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Original Article

The multi-faceted nature of 15 *CFTR* exonic variations: Impact on their functional classification and perspectives for therapy

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ABSTRACT

Background: The majority of variants of unknown clinical significance (VUCS) in the CFTR gene are missense variants. While change on the CFTR protein structure or function is often suspected, impact on splicing may be neglected. Such undetected splicing default of variants may complicate the interpretation of genetic analyses and the use of an appropriate pharmacotherapy.

Methods: We selected 15 variants suspected to impact *CFTR* splicing after *in silico* predictions on 319 missense variants (214 VUCS), reported in the *CFTR*-France database. Six specialized laboratories assessed the impact of nucleotide substitutions on splicing (minigenes), mRNA expression levels (quantitative PCR), synthesis and maturation (western blot), cellular localization (immunofluorescence) and channel function (patch clamp) of the CFTR protein. We also studied maturation and function of the truncated protein, consecutive to in-frame aberrant splicing, on additional plasmid constructs.

Results: Six of the 15 variants had a major impact on *CFTR* splicing by in-frame ($n = 3$) or out-of-frame ($n = 3$) exon skipping. We reclassified variants into: splicing variants; variants causing a splicing defect and the impairment of CFTR folding and/or function related to the amino acid substitution; deleterious missense variants that impair CFTR folding and/or function; and variants with no consequence on the different processes tested.

Conclusion: The 15 variants have been reclassified by our comprehensive approach of *in vitro* experiments that should be used to properly interpret very rare exonic variants of the *CFTR* gene. Targeted therapies may thus be adapted to the molecular defects regarding the results of laboratory experiments.

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1. Introduction

Classifying missense variants as deleterious or benign still remains challenging for geneticists, since the numerous computational algorithms available show variable performance [1], and *in vitro* assays are not always available. If an impairment on the resulting protein structure or function is the most expected outcome of such variants, an impact on splicing should also be considered for a comprehensive insight into their pathogenic mechanism. Indeed, at least 10% of so-called missense variants in all genes involved in genetic diseases are actually splicing variants [2,3]. Pathogenic variations in the *Cystic Fibrosis Transmembrane conductance Regulator* (CFTR) gene may cause Cystic fibrosis (CF) and a wide spectrum of milder phenotypes called CFTR-Related Disorders (CFTR-RD) [4,5]. To date, more than 2100 sequence variations have been reported worldwide in the *CFTR* gene (http://www.genet.sickkids.on.ca/). An efficient international network of geneticists, physicians and researchers have built the CFTR2 database that aims to determine the molecular impact and the severity of the most frequent *CFTR* variants identified in CF patients worldwide [6]. Complementarily, the *CFTR*-France database is a locus-specific database dedicated to rare *CFTR* variants [7] that currently contains a total of 905 different variants, of which 70% are reported in five individuals or fewer. Among these variants, 442 are so-called missenses, of which 147 are classified as disease-causing, 4 as non-disease causing, but the majority ($n = 291$) is still annotated as variants with unknown clinical significance (VUCS), due to the lack of available data and reliable resources. The functional classification (I-VI) [8,9] and the characterization of a minor (*i.e.*, totally disrupted) or residual (*i.e.*, partially disrupted) function of these rare variants now condition the patients' eligibility to highly effective modulator therapies.

Altered splicing consecutive to DNA changes in exonic regions is still widely under-evaluated and this neglected mechanism could change the eligibility to personalized CFTR modulators. In this study, after *in silico* analyses on 319 missense variants reported in the *CFTR*-France database, we selected 15 *a priori* missense variants for which bioinformatics tools suggest an impact of nucleotide changes on splicing of the *CFTR* gene (Table 1). Here, we assessed their molecular impact with a combination of approaches: minigene studies, mRNA quantification, protein maturation and trafficking, protein cellular localization and chloride channel function. We then proposed to classify the variants using the functional classification [8,9] and the clinical classification [10] in regard with epidemiological data, as the two classification are of complementary interest, to decipher the severity of the variants and the molecular defect that can be targeted by the new therapies (Fig. 1).

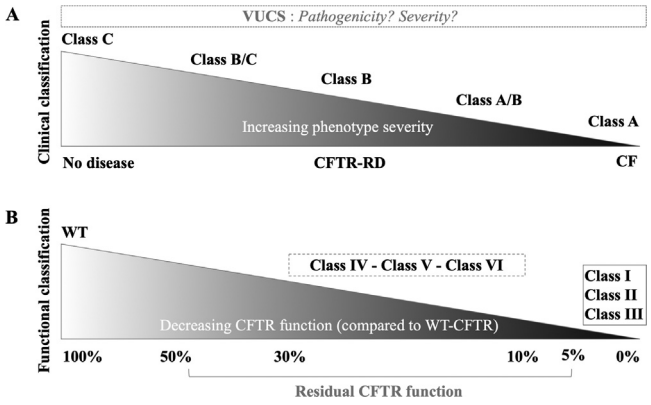

Fig. 1. Two different and complementary classifications for *CFTR* variants.
A) Clinical classification according to the international guidelines of Castellani et al., 2008 [10]. This classification refers to the clinical phenotypes observed in patients carrying the variant of interest **and a CF-causing variant in *trans*** (*i.e.*, on the other allele): (i) Variants identified only in CF patients are in class A, (ii) variants identified only in CFTR-RD patients are in class B, (iii) variants identified both in CF patients and patients with a CFTR-RD are in class A/B, (iv) variants identified in patients with a CFTR-RD and in healthy individuals are in class B/C, (v) variants identified in healthy individuals and/or with a high frequency in the general population are in class C. Variants of uncertain significance cannot, by definition, be classified in the other classes and belong to class D.
B) Functional classification of variants refers to the molecular defect observed or assumed (*e.g.*, class I premature stop codons leading to the synthesis of a truncated protein and its early degradation) [8,9]. The nature of the molecular defect can be correlated to the residual CFTR function from 0% of WT for class I to higher than 30% for some class IV to V variants. In general, patients carrying two variants of class I to III exhibit a CF phenotype, related to the very low level of CFTR function compared to WT-CFTR. Patients carrying at least a variant of class IV to VI, with a residual CFTR function show, in general, milder clinical phenotypes.

2. Methods

2.1. In silico *predictions*

We used Human Splicing Finder [11], MaxEntScan [12] and NNSplice [13] (https://www.fruitfly.org/seq_tools/splice.html) to perform predictions on all variants reported in the *CFTR*-France database prior to the start of the project (data update: 2014). We focused on the predictions of acceptor and donor splice sites to select the variants that possibly have an impact on splicing of *CFTR* pre-mRNA [14].

2.2. Cell cultures

BEAS-2B cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen). HeLa cells were cultured in DMEM + GlutaMAX (Invitrogen). Media were complemented with 10% fetal bovine serum (FBS) or 5% FBS and 1% Ultroser, and 1% penicillin/streptomycin. Cells were maintained at 37 °C in 5% CO_2 humidified atmosphere. For all experiments, cells were transfected with the WT and mutant constructs using the jetPEI® transfection reagent (Polyplus transfection) according to manufacturer's protocol.

2.3. Minigene assays

CFTR exons and their flanking intronic regions were introduced in pcDNA3.1 and pSPL3 minigene systems for splicing analysis. Mutants were obtained by directed mutagenesis with specific primers (QuickChange II Site-Directed Mutagenesis Kit, Agilent technologies). The wild-type (WT) and mutant constructs were transfected in BEAS-2B The splicing patterns were visualized on agarose gels and confirmed by Sanger sequencing after RT-PCR on total RNA from cells, as previously described [14,15].

2.4. CFTR cDNA expression plasmids

We used the expression vector pTracer-CMV containing the CFTR-wild-type (WT) cDNA (called pTracer-CFTR) [16]. Single Nucleotides Variants (SNVs), that lead to the amino acid changes (so called "missense variants"), and deletions consecutive to in-frame splicing alterations (called "CFTR-delEx4", "CFTR-del57bpEx5" and "CFTR-delEx9") were generated by directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Agilent). The WT and mutants were transiently expressed in BEAS-2B (for mRNA quantification, western blot protein analysis and immunofluorescence (IF) assays) and HeLa cells (for western blot and patch-clamp protein analysis).

Table 1
Results of *in silico* predictions, *in vitro* functional experiments and final classification (clinical and functional) proposed for the 15 variants studied.

Variant			Supposed Phenotypic spectrum (*i.e.*, clinical classification [10])	*In silico* predictions		Impact on *CFTR* Splicing *in vitro*	Western Blot experiments: C/(B + C), ratio on wt				Immunofluorescence assays (BEAS-2B)"+": presence"-": absence		Patch Clamp experiments:CFTR function (HeLa)		Final classification proposed	
Legacy name	HGVS cDNA	HGVS protein		on CFTR protein[a]	on *CFTR* Splicing[b]		Missense (BEAS-2B)	Missense (HeLa)	Splicing mutant (BEAS-2B)	Splicing mutant (HeLa)	Missense	Splicing mutant	Missense	Splicing mutant	Clinical [10]	Functional [8,9]
R55K	c.164G>A	p.(Arg55Lys)	B	VUS: probably no impact	low	None	1.1	1.2	NA	NA	+	NA	Equivalent to wt	ND	B/C	NA
R74Q	c.221G>A	p.(Arg74Gln)	B	VUS: probably no impact	low	None	0.7	0.7	NA	NA	+	NA	Equivalent to wt	ND	B/C	NA
E92K	c.274G>A	p.(Glu92Lys)	A/B	VUS: likely no impact	low	Partial exclusion of exon 4 (in-frame)	0	0	0	0	–	–	No channel activity	No channel activity	A	II
I175V	c.523A>G	p.(Ile175Val)	A	VUS: probably no impact	low	total deletion of 57 nt in exon 2 (in-frame)	0.7	0.7	0 (B band only)	0 (B band only)	+	+	Equivalent to wt	No channel activity	A	II
T351S	c.1052C>G	p.(Thr351Ser)	A/B	VUS: probably no impact	low	None	None	None	ND	ND	+	NA	Equivalent to wt	ND	B/C	ND
E403D	c.1209G>C	p.(Glu403Asp)	A	VUS: probable impact	high	Total exclusion of exon 9 (8) (in-frame)	1.5	1.0	0 (B band only)	0 (B band only)	–	–	Equivalent to wt	No channel activity	A	II
M469V	c.1405A>G	p.(Met469Val)	B	VUS: conflicting predictions	low	None	0.6	0.6	NA	NA	–	NA	No channel activity	NA	B	II and III
R560K	c.1679G>A	p.(Arg560Lys)	A	VUS: probable impact	high	Total exclusion of exon 12 (11) (out-of-frame)	0	0	NA	NA	–	NA	No channel activity	NA	A	V
I918M	c.2754T>G	p.(Ile918Met)	B	VUS: probable impact	low	Partial exclusion of exon 17 (15) (=wt) in pSPL3 system / Retention of intron 17 (15) in pcDNA3 system	0.8	1.0	NA	NA	+	NA	Equivalent to wt	NA	B/C	NA

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Table 1
(continued)

Variant			Supposed Phenotypic spectrum (*i.e.*, clinical classification [10])	*In silico* predictions		Impact on *CFTR* Splicing *in vitro*	Western Blot experiments: C/(B + C), ratio on wt				Immunofluorescence assays (BEAS-2B)"+": presence"-": absence		Patch Clamp experiments:CFTR function (HeLa)		Final classification proposed	
Legacy name	HGVS cDNA	HGVS protein		on CFTR protein[a]	on *CFTR* Splicing[b]		Missense (BEAS-2B)	Missense (HeLa)	Splicing mutant (BEAS-2B)	Splicing mutant (HeLa)	Missense	Splicing mutant	Missense	Splicing mutant	Clinical [10]	Functional [8,9]
G970R	c.2908G>C	p.(Gly970Arg)	A	VUS: probable impact	high	Total exclusion of exon 17 (15) (out-of-frame)	None	None	NA	NA	+	NA	Not assessed (see Yu et al., 2012)	NA	A	V
G970V	c.2909G>T	p.(Gly970Val)	A	VUS: probable impact	low	Total exclusion of exon 18 (16) (out-of-frame)	None	None	NA	NA	+	NA	Residual channel activity	NA	A	V
G970D	c.2909G>A	p.(Gly970Asp)	A	VUS: probable impact	low	Partial exclusion of exon 18 (16) (out-of-frame)	None	None	NA	NA	+	NA	Residual channel activity	NA	A	IV
G1069R	c.3205G>A	p.(Gly1069Arg)	B	VUS: likely no impact	low	None	None	None	NA	NA	+	NA	Equivalent to wt	NA	B/C	ND
R1070Q	c.3209G>A	p.(Arg1070Gln)	A	VUS: probable impact	low	Partial exclusion of exon 20 (17b) (=wt)	1.6	1.2	NA	NA	+	NA	Equivalent to wt	NA	B/C	ND
A1364V	c.4091C>T	p.(Ala1364Val)	B	conflicting predictions	low	None	None	None	NA	NA	+	NA	Equivalent to wt	NA	B/C	ND

CF: Cystic Fibrosis; **NA**: Not appropriated; **ND**: not determined; **VUS**: variant of unknown significance (according to the *CFTR*-France classification) - probably no impact on protein; likely no impact on protein; inconsistent/conflicting predictions; probable impact on protein); **WT**: wild type construct.
Clinical classification [10]: A: CF-causing; **B**: CFTR-Related Disorder associated; **C**: No clinical consequences; **A/B**: variants that may cause CF or CFTR-RD phenotypes; **B/C**: non-CF-causing with incomplete penetrance for CFTR-RD; **D**: VUS.
[a]AlignGVGD (Tavtigian et al., 2006), SIFT (Kumar et al. 2009), Polyphen2 (Adzhubei et al., 2010) and MAPP (Stone et al. 2005). [b]HSF [11], MaxEnt [12] and NNSPLICE [13].

2.5. mRNA quantification

Quantitative PCR (qPCR) was performed using *CFTR* specific primers that hybridize on exon 21 and exons 22–23 boundary. Total cellular RNA was extracted from cells transfected with WT or mutants pTracer-CFTR using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. For each transfection, two independent reverse transcriptions with 1 μg of total RNA were conducted using SuperScript® III First-Strand Synthesis System (Invitrogen). qPCR experiments were run in triplicate with 10 ng of cDNA using SYBR Green I PCR Master Mix on the Light Cycler 480 (Roche Diagnostics®). *HPRT* (*Hypoxanthine Guanine Phosphoribosyltransferase*) and beta-actin were used as reference genes and T84 cells endogenous *CFTR* expression was used as a calibrator for normalization. Relative differences in transcripts levels were quantified using the $\Delta\Delta$Ct method [17].

2.6. Western blot

Western blotting was performed on protein lysates from cells transfected with WT or mutants pTracer-CFTR as previously described [18]. Relative intensities of equal areas were compared using the Quantity One® analysis software (Bio-Rad). A ratio C/(C + B) between mutant and WT band intensities was used for Western Blot normalization, relative to lamin A/C; B band corresponding to the Endoplasmic Reticulum (ER) sequestered immature CFTR and C band to the transgolgian or transmembrane mature CFTR.

2.7. Immunofluorescence

BEAS-2B cells were grown on coverslips, transfected with the WT or mutants pTracer-CFTR, and treated 24 h later as previously described [19]. Acquisitions were performed with an Axio Imager 2 and the Zen 2012 software. Fluorescence was collected with a X40/0.75 plan-neofluar lens. The DAPI and A488/FITC fluorescence filter set were fitted to optimize data acquisition and limit bleed-through. At least, ten representative immunostaining images were taken by transfection.

2.8. Patch clamp experiments

Cells were transfected with the WT or mutants pTracer-CFTR. Ionic currents were measured in the broken-patch, whole cell configuration of the patch clamp method as previously described [20]. All experiments were conducted at room temperature (20–25 °C). Results are expressed as means ± standard error (SE) of *n* observations.

2.9. Chemicals

Forskolin (Fsk) and CFTRinh172 were purchased from Sigma. Stock solutions (10 mM) were prepared in dimethyl sulfoxide and used at a final concentration of 10 µM.

2.10. Statistical analysis

Patch Clamp data of CFTR mutants were compared to WT-CFTR using a one-way ANOVA followed by Dunnett's test with GraphPad Prism version 7.0 (GraphPad Software). Wilcoxon-test was performed for mRNA level and western blot data interpretation. Differences were considered significant for p *values* < 0.05.

3. Results

3.1. Five CFTR variants have a major impact on pre-mRNA splicing

Minigene studies showed that the c.523A>G (legacy name I175V) and c.1209G>C (E403D) variants led to aberrant splicing events, since no "full-length" transcripts were observed (Fig 2A). For c.523A>G, the reinforcement of an exonic cryptic donor splice site induced a skipping of the last 57-bp of the exon 5, leading to an in-frame deletion in *CFTR* transcripts. The variant c.1209G>C, located on the last nucleotide of the exon 9, led to an in-frame skipping of the whole exon, due to the alteration of the natural donor splice site.

Major aberrant splicing was also observed for three other variants: c.1679G>A (R560K) and c.2908G>C (G970R) that affect the last nucleotide of exon 12 and 17 respectively, and the c.2909G>T (G970V), located on the first nucleotide of exon 18 (Fig 2B). They all lead to an out-of-frame deletion of the corresponding exon in *CFTR* transcripts, suggesting that these three mutants would never lead to amino acid substitution. Nevertheless, as data obtained *in vitro* should be considered with caution considering quantitative aspects, few residual full-length transcripts, carrying the amino acid substitution, could remain *in vivo*. We thus performed additional experiments to determine a possible additional impact of all missense variants on protein synthesis, trafficking and activity. We also assessed processing, cellular localization and activity of protein lacking 19 and 31 amino acids, due to c.523A>G (exon 5) and c.1209G>C (exon 9) respectively. Of note, all the SNVs tested and in-frame deletions constructs CFTR-delEx4 and CFTR-del57bpEx5 showed no decrease of *CFTR* mRNA. Even if minor changes in mRNA quantity may not be detected, or may be conditioned by the cellular background of the heterologous system, we observed that the CFTR-delEx9 construct was associated to a low level (about 50% compared to wild-type) of the shorter aberrant transcript (Suppl. Fig. S1). These results suggest that CFTR-delEx4 and CFTR-del57bpEx5 rather had an impact on protein synthesis or maturation while absence of the entire exon 9 might additionally affect mRNA quantity.

We did not observe any impact on protein synthesis and activity of the mutant proteins carrying I175V and E403D (missense variants). Even if I175 and E403 are two highly conserved amino acids among orthologs, the absence of impact was suspected by the similarity of physicochemical properties of the WT and mutant amino acids in the Venn Diagram and the absence of deleterious effect predicted by the three-dimensional model [21]. As expected, the two shorter proteins resulting from the aberrant splicing, CFTR-del57bpEx5 and CFTR-delEx9, were not correctly folded (no C band in Western Blot (WB)) (Fig 2A). Of note, IF assays revealed some discrepancies with the results obtained by WB for 2 conditions, CFTR-del57bpEx5 and E403D, with respectively 18% and less than 10% of cells with labeling at the plasma membrane (Fig 2A). However, patch-clamp experiments revealed no chloride channel activity in cells transfected by CFTR-del57bpEx5 and CFTR-delEx9 (containing in-frame deletions), whereas full channel function was conserved for I175V and E403D mutant proteins (Fig 2C and Suppl. Fig. S3). Thus, the two variants c.523A>G and c.1209G>C may be considered as splicing variants leading to immature proteins and should be classified in the class II of *CFTR* variants (Table 1).

The amino acid substitutions (missense variants) R560K, G970R and G970V, consecutive to the variants c.1679G>A, c.2908G>C and c.2909G>T respectively, impaired protein folding and/or function. While the R560K induced a complete folding defect of the mutant CFTR, the G970R and G970V rather led to a gating defect without affecting folding of the mutant protein (Fig 2B). The total disruption of the channel function *in vitro* was previ-

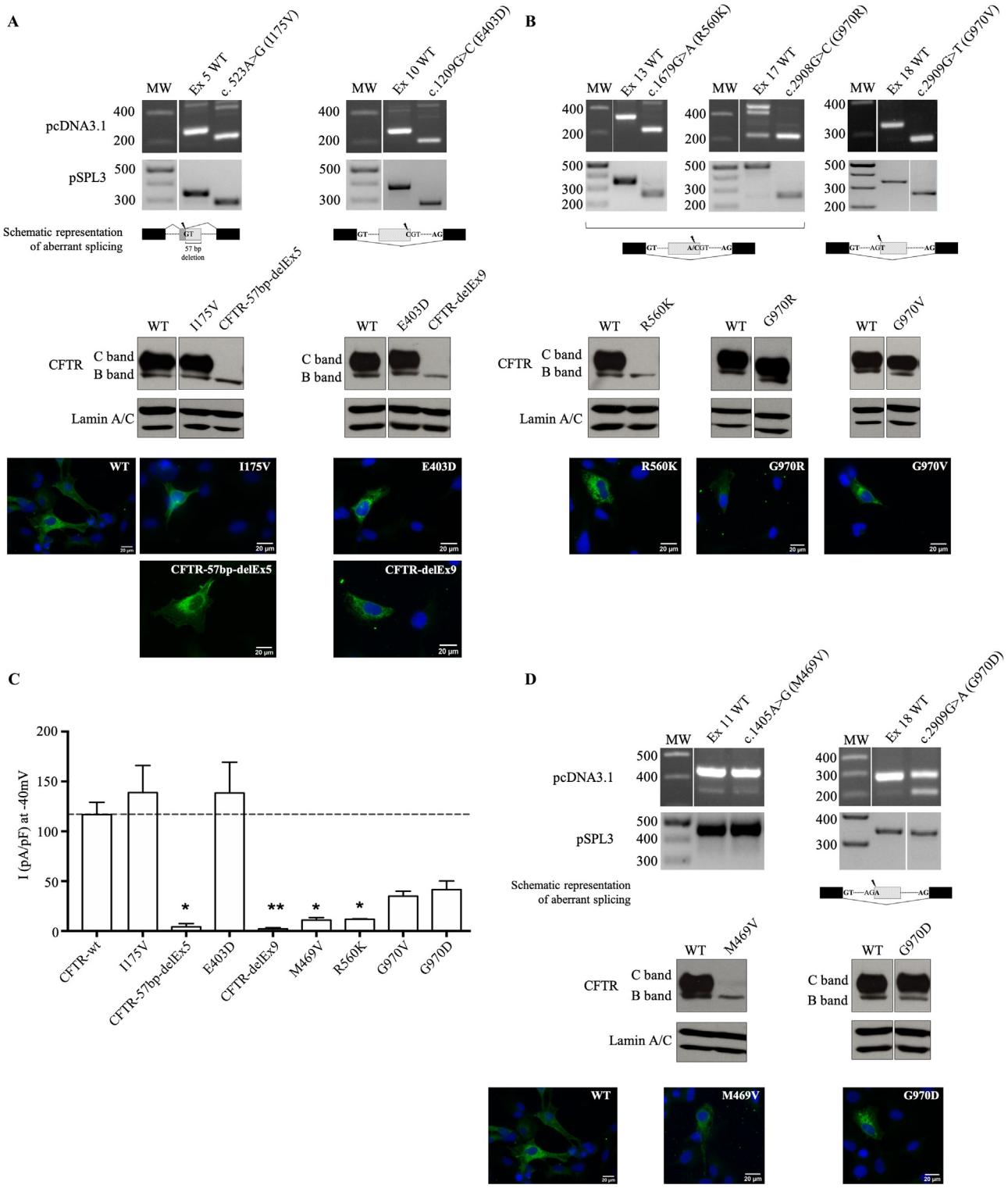

Fig. 2. Results of the functional experiments in BEAS-2B and/or HeLa cell lines.
A) Variants c.523A>G (I175V) and c.1209G>C (E403D). **B)** Variants c.1679G>A (R560K), c.2908G>C (G970R) and c.2909G>T (G970V). **C)** Results of patch clamp experiments for missense variants I175V, E403D, M469V, R560K, G970V and G970D, and the in-frame deletions constructs CFTR-del57bpEx5 and CFTR-delEx9 ($*p < 0.05$ and $**p < 0.01$ denote statistical significance compared to the WT chloride transport CFTR activity). Note: G970R was not assessed as similar functional assays were available in previous studies [22,23,32]. **D)** Variants c.1405A>G (M469V) and c.2909G>A (G970D).
From the top to the bottom: pcDNA3.1 and pSPL3 minigene experiments in the BEAS-2B cell line with the schematic representation of aberrant splicing events, representative western blot experiments (molecular weights: B band approximately 131 kDa and C band approximately 160 kDa, lamin A/C used as loading controls) and immunofluorescence assays in HeLa cells.

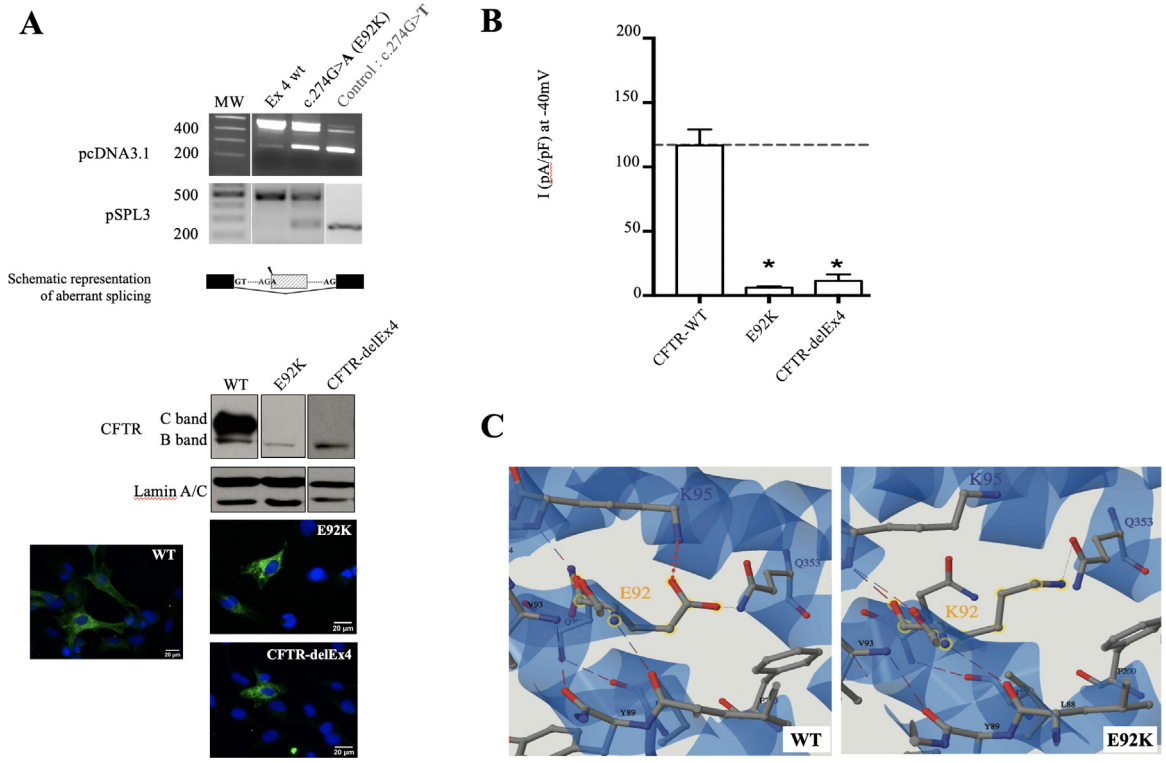

Fig. 3. Results of the functional experiments in BEAS-2B and/or HeLa cell lines for the variant c.274G>A (E92K).
A) From the top to the bottom: pcDNA3.1 and pSPL3 minigene experiments in the BEAS-2B cell line with the schematic representation of aberrant splicing events, representative western blot experiments (molecular weights: B band approximately 131 kDa and C band approximately 160 kDa, lamin A/C used as loading controls) and immunofluorescence assays in HeLa cells for E92K and the in-frame deletion CFTR-delEx4. **B)** Results of patch clamp experiments for missense variants E92K and the in-frame deletion CFTR-delEx4 ($^*p < 0.05$ denotes statistical significance compared to the WT chloride transport CFTR activity). **C)** Structural representation of the wild-type E92 (left) and the E92K mutation (right). The views are centered on the 92 position, highlighted with the yellow label. Residues located at less than 5 Å are labeled and depicted with their full side chains. Residues involved in the salt bridge (K95) and the H-bond (Q353) are labeled in blue. The wild-type E92 is forming a salt bridge with K95 (red dotted line) and a hydrogen bond with Q353 (black dotted line). E92K mutation is predicted to cause the loss of the salt bridge with K95.

ously demonstrated for the G970R variant [22,23] and our patch clamp experiments showed that the variant G970V conserved a residual function (*i.e.*, very low activity) (Fig 2C and Suppl. Fig. S3).

Thus, the three variants c.1679G>A, c.2908G>C and c.2909G>T, should be clearly classified as class V variants due to the out-of-frame aberrant splicing, with insufficient protein synthesis. The experiments performed on R560K, G970R and G970V missense variants showed an impact on protein folding (R560K) or activity (G970R and G970V). However, if residual full-length transcripts might theoretically be present and translated into variant proteins *in vivo*, these results are not representative of the major pathogenic mechanism of the nucleotide changes (*i.e.*, splicing alteration leading to NMD).

3.2. Two variants are exclusive missense variants with an impact on cftr folding and channel function

No significant alteration of splicing was observed for the c.1405A>G (M469V) and c.2909G>A (G970D) variants (Fig 2D) that can be considered as "authentic" missense variants. The variant M469V mainly impacts the folding of the CFTR protein, with a very weak detection of band C in WB experiments and no measured activity, that sorted it in the class II with minor function. The variant c.2909G>A, located at the first nucleotide of the exon 18, led to the deletion of exon 18 (predicted out-of-frame) in less than 50% of transcripts in the pcDNA3.1 construct only. We also observed a small amount of shorter transcripts in the WT construct (Fig 2D). That could be explained by the differences in the strength of the splice sites or the flanking exons used in the two different plasmids. Aberrant transcripts were not visible by agarose gel in the pSPL3 mutant construct but a very small proportion of transcripts lacking the exon 18 was detected by cDNA sequencing, but was considered not-significant. The full-length transcripts containing the c.2909G>A encode for the G970D mutant protein that shows a residual CFTR function, as the channel activity is partially altered without any significant decrease of the mature CFTR (Fig 2C, 2D and Suppl. Fig. S3). This variant can be classified in the class IV. The minor splicing alteration observed in pcDNA3.1 minigenes should be confirmed by the direct study of transcript patterns from nasal epithelial cells of the patients carrying the c.2909G>A variant.

3.3. One variant may have a combined impact on splicing and CFTR folding

The c.274G>A variant, located at the first nucleotide of the exon 4, led to the in-frame deletion of exon 4 in half of the *CFTR* transcripts in the two minigene systems used in this study (Fig 3A). This is concordant with previous observations for another variant at the same position, c.274G>T, that was first considered as a nonsense variant (E92X), but was secondarily reclassified as a splicing variant after the evaluation of transcripts patterns in nasal epithelial cells of a patient [24]. This is concordant with our *in vitro* results and suggests that this position is critical for the recognition of the acceptor splice site of exon 4. Both E92K and E92-delEx4 mutants blocked the maturation of the protein and the *in vitro* channel activity was consecutively about null (Fig 3A, 3B and Suppl. Fig. S3). Regarding the 3D structural model [21], the amino acid Glu92 is involved in the pore construction and participates in a

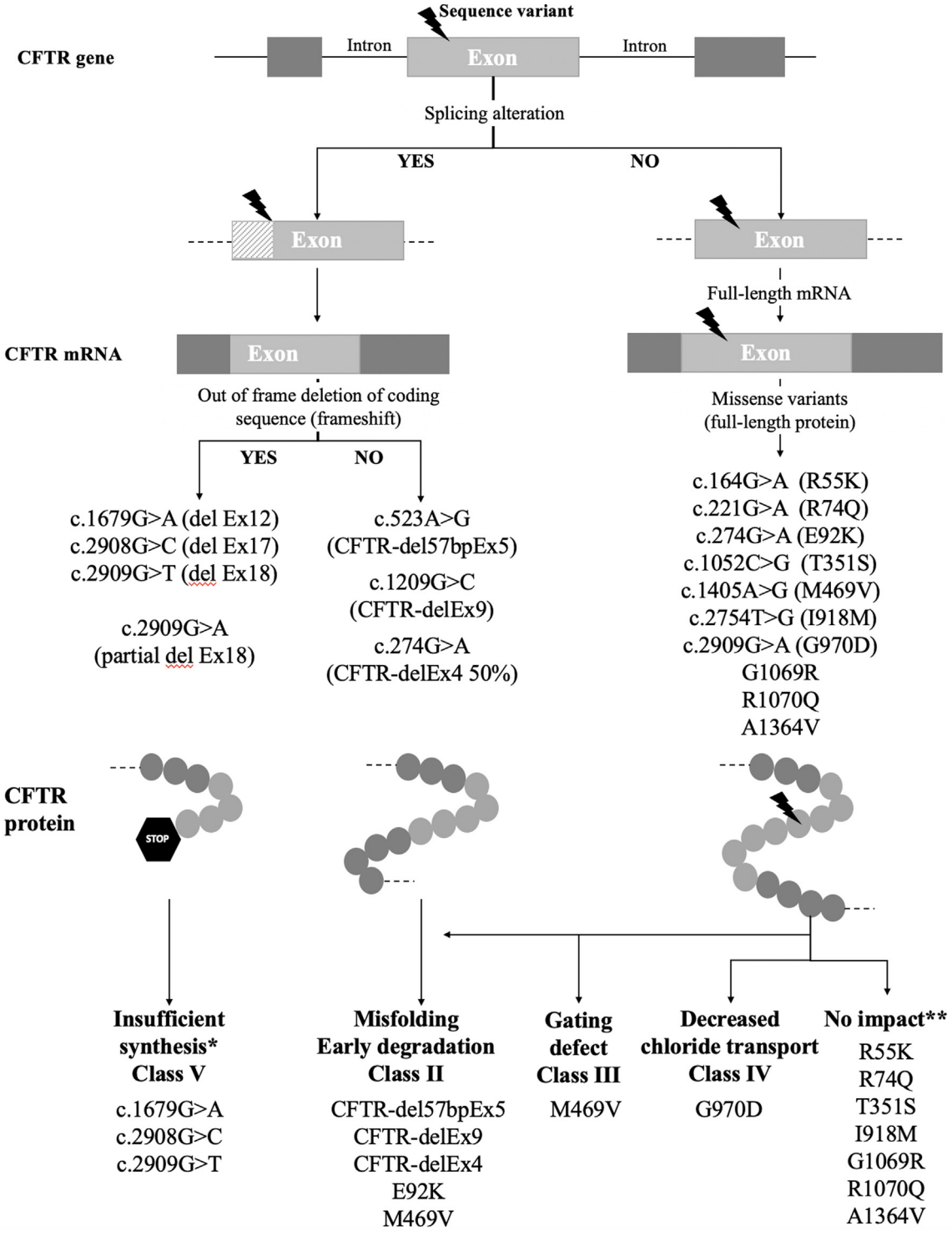

Fig. 4. Summary of the functional consequences of the *CFTR* variants studied and their related functional classification. *Insufficient to no protein synthesis depending on the amount of exon skipping and NMD *in vivo* (not quantified in this study). **No deleterious impact *in vitro* on splicing, CFTR protein synthesis or folding and chloride secretion.

salt bridge (Fig 3C), that may result in a gating defect if a very low proportion of mature protein could be addressed to the plasma membrane *in vivo*. The variant E92K can therefore concomitantly be considered as a class II variant, due to the splicing defect and the amino acid substitution, and possibly class III of the functional classification.

3.4. *Seven variants showed no deleterious consequence* in vitro

The *in vitro* experiments performed on the variants R55K, R74Q, T351S, I918M, G1069R, R1070Q and A1364V did not show any deleterious impact on splicing, mRNA expression level, CFTR protein folding and chloride secretion (Suppl. Figs. S2 and S3).

4. Discussion

The clinical classification of variants is essential to refine genotype-phenotype correlation (CF or CFTR-RD), guide the diagnosis and propose appropriate follow-up to patients [10]. For the rarest variants, collecting clinical data from patients may be challenging and do not allow to determine the class to which they belong. In our study, the results of functional assays associated to clinical data from *CFTR*-France and other available descriptions in the CFTR2 database or in the literature allowed us to refine the phenotypic spectrum of the 15 studied variants. Their clinical and functional classifications ([8–10] and Fig. 1) based on the data collected by our study are detailed in Table 1 and summarized in Fig. 4.

Among variants that disrupt CFTR processes *in vitro*, the classification as CF-causing (class A) was evident for 6 variants, while variants E92K and M469V could be associated with a large spectrum of disorders from CF to CFTR-RD (*i.e.*, class A/B). It was previously reported that CF patients with E92K presented milder phenotypes than other CF patients, with lower sweat test values, no pancreatic insufficiency in at least 50% of patients and less Pseudomonas lung colonization [6]. CFTR-RD phenotypes have been described in *CFTR*-France in two patients carrying c.274G>A (E92K) and a CF-causing variant on the second allele. However, we demonstrated that the variant c.274G>A (E92K) may disrupt three molecular CFTR processes: splicing and protein folding, as shown by a recent study [25], but also possibly CFTR function, according to 3D modeling (Fig. 2C). It would result in a major functional defect, which is in accordance with other observations [6,26]. Discrepancies between *in vitro* assays and clinical data were also raised for the variant M469V, for which functional experiments suggested a major pathogenic effect whereas 3 informative patients were reported with a CFTR-RD phenotype, rather suggesting a residual synthesis and activity of the chloride channel (*e.g.*, one CBAVD with *trans*-segregation of a CF-causing variant in *CFTR*-France). For these two variants, for which *in vitro* data suggest a more severe impact than the phenotype observed in patients, we could hypothesize that a small fraction of full-length mutant proteins with some residual function, not detected *in vitro*, may remain *in vivo* and reach the plasma membrane in some organs. This could be consistent with the high response of E92K to CFTR modulator therapy that combines VX-809 and VX-770 [26].

CFTR chloride channel function was similar to the WT for R55K, R74Q, T351S, I918M, G1069R, R1070Q and A1364V. As the whole cell patch-clamp assays would have detected a reduced membrane stability of the protein, we concluded that these 7 variants had no impact on gating, chloride conductance, and CFTR stability at the membrane. Thus, these variants clearly do not cause CF, but may present with an incomplete penetrance possibly resulting in a CFTR-RD or no disease were classified as B/C. As bicarbonate secretion was not assessed by our patch-clamp experiments, we can't exclude an impact of these variants on bicarbonate transport activity of the mutant proteins [27,28]. We cannot exclude either the presence of a cryptic disease-causing variant, not detected by the current techniques, that could be in complex allele with the variants, to explain patient's phenotype as previously shown for other variants [7,29]. The R1070Q, was mostly reported in complex allele (*cis*-segregation) with the CF-causing variant p.Ser466* in CF patients but reported alone in milder phenotypes [30]. Despite the absence of functional evidence in favor of the pathogenicity of T351S, G1069R and A1364V, combined with the relative high frequency in the healthy population from specific origins (Variant Allele Frequency of 0.0001 in European and Latino populations for T351S and A1364V respectively), they were also classified as B/C, as they were reported in patients with CFTR-RDs in combination with a second *CFTR* pathogenic variant (CF or CFTR-RD causing).

In conclusion, our study emphasizes the importance of assessing the impact on splicing of exonic variants to decipher their pathogenic mechanism, which conditions the efficiency of CFTR therapies *in vivo* [31], as illustrated for the G970R, incorrectly considered as a gating mutation [23,32] and well summarized in a very recent communication from Raraigh *et al.*, [33] in which the data discussed are in accordance with our results.

Nevertheless, the efficacy of CFTR modulators on splicing alterations that maintain in-frame transcripts should be assessed, as a shorter misfolded protein was detected *in vitro*. Further experiments on materials derived from patients to confirm the splicing defects observed *in vitro* as well as testing the efficacy of modulators on primary cultures from the patients carrying those variants must be performed and could support the use of these therapies in previously ineligible patients. Finally, future developments in CF targeted therapy should include splicing modulators that could, in association with correctors and potentiators, correct the basic defect due to out-of-frame splicing alterations related to numbers of variants.

5. Credit authors statement

We would be grateful if you would accept to add two authors in the revised version of the manuscript, F. Degrugillier and G. Le Gac, after discussion with the local coordinators of the two labs concerned, it is justified that they are part of the authors of this manuscript.

Thank you in advance for considering our request.

Anne Bergougnoux, Arnaud Billet, Chandran Ka, Fanny Degrugillier, Marion Heller, Marie-Laure Winter and Vincent Thoreau performed the experiments. Corinne Bareil, Souphata Sasorith and Corinne Thèze performed *in silico* predictions. Caroline Raynal, Corinne Thèze, Corinne Bareil, Magali Taulan-Cadars and Mireille Claustres conceived the project. Marie-Pierre Audrezet, Emmanuelle Girodon, Pascale Fanen, Chadia Mekki, Patricia Fergelot, Marie-Pierre Reboul, Alain Kitzis and Frederic Becq participated to the conception of the project, directed the experiments in their laboratories and contributed to the writing of manuscript. Claude Ferec, Gerald Le Gac, Thierry Bienvenu, Eric Bieth, Véronique Gaston, Guy Lalau, Adrien Pagin, Marie-Claire Malinge, Fabienne Dufernez, Lydie Lemonnier and Michel Koenig contributed to the writing of manuscript. Anne Bergougnoux, Souphata Sasorith and Caroline Raynal wrote the manuscript. All authors have reviewed the manuscript and approved its submission.

Conflict of Interest Statement

The authors declare no conflicts of interest.

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Web Sites

CFTR2 Database - CFTR2, Clinical and Functional Translation of CFTR: https://www.cftr2.org/ (accessed April 13, 2022).

CFTR-France database - CFTR-France: https://cftr.iurc.montp.inserm.fr/cftr/ (accessed April 13, 2022).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2022.12.003.

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