis* with R553X ($n = 2$). None of these deep-intronic variants was associated with additional pathogenic consequence, based on the clinical or the functional data collected in the infants.

3.3. CFTR in vivo or ex vivo functional evaluation of patients carrying VVCC

Nineteen patients underwent ICM and demonstrated a CFTR dependent Cl⁻ transport in the normal range based on Δ Forskolin and the ICM score (Table 1, Supplementary Table S1, Supplementary Fig. S1). Those 4 patients who did not have ICM underwent NPd, which was reliable in 3 of them (P8, P9, P16). Two carried the F508del/D1152H genotype (P8, P9). NPd showed a decreased cAMP dependent Cl⁻ transport in the CF range and a high response

to Amiloride (Supplementary Fig. S2, Panel A). The third one carried the TG11T5 allele in *trans* with R553X and showed normal values (P16) (Supplementary Fig. S2, Panel B). Additionally, NPD was also performed in the patient carrying the F508del/F1052V genotype (P3). This patient had a normal CFTR dependent Cl⁻ transport but an elevated response to Amiloride, indicative of CFTR dysfunction (Supplementary Fig. S2, Panel C).

Altogether, the combination of the genetic and the functional information helped categorize these patients. Four patients were categorized as Pancreatic sufficient CF because of consistent conversion of sweat chloride above 60 mmol/L ($n = 2$; P1 F508del/R117H;7T and P5 TG12T5/TG13T5), or NPD in the CF range (P8 and P9, both carrying the F508del/D1152H genotype). The third F508del/D1152H patient (P7) was classed at high likelihood to develop CF because of high penetrance of D1152H for CF. The 14 remaining patients had a normal ICM for 12 of them (not performed for the last 2). The patients carrying the R31C or the TG11T5 variant (P16 to P23) were classed at low risk to develop CFTR-RD because of the low penetrance of these variants for CFTR-RD while the others were considered at risk for developing CFTR-RD later in life because of high penetrance of their VVCC for CFTR-RD (P2, P3, P4, P6, P10, P11, P12) (Supplementary Table S1).

3.4. Characterization of patients carrying variants of unknown significance

Four patients of the cohort carried a VUS (1609G>A, p.Asp537Asn, D537N; c.1745C>T, p. Thr582Ile, T582I; c.2855T>C, p.Met952Thr, M952T) in *trans* of F508del ($n = 3$) or 3849+10kbC>T ($n = 1$) (Table 1, Supplementary Table S1). The main 3D structures of the mutants and their consequences are described in Supplementary Fig. S3 and Supplementary Table S4.

The D537N variant displays 100% conservation among CFTR orthologs but is present in only 6.88% sequences of NBD1 homologs (Supplementary Table S4, Supplementary Fig. S3). D537N showed a significant impact on the quantity of total CFTR protein (B+C ratio (mean \pm SD) of 33% \pm 23 of the WT) and a moderate impact on CFTR maturation as shown by the decreased quantity of mature mutant protein, based on the C/(B+C) ratio of 83% \pm 6% (Fig. 1C and D). It strongly decreases CFTR activity to 14% \pm 6 of the WT. This residual function is partially potentiated by VX-770 up to 30% \pm 5 (Fig. 1E, Supplementary Fig. 4). The patient carrying D537N in *trans* with F508del had normal CFTR dependent Cl⁻ transport in the intestinal mucosa in contrast to the nasal mucosa where NPD showed a high response to Amiloride while Cl⁻ transport was normal (Fig. 1A and B). This enabled us to categorize this variant as a CFTR-RD causing variant, similar to F1052V.

The T582I variant, also located in NBD1, displays 100% conservation among CFTR orthologs and 22.68% conservation among NBD1 homologs (Supplementary Table S4, Supplementary Fig. S3). Western blot experiments showed a mild impact on the quantity of the CFTR protein with a B + C ratio of 58% \pm 19 but no significant impact on CFTR maturation (Fig. 2B and C). The halide-sensitive functional test showed a decreased CFTR activity at 39% \pm 13 of the WT which was restored to 64% \pm 8 by VX-770 (Fig. 2D). The CFTR dependent Cl⁻ transport was normal in the intestinal mucosa of the patient carrying T582I in *trans* with 3849+10kbC>T (Fig. 2A, Supplementary Fig. S4).

The M952T variant is 100% conserved among orthologs and 51.97% among MSD2 homologs. *In vitro* experiments show that this mutant does not significantly impact protein formation or function (Fig. 3-D) and thus should be considered to be of no clinical consequence. The two patients, carrying M952T in *trans* of F508del had normal Cl⁻ transport in the intestinal mucosa (Fig. 3A, Supplementary Fig. S4).

4. Discussion

This case series of inconclusive cases after NBS for CF investigates the usefulness of a combination of genetic and functional investigations to assess the likelihood of progressing to CF or CFTR-RD in asymptomatic newborns.

We provide here mainly functional insights and some relation with clinical evolution for some genotypes such as D1152H. The polish neonatal screening programs was initiated in region of Warsaw in 2006 and on a nationwide basis in 2009. Therefore, the main limitation of this study is a follow-up time relatively short (mean follow up of 7.7 years, ranging between 4 years and 13 years). Thus the advent of CFTR-RD in these subjects cannot be fully assessed.

Extensive genetic screening led to identify, in *trans* with a CF causing mutation, variants that were initially classified as VVCC. Further consideration of their penetrance for CF or CFTR-RD based on systematic analysis of CFTR2 and CFTR-France as well as their frequency in the general population allowed us to reclassify them as (i) VVCC with an increased penetrance for CF (D1152H); (ii) VVCC with an increased penetrance for CFTR-RD and a low penetrance for CF (R117H;7T, TG12T5; TG13T5; F1052V, L997F, R1162L), and (iii) VVCC with a low penetrance for CFTR-RD (TG11T5 and R31C), concordant with previous studies [17,18]. L997F, R1162L are classed as non CF-causing in CFTR2 but are considered as CFTR-RD causing in CFTR-France, based on epidemiological data, which led us to reclassify them as VVCC. The case of the TG12T5/TG13T5 patient is very illustrative. This patient displayed a conversion of sweat test about 60 mmol/l at the 3 years follow up illustrating the impact of the 5T variant in association with a TG12 or TG13 repeat in babies with inconclusive diagnosis at NBS when in *trans* with a CF causing mutation. Importantly, the 5T variant is not searched in other traditional NBS programs using IRT/DNA panel algorithms and therefore, those babies are "missed".

The genetic classification was extended by whole CFTR gene studies to search for additional CFTR pathogenic deep-intronic variants that would not have been found by conventional sequencing techniques [32,33]. All the new variants found were finally considered as non-pathogenic *per se*, considering infants' phenotypes, concordance with their previously determined genotype, functional investigations, *in silico* predictions and the notable frequency in the general population.

Interestingly, all the patients in this study had a Forskolin response above 50% of the normal ratio. This is probably because CFTR shows a high level of expression in the intestinal mucosa. Therefore, as the transepithelial transport reflects both channel dysfunction and the number of proteins expressed, a high number of CFTR channels displaying a residual activity might result in a globally normal intestinal transepithelial transport. Indeed, in the murine G480C CF animal model, levels of WT-protein as low as 20% were sufficient to normalize transepithelial anion secretion [34]. Thus, it can be hypothesized that a normal ICM Forskolin response indicates a CFTR function at least above 20% and suggests that those patients are at very low risk of developing typical CF disease, but does not rule out a CFTR-RD. In this context, evaluation of CFTR function in the respiratory mucosa brings additional information as it evaluates CFTR activity based on Cl⁻ transport and its impact on ENaC activity [31].

Our measurements individualized 3 situations. First, tracings showing a typical CF profile as observed in the F508del/D1152H patients, indicating a high likelihood of developing CF. This was supported by the fact that the patients had developed CF like symptoms 3 years later. Unfortunately, we could not perform ICM in these patients. However, it is known that patients carrying D1152H in *trans* of a CF causing variant develop CF although less severe than F508del, putting this VVCC in the CF range [35]. More-

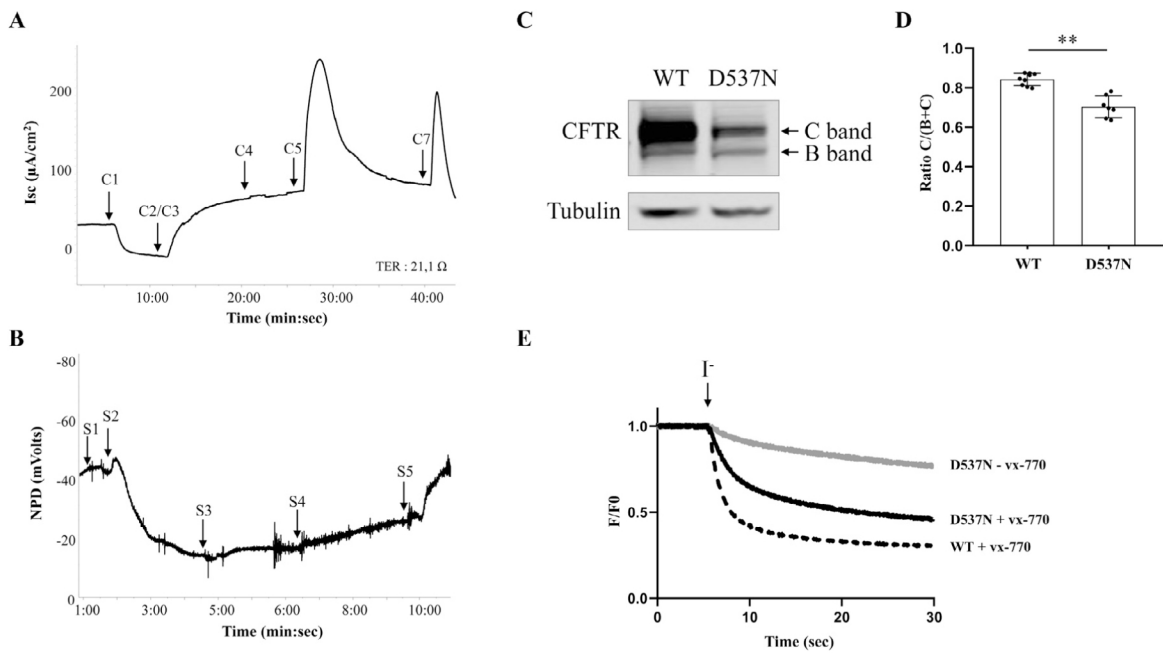


Fig. 1. c.1609G>A - p.Asp537Asn (D537N) CFTR expression and functional experiments A. Short-circuit current (Isc) change of rectal biopsy of Basal Isc (C1) after successive addition in the Ussing chamber of Amiloride (C2,100 μ M), Forskolin (C3, 10 μ M)/IBMX (C4, 100 μ M), Genistein (C5, 10 μ M), Carbachol (C6, 100 μ M), Histamin (C7, 500 μ M). B. Nasal potential difference (NPD) tracing. Baseline PD is measured after perfusion of the nasal epithelium with saline Ringer solution (S1). PD changes are recorded after: perfusion with 100 μ M amiloride in Ringer solution (S2), 100 μ M amiloride in low-chloride solution (S3), 100 μ M amiloride plus 10 μ M isoproterenol in low-chloride solution (S4), 100 μ M amiloride plus 10 μ M isoproterenol plus 100 μ M ATP in low-chloride solution (S5). C. Western blot analysis of WT and D537N CFTR. Fully glycosylated (C band) and core glycosylated CFTR (B band) are indicated with an arrow. Tubulin was probed to assess equal loading amounts. D. Histograms showing the ratio of C band on total CFTR. Data are shown as mean \pm S.D. $n = 9$ for WT and $n = 7$ for D537N with ** indicating $p < 0.01$ (Kruskal-Wallis test). E. Iodide efflux experiments in HEK cells expressing D537N-CFTR after perfusion with CPT-cAMP (100 μ M) without (in gray) or with VX770 (10 μ M, in black). Iodide efflux experiments in HEK cells transfected with WT-CFTR after perfusion with CPT-cAMP (100 μ M) and VX-770 (10 μ M) indicated as a reference (black dashed line).

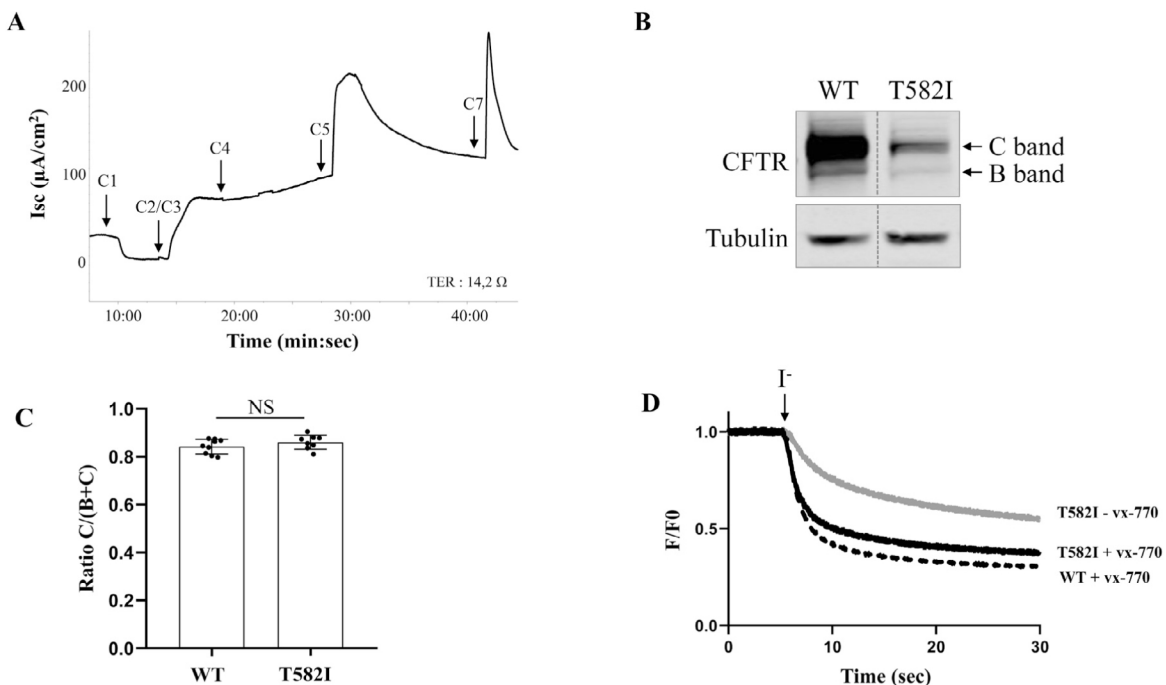


Fig. 2. c.1745C>T - p. Thr582Ile (T582I) CFTR expression and functional experiments A. Short-circuit current (Isc) change of rectal biopsy of Basal Isc (C1) after successive addition in the Ussing chamber of Amiloride (C2,100 μ M), Forskolin (C3, 10 μ M)/IBMX (C4, 100 μ M), Genistein (C5, 10 μ M), Carbachol (C6, 100 μ M), Histamin (C7, 500 μ M). B. Western blot analysis of WT and T582I CFTR. Fully glycosylated (C band) and core glycosylated CFTR (B band) are indicated with an arrow. Tubulin was probed to assess equal loading amounts. The ratio of C band on total CFTR is quantified in C. Data are shown as mean \pm S.D. $n = 9$ for WT and $n = 8$ for T582I with NS indicating $p > 0.05$ (Kruskal-Wallis test). D. Iodide efflux experiments in HEK cells expressing T582I-CFTR after perfusion with CPT-cAMP (100 μ M) without (in gray) or with VX770 (10 μ M, in black). Iodide efflux experiments in HEK cells transfected with WT-CFTR after perfusion with CPT-cAMP (100 μ M) and VX-770 (10 μ M) indicated as a reference (black dashed line).

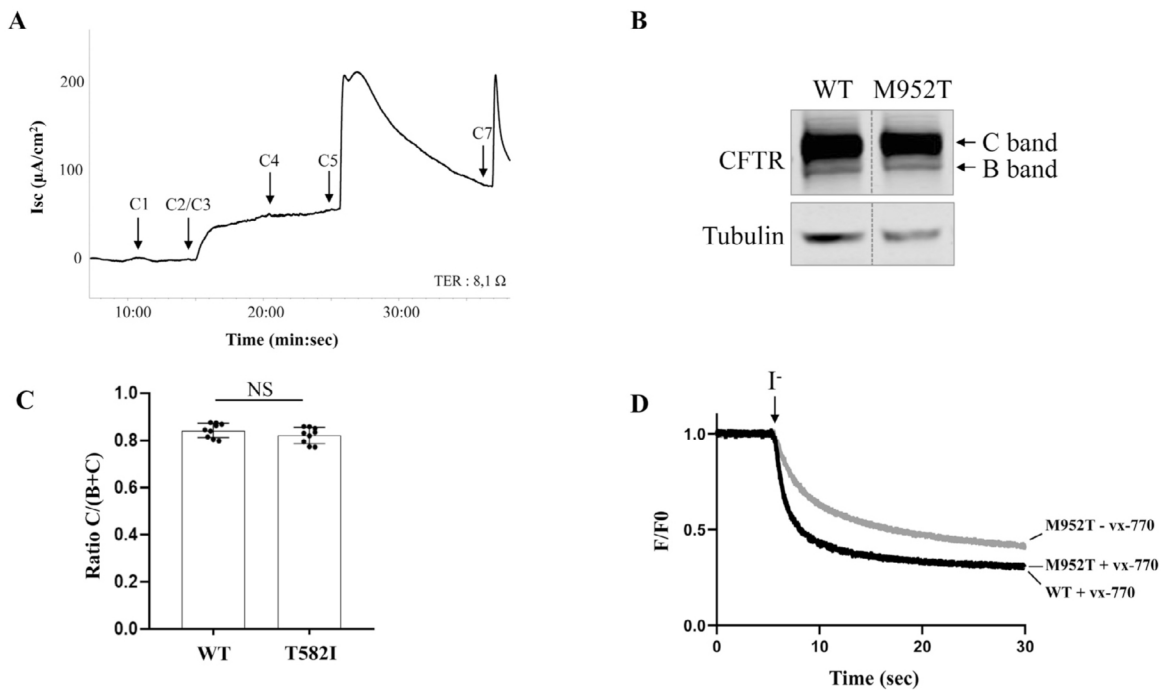


Fig. 3. c.2855T>C - p.Met952Thr (M952T) CFTR expression and functional experiments A. Short-circuit current (Isc) change of rectal biopsy of Basal IsC (C1) after successive addition in the Ussing chamber of Amiloride (C2,100 μ M), Forskolin (C3, 10 μ M)/IBMX (C4, 100 μ M), Genistein (C5, 10 μ M) Carbachol (C6, 100 μ M), Histamin (C7, 500 μ M). B. Western blot analysis of WT and M952T-CFTR. Fully glycosylated (C band) and core glycosylated CFTR (B band) are indicated with an arrow. Tubulin was probed to assess equal loading amounts. C. Histogram showing the ratio of C band on Total CFTR. Data are shown as mean \pm S.D., $n = 9$ for WT and $n = 9$ for M952T with NS indicating $p > 0.05$ (Kruskal-Wallis test). D. Iodide efflux experiments in HEK cells expressing M952T-CFTR after perfusion with CPT-cAMP (100 μ M) without (in gray) or with VX770 (10 μ M, in black). Iodide efflux experiments in HEK cells transfected with WT-CFTR after perfusion with CPT-cAMP (100 μ M) and VX-770 (10 μ M) indicated as a reference (black dashed line).

over, Minso *et al.* reported a CFTR activity in the CF range in intestine in a D1152H homozygous patient [36]. Second, tracings with responses in the normal range in patients carrying variants with low penetrance for CFTR-RD, such as TG11T5. This suggests that these patients are at an extremely low risk, if any, of developing a CFTR-RD. Consistently, no CF symptoms were reported in these patients at a mean age of 8 years. This confirms the observation by Aalbers *et al.* that the TG11T5 variant is associated with lower sweat chloride, less frequently abnormal NPD and less progression of symptoms compared to TG12T5 and TG13T5 variants [13,37]. This observation might be translated to the patients carrying R31C and more generally for variants with a very low penetrance for CFTR-RD based on their frequency in the general population [17,38]. Third, and finally, patients with NPD tracings showing an increased response to Amiloride but a normal or subnormal Cl^- transport, such as in the patient carrying F508del in *trans* with F1052V or D537N. Similar profiles with an isolated ENaC hyperactivity have already been reported in patients carrying CFTR-RD mutations and were significantly improved by Ivacaftor treatment [11,39]. These results, together with the observation of ICM in the normal range, suggest a partial CFTR dysfunction above 20% of the WT but a remaining possibility of developing CFTR-RD in these patients.

Three very rare variants were fully characterized for the first time. *In vitro* functional studies by western blot experiments showed normal maturation and halide transport assay for M952T which suggests a normal function. However, this mutant has been associated with CBAVD in combination with a CF-causing variant. This suggests that this variant could have some pathogenic effect, but with a low penetrance for CFTR-RD [40]. The 2 patients carrying this variant in *trans* of F508del were thus considered at very low risk to develop CFTR-RD. In contrast, T582I displayed a moderate defect in CFTR expression but a defective function at 39% of

the normal, which increases to 64% upon ivacaftor. Lastly, D537N demonstrated a defective maturation, which suggests that this mutant affects protein folding and is probably associated with a defective number of CFTR proteins at the apical membrane. The patient's NPD profile demonstrated residual Cl^- activity but an increased response to Amiloride indicative of CFTR dysfunction, yielding a functional profile similar to F1052V.

These observations lead to general considerations in the context of unconcluded diagnosis after NBS. Although ICM may provide very valuable insight if abnormal given its specificity, it is not sensitive enough to detect variants which retain activity above 20% of the WT. In patients carrying variants with residual function between 10 and 20% of CFTR activity relative to wild-type, CFTR functional assays in the respiratory mucosa are more sensitive. This range of activity is often associated with variable expressivity and penetrance [40]. Indeed, as CFTR activity increases, the relation with ion transport in the respiratory and the sweat epithelium is nonlinear and may be also influenced by environmental factors or other ion transporter defects. In this context, evidence of an elevated Δ Amiloride response suggests CFTR dysfunction with residual or even normal Cl^- transport. The threshold to discriminate between CF and CFTR-RD is currently unknown, but it is likely that a residual CFTR activity above 10% of the normal is consistent with the diagnosis of CFTR-RD [41].

Hence, our study helps refine the strategy of diagnosis in these unconcluded cases at NBS. If the genotype combines a CF-causing variant in *trans* of a VVCC, this indicates a high likelihood to evolve towards CF or a CFTR-RD, even if the probability cannot be stated because the penetrance of CF or CFTR-RD has not been defined for most of the variants. Functional measurement should be performed to characterize a VUS, and may be also proposed to assess the functional impact of a variant associated with a CFTR-RD, whose penetrance for CF is unknown. If ICM is abnormal this indicates

a CFTR activity below 20%. If it is normal, it does not rule out a genotype with residual activity, that could be compatible with an evolution toward a CFTR-RD. In this respect, CFTR activity should be investigated in the respiratory mucosa of the patient. If CFTR activity is decreased, this does not mean that those patients will have a 100% probability to develop symptoms later in life, in adulthood or even at older ages, but a decreased CFTR activity is clearly a sign of vigilance requiring continued follow-up in a CF center and preventive care. As NPD can be challenging in babies, a potential interesting test might be the evaluation of CFTR activity in primary nasal cells collected by a simple nasal brushing, which may be more feasible in young patients. CFTR activity in this model was shown to be correlated to *in vivo* NPD and might therefore provide reliable insight [42,43]. These primary cultures could also be used to test the correction of CFTR function by modulators and help to tailor the potential treatment of patients considered at risk to develop CF, early in life.

5. Conclusion

It is crucial to detect which children with an inconclusive diagnosis at NBS are at increased likelihood for conversion to CF or CFTR-RD in order to best guide care and follow-up. We show here that combination of clinical follow-up, comprehensive CFTR gene analysis, *in vitro* and *in vivo* functional evaluation help reclassify all these children with inconclusive diagnosis at NBS.

Supplementary Fig. S1. Intestinal Short-circuit Current of a rectal biopsy from a subject carrying the F508del/TG11T5 genotype. Change in the Short-circuit Current (Isc) after sequential addition of different pharmacological agonists: Amiloride 100 μ M (apical side); Forskolin 10 μ M and IBMx 100 μ M (apical and basal side); Genistein 10 μ M (apical and the basal side) Carbachol 100 μ M (basal side); Histamin 500 μ M (basal side).

Declaration of Competing Interest

All authors have disclosed their conflict of Interest.

CRediT authorship contribution statement

Aurelie Hatton: Writing – review & editing. **Anne Bergougnoux:** Writing – review & editing. **Katarzyna Zybert:** Writing – review & editing. **Benoit Chevalier:** Writing – review & editing. **Myriam Mesbahi:** Writing – review & editing. **Jean Pierre Altéri:** Writing – review & editing. **Katarzyna Walicka-Serzysko:** Writing – review & editing. **Magdalena Postek:** Writing – review & editing. **Magali Taulan-Cadars:** Writing – review & editing. **Aleksander Edelman:** Writing – review & editing. **Alexandre Hinzpeter:** Writing – review & editing. **Mireille Claudres:** Writing – review & editing. **Emmanuelle Girodon:** Writing – review & editing. **Caroline Raynal:** Writing – review & editing. **Isabelle Sermet-Gaudelus:** Writing – review & editing. **Dorota Sands:** Writing – review & editing.

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