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A New Targeted *CFTR* Mutation Panel Based on Next-Generation Sequencing Technology

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Searching for mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*) is a key step in the diagnosis of and neonatal and carrier screening for cystic fibrosis (CF), and it has implications for prognosis and personalized therapy. The large number of mutations and genetic and phenotypic variability make this search a complex task. Herein, we developed, validated, and tested a laboratory assay for an extended search for mutations in *CFTR* using a next-generation sequencing—based method, with a panel of 188 *CFTR* mutations customized for the Italian population. Overall, 1426 dried blood spots from neonatal screening, 402 genomic DNA samples from various origins, and 1138 genomic DNA samples from patients with CF were analyzed. The assay showed excellent analytical and diagnostic operative characteristics. We identified and experimentally validated 159 (of 188) *CFTR* mutations. The assay achieved detection rates of 95.0% and 95.6% in two large-scale case series of CF patients from central and northern Italy, respectively. These detection rates are among the highest reported so far with a genetic test for CF based on a mutation panel. This assay appears to be well suited for diagnostics, neonatal and carrier screening, and assisted reproduction, and it represents a considerable advantage in CF genetic counseling. *(J Mol Diagn 2017, 19: 788—800; http://dx.doi.org/10.1016/j.jmoldx.2017.06.002)*

Cystic fibrosis (CF) is a chronic, life-threatening genetic disease caused by loss-of-function mutations in the CF transmembrane conductance regulator gene (*CFTR*).[1,2]

Notwithstanding the considerable ethnic and geographic variability in the frequency of CF, the mean incidence of 1 in 2500 live births makes CF the most frequent severe autosomal recessive disease in the white population.[3] CF is characterized by wide genetic and clinical heterogeneity, which complicates diagnosis, prognosis, and therapy. From birth to adulthood, there is considerable variability in the severity and rate of disease progression in CF, with varying clinical presentations and different organs involved at different ages.[4] Often, great phenotypic variability arises from even a single *CFTR* genotype.[5,6]

More than 2000 different *CFTR* sequence variations, including CF-causing mutations and polymorphisms, have been reported (CFTR1 database, *http://www.genet.sickkids.on.ca*, last accessed May 15, 2017). Also in limited geographic areas, *CFTR* shows a complete catalog of mutation types in exons, introns, the 5′-flanking region, and the 3′-untranslated region, that is, point mutations, small insertions and deletions, complex alleles, and large genomic rearrangements.[5,7–12] This genetic heterogeneity greatly affects the allele *detection rate* (DR) of genetic tests, which is defined as the overall frequency of mutant alleles (of all of the mutant alleles present), as evidenced by a genetic test. For example, the DRs of the 23-mutation panel established by the American Congress of Obstetrician and Gynecologist and the

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American College of Medical Genetics differ substantially between various geographic regions, ranging from 49% to 94%.[13,14] This genetic heterogeneity is further enhanced if various well-recognized clinical forms of CF, such as classic CF, CFTR-related disorders, and congenital bilateral absence of vas deferens, are taken into consideration.[15,16] The same genetic test may have very different DRs, depending on its application in these different clinical forms.[17] In this article, CF refers to the classic form of the disease and excludes CFTR-related disorders and congenital bilateral absence of vas deferens, which will be specifically mentioned when appropriate.

Limiting the scope to CF, but considering geographic areas with high genetic heterogeneity, an extended Sanger sequencing protocol of the 5′-flanking region, 27 exons and proximal intronic flanking regions, plus selected deep intronic zones for specific intron mutations in *CFTR*, have shown DRs of up to 97%.[5,17] An additional search for large *CFTR* rearrangements showed a DR increment of approximately 2%.[5,9,17] The high DRs of approaches with extended genetic characterization seem to be confirmed by next-generation sequencing (NGS) technology, which has been recently used for sequence and copy number variation (CNV) analyses of *CFTR*.[18–27] NGS has been used mainly in validation studies and has been performed on a limited number of samples in comparisons of the performance of NGS to those of classic Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA). Although the NGS approach provides an obvious reduction in cost per base, and an actual possibility of multiple-gene analysis for multigenic disorders, the suitability of this approach in monogenic disorders is still debated. In particular, the classic sequencing approach and MLPA have shown excellent laboratory operative characteristics for *CFTR*.[5,17,28,29]

Despite the excellent operative characteristics of experimental approaches based on both extended sequencing and large rearrangement search, which are welcomed for diagnostic purposes, these approaches may not be suitable for other health programs, such as neonatal and carrier screening. In fact, in addition to the obvious problems of cost and time, the functional characterization of all of the possibly identified sequence variations and the subsequent assignment of their clinical significance[30] may be impossible tasks. Consequently, several efforts have been made to develop experimental approaches based on selected panels of known CF-causing mutations.[31,32] In this case, the DR of the selected panel of mutations in populations of various ethnographic origins and/or with various CF clinical forms invariably arose as a crucial variable.[17] NGS platforms seem to be particularly suited to the development of mutation panels with high a mutation number and, consequently, a high DR. However, at the moment, only a small number of articles have addressed specific *CFTR* mutation panels based on NGS approaches.[33]

In this work, we developed, validated, and tested a high-throughput, NGS-based approach using a customized mutation panel containing 188 CF-causing mutations in *CFTR*. We analyzed 1828 subjects referred for diagnosis and a case series of 1138 patients with CF (646 patients from northern Italy and 492 patients from central Italy). We called this assay *188-CF-NGS*. This approach revealed DRs of 95.0% and 95.6% in CF patients from central and northern Italy, respectively. Also in geographic regions with high genetic heterogeneity (such as Italy), a suitably customized and reasonably large panel of *CFTR* mutations, together with an NGS-based approach, allows the attainment of a high DR that is definitely suited for diagnostic purposes, at least in CF, and for neonatal and carrier screening.

Materials and Methods

Study Design and Case Series

We designed a specific, customized panel of 188 CF-causing mutations, described in Supplemental Table S1. The mutations were included based on their high frequency in the Italian population and their documented associations with CF. Frequency information was obtained using the data on the frequency of CF mutations in Italian patients in the literature,[5,34–39] from the 2010 Report of the Italian CF Register (*http://www.registroitalianofibrosicistica.it*, last accessed January 31, 2017), as well as from personal communication with some Italian CF centers and laboratories. The characterization of the mutations as CF causing was obtained from data on the functional effect from the North American CFTR2 (Clinical and Functional Translation of CFTR, *https://cftr2.org*, last accessed January 31, 2017) project[40] and from the literature.[5,34,35,41] With this panel of *CFTR* mutations, we optimized an NGS-based assay we called *188-CF-NGS*.

Our assay underwent six steps of validation (Figure 1). If allowed by the overall number of mutant alleles, each mutation was validated in at least three independent samples. Also, possible differences in quality, analytical sensitivity, and analytical specificity of the 188-CF-NGS assay between analysis of dried blood spots (DBSs) and analysis of genomic DNA were evaluated.

The first validation step was the analysis of 48 selected DBSs (referred to the Newborn Screening Laboratory, ASST Fatebenefratelli Sacco—PO Ospedale dei Bambini "V. Buzzi," Milan, Italy) that were previously investigated by mass spectrometry assay (*Mass Spectrometry Assay*). The DBSs were obtained from neonates screened as positive through the immunoreactive trypsinogen assay. The composition of this subset of DBS samples was as follows: eight homozygotes, 26 compound heterozygotes, four heterozygotes, and 10 with no *CFTR* mutation.

The second validation step was the analysis of 24 genomic DNA samples extracted from peripheral blood (referred to the Medical Genetics Laboratory, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy), which had been genotyped by confirmatory methods (*Confirmatory Methods*). The subset of 24 genomic DNA samples consisted of nine compound heterozygotes, eight heterozygotes, and seven with no *CFTR* mutation.

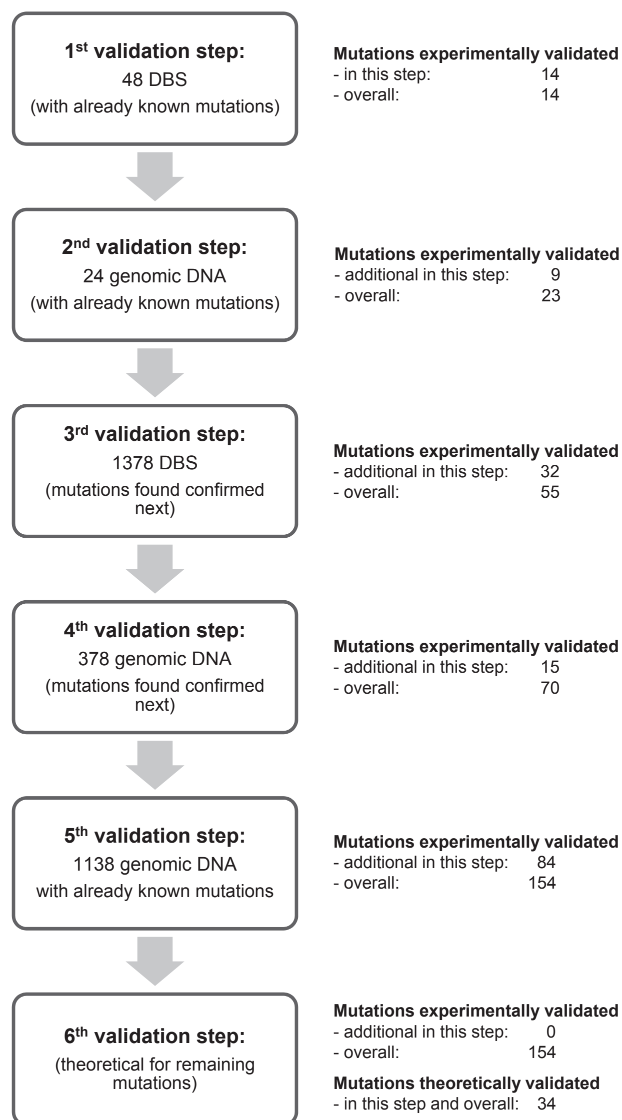

Figure 1 Study workflow of the 188-CF-NGS assay. The validation steps, specimens used, and number of mutations validated at each step, as well as overall, are reported. DBS, dried blood spots.

In the third validation step, 1378 DBSs from neonates (referred to the Newborn Screening Laboratory, Azienda Socio Sanitaria Territoriale Fatebenefratelli Sacco—PO Ospedale dei Bambini "V. Buzzi," Milan, Italy) with elevated levels of immunoreactive trypsinogen at neonatal screening were analyzed for mutations using our 188-CF-NGS assay. All of the identified mutations were subsequently confirmed by confirmatory methods (*Confirmatory Methods*).

In the fourth validation step, 378 genomic DNA samples from subjects referred for genetic analysis (to the Medical Genetics Laboratory, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy) were analyzed using the 188-CF-NGS assay. A subset of 325 subjects underwent carrier screening or carrier testing (211 for family history, 98 as partners of carriers, and 16 for consanguinity), and another subset (53 subjects) were analyzed for hyperechogenic bowels. All of the identified mutations were subsequently confirmed by confirmatory methods (*Confirmatory Methods*).

To complete the experimental validation of all of the mutations available in our case series, we applied a fifth validation step. We used samples from a case series comprising 1138 patients affected by CF and diagnosed according to the present guidelines[41]: 646 from northern Italy (referred to the Lombardia Regional Reference CF Center) and 492 from central Italy (referred to the Lazio Regional Reference CF Center). Patients with clinical manifestations associated with CFTR dysfunction but whose conditions did not fulfill the diagnostic criteria for CF (namely, CFTR−related disorders or congenital bilateral absence of vas deferens[15]) were not included in the case series. In the cohort from northern Italy, there were 149 patients (23%) with pancreatic sufficiency and 497 patients (77%) with pancreatic insufficiency. The age range was 2 months to 65 years. In the cohort from central Italy, there were 138 patients (28%) with pancreatic sufficiency and 354 patients (72%) with pancreatic insufficiency. The age range was 6 months to 58 years. All of the patients had undergone genetic studies, and their mutations had been identified/confirmed by confirmatory methods (*Confirmatory Methods*). This case series was also used for calculating the DRs of the 188-CF-NGS assay (in Lombardia and Lazio regions).

In the sixth validation step, all of the mutations not validated in steps 1 to 4 and not present in our case series underwent theoretical validation, also by a check of the high coverage of the corresponding targeted regions. We did not detect false-negatives related to these mutations.

All subjects provided informed consent to use the deidentified biological samples for scientific study.

DNA Extraction

Genomic DNA from peripheral blood samples, collected in EDTA tubes, was extracted from 400 μL of blood using a QiaSymphony DSP DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's automated protocol. For the DBS samples, DNA was extracted from three punches of 3 mm from 226 Spot Saver Cards (catalog number GR2261003; PerkinElmer, Waltham, MA) using a QIAamp DNA Investigator Kit (Qiagen), according to the manufacturer's protocol, with 50 μL of distilled water for the final elution step. For evaluation of the stability of the extract, all of the samples were stored at 4°C, except for four DBSs, which were extracted in duplicate and stored at −20°C; the obtained data did not reveal any differences.

Confirmatory Methods

The detection and characterization of all *CFTR* mutations in all of the subjects included in this study were also performed

with methods alternative to the 188-CF-NGS assay. These methods were applied before 188-CF-NGS in validation steps 1, 2, and 5 and after 188-CF-NGS in validation steps 3 and 4 (Figure 1). We called this group of methods *confirmatory methods*. Overall, each mutation that was identified by the 188-CF-NGS assay was always detected, in the same sample, by at least one other confirmatory method. A list of confirmatory methods and their brief descriptions are reported in the following sections.

Mass Spectrometry Assay

A mass spectrometry assay was conducted using a Myriapod Cystic Fibrosis Kit SQ080 (Sequenom, San Diego, CA), which can detect 80 mutations (including eight deletions) and the T_n tract of *CFTR*, according to the manufacturer's protocol. Briefly, DNA was amplified by multiplex PCR using eight different mixes of primers to obtain fragments corresponding to all of the regions of interest. The PCR products were treated with shrimp alkaline phosphatase (included in the SQ080 kit) to remove residual nucleotides. Then, a primer extension reaction (iPlex) with mass-modified nucleotides was performed, providing wild-type and mutant analytes of known mass. After purification and chip loading, analysis by the mass spectrometry platform resolved each analyte by its molecular weight. The obtained data were analyzed using a MassArray Analyser4 device and iGenetics Myriapod software version 4.0 (Diatech Pharmacogenetics, Jesi, Italy). The mass spectrometry assay was used for genetic characterization of the 48 DBS samples before the 188-CF-NGS assay preliminary validation (first validation step, *Study Design and Case Series*).[42]

Sanger Sequencing

The regions of interest were amplified by PCR using Sensoquest Thermal Cyclers (Biomedizinische Elektronik, Hamburg, Germany). The PCR products were subsequently subjected to automated purification with a Biomek 3000 device (Beckman Coulter, Nyon, Switzerland) and Agentcourt chemistry (Beckman Coulter), followed by automated cycle sequencing with the Big Dye Terminator Cycle Sequencing Kit version 1.1 (Life Technologies, New York, NY).[29] Electrophoresis was performed on the ABI PRISM 3130*xl* platform (Life Technologies). Data analysis was performed with Sequencing Analysis software version 5.3.1 (Life Technologies) and then visualized with SeqScape software version 2.1.1 (Life Technologies).[43] In addition to the prior genetic characterization of the 24 genomic samples used for NGS preliminary validation (second validation step, *Study Design and Case Series*), and the prior genetic characterization of a subset of the case series of 1138 CF patients (fifth validation step, *Study Design and Case Series*), Sanger sequencing was also used as a confirmatory tool for the identified mutations in the 188-CF-NGS assay (those which were suitable to be characterized by sequencing) in the third and fourth validation steps.

CF-OLA Assay

The CF-OLA assay (Abbott, Wiesbaden, Germany) was used for the prior genetic characterization of a subset of the case series of 1138 CF patients (fifth validation step, *Study Design and Case Series*) according to the manufacturer's protocol. Electrophoresis was performed on the ABI PRISM 3130*xl* platform.

CFTR Core and CFTR Italia Assays

The CFTR Core and CFTR Italia version 2 assays (Devyser, Hagersten, Sweden) were used for the prior genetic characterization of a subset of the case series of 1138 CF patients (fifth validation step, *Study Design and Case Series*). They were also used to confirm and characterize the macrodeletions found by the 188-CF-NGS assay (those which were included in these Devyser assays) in the third and fourth validation steps. They were applied following the manufacturer's protocol. Electrophoresis was performed on the ABI PRISM 3130*xl* platform.

RDB-Inno LiPA CFTR17+Tn Update, CFTR19, CFTR Italian Regional and CFTR Deletions +6

RDB-Inno LiPA CFTR17+Tn Update, CFTR19, CFTR Italian Regional and CFTR Deletions +6 (Fujirebio Europe, Gent, Belgium) were used for the prior genetic characterization of a subset of the case series of 1138 CF patients (fifth validation step, *Study Design and Case Series*) according to the manufacturer's protocol.

MLPA

MLPA was performed by using the Salsa MLPA P091-D1 CFTR probe mix and reagent kit according to the manufacturer's protocol (MRC-Holland, Amsterdam, the Netherlands). Electrophoresis was performed on the ABI PRISM 3130*xl* platform. The data analysis was performed using the Coffalyser.net software version 140721.1958 (MRC-Holland). MLPA was used for the prior macrodeletion and macroduplication detection in samples from a subset of the case series of 1138 CF patients (fifth validation step, *Study Design and Case Series*). MLPA was also used to confirm the macrodeletions and macroduplications that were identified by the 188-CF-NGS assay (in the third and fourth validation steps) and were not included in the Devyser assays. In these cases, the breakpoints were characterized by Sanger sequencing.

NGS

The NGS experiments were performed using the commercial CFTR MASTR Dx version 2 assay (Multiplicom, Niel, Belgium). This assay enabled the targeted, multiplex PCR amplification of all coding regions, selected intronic regions, and part of the promoter region of *CFTR*, in a limited number of PCR reactions. In the first step, all targeted regions were amplified in two separate multiplex PCR amplification reactions per individual, using a hot-start

DNA polymerase. In the second step, a second round of PCR, which allowed tagging of the amplicons with molecular identifiers and MiSeq adaptors, was performed. The resulting tagged amplicons were visualized for quality control through fluorescent labeling and capillary electrophoresis on ABI PRISM 3130*xl* and were then pooled per individual. Each pooled amplicon library was subsequently purified from small residual DNA fragments with AMPure beads (Beckman Coulter), and the DNA concentration was determined by the Qubit dsDNA BR Assay Kit (Life Technologies). Next, purified and individually tagged amplicon libraries were pooled in equimolar samples (9 pmol/L each), along with 5% PhiX as a control (Illumina, San Diego, CA). The resulting amplicon pools were further processed on a MiSeq System with the 2 × 250 reagent kit and the MiSeq Reagent Nano kit version 2 (Illumina) according to the manufacturer's recommendations.

NGS Data Analysis

Data produced by the 188-CF-NGS assay were analyzed starting from the FastQ files generated by the MiSeq System (Illumina) using Data Driven Medicine software version 4.2.5 (Sophia Genetics, Ecublens, Switzerland).

The FastQ files were automatically uploaded and processed using patented advanced algorithms, generating a report of the entire mutation panel. For each mutation, the system specified the legacy name, its status (present/absent), the reference used for the alignment, the number of reads, and the chromosome position. When a mutation was present, the platform detailed the exon (and codon) or intron position, Human Genome Variation Society nomenclature at the DNA level and, when indicated, at the protein level, as well as the variant frequency percentage to define zygosity.

Results

Quality Metrics of the Runs

All of the runs yielded a median cluster density of 887 K/mm^2 (minimum 577, maximum 1080).

The mean base-call quality in each sequencing cycle was very high and typically declined slightly toward the end of the read. The distribution of mean read qualities in each data set resulted in a peak of approximately 35 to 40. The distribution of raw read lengths resulted in one or more peaks at the end of the x-scale. Regarding mapping quality, most of the mappings were of high quality, with one peak of approximately 40. If a read could not be aligned over its full length, some bases were soft-clipped at the borders of the read. However, a very small number of bases (4.50% on average) were clipped.

The mean numbers of bases and reads generated in the runs were 631.41 Mbp and 2,408,900, respectively. A read was categorized as unmapped (mean, 0.60%) if it did not align to the genome, as off target (mean, 0.31%) if it was aligned but not within the targeted amplicons, and as on target (mean, 99.09%) if it was mapped within the amplicon regions. None of the samples in any run had <85% of reads mapping to the amplicon regions. The mean percentage of bases called incorrectly at any one cycle was 1.33% (error rate), whereas the mean percentage of bases with a quality score of 30 or higher was 95.07%. The median percentage of single clusters with a clear signal in the bases sequenced, indicated by passing filter percentage, was 95.60%.

An important prerequisite for reliable variant detection from NGS data was the coverage of targeted regions. The *base coverage* was defined as the number of reads covering a base. The *coverage of an amplicon* on a targeted region was defined as its mean base coverage. There was no region that fell within amplicons with a coverage of <50. In fact, the mean coverage achieved in the samples subjected to NGS analysis was 638 reads for each amplicon. In addition, the minimum/maximum respective values were 147/3303.

CFTR Mutations Detected by the 188-CF-NGS Assay and Validation

In Supplemental Table S1, the mutations detected by the 188-CF-NGS assay are indicated according to both the old nomenclature (legacy name) and new nomenclature (Human Genome Variation Society), whereas in the text they are indicated according to legacy name only. In Table 1, each mutation is assigned to the validation step in which it was first identified, although several mutations were identified several times in different validation steps. To date, we have analyzed 1828 samples and used a case series comprising 1138 CF patients (*Materials and Methods*) (Figure 1). For validation steps 1 and 2, 48 DBS and 24 genomic DNA samples with known mutations, respectively, were analyzed. For validation steps 3 and 4, 1378 DBSs from neonatal screening and 378 genomic DNA samples with unknown mutations, respectively, were analyzed. For validation step 5, samples from a case series of 1138 CF patients with known *CFTR* genotype were used. Step 6 was a theoretical validation of the mutations that were not found in steps 1 to 4 and were not represented in our case series.

In the 48 DBSs (validation step 1) (Table 1), we selected one small deletion (F508del), one small insertion (4016insT), four nonsense mutations (G542X, Q552X, R1162X, and W1282X), six missense mutations (T338I, D1152H, N1303K, R1066H, R117C, and R117H), and two splicing mutations (1898+1G>A and 3849+10kbC>T). In this step we validated 14 different mutations.

The analysis of the 24 genomic DNA samples (validation step 2) (Table 1) identified the following mutations: four small deletions (F508del, 1782delA, 4382delA, and 1002-1110_1113delTAAG), four nonsense mutations (E585X, G542X, Q552X, and R1162X), eight missense mutations (D1152H, G1244E, L1077P, L206W, N1303K, R1066H,

R117C, and R347P), and two splicing mutations (1717-1G>A and 3849+10kbC>T). In this step, we validated 18 different mutations, 9 of which were in addition to those from the previous step. In the first two steps, we validated a total of 23 different *CFTR* mutations.

All of the mutations found by the 188-CF-NGS assay within the first and second validation steps were correctly identified with respect to a prior genetic characterization of the same samples by a confirmatory method (*Materials and Methods*).

Among the 1378 samples selected by neonatal screening with elevated levels of immunoreactive trypsinogen (validation step 3) (Table 1), we identified 28 CF neonates (6 homozygotes and 22 compound heterozygotes) and 141 heterozygotes, with a negative sweat test. In 9 CF newborns (1 homozygote and 8 compound heterozygotes) and in 29 heterozygotes, we were able to identify 16 mutations that would not have been detected with other methods because they were not present in the mutation panels that we previously used for genetic analysis of newborns who were positive on neonatal screening. An overall number of 49 different mutations were found, 32 of which were additional to those identified using the previous two validation steps. In these three steps, we validated a total of 55 mutations.

Among the 378 genomic DNA samples (validation step 4) (Table 1), we identified 93 heterozygotes. Overall, 36 different mutations were found, 15 of which were additional to those identified using the previous three validation steps. In these four steps, we validated a total of 70 *CFTR* mutations.

In the third and fourth validation steps, all of the mutations found by the 188-CF-NGS assay were confirmed in a subsequent genetic characterization of the same samples by a confirmatory method (*Materials and Methods*).

In samples from the case series of 1138 CF patients (validation step 5) (Table 1), 84 mutations that were not validated in the previous four steps were selected, and samples from the corresponding patients were analyzed by the 188-CF-NGS assay for validation. In all of the samples analyzed in this step, all of the mutations found by the 188-CF-NGS assay were correctly identified with respect to a prior genetic characterization of the same samples by a confirmatory method (*Materials and Methods*). In all of the five validation steps, we validated a total of 154 different *CFTR* mutations of the 188 included in the 188-CF-NGS assay.

After the five validation steps, an overall number of 34 mutations were left with only theoretical validation (validation step 6) (Table 1).

Finally, the 188-CF-NGS mutation panel consisted of 188 *CFTR* mutations, 154 of which were experimentally validated and 34 were theoretically validated.

Macrodeletions, Macroduplications, and Complex Alleles in the 188-CF-NGS Assay

The 188-CF-NGS assay can detect each macrodeletion and macroduplication listed in Supplemental Table S1. If the macrodeletion or macroduplication breakpoints are not known, a general Human Genome Variation Society notation is used. By contrast, whenever a macrodeletion or a macroduplication with characterized breakpoints exists, the specific Human Genome Variation Society name is reported. With this method, it may not be possible to directly infer the breakpoints of the identified macrodeletion or macroduplication. In these cases, further studies are needed to complete the structural characterization.

Some of the mutations included in the 188-CF-NGS assay have also been found within complex alleles. A brief description of these mutations and the corresponding complex alleles, as well as their prevalences in this case series of CF patients, are reported in Supplemental Table S2.

Operative Characteristics of the Assay

All of the 154 experimentally validated mutations, both those that were already known to be present and those that were first revealed by the 188-CF-NGS assay and then confirmed by a confirmatory method, were correctly revealed in every tested sample and replicate. Similarly, every wild-type sample and replicate was correctly characterized as negative by the 188-CF-NGS assay. Consequently, we can assign a value of 100% to the analytical sensitivity and analytical specificity of the 188-CF-NGS assay. None of the replicated samples showed discrepant results, allowing an estimation of 100% reproducibility.

The DRs (diagnostic sensitivity) of the 188-CF-NGS assay were 95.0% in the Lazio case series and 95.6% in the Lombardia case series (Table 1). As we found no false-positive results, we also report 100% overall diagnostic specificity.

The pipeline was fully automated. From an experimental point of view, the turnaround time of the platform for the complete assay in a single NGS analytical run of up to 52 samples simultaneously was 3 working days.

Discussion

This study is one of the first to apply a customized, geographically matched panel for CF disease-causing mutations using NGS technology. We validated this NGS-based assay (which we called the 188-CF-NGS assay), generating a customized panel of 188 *CFTR* mutations. This assay showed DRs of 95.0% and 95.6% in central and northern Italy, respectively, as deduced from the analysis of samples from two large-scale case series of Italian patients with CF. This assay also showed excellent values of all of the other analytical and diagnostic operative characteristics.

Due to the complex genetics and genotype–phenotype relationship of CF, an unequivocal mutation search strategy has not been delineated yet. It has been evidenced that a unique genetic test suitable for diagnostic and neonatal and carrier screening programs, as well as for all clinical forms

Table 1 Characteristics and Frequency of Mutations Included in the 188-CF-NGS Assay

Legacy name	Validation step	Type of mutation	Classification in CFTR2	Prevalence CF (PI + PS) Lazio		Prevalence CF (PI + PS) Lombardia	
				n	Frequency	*n*	Frequency
CFTR-dup 1—3	5	Duplication	ND	0	0.000	1	0.001
M1V	5	Missense	CF-causing	1	0.001	3	0.002
CFTRdele1	5	Deletion	CF-causing	0	0.000	1	0.001
P5L	3	Missense	ND	2	0.002	1	0.001
L15P	5	Missense	ND	0	0.000	1	0.001
175insT	4	Insertion	ND	0	0.000	0	0.000
186-13C>G	6	Splicing	ND	0	0.000	0	0.000
CFTRdele2,3	5	Deletion	CF-causing	3	0.003	3	0.002
CFTRdele2ins182	6	Deletion/Insertion	ND	0	0.000	0	0.000
CFTRdele2	5	Deletion	CF-causing	3	0.003	6	0.005
Q39X	6	Nonsense	CF-causing	0	0.000	0	0.000
296+2T>G	5	Splicing	ND	1	0.001	1	0.001
E60X	6	Nonsense	CF-causing	0	0.000	0	0.000
L61P	6	Missense	ND	0	0.000	0	0.000
R75X	5	Nonsense	CF-causing	0	0.000	1	0.001
G85E	3	Missense	CF-causing	34	0.035	13	0.010
394delTT	5	Deletion	CF-causing	0	0.000	1	0.001
406-1G>C	5	Splicing	ND	0	0.000	1	0.001
CFTRdele4	3	Deletion	ND	0	0.000	1	0.001
CFTRdele4-10	5	Deletion	ND	0	0.000	1	0.001
E92K	6	Missense	CF-causing	0	0.000	0	0.000
K95E	5	Missense	ND	0	0.000	1	0.001
457TAT>G	5	Deletion/Insertion	CF-causing	0	0.000	2	0.002
D110H	5	Missense	CF-causing	5	0.005	0	0.000
D110E	6	Missense	ND	0	0.000	0	0.000
R117C	1	Missense	CF-causing	3	0.003	5	0.004
R117H	1	Missense	Varying	0	0.000	3	0.002
R117L	5	Missense	ND	6	0.006	0	0.000
G126D	3	Missense	ND	1	0.001	0	0.000
541delC	5	Deletion	CF-causing	0	0.000	3	0.002
541del4	6	Deletion	ND	0	0.000	0	0.000
546insCTA	5	Insertion	ND	0	0.000	1	0.001
H139R	5	Missense	ND	4	0.004	0	0.000
H147P	4	Missense	ND	0	0.000	0	0.000
574delA	3	Deletion	CF-causing	3	0.003	0	0.000
621+1G>T	5	Splicing	CF-causing	8	0.008	1	0.001
663insT	5	Insertion	ND	0	0.000	1	0.001
G178R	5	Missense	CF-causing	3	0.003	3	0.002
E193K	3	Missense	ND	0	0.000	0	0.000
711+1G>T	5	Splicing	CF-causing	5	0.005	3	0.002
711+3A>G	4	Splicing	CF-causing	1	0.001	5	0.004
711+5G>A	5	Splicing	CF-causing	0	0.000	7	0.005
H199R	5	Missense	ND	1	0.001	0	0.000
P205S	6	Missense	CF-causing	0	0.000	0	0.000
L206W	2	Missense	CF-causing	0	0.000	3	0.002
G213E	5	Missense	ND	0	0.000	3	0.002
Q220X	5	Nonsense	CF-causing	1	0.001	0	0.000
852del22bp	3	Deletion	CF-causing	1	0.001	6	0.005
875+1G>A	6	Splicing	ND	0	0.000	0	0.000
C276X	5	Nonsense	CF-causing	2	0.002	0	0.000
991del5	5	Nonsense	CF-causing	1	0.001	0	0.000
1002-1110_1113delTAAG	2	Deletion	ND	0	0.000	3	0.002
1002-1111A>C	4	Splicing	ND	0	0.000	0	0.000
A309D	5	Missense	ND	0	0.000	1	0.001
1078delT	5	Deletion	CF-causing	0	0.000	1	0.001

(*table continues*)

Table 1 (*continued*)

Legacy name	Validation step	Type of mutation	Classification in CFTR2	Prevalence CF (PI + PS) Lazio		Prevalence CF (PI + PS) Lombardia	
				n	Frequency	*n*	Frequency
R334W	3	Missense	CF-causing	8	0.008	8	0.006
R334L	5	Missense	ND	5	0.005	0	0.000
T338I	1	Missense	CF-causing	5	0.005	7	0.005
R347H	5	Missense	CF-causing	3	0.003	2	0.002
R347P	2	Missense	CF-causing	7	0.007	7	0.005
R352W	3	Missense	ND	0	0.000	0	0.000
R352Q	3	Missense	CF-causing	0	0.000	14	0.011
Q353X	6	Nonsense	ND	0	0.000	0	0.000
1248+1G>A	5	Splicing	CF-causing	0	0.000	2	0.002
1249-1G>A	5	Splicing	CF-causing	0	0.000	1	0.001
1259InsA	3	Insertion	CF-causing	1	0.001	1	0.001
1497delGG	6	Deletion	CF-causing	0	0.000	0	0.000
V456A	6	Missense	ND	0	0.000	0	0.000
G463D	4	Missense	ND	0	0.000	0	0.000
K464N	5	Missense	ND	0	0.000	2	0.002
S466X(TAA)	3	Nonsense	CF-causing	0	0.000	0	0.000
S466X(TGA)	5	Nonsense	CF-causing	3	0.003	0	0.000
1565delCA	6	Deletion	ND	0	0.000	0	0.000
Q493X	6	Nonsense	ND	0	0.000	0	0.000
E504X	5	Nonsense	ND	0	0.000	1	0.001
[delta]I507	5	Deletion	CF-causing	0	0.000	1	0.001
[delta]F508	1	Deletion	CF-causing	440	0.447	608	0.471
1677delTA	3	Deletion	CF-causing	0	0.000	3	0.002
Q525X	6	Nonsense	CF-causing	0	0.000	0	0.000
1706del17	6	Deletion	ND	0	0.000	0	0.000
CFTRdele11	4	Deletion	CF-causing	0	0.000	0	0.000
1716+18672A>G	5	Splicing	ND	0	0.000	4	0.003
1717-8G>A	5	Splicing	CF-causing	2	0.002	1	0.001
1717-1G>A	2	Splicing	CF-causing	15	0.015	41	0.032
G542X	1	Nonsense	CF-causing	43	0.044	64	0.050
S549R(A>C)	5	Missense	CF-causing	12	0.012	0	0.000
S549N	5	Missense	CF-causing	2	0.002	1	0.001
S549R(T>G)	4	Missense	CF-causing	7	0.007	0	0.000
1782delA	2	Deletion	CF-causing	0	0.000	4	0.003
1784delG	5	Deletion	ND	0	0.000	1	0.001
G551D	5	Missense	CF-causing	1	0.001	0	0.000
Q552X	1	Nonsense	CF-causing	1	0.001	1	0.001
R553X	5	Nonsense	CF-causing	13	0.013	12	0.009
V562I	3	Missense	ND	0	0.000	1	0.001
Y563X	5	Nonsense	ND	0	0.000	2	0.002
L571S	5	Missense	ND	1	0.001	1	0.001
1845delAG/1846delGA	5	Deletion	ND	0	0.000	1	0.001
D579G	3	Missense	Varying	1	0.001	5	0.004
1874insT	6	Insertion	ND	0	0.000	0	0.000
E585X	2	Nonsense	CF-causing	3	0.003	11	0.009
S589I	4	Missense	ND	0	0.000	0	0.000
1898+1G>A	1	Splicing	CF-causing	0	0.000	5	0.004
1898+3A>G	6	Splicing	CF-causing	0	0.000	0	0.000
1898+5G>T	5	Splicing	ND	0	0.000	1	0.001
S589R	4	Missense	ND	0	0.000	0	0.000
K598X	5	Nonsense	ND	0	0.000	1	0.001
1949del84	4	Deletion	ND	0	0.000	0	0.000
H609R	5	Missense	ND	0	0.000	2	0.002
A613T	5	Missense	ND	1	0.001	1	0.001
D614G	4	Missense	Varying	6	0.006	0	0.000

(*table continues*)

Table 1 (*continued*)

Legacy name	Validation step	Type of mutation	Classification in CFTR2	Prevalence CF (PI + PS) Lazio		Prevalence CF (PI + PS) Lombardia	
				n	Frequency	*n*	Frequency
H620P	5	Missense	ND	0	0.000	1	0.001
2118del4	3	Deletion	CF-causing	0	0.000	1	0.001
2143delT	5	Deletion	CF-causing	1	0.001	0	0.000
G673X	6	Nonsense	CF-causing	0	0.000	0	0.000
2183AA>G	3	Deletion/Insertion	CF-causing	12	0.012	20	0.015
2184delA	6	Deletion	CF-causing	0	0.000	0	0.000
2184insA	5	Insertion	CF-causing	6	0.006	2	0.002
2185insC	6	Insertion	CF-causing	0	0.000	0	0.000
R709X	5	Nonsense	CF-causing	3	0.003	0	0.000
Q720X	5	Nonsense	ND	0	0.000	1	0.001
2307insA	6	Insertion	CF-causing	0	0.000	0	0.000
L732X	5	Nonsense	CF-causing	2	0.002	1	0.001
R764X	5	Nonsense	CF-causing	1	0.001	0	0.000
R785X	3	Nonsense	CF-causing	0	0.000	1	0.001
2585delT	6	Deletion	CF-causing	0	0.000	0	0.000
E822X	4	Nonsense	CF-causing	0	0.000	0	0.000
E831X	5	Nonsense	CF-causing	1	0.001	2	0.002
Y849X	5	Nonsense	CF-causing	1	0.001	0	0.000
R851X	5	Nonsense	CF-causing	0	0.000	1	0.001
2711delT	5	Deletion	CF-causing	0	0.000	1	0.001
CFTRdele14b-17b	5	Deletion	CF-causing	1	0.001	5	0.004
W882X	5	Nonsense	ND	0	0.000	1	0.001
2789+5G>A	3	Splicing	CF-causing	40	0.041	39	0.030
2790-2A>G	5	Splicing	ND	1	0.001	0	0.000
2811G>T	6	Splicing	ND	0	0.000	0	0.000
S912X	3	Nonsense	CF-causing	1	0.001	0	0.000
2909delT	6	Deletion	ND	0	0.000	0	0.000
S945L	5	Missense	CF-causing	2	0.002	1	0.001
G970R	6	Missense	CF-causing	0	0.000	0	0.000
CFTRdele17a-18	3	Deletion	CF-causing	4	0.004	2	0.002
3121-2A>T	6	Splicing	CF-causing	0	0.000	0	0.000
3121-1G>A	6	Splicing	CF-causing	0	0.000	0	0.000
3132delTG	6	Deletion	CF-causing	0	0.000	0	0.000
A1006E	5	Missense	ND	0	0.000	1	0.001
[V562I;A1006E]	5	Complex Allele	ND	9	0.009	1	0.001
Q1012X	4	Nonsense	ND	0	0.000	0	0.000
3199del6	6	Deletion	ND	0	0.000	0	0.000
Y1032C	3	Missense	ND	0	0.000	0	0.000
CFTRdele17b	5	Deletion	ND	0	0.000	1	0.001
3272-26A>G	3	Splicing	CF-causing	1	0.001	2	0.002
H1054D	5	Missense	CF-causing	0	0.000	1	0.001
L1065P	5	Missense	CF-causing	6	0.006	0	0.000
R1066C	3	Missense	CF-causing	5	0.005	9	0.007
R1066H	1	Missense	CF-causing	1	0.001	23	0.018
F1074L	3	Missense	Varying	0	0.000	1	0.001
L1077P	2	Missense	CF-causing	12	0.012	15	0.012
M1101K	5	Missense	CF-causing	0	0.000	2	0.002
E1104X	3	Nonsense	CF-causing	0	0.000	1	0.001
W1145X	5	Nonsense	ND	0	0.000	1	0.001
D1152H	1	Missense	Varying	7	0.007	23	0.018
R1158X	3	Nonsense	CF-causing	0	0.000	10	0.008
[R334W;R1158X]	3	Complex Allele	ND	0	0.000	0	0.000
R1162X	1	Nonsense	CF-causing	3	0.003	4	0.003
3659delC	4	Deletion	CF-causing	0	0.000	2	0.002
S1206X	5	Nonsense	ND	1	0.001	1	0.001

(*table continues*)

Table 1 (*continued*)

Legacy name	Validation step	Type of mutation	Classification in CFTR2	Prevalence CF (PI + PS) Lazio		Prevalence CF (PI + PS) Lombardia	
				n	Frequency	*n*	Frequency
M1210K	5	Missense	ND	0	0.000	2	0.002
I1234V	5	Missense	CF-causing	3	0.003	0	0.000
3849+10kbC>T	1	Splicing	CF-causing	9	0.009	21	0.016
G1244E	2	Missense	CF-causing	4	0.004	8	0.006
3876delA	6	Deletion	CF-causing	0	0.000	0	0.000
3878delG	5	Deletion	CF-causing	0	0.000	1	0.001
S1251N	5	Missense	CF-causing	0	0.000	1	0.001
3905insT	3	Insertion	CF-causing	0	0.000	0	0.000
D1270N	3	Missense	Varying	1	0.001	0	0.000
W1282X	1	Nonsense	CF-causing	47	0.048	19	0.015
Q1291R	3	Missense	ND	3	0.003	0	0.000
CFTRdele21	5	Deletion	ND	0	0.000	1	0.001
4015delA	5	Deletion	CF-causing	0	0.000	3	0.002
4016insT	1	Insertion	CF-causing	1	0.001	15	0.012
4040delA	5	Deletion	CF-causing	2	0.002	0	0.000
N1303K	1	Missense	CF-causing	61	0.062	64	0.050
Q1313X	6	Nonsense	CF-causing	0	0.000	0	0.000
CFTRdele22-24	5	Deletion/Insertion	CF-causing	7	0.007	3	0.002
CFTRdele22-23	4	Deletion	CF-causing	1	0.001	4	0.003
G1349D	3	Missense	CF-causing	2	0.002	1	0.001
4209TGTT>AA	6	Deletion/Insertion	CF-causing	0	0.000	0	0.000
H1375P	5	Missense	ND	3	0.003	2	0.002
4382delA	2	Deletion	CF-causing	0	0.000	6	0.005
S1455X	3	Nonsense	ND	1	0.001	1	0.001
E1473X	5	Nonsense	ND	0	0.000	2	0.002
Detected alleles				935		1235	
Unknown alleles				49		57	
Overall alleles				984		1292	
Detection rate					0.950 (95.0%)		0.956 (95.6%)

The mutations are listed only in old nomenclature (legacy name; refer to Supplemental Table S1 for Human Genome Variation Society nomenclature). The Validation step column refers to the following validation procedures (*Materials and Methods*): 1 = validated in the first step by the selected 48 dried blood spots (DBSs); 2 = validated in the second step by the selected 24 genomic DNA; 3 = validated in the third step by the subsequent 1378 DBSs; 4 = validated in the fourth step by the subsequent 378 genomic DNA; 5 = validated in the fifth step by the 1138 cystic fibrosis (CF) patient case series; 6 = validated only at theoretic level. The type of mutations and the functional classification according to CFTR2 database is reported. The prevalence of each mutation in both the Lazio and Lombardia case series is reported as number (*n*) and frequency of alleles; the corresponding DRs are also shown.

CF-causing, mutation that causes classic form of CF; ND, mutation not reported in the CFTR2 database; PI, pancreatic insufficiency; PS, pancreatic sufficiency; Varying, mutation with variable clinical effect.

of CF, does not exist.[17] A multistep genetic approach is usually applied for diagnostic purposes.[5,17] The first step is to search for a panel of frequent *CFTR* mutations. The DRs of CF mutation panels are different in diverse populations[33] and depend on the clinical form.[17] Consequently, this step leaves a variable proportion of alleles with no mutation detected. To characterize these unknown alleles, a second step of sequencing (usually sequencing of all of the exons, adjacent intronic zones, and proximal 5′-flanking region of *CFTR*) is applied. Sequencing of *CFTR*, when applied for diagnostic purposes based on either classic Sanger sequencing or NGS, is reported to detect approximately 97% of disease-causing mutations, at least in CF.[5,17,44] In CFTR—related disorders and congenital bilateral absence of vas deferens, even sequencing may have a low DR.[5,17,44] Those mutations possibly not recognized, for example macrodeletions and macroduplications, are usually investigated in a third step of mutation search to reveal CNVs. It is expected that this step will add up to 2% to the DR. Although a sequencing protocol, possibly followed by a CNV search, may be suitable for diagnostic purposes, it does not appear to be recommendable for carrier or neonatal screening. This is especially true if a powerful approach, such as NGS, is used.[45] The DR of an extended mutation panel by NGS, including frequent and characterized CNVs, usually is sufficiently high for carrier and neonatal screening purposes. On the other hand, sequencing (and in some cases also CNV analysis) may identify not only disease-causing mutations possibly undetected by commonly used panels, but also novel or rare *CFTR* variants the clinical significance of which has not been assessed, or variants associated with a broad phenotypic spectrum. In addition, novel CNVs

usually need further structural characterization. When mutation-identification assays, such as sequencing, are used, novel variants are identified in an estimated ∼1 in 500 samples that are tested for carrier status.[46] In addition, to limit costs and time, even for diagnostic purposes in CF, it may be suitable to start the mutation search with a mutation panel. For these reasons, protocols based on mutation panels still have a role in mutational searches performed for both CF diagnostics and screening purposes, even in the NGS era.

The DR reached by our 188-CF-NGS assay is among the highest achieved by a genetic test for CF based on a mutation panel (level I assay) in a population with high genetic heterogeneity of *CFTR*. For example, the Food and Drug Administration−approved MiSeqDX Cystic Fibrosis 139-variant assay (Illumina), an NGS mutation panel that was not specifically optimized for central or northern Italy, shows DRs considerably lower than that of our assay in both regions of Italy (central, 86.8%; northern, 86.9%), as deduced from the analysis of data from our case series. This result highlights that, despite the power of extended mutation panels based on NGS technology, customization is still required to achieve maximal DRs in specific geographic areas and, conceivably, in ethnicities and clinical forms. Moreover, there may be geographic areas with even higher genetic heterogeneity than that of Italy where a similarly high DR may not be reached, even with a widely targeted NGS mutation panel. Although the definition of a mutation search as a first-level test is irrespective of the number of mutations included in the panel, it is evident that NGS greatly enhances this approach by using wider mutation panels. Owing to this enhancement, the gap between the DRs shown by the same mutation panel in different geographic areas, ethnicities, and CF clinical forms will decrease. However, due to rare and often individual mutations[17] and high geographic heterogeneity, the use of only one large mutation panel that is suitable for all purposes remains unlikely.

A common problem of mutation panels is that of complex alleles, which contain more than one mutation in *cis*. It is becoming clear that complex alleles occur more frequently than is usually recognized. We chose to include at least one mutation of the most frequent complex alleles in the 188-CF-NGS assay and to supply a list of mutations that would have been searched for in cases in which a potentially associated mutation was found. It is obvious that the complete panel of mutations within complex alleles can be easily added to the Data Driven Medicine platform (Sophia Genetics). Presently, the Data Driven Medicine platform lets users unmask the analysis of the $(TG)_mT_n$ variant tract when one of the following mutations is detected: R117C, R117H, K464N, V562I, A1006E, or 2184insA (Supplemental Table S2).

The validation of the 188-CF-NGS assay on DBSs showed excellent operative characteristics that are fully comparable to those of genomic DNA samples. This result makes the assay also suitable for the genetic step of neonatal screening programs, such as the immunoreactive trypsinogen/DNA/immunoreactive trypsinogen protocol.[47,48]

Another characteristic that makes the 188-CF-NGS assay attractive is the possibility that when no mutations or only one mutation is identified in individuals potentially affected by CF, a different profiling analysis can be applied with the Data Driven Medicine platform, unmasking the complete sequence of *CFTR*. Furthermore, the *CFTR* sequence acquired by this NGS approach can reveal CNVs by analyzing the number of reads, including the identification of *CFTR* macrodeletions and macroduplications. The possibility of applying all three steps of the mutation search (also combining the second and third steps) according to the requirements of the applied protocol and by only switching software settings represent considerable enhancements with respect to previous multistep approaches. Improved *CFTR* genotyping is expected from the 188-CF-NGS assay due to the speediness, simplicity, and high-throughput nature of the assay, as well as its predictable, enhanced identification of complex alleles.

As NGS methods are still in the experimental phase, confirmation by an independent method seems to be appropriate. Depending on the type of mutation evidenced, an appropriate confirmatory method (*Materials and Methods*) was applied. There was perfect concordance between the data obtained with the 188-CF-NGS assay and the confirmatory methods. Although it may still be untimely to abandon the confirmatory procedure, these results further highlight the reliability of NGS-based approaches as stand-alone methods.

A turnaround time of 3 working days for up to 52 samples seems suitable for overcoming the wait-time issues related to the collection of an appropriate number of samples. From a data analysis point of view, the type of analysis can be selected according to clinical indications. The algorithm of the Data Driven Medicine platform was specifically adapted to our needs to accurately perform DNA sequence assembly, alignment to the reference genome, variant calling, and variant annotation in a short time for level I analysis (panel), and one can easily proceed, if indicated, to analysis levels II (sequence) and III (macrodeletions/macroduplications). Also, the $(TG)_mT_n$ tracts can be easily analyzed if needed.

In conclusion, the 188-CF-NGS assay is a robust method that is easy to handle and that allows the detection of approximately 95% of the mutations in the Italian population, thus achieving a marked increase in DR compared with those of other NGS-based mutation panel methods. This result highlights the concept that despite the high number of *CFTR* mutations that can be searched by NGS methods, the customization of mutation panels for a geographic area and, conceivably, for ethnicities and clinical forms, is still advisable. The availability of an extended mutation panel with a high DR is useful for limiting the use of sequencing and for avoiding the detection of DNA variants with unclear functional meaning. It enhances the ability to obtain an early, complete definition of mutated genotypes in CF patients, providing a meaningful genotypic-oriented view of *CFTR* genetics.[5] Our assay is well suited for CF diagnostic

purposes, neonatal screening, and carrier screening, and has also been finalized for assisted reproductive technology. It might also show good performance for CFTR-related disorders and congenital bilateral absence of vas deferens; however, experimental verification seems appropriate. Experimental approaches based on extended panels of mutations for NGS, such as the one described here, greatly facilitate the task of finding mutations, providing a better definition of the genotype−phenotype relationship. Overall, these findings clearly have positive implications for the genetic counseling of CF. Finally, the synergy between NGS approaches and the actual possibility of personalized CF therapy is anticipated to produce unparalleled advantages for CF patients. NGS approaches help to reduce possibly incomplete genotyping, which may hamper the therapeutic response to a mutation-specific therapy. This enhanced knowledge of *CFTR* genetics may finally allow the physician to apply both predictive and preventive medicine in CF.[49]

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.jmoldx.2017.06.002*.

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Supplemental Table S1. List of mutations included in the 188-CF-NGS assay.

Old nomenclature (legacy name)		New nomenclature (HGVS name)		
Position	***Notation***	***Position***	***Nucleotidic notation***	***Aminoacidic notation***
Promoter - Intron 3	CFTR-dup 1-3	Promoter - Intron 3	c.1-6186_273+507dup35741	-
Exon 1	M1V	Exon 1	c.1A>G	p.Met1Val
Exon 1 - Intron 1	CFTRdele1	Exon 1 - Intron 1	c.4_53+69delins299	-
Exon 1	P5L	Exon 1	c.14C>T	p.Pro5Leu
Exon 1	L15P	Exon 1	c.44T>C	p.Leu15Pro
Exon 1	175insT	Exon 1	c.43_44insT	p.Ser18Glnfs*27
Intron 1	186-13C>G	Intron 1	c.54-13C>G	-
Intron 1 - Intron 3	CFTRdele2,3	Intron 1 - Intron 3	c.54-5940_273+10250del21080	p.Ser18Argfs*16
Intron 1 - Intron 2	CFTRdele2ins182	Intron 1 - Intron 2	c.54-5811_164+2186del8108ins182	-
Intron 1 - Intron 2	CFTRdele2	Intron 1 - Intron 2	c.54-1161_164+1603del2875	-
Exon 2	Q39X	Exon 2	c.115C>T	p.Gln39*
Intron 2	296+2T>G	Intron 2	c.164+2T>G	-
Exon 3	E60X	Exon 3	c.178G>T	p.Glu60*
Exon 3	L61P	Exon 3	c.182T>C	p.Leu61Pro
Exon 3	R75X	Exon 3	c.223C>T	p.Arg75*
Exon 3	G85E	Exon 3	c.254G>A	p.Gly85Glu
Exon 3	394delTT	Exon 3	c.262_263delTT	p.Leu88Ilefs*22
Intron 3	406-1G>C	Intron 3	c.274-1G>C	-
? - Exon 4 - ?	CFTRdele4	? - Exon 4 - ?	c.(273+1_274-1)_(489+1_490-1)del	-
? - Exon 4 - Exon 10 - ?	CFTRdele4-10	? - Exon 4- Exon 11 - ?	c.(273+1_274-1)_(1584+1_1585-1)del	-
Exon 4	E92K	Exon 4	c.274G>A	p.Glu92Lys
Exon 4	K95E	Exon 4	c.283A>G	p.Lys95Glu
Exon 4	457TAT>G	Exon 4	c.325_327delTATinsG	p.Tyr109Glyfs*4
Exon 4	D110H	Exon 4	c.328G>C	p.Asp110His
Exon 4	D110E	Exon 4	c.330C>A	p.Asp110Glu
Exon 4	R117C	Exon 4	c.349C>T	p.Arg117Cys
Exon 4	R117H	Exon 4	c.350G>A	p.Arg117His
Exon 4	R117L	Exon 4	c.350G>T	p.Arg117Leu
Exon 4	G126D	Exon 4	c.377G>A	p.Gly126Asp
Exon 4	541delC	Exon 4	c.409delC	p.Leu137Serfs*16
Exon 4	541del4	Exon 4	c.409_412delCTCC	p.Leu137Tyrfs*15
Exon 4	546insCTA	Exon 4	c.414_415insCTA	p.Leu139*
Exon 4	H139R	Exon 4	c.416A>G	p.His139Arg
Exon 4	H147P	Exon 4	c.440A>C	p.His147Pro
Exon 4	574delA	Exon 4	c.442delA	p.Ile148Leufs*5
Intron 4	621+1G>T	Intron 4	c.489+1G>T	-
Exon 5	663insT	Exon 5	c.531_532insT	p.Gly178Trpfs*5
Exon 5	G178R	Exon 5	c.532G>A	p.Gly178Arg
Exon 5	E193K	Exon 5	c.577G>A	p.Glu193Lys
Intron 5	711+1G>T	Intron 5	c.579+1G>T	-
Intron 5	711+3A>G	Intron 5	c.579+3A>G	-
Intron 5	711+5G>A	Intron 5	c.579+5G>A	-

Table S1 – follows

Old nomenclature (legacy name)		New nomenclature (HGVS name)		
Position	***Notation***	***Position***	***Nucleotidic notation***	***Aminoacidic notation***
Exon 6a	H199R	Exon 6	c.596A>G	p.His199Arg
Exon 6a	P205S	Exon 6	c.613C>T	p.Pro205Ser
Exon 6a	L206W	Exon 6	c.617T>G	p.Leu206Trp
Exon 6a	G213E	Exon 6	c.638G>A	p.Gly213Glu
Exon 6a	Q220X	Exon 6	c.658C>T	p.Gln220*
Exon 6a	852del22bp	Exon 6	c.720_741delAGGGAGAATGATGATGAAGTAC	p.Gly241Glufs*13
Intron 6a	875+1G>A	Intron 6	c.743+1G>A	-
Exon 6b	C276X	Exon 7	c.828C>A	p.Cys276*
Exon 6b	991del5	Exon 7	c.859_863delAACTT	p.Asn287Lysfs*19
Intron 6b	1002-1110_1113delTAAG	Intron 7	c.870-1113_870-1110delGAAT	-
Intron 6b	1002-1111A>C	Intron 7	c.870-1111A>C	-
Exon 7	A309D	Exon 8	c.926C>A	p.Ala309Asp
Exon 7	1078delT	Exon 8	c.948delT	p.Phe316Leufs*12
Exon 7	R334W	Exon 8	c.1000C>T	p.Arg334Trp
Exon 7	R334L	Exon 8	c.1001G>T	p.Arg334Leu
Exon 7	T338I	Exon 8	c.1013C>T	p.Thr338Ile
Exon 7	R347H	Exon 8	c.1040G>A	p.Arg347His
Exon 7	R347P	Exon 8	c.1040G>C	p.Arg347Pro
Exon 7	R352W	Exon 8	c.1054C>T	p.Arg352Trp
Exon 7	R352Q	Exon 8	c.1055G>A	p.Arg352Gln
Exon 7	Q353X	Exon 8	c.1057C>T	p.Gln353*
Intron 7	1248+1G>A	Intron 8	c.1116+1G>A	-
Intron 7	1249-1G>A	Intron 8	c.1117-1G>A	-
Exon 8	1259InsA	Exon 9	c.1127_1128insA	p.Gln378Alafs*4
Exon 9	1497delGG	Exon 10	c.1365_1366delGG	p.Val456Cysfs*25
Exon 9	V456A	Exon 10	c.1367T>C	p.Val456Ala
Exon 9	G463D	Exon 10	c.1388G>A	p.Gly463Asp
Exon 9	K464N	Exon 10	c.1392G>T	p.Lys464Asn
Exon 10	S466X(TAA)	Exon 11	c.1397C>A	p.Ser466*
Exon 10	S466X(TGA)	Exon 11	c.1397C>G	p.Ser466*
Exon 10	1565delCA	Exon 11	c.1433_1434delCA	p.Ser478*
Exon 10	Q493X	Exon 11	c.1477C>T	p.Gln493*
Exon 10	E504X	Exon 11	c.1510G>T	p.Glu504*
Exon 10	[delta]I507	Exon 11	c.1519_1521delATC	p.Ile507del
Exon 10	[delta]F508	Exon 11	c.1521_1523delCTT	p.Phe508del
Exon 10	1677delTA	Exon 11	c.1545_1546delTA	p.Tyr515*
Exon 10	Q525X	Exon 11	c.1573C>T	p.Gln525*
Exon 10	1706del17	Exon 11	c.1574_1590delAACTAGAAGAGGACATC	p.Gln525Leufs*37
? - Exon 11 - ?	CFTRdele11	? - Exon 12 - ?	c.(1584+1_1585-1)_(1679+1_1680-1)del	-
Intron 10	1716+18672A>G	Intron 11	c.1584+18672A>G	-
Intron 10	1717-8G>A	Intron 11	c.1585-8G>A	-
Intron 10	1717-1G>A	Intron 11	c.1585-1G>A	-
Exon 11	G542X	Exon 12	c.1624G>T	p.Gly542*
Exon 11	S549R(A>C)	Exon 12	c.1645A>C	p.Ser549Arg
Exon 11	S549N	Exon 12	c.1646G>A	p.Ser549Asn
Exon 11	S549R(T>G)	Exon 12	c.1647T>G	p.Ser549Arg
Exon 11	1782delA	Exon 12	c.1650delA	p.Gly551Valfs*8
Exon 11	1784delG	Exon 12	c.1652delG	p.Gly551Valfs*8
Exon 11	G551D	Exon 12	c.1652G>A	p.Gly551Asp
Exon 11	Q552X	Exon 12	c.1654C>T	p.Gln552*
Exon 11	R553X	Exon 12	c.1657C>T	p.Arg553*

Table S1 – follows

Old nomenclature (legacy name)		New nomenclature (HGVS name)		
Position	***Notation***	***Position***	***Nucleotidic notation***	***Aminoacidic notation***
Exon 12	V562I	Exon 13	c.1684G>A	p.Val562Ile
Exon 12	Y563X	Exon 13	c.1689C>G	p.Tyr563*
Exon 12	L571S	Exon 13	c.1712T>C	p.Leu571Ser
Exon 12	1845delAG/1846delGA	Exon 13	c.1713_1714delAG	p.Asp572Leufs*16
Exon 12	D579G	Exon 13	c.1736A>G	p.Asp579Gly
Exon 12	1874insT	Exon 13	c.1739_1740insT	p.Leu581Phefs*8
Exon 12	E585X	Exon 13	c.1753G>T	p.Glu585*
Exon 12	S589I	Exon 13	c.1766G>T	p.Ser589Ile
Intron 12	1898+1G>A	Intron 13	c.1766+1G>A	-
Intron 12	1898+3A>G	Intron 13	c.1766+3A>G	-
Intron 12	1898+5G>T	Intron 13	c.1766+5G>T	-
Exon 13	S589R	Exon 14	c.1767C>A	p.Ser589Arg
Exon 13	K598X	Exon 14	c.1792A>T	p.Lys598*
Exon 13	1949del84	Exon 14	c.1817_1900delAAATGGAACATTTAAAGAAAGCTGACAAAATATTAATTTTGCATGAAGGTAGCAGCTATTTTTATGGGACATTTTCAGAACTCC	p.Met607_Gln634del
Exon 13	H609R	Exon 14	c.1826A>G	p.His609Arg
Exon 13	A613T	Exon 14	c.1837G>A	p.Ala613Thr
Exon 13	D614G	Exon 14	c.1841A>G	p.Asp614Gly
Exon 13	H620P	Exon 14	c.1859A>C	p.His620Pro
Exon 13	2118del4	Exon 14	c.1986_1989delAACT	p.Thr663Argfs*8
Exon 13	2143delT	Exon 14	c.2012delT	p.Leu671*
Exon 13	G673X	Exon 14	c.2017G>T	p.Gly673*
Exon 13	2183AA>G	Exon 14	c.2051_2052delAAinsG	p.Lys684Serfs*38
Exon 13	2184delA	Exon 14	c.2052delA	p.Lys684Asnfs*38
Exon 13	2184insA	Exon 14	c.2052_2053insA	p.Gln685Thrfs*4
Exon 13	2185insC	Exon 14	c.2053_2054insC	p.Gln685Profs*4
Exon 13	R709X	Exon 14	c.2125C>T	p.Arg709*
Exon 13	Q720X	Exon 14	c.2158C>T	p.Gln720*
Exon 13	2307insA	Exon 14	c.2175_2176insA	p.Glu726Argfs*4
Exon 13	L732X	Exon 14	c.2195T>G	p.Leu732*
Exon 13	R764X	Exon 14	c.2290C>T	p.Arg764*
Exon 13	R785X	Exon 14	c.2353C>T	p.Arg785*
Exon 13	2585delT	Exon 14	c.2453delT	p.Leu818Trpfs*3
Exon 13	E822X	Exon 14	c.2464G>T	p.Glu822*
Exon 14a	E831X	Exon 15	c.2491G>T	p.Glu831*
Exon 14a	Y849X	Exon 15	c.2547C>A	p.Tyr849*
Exon 14a	R851X	Exon 15	c.2551C>T	p.Arg851*
Exon 14a	2711delT	Exon 15	c.2583delT	p.Phe861Leufs*3
Intron 14a - Intron 17b	CFTRdele14b-17b	Intron 15 - Intron 20	c.2620–674_3367+198del9858	-
Exon 14b	W882X	Exon 16	c.2645G>A	p.Trp882*
Intron 14b	2789+5G>A	Intron 16	c.2657+5G>A	-
Intron 14b	2790-2A>G	Intron 16	c.2658-2A>G	-
Exon 15	2811G>T	Exon 17	c.2679G>T	p.Gly893=
Exon 15	S912X	Exon 17	c.2735C>A	p.Ser912*
Exon 15	2909delT	Exon 17	c.2777delT	p.Leu926Cysfs*16
Exon 15	S945L	Exon 17	c.2834C>T	p.Ser945Leu
Exon 15	G970R	Exon 17	c.2908G>C	p.Gly970Arg
Intron 16 - Intron 18	CFTRdele17a-18	Intron 18 - Intron 21	c.2988+1173_3468+2111del8600	-
Intron 16	3121-2A>T	Intron 18	c.2989-2A>T	-
Intron 16	3121-1G>A	Intron 18	c.2989-1G>A	-

Table S1 – follows

Old nomenclature (legacy name)		New nomenclature (HGVS name)		
Position	***Notation***	***Position***	***Nucleotidic notation***	***Aminoacidic notation***
Exon 17a	3132delTG	Exon 19	c.3002_3003delTG	p.Val1001Aspfs*45
Exon 17a	A1006E	Exon 19	c.3017C>A	p.Ala1006Glu
Exon 12; Exon 17a	[V562I;A1006E]	Exon13; Exon 19	c.[1684G>A;3017C>A]	p.[Val562Ile;Ala1006Glu]
Exon 17a	Q1012X	Exon 19	c.3034C>T	p.Gln1012*
Exon 17a	3199del6	Exon 19	c.3067_3072delATAGTG	p.Ile1023_Val1024del
Exon 17a	Y1032C	Exon 19	c.3095A>G	p.Tyr1032Cys
? - Exon 17b - ?	CFTRdele17b	? - Exon 20 - ?	c.(3139+1_3140-1)_(3367+1_3368-1)del	-
Intron 17a	3272-26A>G	Intron 19	c.3140-26A>G	-
Exon 17b	H1054D	Exon 20	c.3160C>G	p.His1054Asp
Exon 17b	L1065P	Exon 20	c.3194T>C	p.Leu1065Pro
Exon 17b	R1066C	Exon 20	c.3196C>T	p.Arg1066Cys
Exon 17b	R1066H	Exon 20	c.3197G>A	p.Arg1066His
Exon 17b	F1074L	Exon 20	c.3222T>A	p.Phe1074Leu
Exon 17b	L1077P	Exon 20	c.3230T>C	p.Leu1077Pro
Exon 17b	M1101K	Exon 20	c.3302T>A	p.Met1101Lys
Exon 17b	E1104X	Exon 20	c.3310G>T	p.Glu1104*
Exon 18	W1145X	Exon 21	c.3435G>A	p.Trp1145*
Exon 18	D1152H	Exon 21	c.3454G>C	p.Asp1152His
Exon 19	R1158X	Exon 22	c.3472C>T	p.Arg1158*
Exon 7, Exon 19	[R334W;R1158X]	Exon 8, Exon 22	c.[1000C>T;3472C>T]	p.[Arg334Trp;Arg1158*]
Exon 19	R1162X	Exon 22	c.3484C>T	p.Arg1162*
Exon 19	3659delC	Exon 22	c.3528delC	p.Lys1177Serfs*15
Exon 19	S1206X	Exon 22	c.3617C>G	p.Ser1206*
Exon 19	M1210K	Exon 22	c.3629T>A	p.Met1210Lys
Exon 19	I1234V	Exon 22	c.3700A>G	p.Ile1234Val
Intron 19	3849+10kbC>T	Intron 22	c.3717+12191C>T	-
Exon 20	G1244E	Exon 23	c.3731G>A	p.Gly1244Glu
Exon 20	3876delA	Exon 23	c.3744delA	p.Lys1250Argfs*9
Exon 20	3878delG	Exon 23	c.3747delG	p.Lys1250Argfs*9
Exon 20	S1251N	Exon 23	c.3752G>A	p.Ser1251Asn
Exon 20	3905insT	Exon 23	c.3773_3774insT	p.Leu1258Phefs*7
Exon 20	D1270N	Exon 23	c.3808G>A	p.Asp1270Asn
Exon 20	W1282X	Exon 23	c.3846G>A	p.Trp1282*
Exon 20	Q1291R	Exon 23	c.3872A>G	p.Gln1291Arg
? - Exon 21 - ?	CFTRdele21	? - Exon 23 - ?	c.(3873+1_3874-1)_(3963+1_3964-1)del	-
Exon 21	4015delA	Exon 24	c.3883delA	p.Ile1295Phefs*33
Exon 21	4016insT	Exon 24	c.3884_3885insT	p.Ser1297Phefs*5
Exon 21	4040delA	Exon 24	c.3908delA	p.Asn1303Thrfs*25
Exon 21	N1303K	Exon 24	c.3909C>G	p.Asn1303Lys
Exon 21	Q1313X	Exon 24	c.3937C>T	p.Gln1313*
Intron 21 - 3'-UTR	CFTRdele22-24	Intron 24 - 3'-UTR	c.3964–3890_4443+3143del9454ins5	-
Intron 21 - Intron 23	CFTRdele22-23	Intron 24 - Intron 26	c.3964–78_4242+577del1532	-
Exon 22	G1349D	Exon 25	c.4046G>A	p.Gly1349Asp
Exon 22	4209TGTT>AA	Exon 25	c.4077_4080delTGTTinsAA	p.Val1360Thrfs*3
Exon 22	H1375P	Exon 25	c.4124A>C	p.His1375Pro
Exon 24	4382delA	Exon 27	c.4251delA	p.Glu1418Argfs*14
Exon 24	S1455X	Exon 27	c.4364C>G	p.Ser1455*
Exon 24	E1473X	Exon 27	c.4417G>T	p.Glu1473*

The mutations are listed in old nomenclature (legacy name) and HGVS nomenclature at nucleotidic and, whenever possible, aminoacidic level. The position within the *CFTR* gene is also indicated. If the breakpoints of a CFTR macrodeletion are not known, preceding and following introns are noted with a question mark.

Supplemental Table S2. List of mutations included in the 188-CF-NGS assay and previously found also within complex alleles.

Mutation (legacy name)	Old nomenclature (legacy name)		New nomenclature (HGVS name)		Prevalence of complex allele in CF(PI+PS)	
	Complex allele	Respective position	Complex allele	Respective position	Lazio	Lombardia
296+2T>G	[L24F;296+2T>G]	exon 2, intron 2	c.[72G>C;164+2T>G]	exon 2, intron 2	0.001[*]	0
R75X	[125G>C;R75X]	promoter, exon 3	c.[-8G>C;223C>T]	promoter, exon 3	0	0
R117C	[R117C;(TG)mT5]	exon 4, intron 8	c.[349C>T;1210-34TG[m];1210-12T[5]]	exon 4, intron 9	0	0
R117H	[129G>C;R117H]	promoter, exon 4	c.[-4G>C;350G>A]	promoter, exon 4	0	0
	[R117H;(TG)mT5]	exon 4, intron 8	c.[350G>A;1210-34TG[m];1210-12T[5]]	exon 4, intron 9	0	0
R117L	[R117L;L997F]	exon 4, exon 17a	c.[350G>T;2991G>C] p.[Arg117Leu;Leu997Phe]	exon 4, exon 19	0.006 †	0
R334W R1158X	[R334W;R1158X]	exon 7, exon 19	c.[1000C>T;3472C>T] p.[Arg334Trp;Arg1158*]	exon 8, exon 22	0	0
R347H	[R347H;D979A]	exon 7, exon 16	c.[1040G>A;2936A>C] p.[Arg347His;Asp979Ala]	exon 8, exon 18	0	0
R352W	[R352W;P750L]	exon 7, exon 13	c.[1054C>T;2249C>T] p.[Arg352Trp;Pro750Leu]	exon 8, exon 14	0	0
K464N	[(TG)11T5;K464N]	intron 8, exon 9	c.[1210-34TG[11];1210-12T[5];1392G>T]	intron 9, exon 10	0	0
S466X(TGA)	[S466X(TGA);R1070Q]	exon 10, exon 17b	c.[1397C>G;3209G>A] p.[Ser466*;Arg1070Gln]	exon 11, exon 20	0.003 ‡	0
E504X	[E504X;1898+73T>G]	exon 10, intron 12	c.[1510G>T;1766+73T>G]	exon 11, intron 13	0	0.001[*]
[delta]F508	[delta]F508;R553Q]	exon 10, exon 11	c.[1521_1523delCTT;1658G>A] p.[Phe508del;Arg553Gln]	exon 11, exon 12	0	0
	[delta]F508;I1027T]	exon 10, exon 17a	c.[1521_1523delCTT;3080T>C] p.[Phe508del;Ile1027Thr]	exon 11, exon 19	0	0
	[A238V;[delta]F508]	exon 6a, exon 10	c.[713C>T;1521_1523delCTT] p.[Ala238Val;Phe508del]	exon 6, exon 11	0	0
S549N	[R75Q;S549N]	exon 3, exon 11	c.[224G>A;1646G>A] p.[Arg75Gln;Ser549Asn]	exon 3, exon 12	0	0
S549R(T>G)	[-102T>A;S549R(T>G)]	promoter, exon 11	c.[-234T>A;1647T>G]	promoter, exon 12	0	0
V562I A1006E	[(TG)11T5;V562I;A1006E]	intron 8, exon 12, exon 17a	c.[1210-34TG[11];1210-12T[5];1684G>A;3017C>A]	intron 9, exon 13, exon 19	0.009 §	0.001¶
H609R	[I148T;H609R]	exon 4, exon 13	c.[443T>C;1826A>G] p.[Ile148Thr;p.His609Arg]	exon 4, exon 14	0	0.001 ‖
2184insA	[(TG)11T5;2184insA]	intron 8, exon 13	c.[1210-34TG[11];1210-12T[5];2052_2053insA]	intron 9, exon 14	0	0
S912X	[M348K;S912X]	exon 7, exon 15	c.[1043T>A;2735C>A] p.[Met348Lys;Ser912*]	exon 8, exon 17	0.001[*]	0
3199del6	[I148T;3199del6]	exon 4, exon 17a	c.[443T>C;3067_3072delATAGTG] p.[Ile148Thr;Ile1023_Val1024del]	exon 4, exon 19	0	0
3849+10kbC>T	[R668C;3849+10kbC>T]	exon 13, intron 19	c.[2002C>T;3717+12191C>T]	exon 14, intron 22	0	0
S1251N	[F508C;S1251N]	exon 10, exon 20	c.[1523T>G;3752G>A] p.[Phe508Cys;Ser1251Asn]	exon 11, exon 23	0	0
D1270N	[R74W;V201M;D1270N]	exon 3, exon 6a, exon 20	c.[220C>T;601G>A;3808G>A] p.[Arg74Trp;Val201Met;Asp1270Asn]	exon 3, exon 6, exon 23	0.001[*]	0
	[R74W; R1070W;D1270N]	exon 3, exon 17b, exon 20	c.[220C>T;3208C>T;3808G>A] p.[Arg74Trp;Arg1070Trp;Asp1270Asn]	exon 3, exon 20, exon 23	0	0

*Only the complex allele was found (once). †Only the complex allele was found (6 times). ‡Only the complex allele was found (3 times).
§Only the complex allele was found (9 times). ¶The complex allele was found once, but the mutations were also found in simple allele(s).
‖The complex allele was found once, but the mutation was also found in simple allele(s).
The complex alleles are listed in old nomenclature (legacy name) and HGVS nomenclature at nucleotidic and, whenever possible, amino acid level. Their position within the *CFTR* gene and their frequency in the case series studied are also reported. PI, pancreatic insufficiency; PS, pancreatic sufficiency.