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Original Article

Severity of the S1251N allele in cystic fibrosis is affected by the presence of the F508C variant *in cis*

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ABSTRACT

Background: In cystic fibrosis (CF), genotype-phenotype correlation is complicated by the large number of *CFTR* variants, the influence of modifier genes, environmental effects, and the existence of complex alleles. We document the importance of complex alleles, in particular the F508C variant present *in cis* with the S1251N disease-causing variant, by detailed analysis of a patient with CF, with the [S1251N;F508]/G542X genotype and a very mild phenotype, contrasting it to that of four subjects with the [S1251N;F508C]/F508del genotype and classical CF presentation.
Methods: Genetic differences were identified by Sanger sequencing and CFTR function was quantified using rectal organoids in rectal organoid morphology analysis (ROMA) and forskolin-induced swelling (FIS) assays. *CFTR* variants were further characterised in CF bronchial epithelial (CFBE) cell lines. The impact of involved amino acid changes in the CFTR 3D protein structure was evaluated.
Results: Organoids of the patient [S1251N;F508] with mild CF phenotype confirmed the CF diagnosis but showed higher residual CFTR function compared to the four others [S1251N;F508C]. CFBE cell lines showed a decrease in [S1251N;F508C]-CFTR function but not in processing when compared to [S1251N;F508]-CFTR. Analysis of the 3D CFTR structure suggested an additive deleterious effect of the combined presence of S1251N and F508C with respect to NBD1-2 dimerisation.
Conclusions: *In vitro* and *in silico* data show that the presence of F508C *in cis* with S1251N decreases CFTR function without affecting processing. Complex *CFTR* alleles play a role in clinical phenotype and their identification is relevant in the context of personalised medicine for each patient with CF.

1. Introduction

More than 2000 variants in the *CFTR* gene have been reported, of which 466 have been functionally characterised in the CFTR2 database [1]. Of these, 382 variants have been classified as CF-causing, 24 as non-CF-causing, 49 variants are associated with varying clinical consequences, and for 11 the significance is un-

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known [1]. This however provides a "simple view", just assessing one variant at a time. Indeed, two or more variants *in cis*, known as complex alleles, can have an additive disease-causing effect, or one variant can reduce the disease severity caused by the other, or one can be neutral and the other disease-causing [2–5]. Complex alleles can be overlooked as full *CFTR* sequencing is often reserved for cases where less than two variants are found with "first-line" mutation panels [6]. Complex alleles, along with the number of variants, the influence of modifier genes, and environmental effects, thus complicate the genotype-phenotype correlation in CF [7].

A patient of mixed Belgian/Asian descent was diagnosed with CF at the age of 15 years with an unusually mild disease course. Screening for frequent *CFTR* variants showed a S1251N and a G542X variant, confirmed to be *in trans* via parental genotyping. Sequencing showed absence of the F508C variant *in cis* with S1251N. S1251N is a class III CF-causing variant located in the Walker A motif of NBD2, reducing chloride transport to <10% of normal with most patients being pancreatic insufficient (PI) and having a sweat chloride concentration (SCC) in the CF range [1,8,9].

To understand the unusual phenotype-genotype association, we compared the repercussions of the presence or absence of F508C on the S1251N variant, *in vivo* (clinical data) and *in vitro* (residual CFTR function and response to CFTR modulators in organoids), as well as in human CF bronchial epithelial (CFBE) cell constructs and *in silico* by analysis of the CFTR 3D protein structure.

2. Materials and Methods

2.1. Subjects

The index patient (patient 1) presented with the unusual phenotype consisting only of recurrent pancreatitis, without clinical lung disease with normal lung imaging (chest CT), normal lung function (dynamic spirometry and lung clearance index), and a SCC in the intermediate range. Nasal potential difference measurements indicated CFTR dysfunction (Wilschanski indices 0.30 and 0.51) (figure S1). Initial work-up showed no other aetiology for the recurrent pancreatitis (normal anatomy, negative antinuclear antibodies, normal IgG4, no *SPINK1* mutation). Genetic analysis showed the [S1251N;F508]/G542X genotype. Patients 2-5 were followed at the University Hospital Leuven CF centre, with the known S1251N/F508del genotype.

This study was approved by the University Hospital Leuven Ethics Review Board (S56329). All patients/parents gave written informed consent and/or assent. Rectal tissue was obtained during a routine visit.

2.2. Next Generation Sequencing

DNA was extracted from peripheral blood of patient 1 and both parents. A HaloPlex DNA enrichment assay (Agilent Technologies) was designed targeting a 499.999kbp region encompassing the *CFTR* locus (chr7:117027043-117527041), 482.98kbp total target bases analysable, 486.05kbp total sequenceable design size, 96.60% target coverage. The MiSeq Reagent Kit v2 (Illumina) was used for sequencing template preparation; 2 × 150 paired-end sequencing was performed on a MiSeq apparatus (Illumina). Bio-informatic analysis was done with CLC Genomics Workbench 9.0.1-11.0.1 (CLC Bio, Qiagen). The reference sequence and annotations were H. sapiens, hg19, GRCh37 (Feb.2009; 1-based) build.

The *CFTR* alleles including introns and flanking intergenic regions (including the promoter), were targeted for sequencing with a highly parallel sequencing assay and compared to database patients with CF (PwCF) carrying a S1251N variant. The parents of patient 1 were sequenced to determine the complete S1251N haplotype background by segregation analysis. The sequencing data from three S1251N/F508del database patients was compared to the haplotypes in F508del homozygous patients, to find out the complete S1251N haplotype in these database patients.

2.3. Organoid production and analysis of organoid morphology and CFTR function

Organoids were cultured from crypts isolated from rectal biopsies as described before [10]. Baseline organoid morphology was assessed using rectal organoid morphology analysis (ROMA) [11]. Residual CFTR activity was quantified as the AUC of the response curve to increasing forskolin concentrations (0.008, 0.02, 0.05, 0.128, 0.32, 0.8, 2 and 5 µM), plotted as the AUC of the time vs organoid surface increase curve over 60 minutes at each of the forskolin concentrations ('AUC of the AUC', figure S2) [10,12]. To assess their response to modulators, organoids were incubated overnight with 3 μM lumacaftor (VX-809, SelleckChem) and activated at time zero with 3 μM ivacaftor (VX-770, SelleckChem) before measurement of AUC's as described before. The mean of 3 independent experiments with every condition in duplicate is reported.

2.4. Genomic DNA extraction and Sanger sequencing

Genomic DNA was extracted from organoids. Exon 10, 11 and 20 were amplified by PCR (primers and conditions in table S1), and PCR products purified for Sanger sequencing. Legacy names/numeration are used (nomenclature in table S2).

2.5. RNA extraction and CFTR transcripts analysis

Total RNA was extracted from organoids and cDNA was produced. *CFTR* transcripts analysis was done by RT-PCR amplification as described before [13], with small alterations (table S1).

2.6. CFBE cell line production

CFBE cell lines stably expressing S1251N-, F508C-, [S1251N;F508C]-, and wild type (wt)-CFTR were produced. After mutagenesis to introduce the variants into the *CFTR* cDNA and confirmation by Sanger sequencing, lentiviral vector particles (pLV) were used to transduce the parental cell line CFBE41o-[14]. Transduction efficiency was confirmed by Western blot.

2.7. Immunoblotting

Immunoblotting on the transduced cell lines was performed to assess CFTR expression and processing. For CFTR protein detection, cells were lysed in Laemmli buffer supplemented with complete protease inhibitor tablets (Roche). Total protein was analysed by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). CFTR protein was detected with anti-CFTR monoclonal antibody 596 (CFF) at a 1:3000 dilution. As loading control, calnexin detected by anti-calnexin antibody (1:3000 dilution) (BD Biosciences) was used. The secondary antibody was horseradish peroxidase-labelled anti-mouse at 1:3000 (Bio-Rad). Images were acquired using ChemiDoc XRS+ imaging system BioRad and further processed by Image lab 4.0.

2.8. CFTR channel function in CFBE Cells

Transduced cells were grown in Snapwell inserts for 8-10 days and mounted in modified micro-Ussing chambers when the monolayers' transepithelial electrical resistance was >450 Ω.cm2. Recordings were performed as previously described [15,16].

2.9. Analysis of the CFTR 3D protein structure

The experimental 3D structure of the human CFTR protein (wild-type, phosphorylated, ATP-bound form) was extracted from PDB, and manipulated using UCSF Chimera [17–19].

2.10. Statistics

GraphPad and STAT were used for figures and statistical analysis (generalised linear models and unpaired t tests).

3. Results

3.1. CFTR gene analysis

Apart from some rare variants never reported before in the SNP141 database, the S1251N *CFTR* haplotype from the three database PwCF with the F508del/S1251N genotype were all identical, suggesting a single ancestral origin. The S1251N variant in patient 1 was found on a completely different *CFTR* haplotype background. The differences were mostly common SNPs, most notably the absence of F508C (table S3). This allele was inherited from the patient's Sri Lankan mother.

We hypothesised that the lack of F508C in the S1251N allele would influence the function of the mutant CFTR and consequently modulate clinical presentation and disease severity. We collected clinical data including the responses to modulators of patient 1, and of four S1251N/F508del PwCF followed in our clinic (patients 2-5).

3.2. Clinical data before and after initiation of modulator treatment

Patient 1 had a normal baseline ppFEV1 with no change after 6 months of modulator treatment, while SCC remained in the intermediate range (Table 1). A change in frequency of pancreatitis was difficult to assess given the low number of episodes. Patient 1 eventually decided to stop ivacaftor due to lack of perceived benefit.

The ppFEV1 of patients 2-4 increased with a mean of 15% and their SCC decreased with a mean of 69 mmol/L after initiation of modulators. Patient 5 started with a low ppFEV1 of 40%, which rose by 5%; data on SCC after treatment was not available (table 1).

To determine if these differences in responses were related to differences in CFTR function, we produced organoids for these 5 patients.

3.3. Baseline organoid morphology

For all patients, organoid morphology was suggestive of CF, confirmed by ROMA indices (CI 0.59, IR 1.16) in the CF range (figures S3A and S3B), supporting the diagnosis of CF also in patient 1 [11].

3.4. Residual CFTR function

In patient 1, residual CFTR function measured as AUC at 0.8 µM Fsk was 37-fold higher compared to patients 2-5 (figure 1, $p<0.001$). Residual function expressed as the AUC of AUC values over the full range of forskolin concentrations (figure 1 and S2) was significantly higher in patient 1 compared to patients 2-5 ($p<0.001$). No differences were observed between the four patients with the [S1251N;F508C];F508del genotype (figure 1).

3.5. Responsivity of organoids to modulators

Forskolin-induced swelling of organoids after addition of ivacaftor was similar in all patients (figure 2A-C and S4). Lumacaftor,

Table 1
Clinical characterisation of the S1251N patients and clinical trial results before and after 6 months of treatment.

Patient ID	Genotype	Treatment	FEV1%			Sweat chloride concentration (mmol/L)			Pancreatic status	Age at diagnosis (years)	CT thorax
			Start	Plus 6 months	Change	Start	Plus 6 months	Change			
1	G542X/S1251N	Kalydeco™	97	99	2	47.5	33.5	-14	PS	15	normal
2	F508del/S1251N	Kalydeco™	60	80	20	84	20	-64	PS	0.1	bronchiectasis
3	F508del/S1251N	Kalydeco™	95	106	11	88	38	-50	PI	0.7	bronchial wall thickening
4	F508del/S1251N	Kalydeco™	55	69	14	103	11	-92	PI -> PS*	0.4	bronchial wall thickening
5	F508del/S1251N	Orkambi™	40	45	5	95	ND	ND	PI	0.2	bronchiectasis

PS-pancreatic sufficient, PI-pancreatic insufficient; * patient changed from pancreatic insufficient to sufficient during treatment with Kalydeco

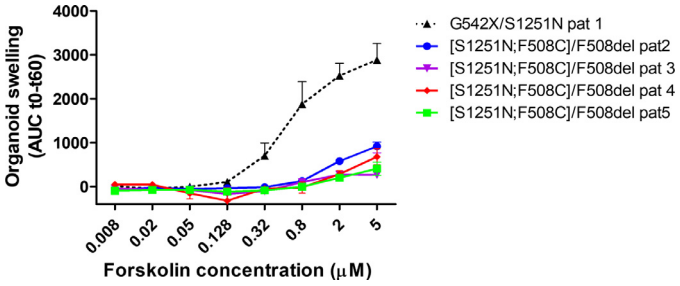

Figure 1. Assessment of CFTR residual function by FIS assays in rectal organoids from the five patients. Swelling of organoids in response to stimulation with increasing forskolin concentrations is plotted. Each data point is the mean (SEM) of the area under the curve (AUC) of the normalised total organoid area over a 1 hour measurement with intervals of 10 minutes, using the average of duplicate measurements on three independent experiments.

alone or in combination with ivacaftor, had no additional effect. When correcting for residual CFTR function (swelling with forskolin alone), the effect of ivacaftor in organoids of patient 1 was about half of the response of patients 2-5 (figure 2D).

3.6. CFTR *genomic and transcript analysis in organoids*

Sanger sequencing of exon 10 confirmed the presence of F508del and F508C in patients 2-5 and the lack thereof in patient 1 (figure S5). SNPs found in exon 10 are shown in table S4.

Sequencing of exon 20 confirmed the presence of the S1251N variant in all five patients and sequencing of exon 11 the presence of G542X in patient 1.

RT-PCR analyses of RNA covering the complete *CFTR* coding sequence showed no differences between patients 1 and 2-5. No alternative splicing was detected except for exon 9 skipping, observed in all (figure S6). Sequencing of RT-PCR fragments including exon 11 in patient 1 did not detect G542X transcripts, indicating nonsense mediated mRNA decay (NMD).

3.7. *CFTR processing and function in CFBE cells*

To better understand the impact of the two variants alone or in combination, without the confounding effect of the second allele, CFBE cell lines stably expressing S1251N-, F508C-, [S1251N;F508C]-, and wt-CFTR pLV were produced.

No differences were found in processing (relative amount of mature and immature forms) between CFBE cells expressing S1251N-, F508C-, and [S1251N;F508C]-CFTR (figure 3A-B), but the double mutant leads to a decrease in the total amount of CFTR (figure S7A-B).

Transepithelial chloride transport assessed in Ussing chambers showed that activation of CFTR by forskolin in cells expressing

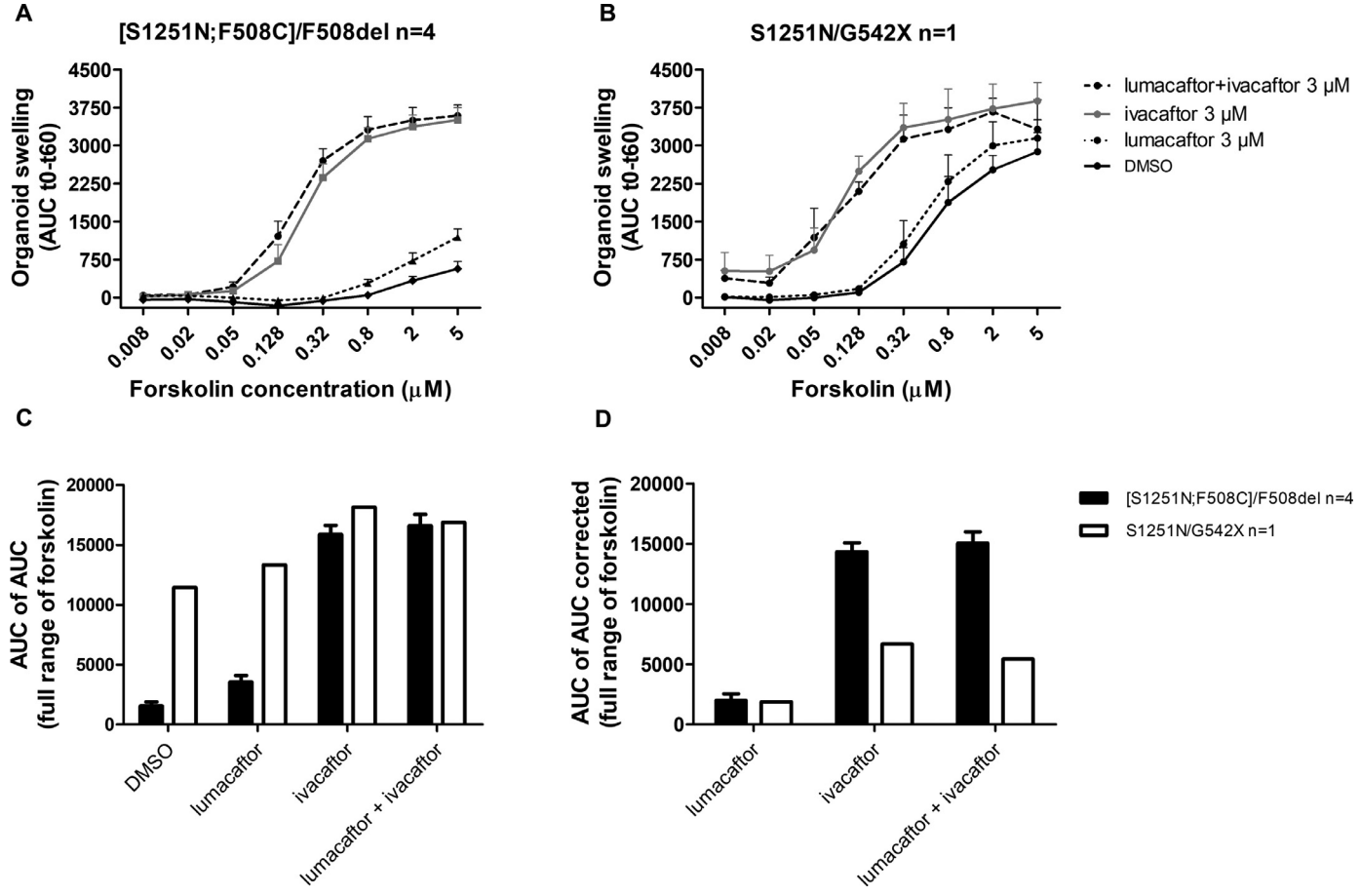

Figure 2. Rescue of CFTR function using modulators in rectal organoids. (A-B) FIS assay showing the swelling of organoids in response to stimulation with increasing forskolin concentrations and exposition to CFTR modulators ivacaftor and/or lumacaftor. Each data point is the mean (SEM) of the area under the curve (AUC) of the normalised total organoid area over a 1 hour measurement with intervals of 10 minutes, using the average of duplicate measurements on three independent experiments. **(C-D)** Quantification of the response to the full range of forskolin concentrations with or without modulators ('AUC of AUC (t0-60)') of the response curve obtained by the FIS assay plotted in figure 1A and 2B, either uncorrected (C) and corrected (D) for the response to forskolin alone. In A, C, and D, data is presented as mean and SEM for each genotype (n=4 for [S1251N;F508C]/F508del genotype and n=1 for S1251N/G542X. In B, data is presented as mean and SEM of the three independent experiments for genotype S1251N/G542X.

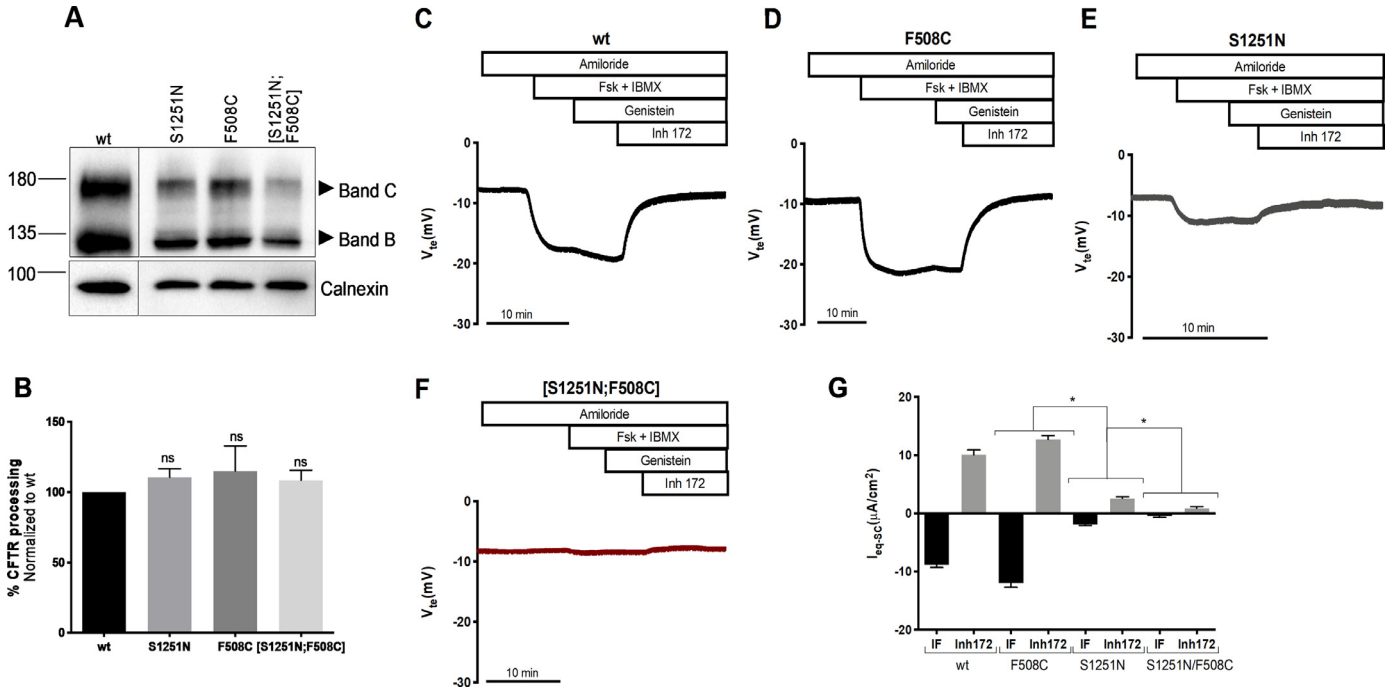

Figure 3. Assessment of CFTR processing and function in CFBE cells stably expressing wild type (wt), S1251N-, F508C- and [S1251N;F508C]-CFTR (A) Representative Western blot of CFBE cells stably expressing the different variants. **(B)** Quantification of CFTR processing in stably transfected CFBE cells. For each condition, densitometry was used to calculate the percentage of mature CFTR (band C) vs total CFTR expressed. Data were normalised to the efficiency of processing of wt-CFTR and is shown as mean ± SEM (n=3). Images were acquired using ChemiDoc XRS+ imaging system BIO-RAD and processed using Image lab 4.0 software. ns = no significant difference compared with wt ($p>0.01$, unpaired t test). Original Ussing chamber (open-circuit) recordings showing transepithelial voltage measurements (Vte) in CFBE cells stably transduced with **(C)** wt-CFTR, **(D)** F508C-CFTR, **(E)** S1251N-CFTR and **(F)** [S1251N/F508C]-CFTR. A low chloride Ringer solution was used at apical side to establish a Cl- gradient. Negative transepithelial voltage (Vte) deflections were observed following the addition of apical forskolin (2 μM) and IBMX (100 μM) and were fully reverted by addition of the specific CFTR inhibitor Inh172 (30 μM). **(G)** Quantification of the equivalent short-circuit current (Ieq-sc) after apical stimulation with FSK+IBMX. Asterisks indicate significant difference compared with the previous condition (S1251N vs F508C and [S1251;F508C] vs S1251N, $p<0.01$, unpaired t test).

F508C-CFTR elicited a lumen-negative response similar to that of wt-CFTR that was further potentiated by treatment with genistein and inhibited with the specific CFTR inhibitor Inh172. In cells expressing S1251N-CFTR, a clear response to forskolin was observed but at lower levels. In cells expressing [S1251N;F508C]-CFTR, hardly any response to forskolin and IBMX was observed (figure 3C-G). These differences were significant even after normalisation for the total amount of CFTR (figure S7C).

3.8. Analysis of the CFTR 3D protein structure

S1251 is a conserved amino acid residue in the NBD2 Walker A motif, which directly participates in ATP binding (ATP β-phosphate and magnesium) at the canonical ATP-binding site (light green area in figure 4). S1251N was thus predicted to impair CFTR gating by affecting ATP binding and NBD dimerisation [20]. F508 participates in a network of interacting residues, involving aromatic and basic side chains belonging to NBD1 (R560, M498, W496) and to the fourth intracellular loop (ICL4) of Membrane-Spanning Domain 2 (MSD2) (F1068, R1070) (blue area in figure 4). By disrupting cation-π interaction with R560 and H-bonds made by R560 Nη atoms with the W496 main chain oxygen, F508C could lead to enhancement of conformational sampling of the NBD1 Q-loop (Q493), which is connected to the non-canonical ATP-binding site (dark green area in figure 4). In addition, by weakening the NBD1-ICL4 interface, F508C could also disrupt the salt bridge between ICL4 R1060 and ICL2 E267 (pink area in figure 4), thereby affecting the whole dimer interface, as well as the canonical ATP-binding site via ICL2 Q270. Both mechanisms could in turn affect NBD dimerisation at the level of the two ATP-binding sites.

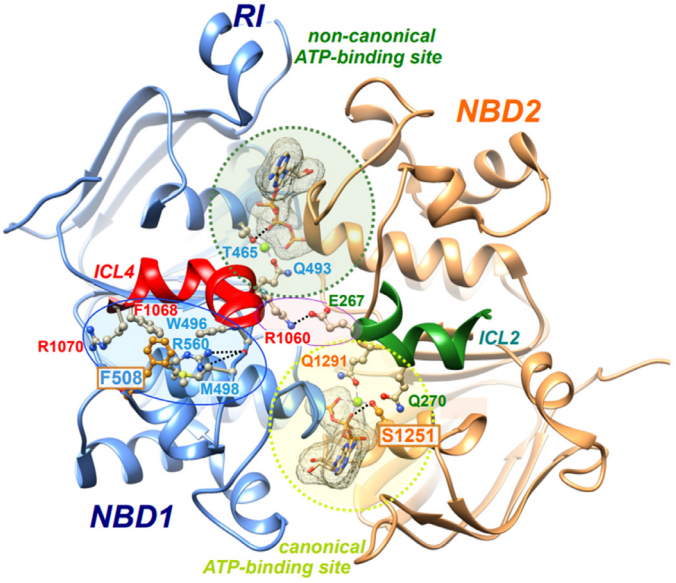

Figure 4. Analysis of the 3D structure in light of the [S1251N;F508C] complex allele. Ribbon representation of the experimental 3D structure of human, wild-type CFTR (phosphorylated, ATP-bound – pdb 6MSM, [17] on which are reported in a ball-and-stick representation the positions of F508 and S1251, as well as critical amino acids. Amino acid labels are coloured according to the domain to which they belong: blue-NBD1, orange-NBD2, green-ICL2 (MSD1), red-ICL4 (MSD2). The ATP-binding sites are highlighted with green circles, whereas the NBD1:ICL4 and ICL4:ICL2 interfaces are depicted in blue and pink, respectively. RI stands for regulatory insertion (non-canonical ATP-binding site).

4. Discussion

We comprehensively assessed the role of the complex allele [S1251N;F508C] which is associated with a typical CF presentation, by comparing it to the milder phenotype in a patient with an unusual [S1251N;F508] allele, thus without the F508C variant. We quantified the higher residual CFTR function of [S1251N;F508]-CFTR in patient-derived organoids and confirmed the mitigating effect of the absence of F508C in CFBE cells. This seems to be mediated by an improved CFTR function rather than a milder processing defect.

The G542X variant *in trans* with the S1251N allele without F508C could not explain these differences. This variant is not associated with SCC in the intermediate range nor with significant residual CFTR function in organoids [1,12]. RT-PCR did not detect G542X transcripts in this patient, which points to NMD below the level detectable by sequencing.

We focused this study on the hypothesis that the presence of F508C *in cis* would affect S1251N-CFTR activity. Analysis in both patient-derived organoids and CFBE cell lines confirmed this effect. However, we cannot rule out possible effects of other SNPs that may be different between these patients, especially since only one patient with the [S1251N;F508] genotype was analysed. For example, this patient was homozygous for M470V, while the other four were heterozygous.

The additive disease-causing effect of variants *in cis* has already been demonstrated for several complex alleles, including [R117H;5T] and [R117L;L997F] [5,21]. By itself, F508C is reported as non-CF-causing [1]. It has however been linked to congenital bilateral absence of the vas deferens [22,23]. *In vitro* data also suggest a role in channel gating, with F508C-CFTR exhibiting an increased channel mean closed time, which thus could exacerbate the effect of S1251N as a gating mutation [24]. Our findings corroborate an earlier suggestion of a synergistic negative effect on CFTR function for the complex allele [S1251N;F508C] [25].

Modelling the impact of F508C on the experimental 3D structure of the human CFTR protein suggests that this variant could affect the non-canonical ATP-binding site by enhancing conformational sampling of the NBD1 Q-loop, similarly to what was observed with F508del [26]. In addition to the deleterious effect of the S1251N variant at the canonical ATP-binding site, this could impair ATP binding at this site and thus lower the reopening probability. Alternatively, or additionally, by disturbing the NBD1:ICL4 interface, F508C may affect the whole cytosolic dimer interface at the level of the internal helices of ICLs. In particular, a salt bridge between ICL4 R1060 and ICL2 E267 is directly involved in tight association of the ICLs and mutation of these amino acids affect gating [27,28]. An allosteric network could connect the F508 position to the canonical binding site through this pathway, as E267 is directly linked to the canonical binding site through Q270, providing a possible additive negative effect. This *in silico* analysis further strengthens the interpretation of *in vitro* results.

An important question is whether our findings could impact therapeutic choices for modulators, expensive medications to be given lifelong. The response to ivacaftor in organoids of the patient with the S1251N without F508C allele was smaller compared to organoids with the [S1251N;F508C] allele, in essence due to the higher residual CFTR function. This was mirrored by an unclear benefit, as lower basal SCC and absence of clinical lung disease limited possible treatment benefits. The patient did not feel any subjective improvement and eventually decided to discontinue the treatment. Complex alleles could thus impact the risk- and cost-benefit balance of modulators. *In vivo* biomarkers such as SCC have shortcomings as they do not reflect the pulmonary condition and have high intra-person variability, but can be informative in many cases [29]. *In vitro* assessment in primary patient cells, such as rectal or nasal organoids, is also promising. A pragmatic approach could be the measurement of individual responses to modulators in patient tissue (sweat gland and organoids) to strive towards personalised medicine.

Our results underline the importance of comprehensive genetic analysis in PwCF. Genetic analysis is often limited to screening for the most frequent variants with commercial or in-house *CFTR* mutation panels, without further assessment of other associated variants or complex alleles when two disease-causing mutations have been found. A more in-depth genetic analysis to detect complex alleles could be a first step. However, interpretation of interactions between variants *in cis* would require the (re-)evaluation of many patients within international collaborations, as many variant combinations will be even more rare than the rare "single" variants already described.

The S1251N variant is rare in Europe overall but accounts for 1.4% of alleles in the Belgian and 1.2% in the Dutch CF registry (https://www.sciensano.be/en/biblio/annual-report-belgian-cystic-fibrosis-registry-2019 and https://ncfs.nl/onderzoek-naar-taaislijmziekte/dutch-cf-registry). It is not known how common the absence of F508C *in cis* with S1251N is.

In the CFTR2 database, there are 72 patients with S1251N plus a PI-CF-causing variant in *trans* with a reported SCC. Of those, 7 have a SCC below 60 mmol/L, are pancreatic sufficient (PS), have better-than-average lung function (KNoRMA Z-score 1.13), and their mean age at diagnosis is 12 years (range 0-48 years) (G. Cutting personal communication). CFTR2 combines data from several national CF registries with possible incomplete data and enrichment of more severe cases.

In the *CFTR*-France database (https://cftr.iurc.montp.inserm.fr/cftr), which also includes patients not meeting full CF disease criteria, 18 patients carrying S1251N are described. One has another CF-causing variant *in trans* but intermediate SCC on repeated measurements. In the clinical trial assessing the effect of ivacaftor in non-G551D gating mutations, all 8 patients with the S1251N/F508del genotype had a SCC in the CF range. At least one of the patients had no significant improvement in SCC after starting ivacaftor (-7 mmol/L), while participants with other gating mutations had an improvement of at least 35 mmol/L [30]. These data could indicate high residual function in a small proportion of PwCF carrying S1251N, potentially related to absence of F508C *in cis*. Hence, when S1251N is detected, we argue that assessing F508C status is important both in the context of a clinical CF diagnosis or after newborn screening.

In conclusion, we report the importance of assessing the complex allele [S1251N;F508C] in PwCF and a S1251N allele without the F508C variant. With high residual CFTR function and unclear additional benefit from treatment with ivacaftor, the S1251N allele without the F508C variant seems to result in less severe CFTR dysfunction than [S1251N;F508C]. Identification of complex alleles could impact decisions regarding modulator treatment, and illustrate the utility of a "personalised medicine" approach.

Credit author statement

Senne Cuyx: Conceptualisation, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review and editing, Visualisation, Funding acquisition

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2022.05.013.

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