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p.Ser1235Arg should no longer be considered as a Cystic Fibrosis mutation: results from a large collaborative study

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48 **Abstract**

49 Among the 1700 mutations reported in the cystic fibrosis transmembrane conductance
50 regulator (*CFTR*) gene, a missense mutation, p.Ser1235Arg is a relatively frequent
51 finding. To clarify its clinical significance, we collected data from 104 subjects
52 heterozygous for the mutation p.Ser1235Arg from the French CF network, addressed
53 for various indications including classical CF, atypical phenotypes or carrier screening
54 in subjects with or without a family history. Among them, twenty-six patients (five
55 having CF, ten CBAVD -Congenital Absence of the Vas Deferens- and eleven with CF-
56 like symptoms) and fourteen healthy subjects were compound heterozygous for a
57 second *CFTR* mutation. An exhaustive *CFTR* gene analysis identified a second
58 mutation in *cis* of p.Ser1235Arg in all CF patients and in 81.8% CBAVD patients.
59 Moreover, epidemiological data from more than 2100 individuals found a higher
60 frequency of p.Ser1235Arg in the general population than in CF or CBAVD patients.
61 These data, added to the fact that *in silico* analysis and functional assays suggest a
62 benign nature of this substitution gives several lines of evidence against an association
63 of p.Ser1235Arg with CF or CBAVD.

64

65 **Introduction**

66 Cystic Fibrosis (CF, MIM#219700), one of the most frequent life-shortening
67 recessively inherited diseases, is caused by alterations in the *CFTR* gene (*Cystic*
68 *Fibrosis Transmembrane Conductance Regulator*; MIM#602421), that result in loss or
69 impairment of CFTR-mediated ion transport across epithelial cell membrane. The
70 clinical expression and severity of CF are highly variable. Severe phenotypes are
71 usually associated with high concentrations of sweat chloride, early onset of pancreatic
72 insufficiency (PI), meconium ileus at birth and severe lung disease, whereas mild
73 phenotypes are associated with lower sweat chloride concentrations, pancreatic
74 sufficiency (PS) and variable lung disease. Mutations in the *CFTR* gene are also
75 involved in atypical phenotypes¹ such as CBAVD (Congenital Bilateral Absence of the
76 Vas Deferens; MIM # 277180), mild pulmonary diseases or ICP (Idiopathic Chronic
77 Pancreatitis).

78 Since the cloning of the *CFTR* gene, a wide spectrum of molecular abnormalities
79 (more than 1700 mutations, variants or polymorphisms) has already been reported to the
80 Cystic Fibrosis Genetic Analysis Consortium (<http://www.genet.sickkids.on.ca/cftr/>).
81 However, a clear statement on the pathogenicity of a mutation is difficult to obtain, in
82 particular for missense mutations^{2,3}. Moreover, the existence of at least two mutations
83 or sequence variations on the same allele, named complex alleles, complicates genetic
84 counseling⁴⁻¹¹. p.Ser1235Arg (3837T>G or c.3705T>G), initially reported by Cuppens
85 *et al.*¹² with a second mutation on the same allele, p.Gly628Arg, is located in a poorly
86 conserved region in the second nucleotide binding fold (NBF2). Earlier reports failed to
87 establish a clear impact of p.Ser1235Arg on the phenotype severity¹³⁻¹⁶. The aim of this
88 study was to determine whether we should consider p.Ser1235Arg as a possible CF-

89 associated mutation or reclassify it as a neutral polymorphism. Thus, we implemented a
90 large collaborative study to repertory the patients or individuals bearing the
91 p.Ser1235Arg mutation from nine French laboratories and compared their phenotype,
92 genotype and associated *CFTR* haplotypes. To predict the potential effect of this
93 sequence variation on the CFTR protein, *in silico* analyses using various programs and
94 functional studies were performed. Here, we gather lines of evidence that p.Ser1235Arg
95 should be no longer considered as a CF mutation.

96

97 **Materials and Methods**

98 ***Patients and individuals***

99 Data were collected from one hundred and four subjects heterozygous or compound
100 heterozygous for the p.Ser1235Arg mutation registered from the French CF network of
101 molecular genetics laboratories. Among them, sixty-seven were referred for diagnosis:
102 classical CF (six unrelated patients), CAVD (ten individuals with bilateral and one with
103 unilateral Congenital Absence of the Vas Deferens), fetal suspicion of CF (eight fetuses
104 with abnormal ultrasound signs of bowel anomalies) and forty-two individuals
105 presenting CF-related symptoms (according to the International Classification of
106 Disease (ICD) in the section “Cystic Fibrosis and Related Disorders” [Meeting report,
107 2002]) with normal or borderline sweat chloride values including genital (five
108 individuals), respiratory (twenty-three individuals) or digestive (fourteen individuals)
109 symptoms. Thirty-seven healthy subjects were referred for carrier screening including
110 fourteen individuals with a positive family history and twenty-three partners of CF
111 patients or carriers. Written consents to the genetic study were obtained from the
112 patients and/or their family and from healthy subjects.

113

114 ***Molecular epidemiological study***

115 To evaluate the p.Ser1235Arg frequency in the general population, we screened for
116 2114 samples: 929 anonymized dried-blood spot of neonates presenting positive or
117 negative IRT (Immuno Reactive Trypsinogen) at day 3 obtained from the center for
118 neonatal screening in Montpellier, South of France and 1185 genomic DNA from CF
119 patient's or relative's partners from the cohort of the Southern France (Laboratory of
120 molecular genetics of Montpellier) or from the cohort of the Northern France
121 (Laboratory of molecular genetics of Lille).

122

123 ***Mutation Nomenclature***

124 Gene variants at the protein level were named as recommended in the Human Genome
125 Variation Society web page (<http://www.hgvs.org/mutnomen/>). For variations described
126 at the nucleotide level, the A of the ATG translation start codon was numbered as +133
127 in accordance with the current *CFTR* gene numbering based on cDNA sequence
128 (GenBank NM_000492.3) and on the CF mutation database. These variations were also
129 given in parentheses following the approved nomenclature format (A of the ATG
130 translation start codon as +1).

131

132 ***CFTR Genotype Analysis***

133 **Genomic DNA**

134 Genomic DNA was prepared from peripheral blood leukocytes or from amniotic
135 liquid according to standard protocols. *Patients:* The *CFTR* gene coding and flanking
136 regions were analyzed by PCR amplification followed by DGGE (Denaturing Gel

137 Gradient Electrophoresis), SSCA (Single Strand Conformation Analysis), DHPLC
138 (Denaturing High Performance Liquid Chromatography) or heteroduplex analysis
139 followed by sequencing each abnormal pattern detected to characterize the variants. If
140 p.Ser1235Arg was found alone, a screening for large *CFTR* rearrangements was
141 performed using a semi-quantitative fluorescent multiplex PCR assay. *Patients'*
142 *relatives*: A screening for the familial mutations was implemented. *Patients' partners*:
143 In primary screening, five to twelve exons, including exons 13 and 19, were screened by
144 laboratories.

145 To determine the haplotype associated with the p.Ser1235Arg allele, 6 microsatellite
146 markers (IVS1(CA), IVS8(CA), IVS8(TG)m, IVS8T(n), IVS17b(TA), IVS17b(CA))
147 and one biallelic marker (p.Met470Val or 1540A>G(c.1408A>G)) were investigated.

148 **Guthrie cards**

149 Spots of 3 mm diameter, punched from anonymized cards, were distributed in 96-well
150 plates and DNA was extracted using methanol extraction¹⁷. p.Ser1235Arg was screened
151 for using DHPLC technology and each abnormal pattern was confirmed by sequencing
152 analysis.

153

154 ***Computer-assisted analysis***

155 To predict the potential pathogenicity of p.Ser1235Arg, we used PolyPhen® and
156 SIFT® softwares. The PolyPhen® program (<http://coot.embl.de/PolyPhen/>) is based on
157 the sequence homology and the mapping of the substitution site to known protein 3-
158 dimensional structures. Results are given as "benign", "possibly damaging", "probably
159 damaging", or "unknown". The SIFT® program (<http://blocks.fhrc.org/sift/>) uses
160 sequence homology to predict whether an amino acid substitution will affect protein

161 function and hence, potentially alters phenotype. Results are reported as "deleterious or
162 not". Consequences on the splicing machinery were evaluated by Human Splicing
163 Finder tool (<http://www.umd.be/HSF>). This program integrates all available matrices to
164 identify ESE, ESS, ISE and ISS motifs: ESE Finder ¹⁸, RESCUE ESE ¹⁹, Silencer
165 motifs from Sironi et al. ²⁰, ESS decamers from Wang et al. ²¹ and new algorithms to
166 calculate the consensus values of potential splice sites and search for Branch points. To
167 gain some insight into the potential effect of the p.Ser1235Arg mutation from a
168 structural point of view, we used the experimental structures of MJ0796 (in complex
169 with ATP, pdb identifier 112t) as template for modeling as previously described by
170 Eudes et al. ²². The MJ0796 structure was visualized with MBT SimpleViewer software.
171 The alignment of sequences of the two CFTR NBDs, other ABC transporters family and
172 MJ0796 was obtained by ClustalW program and visualized using Jalview 2.3 tools.

173

174 *In vitro functional studies*

175 **CFTR expression plasmids, Site-Directed Mutagenesis, minigene constructs and** 176 **cells**

177 A plasmid expressing the human *CFTR* gene was constructed by placing the full-length
178 *CFTR* cDNA coding sequence obtained from pTG5985 plasmid, a gift from Transgene
179 SA, in the pcDNA3(+) expression vector. The p.Ser1235Arg mutation was inserted into
180 the pcDNA3-CFTR vector by site-directed mutagenesis using QuickChange® II Site-
181 Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions.

182 The human *CFTR* genomic region encompassing the exon 19 with the flanking intronic
183 sequences, was amplified from a patient heterozygous for the p.Ser1235Arg using
184 primers FOR 5'AATTCTGGAGCTCGAGCAGGCCTATACAGAGCCCATT-3' and

185 REV 5' CTCTTAATTTGCTAGCCATTTTCAAGATGGGAAATCTAAAACA-3'
186 (which introduce NheI and XhoI sites in the PCR product). After enzymatic digestion,
187 the PCR product was subcloned into the *XhoI/NheI* digested pSPL3 plasmid, kindly gift
188 by Dr. S. Tuffery-Giraud (Laboratory of Molecular Genetics, Montpellier, France). One
189 clone containing the wild-type allele and one with the mutation were retained for
190 expression experiments. All plasmid constructs were fully sequenced.

191 Beas2B, a human bronchial epithelial cell line expressing CFTR and the monkey kidney
192 fibroblast cells COS-7, which does not express endogenous CFTR, were cultured as
193 previously described ²³. Cells were seeded at a density of 200, 000 cells/2 ml of
194 medium in 6-well dishes. After 24h, cells were transfected with Polyfect transfection
195 reagent (Qiagen, France) according to the manufacturer's recommendations. All
196 experiments were repeated at least twice.

197 **RNA extraction and RT-PCR**

198 Total RNA was extracted using RNeasy Plus kit (Qiagen, France). Reverse transcription
199 was performed with 1 µg of total RNA as previously described ²³. 1 µl of the cDNA
200 synthesis reaction was used for PCR analyses using a mixture of 10 pmol of both
201 SD6/SA2 primers and β-actin primers, 0.5 U of Taq DNA polymerase (New England
202 Bio Labs Inc) and 10 mM of dNTPs in a 25 µl volume. Amplification was performed
203 with an initial denaturation step at 94°C for 5 min, 35 cycles of 94°C 30s, 57°C 30s,
204 72°C 90s, followed by a final extension step 72°C 7 min. PCR products were visualized
205 on 1.5 % agarose gel. Each PCR product was purified and fully sequenced.

206 **Whole cells extracts and Western-Blot**

207 Whole cells extracts from 6-well dishes were extracted and resuspended in Laemmli
208 buffer. Proteins were separated by SDS-PAGE in a 5% acrylamide gel, transferred in a

209 PVDF membrane and immunoblotted with antibody against CFTR (MM13-4 from
210 Millipore).

211

212 *Statistical analysis*

213 Statistical analyses were performed using InStat® software. Chi-square test was used to
214 compare differences between general population vs CF patients or general population vs
215 CBAVD patients. P-values < 0.05 were considered to indicate statistical significance.

216

217 **Results**

218 **Genotype/phenotype description of individuals carrying p.Ser1235Arg**

219 Among 104 subjects from 91 unrelated families reported by the nine French
220 laboratories, 6 patients are affected with classical CF (Table 1A). Five of them were
221 referred for severe disease with diagnosis before one year, positive sweat test,
222 pancreatic insufficiency and persistent respiratory infections, and one had a mild
223 phenotype with a borderline sweat test. Complete scanning of the 27 exons and their
224 boundaries identified a second mutation on the same allele for all of them: 1)
225 p.Arg785X, located in exon 13, predicting a truncated protein; 2) 875+1G>A
226 (c.743+1G>A) affecting the highly conserved splice donor site sequence; 3) the
227 (TG)13(T)5 allele which promotes CFTR exon 9 skipping in humans²⁴⁻²⁶.

228 Ten patients were referred for CBAVD (Congenital Bilateral Absence of Vas
229 Deferens) and one for CUAVD (Congenital Unilateral Absence of Vas Deferens)
230 without renal anomalies. The (TG)13(T)5 allele was found in 9 subjects with
231 p.Ser1235Arg (Table 1A). Of these, eight were compound heterozygous for

232 p.Phe508del, and two for mutations associated with CF-related diseases^{3,27},
233 [p.Arg117His;T7] and p.Arg1070Trp (<http://www.genet.sickkids.on.ca/cftr/>).

234 Among 42 patients with monosymptomatic disease carrying p.Ser1235Arg (Table
235 1A), eight cases (19%) were compound heterozygotes, seven for a severe mutation and
236 one for the intronic variant 406-6T>C (c.274-6T>C). Of these, five harbored the
237 (TG)13(T)5 allele associated in *cis* with p.Ser1235Arg. Thirty-four individuals (81%)
238 were heterozygous for p.Ser1235Arg: 5 with reduced sperm quality, 16 with
239 sinopulmonary problems such as bronchiectasis, asthma, nasal polyposis, chronic
240 obstructive pulmonary disease, and 13 with digestive symptoms (meconium ileus at
241 birth, pancreatitis, liver disease). One of them, referred for bronchitis and recurrent
242 infections without bronchiectasis, had a positive sweat test (102 mmol/l).

243 Eight fetal samples were referred for risk calculation in the cohort of
244 hyperechogenic fetal bowel. Among them, five carried only p.Ser1235Arg and were
245 considered simple heterozygote because extensive scanning did not reveal a second CF
246 mutation²⁸. Three cases were compound heterozygous for another mutation. In the first
247 case, a moderate hyperechogenicity was detected at 22 weeks' pregnancy, which
248 decreased at 31. The fetus harbored the p.Phe508del mutation on the other allele and
249 parents decided to continue pregnancy. The second carried the rare missense mutation
250 p.Val920Met and depressed values of digestive enzymes were observed at 17 weeks of
251 pregnancy. In the last case, the fetus carried the complex allele
252 [p.Gly576Ala ;p.Arg668Cys], considered as a mild mutation involved in CBAVD or
253 disseminated bronchiectasis phenotypes in adults²⁹⁻³¹. Although the fetus presented
254 hyperechogenic fetal bowel, the parents were reassured regarding the risk of classical
255 CF. Unfortunately, no data were available on the outcome of these pregnancies.

256 Among 37 individuals tested for cascade carrier screening, 14 were compound
257 heterozygous for p.Ser1235Arg and another CFTR mutation (Table 1B), all being
258 relatives of patients or carriers. Of these, twelve harbored in *trans* a CF-causing
259 mutation. No symptom of CF was notified although sweat test was not performed.
260 Notably, cases 4 and 5 were two fertile fathers carrying a Class I mutation (severe)
261 associated in *trans* of p.Ser1235Arg.

262

263 ***Frequencies of p.Ser1235Arg in the general, CF and CBAVD French populations***

264 Thirty-two p.Ser1235Arg alleles were detected in 2114 subjects of the general
265 population representing an allelic frequency of 0.76% (Table 2). None of them had the
266 p.Arg785X mutation. This allele frequency was compared with those of patients
267 collated in a French study affected with CF (8/7420 alleles) or CBAVD (7/1626 alleles),
268 0.11% and 0.43%, respectively ³². By χ^2 statistical analysis, the p.Ser1235Arg allelic
269 frequency is significantly higher in the general population than in CF patients (0.76%
270 vs. 0.11%, $p < 0.001$) but not statistically different from the CBAVD group (0.76% vs.
271 0.43%, $p > 0.1$). Recently, six CF patients were re-analyzed. Among them, five with
272 severe disease, harbored the p.Arg785X mutation, while another presenting a mild
273 phenotype had the complex allele [p.Ser1235Arg;T5]. When the p.Ser1235Arg
274 frequencies in CF, CBAVD and the general population are compared to frequencies of
275 p.Phe508del (Table 2), it appears that p.Ser1235Arg is as frequent as p.Phe508del
276 ($p=0.91$) in the general population (respectively 0.76% vs. 0.82%); however, its
277 implication in classical CF is significantly different (0.11% vs. 67.2% ($p < 0.0001$) and
278 2.86% ($p < 0.0001$) respectively).

279

280 ***p.Ser1235Arg haplotype backgrounds***

281 Haplotypic markers and familial segregation were completed in thirty-six cases
282 (Table 3). The analysis of haplotypes based on four intragenic *CFTR* markers
283 (IVS1(CA), IVS8(CA), M470V, IVS17b(CA)) revealed that p.Ser1235Arg is always
284 present on the same haplotype 26-17-M-13. This haplotype associated with the
285 polyvariant (TG)12(T)7 represents 77.8% of cases. Only the IVS17b(TA) microsatellite
286 marker shows a large variability in this study ranging from 7 to 44 repeats. However,
287 three complete haplotypes differing only by one repeat in the IVS17b(TA) (35, 36 and
288 37) accounted for more than 63% of the cases. The p.Arg785X found in CF patients in
289 *cis* of the p.Ser1235Arg occurs on a single haplotype 26-17-12-7-M-35-13. The
290 complex allele [p.Ser1235Arg;(TG)13(T)5] characteristic of CBAVD, was also found to
291 be carried on a unique haplotype (26-17-13-5-M-33-13).

292

293 ***In silico predictions and functional impact on the CFTR protein***

294 We evaluated the degree of conservation of serine 1235 residue using alignment
295 of the Nucleotide Binding Domains of the human *CFTR* with those of ABC
296 transporters, whose the *CFTR* belongs (Figure 1A). The results show that the serine
297 1235 is poorly conserved in the ABC transporter family, suggesting that amino acid
298 change in this position is possible. Serine 1235 residue located after a β strand is not
299 involved in anchor or structural points³³. The analysis of its position on the 3D
300 structure of MJ0796 (Figure 1B) previously used as template for modeling the *CFTR*
301 NBDs²², points that the Serine 1235 is located on the exterior surface of the NBD2.
302 Therefore, this amino acid does not participate to the NBD1/NBD2 heterodimer

303 formation and to the ATP binding and hydrolysis. The limited effect of this amino acid
304 change was confirmed using SIFT® and PolyPhen® programs. To evaluate the
305 functional impact of the mutation on the CFTR maturation, we performed Western-blot
306 analysis on whole cell extracts from COS-7 cells transfected with either wild-type or the
307 p.Ser1235Arg constructs (figure 1C). The results show the presence of the fully-
308 glycosylated CFTR protein (Band C) with the p.Ser1235Arg construct, suggesting that
309 the maturation of the pSer1235Arg-CFTR protein is not altered. These results suggest
310 that p.Ser1235Arg has no functional impact on the CFTR protein.

311

312 ***Functional impact of the pSer1235Arg on the splicing***

313 According to the Splicing Sequences Finder tools³⁴, p.Ser1235Arg does not
314 seem to impact the splicing of *CFTR* transcript. To verify this prediction, we used a
315 minigene-based approach. Constructs containing the wild type and mutant *CFTR* exon
316 19 and the intron-exon boundaries were inserted in the pSPL3 vector (figure 2A). After
317 transfection in Beas2B cell line, RNAs were extracted and subjected to RT-PCR. As
318 shown in figure 2B, compared to the wild-type, the presence of the pSer1235Arg
319 alteration does not modify the exon 19 inclusion in the mRNA. Together, these data
320 suggest that pSer1235Arg has no effect on the *CFTR* mRNA splicing.

321

322 **Discussion**

323 Since its initial description in 1993, the clinical significance of the p.Ser1235Arg
324 mutation remains unclear. This ignorance makes genetic counseling very difficult,
325 especially in the context of prenatal diagnosis. Through genotype/phenotype analysis of
326 patients or individuals carrying p.Ser1235Arg, analyzed by the French network and

327 functional analysis, we present several lines of evidence that argue against an
328 association of p.Ser1235Arg with CF or CBAVD although a partial penetrance of
329 p.Ser1235Arg could not be ruled out.

330 First, the presence of a complex allele [p.Ser1235Arg;class I mutation] in *trans*
331 of a severe mutation in five patients presenting severe disease (Table 1A) strongly
332 suggests that p.Ser1235Arg alone cannot be considered as a CF-causing mutation.
333 Moreover, among the eleven patients referred for CAVD, nine are compound
334 heterozygous for a mutation and the complex allele [p.Ser1235Arg;(TG)13(T)5]. The
335 (TG)13(T)5 allele without the contribution of p.Ser1235Arg is sufficient to result in
336 male infertility or in mild phenotypes^{24,25,35}. Among the two patients with p.Ser1235Arg
337 as single allele, one has the mutation p.Phe508del in *trans* while no other mutation was
338 identified in the second one. The possibility of mutations in intronic regions
339 undetectable by our methods cannot be excluded.

340 Second, we present 14 asymptomatic individuals (Table 1B), of whom 12
341 carried a severe and two a mild mutation in *trans*. Three, carrying the complex allele
342 [p.Ser1235Arg; (TG)13(T)5], are sisters or brother of CBAVD or CUAVD patients and
343 no respiratory or digestive symptom was reported. In females, allele (T)5 has been
344 shown to have a low penetrance³⁶ while no urogenital data was available for the male
345 because of absence of parental project. Three adult males are compound heterozygous
346 for p.Ser1235Arg and a severe mutation; two (cases 4 and 5) are biological fathers of
347 CF children and transmitted the severe mutation. Case 7 to 11 are patients' mothers or
348 relatives with no evidence of CF symptoms. Cases 12 and 13 are non-symptomatic
349 sister and first-cousin of a patient with respiratory symptoms whose CF diagnostic
350 could be discussed because symptoms are close to asthma.

351 Third, the frequency of the p.Ser1235Arg in the general population (Table 2) is
352 higher than in CF and CBAVD patients, in accordance with previous reports^{14,37}, and is
353 close to the p.Phe508del frequency (0.82%). By contrast, in CF or CBAVD groups, the
354 p.Ser1235Arg frequency is significantly lower than p.Phe508del.

355 In the functional point of view, results from alignment show that this residue is
356 poorly conserved and occurs in a region not implicated in known CFTR functions.
357 Moreover, *in silico* predictions suggest that this nucleotide change is not critical for the
358 mRNA splicing and the CFTR function. These data are confirmed by functional
359 studies, which showed that p.Ser1235Arg does not alter the *CFTR* mRNA correct
360 splicing and the protein maturation. These data are consistent with previous functional
361 study of this locus in combination with alleles found at p.Met470Val and p.Gly628Arg
362 loci¹³. Wei *et al.* demonstrated that the p.Ser1235Arg CFTR protein does not cause
363 change in the chloride transport activity. Besides, the p.Gly628Arg/p.Ser1235Arg
364 mutant protein induces a significantly lower cAMP dependent chloride transport
365 activity than the p.Gly628Arg mutant protein, showing the major importance of genetic
366 background, particularly for missense mutations.

367

368 In a second step, we determined the haplotypes linked to p.Ser1235Arg and
369 found the same haplotype in 72.4% of cases. Only the IVS17b(TA) marker presents a
370 large variability although three haplotypes, deriving one from the other by addition or
371 deletion of one dinucleotide (35, 36 or 37 repeats), represent more than 55% of the
372 alleles. Besides, IVS1(CA) 26, a rare allele in the European populations (4.2% vs 80%
373 for alleles 22, 23 ad 24)³⁸, is strictly linked to p.Ser1235Arg. In CBAVD patients, the
374 haplotype 26-17-13-5-M-33-13 was found in 85.7% of p.Ser1235Arg alleles. This data

375 suggests the further change of the (TG)12(T)7 to (TG)13(T)5 on a chromosome bearing
376 the alteration p.Ser1235Arg on a background IVS17b(TA)33. Together, this data
377 suggests that p.Ser1235Arg could derive from a founding event and haplotypes
378 differing by a single microsatellite could depend on slippage phenomena, as already
379 proposed³⁹.

380

381 In conclusion, there are now strong lines of evidence against a severe deleterious
382 effect of p.Ser1235Arg and its association with classical CF or CBAVD disease.
383 However, a partial penetrance and/or the presence of other sequence alterations in non-
384 screened regions particularly in patients with atypical or mild symptoms of cystic
385 fibrosis^{27,40,41} cannot be ruled-out. This data highlights the importance of searching for
386 a complex allele whenever p.Ser1235Arg is identified. The absence of clinical effects in
387 healthy individuals compound heterozygous for the p.Ser1235Arg and another severe
388 mutation is helpful to provide adequate genetic counseling to the families with regard to
389 its transmission in offspring.

390

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394

395 **Conflict of interest.**

396 The authors declare no conflict of interest.

397

398 **References**

399

400 1. Kerem E: Atypical CF and CF related diseases. *Paediatr Respir Rev* 2006; **7**

401 **Suppl 1**: S144-146.

402

403 2. Cotton RG, Scriver CR: Proof of "disease causing" mutation. *Hum Mutat* 1998;

404 **12**: 1-3.

405

406 3. Castellani C, Cuppens H, Macek M, Jr. *et al*: Consensus on the use and

407 interpretation of cystic fibrosis mutation analysis in clinical practice. *J Cyst*

408 *Fibros* 2008; **7**: 179-196.

409

410 4. Savov A, Angelicheva D, Balassopoulou A, Jordanova A, Noussia-Arvanitakis

411 S, Kalaydjieva L: Double mutant alleles: are they rare? *Hum Mol Genet* 1995; **4**:

412 1169-1171.

413

414 5. Fanen P, Clain J, Labarthe R *et al*: Structure-function analysis of a double-

415 mutant cystic fibrosis transmembrane conductance regulator protein occurring in

416 disorders related to cystic fibrosis. *FEBS Lett* 1999; **452**: 371-374.

417

418 6. Romey MC, Guittard C, Chazalotte JP *et al*: Complex allele [-

419 102T>A+S549R(T>G)] is associated with milder forms of cystic fibrosis than

420 allele S549R(T>G) alone. *Hum Genet* 1999; **105**: 145-150.

421

- 422 7. Clain J, Fritsch J, Lehmann-Che J *et al*: Two mild cystic fibrosis-associated
423 mutations result in severe cystic fibrosis when combined in cis and reveal a
424 residue important for cystic fibrosis transmembrane conductance regulator
425 processing and function. *J Biol Chem* 2001; **276**: 9045-9049.
426
- 427 8. Rohlfes EM, Zhou Z, Sugarman EA *et al*: The I148T CFTR allele occurs on
428 multiple haplotypes: a complex allele is associated with cystic fibrosis. *Genet*
429 *Med* 2002; **4**: 319-323.
430
- 431 9. Clain J, Lehmann-Che J, Girodon E *et al*: A neutral variant involved in a
432 complex CFTR allele contributes to a severe cystic fibrosis phenotype. *Hum*
433 *Genet* 2005; **116**: 454-460.
434
- 435 10. Niel F, Legendre M, Bienvenu T *et al*: A new large CFTR rearrangement
436 illustrates the importance of searching for complex alleles. *Hum Mutat* 2006; **27**:
437 716-717.
438
- 439 11. Claustres M, Altieri JP, Guittard C, Templin C, Chevalier-Porst F, Des Georges
440 M: Are p.I148T, p.R74W and p.D1270N cystic fibrosis causing mutations?
441 *BMC Med Genet* 2004; **5**: 19.
442
- 443 12. Cuppens H, Marynen P, De Boeck C, Cassiman JJ: Detection of 98.5% of the
444 mutations in 200 Belgian cystic fibrosis alleles by reverse dot-blot and

- 445 sequencing of the complete coding region and exon/intron junctions of the
446 CFTR gene. *Genomics* 1993; **18**: 693-697.
- 447
- 448 13. Wei L, Vankeerberghen A, Jaspers M, Cassiman J, Nilius B, Cuppens H:
449 Suppressive interactions between mutations located in the two nucleotide
450 binding domains of CFTR. *FEBS Lett* 2000; **473**: 149-153.
- 451
- 452 14. Monaghan KG, Feldman GL, Barbarotto GM, Manji S, Desai TK, Snow K:
453 Frequency and clinical significance of the S1235R mutation in the cystic fibrosis
454 transmembrane conductance regulator gene: results from a collaborative study.
455 *Am J Med Genet* 2000; **95**: 361-365.
- 456
- 457 15. Dequeker E, Stuhmann M, Morris MA *et al*: Best practice guidelines for
458 molecular genetic diagnosis of cystic fibrosis and CFTR-related disorders--
459 updated European recommendations. *Eur J Hum Genet* 2009; **17**: 51-65.
- 460
- 461 16. Oca F, Dreux S, Gerard B *et al*: Amniotic fluid digestive enzyme analysis is
462 useful for identifying CFTR gene mutations of unclear significance. *Clin Chem*
463 2009; **55**: 2214-2217.
- 464
- 465 17. Caggana M, Conroy JM, Pass KA: Rapid, efficient method for multiplex
466 amplification from filter paper. *Hum Mutat* 1998; **11**: 404-409.
- 467

- 468 18. Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR: ESEfinder: A web resource
469 to identify exonic splicing enhancers. *Nucleic Acids Res* 2003; **31**: 3568-3571.
470
- 471 19. Fairbrother WG, Yeh RF, Sharp PA, Burge CB: Predictive identification of
472 exonic splicing enhancers in human genes. *Science* 2002; **297**: 1007-1013.
473
- 474 20. Sironi M, Menozzi G, Riva L *et al*: Silencer elements as possible inhibitors of
475 pseudoexon splicing. *Nucleic Acids Res* 2004; **32**: 1783-1791.
476
- 477 21. Wang Z, Rolish ME, Yeo G, Tung V, Mawson M, Burge CB: Systematic
478 identification and analysis of exonic splicing silencers. *Cell* 2004; **119**: 831-845.
479
- 480 22. Eudes R, Lehn P, Ferec C, Mornon JP, Callebaut I: Nucleotide binding domains
481 of human CFTR: a structural classification of critical residues and disease-
482 causing mutations. *Cell Mol Life Sci* 2005; **62**: 2112-2123.
483
- 484 23. Rene C, Taulan M, Iral F *et al*: Binding of serum response factor to cystic
485 fibrosis transmembrane conductance regulator CArG-like elements, as a new
486 potential CFTR transcriptional regulation pathway. *Nucleic Acids Res* 2005; **33**:
487 5271-5290.
488
- 489 24. Disset A, Michot C, Harris A, Buratti E, Claustres M, Tuffery-Giraud S: A T3
490 allele in the CFTR gene exacerbates exon 9 skipping in vas deferens and

- 491 epididymal cell lines and is associated with Congenital Bilateral Absence of Vas
492 Deferens (CBAVD). *Hum Mutat* 2005; **25**: 72-81.
- 493
- 494 25. Groman JD, Hefferon TW, Casals T *et al*: Variation in a repeat sequence
495 determines whether a common variant of the cystic fibrosis transmembrane
496 conductance regulator gene is pathogenic or benign. *Am J Hum Genet* 2004; **74**:
497 176-179.
- 498
- 499 26. Claustres M: Molecular pathology of the CFTR locus in male infertility. *Reprod*
500 *Biomed Online* 2005; **10**: 14-41.
- 501
- 502 27. Feldmann D, Couderc R, Audrezet MP *et al*: CFTR genotypes in patients with
503 normal or borderline sweat chloride levels. *Hum Mutat* 2003; **22**: 340.
- 504
- 505 28. Muller F, Simon-Bouy B, Girodon E, Monnier N, Malinge MC, Serre JL:
506 Predicting the risk of cystic fibrosis with abnormal ultrasound signs of fetal
507 bowel: results of a French molecular collaborative study based on 641
508 prospective cases. *Am J Med Genet* 2002; **110**: 109-115.
- 509
- 510 29. Bombieri C, Benetazzo M, Saccomani A *et al*: Complete mutational screening
511 of the CFTR gene in 120 patients with pulmonary disease. *Hum Genet* 1998;
512 **103**: 718-722.
- 513

- 514 30. Casals T, De-Gracia J, Gallego M *et al*: Bronchiectasis in adult patients: an
515 expression of heterozygosity for CFTR gene mutations? *Clin Genet* 2004; **65**:
516 490-495.
- 517
- 518 31. Pallares-Ruiz N, Carles S, Des Georges M *et al*: Complete mutational screening
519 of the cystic fibrosis transmembrane conductance regulator gene: cystic fibrosis
520 mutations are not involved in healthy men with reduced sperm quality. *Hum*
521 *Reprod* 1999; **14**: 3035-3040.
- 522
- 523 32. Claustres M, Guittard C, Bozon D *et al*: Spectrum of CFTR mutations in cystic
524 fibrosis and in congenital absence of the vas deferens in France. *Hum Mutat*
525 2000; **16**: 143-156.
- 526
- 527 33. Callebaut I, Eudes R, Mornon JP, Lehn P: Nucleotide-binding domains of
528 human cystic fibrosis transmembrane conductance regulator: detailed sequence
529 analysis and three-dimensional modeling of the heterodimer. *Cell Mol Life Sci*
530 2004; **61**: 230-242.
- 531
- 532 34. Desmet F, Hamroun D, Lalande M, Collod-Bérout G, Claustres M, Bérout C:
533 Human Splicing Finder: an online bioinformatics tool to predict splicing signals.
534 *Nucleic Acids Res* 2009; **37**: e67.
- 535
- 536 35. Cuppens H, Lin W, Jaspers M *et al*: Polyvariant mutant cystic fibrosis
537 transmembrane conductance regulator genes. The polymorphic (Tg)m locus

538 explains the partial penetrance of the T5 polymorphism as a disease mutation. *J*
539 *Clin Invest* 1998; **101**: 487-496.

540

541 36. Sun W, Anderson B, Redman J *et al*: CFTR 5T variant has a low penetrance in
542 females that is partially attributable to its haplotype. *Genet Med* 2006; **8**: 339-
543 345.

544

545 37. Modiano G, Bombieri C, Ciminelli BM *et al*: A large-scale study of the random
546 variability of a coding sequence: a study on the CFTR gene. *Eur J Hum Genet*
547 2005; **13**: 184-192.

548

549 38. Mateu E, Calafell F, Bonne-Tamir B *et al*: Allele frequencies in a worldwide
550 survey of a CA repeat in the first intron of the CFTR gene. *Hum Hered* 1999;
551 **49**: 15-20.

552

553 39. Claustres M, Desgeorges M, Moine P, Morral N, Estivill X: CFTR haplotypic
554 variability for normal and mutant genes in cystic fibrosis families from southern
555 France. *Hum Genet* 1996; **98**: 336-344.

556

557 40. Castellani C, Benetazzo MG, Tamanini A, Beghini A, Mastella G, Pignatti P:
558 Analysis of the entire coding region of the cystic fibrosis transmembrane
559 regulator gene in neonatal hypertrypsinaemia with normal sweat test. *J Med*
560 *Genet* 2001; **38**: 202-205.

561

- 562 41. Reboul MP, Laharie D, Amouretti M, Lacombe D, Iron A: Isolated idiopathic
563 chronic pancreatitis associated with a compound heterozygosity for two
564 mutations of the CFTR gene. *Gastroenterol Clin Biol* 2003; **27**: 821-824.
565
- 566 42. Robertson NH, Weston SL, Kelly SJ *et al*: Development and validation of a
567 screening test for 12 common mutations of the cystic fibrosis CFTR gene. *Eur*
568 *Respir J* 1998; **12**: 477-482.
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573 **Titles and legends to figures**

574 Figure1: Structural and processing impact of p.Ser1235Arg mutation on the CFTR
575 protein.

576 A: Conservation of residues close to Ser1235 (blue square) in the two NBD sequences
577 of human CFTR and other ABC transporters. Positions always occupied by
578 hydrophobic amino acids (V, I, L, M, F, Y, W) are shaded blue. Conserved glycine and
579 lysine are colored in orange and in red, respectively.

580 B: Localization of the serine 1235 on the 3D-modelization of the NBD1/NBD2
581 heterodimer .

582 C: COS-7 cell line were transfected with either wild-type or p.Ser1235Arg CFTR
583 constructs. 48 hours post-tranfection, whole-cell proteins were extracted and subjected
584 to a Western-blot analysis. Experiments were repeated three times.

585

586 Figure 2: Functional impact on the *CFTR* mRNA splicing

587 A: Schema of the minigene constructs in pSPL3 vector. The exon 19 and exon-intron
588 junctions were inserted in the multiple cloning site of the pSPL3 vector between the
589 exon A and B from the β -globin by PCR-restriction.

590 B: RT-PCR analysis of mRNA of cell expressing Wild-Type⁴² or
591 p.Ser1235Arg(S1235R) constructs. Beas2B cells were transfected with either empty,
592 wild-type or p.Ser1235Arg pSPL3 vector. After 48 hours, total RNA were extracted and
593 cDNA were synthesized. PCR was performed using SA2 and SD6 primers amplifying
594 the region between exon A and B. On the right, the composition of each transcript was
595 indicated. Each experiment was repeated at least three times.

596

597 Table 1A Genotype and phenotype of CF, CBAVD and CF-like patients carrying the
 598 p.Ser1235Arg mutation.

Genotype		phenotype	No of subjects
allele 1	allele 2		
p.Ser1235Arg;p.Arg785X	p.Phe508del	severe CF	2
p.Ser1235Arg;p.Arg785X	NA ¹	severe CF	1
p.Ser1235Arg;875+1G>A (c.743+1C>A)	3629delT (c.3497delT)	severe CF	1
p.Ser1235Arg;p.Arg785X	p.Gly542X	severe CF	1
p.Ser1235Arg;(TG)13(T)5	p.Gly551Asp	mild CF	1
p.Ser1235Arg;(TG)13(T)5	p.Phe508del	CBAVD	6
p.Ser1235Arg;(TG)13(T)5	p.Arg1070Trp	CBAVD	1
p.Ser1235Arg;(TG)13(T)5	p.Arg117His; (T)7	CBAVD	1
p.Ser1235Arg	p.Phe508del	CBAVD	1
p.Ser1235Arg	-	CBAVD	1
p.Ser1235Arg;(TG)13(T)5	p.Phe508del	CUAVD	1
		<u>Suspicion CF/mild phenotype:</u>	
p.Ser1235Arg	-	genital symptoms	5
p.Ser1235Arg	-	respiratory symptoms	16
p.Ser1235Arg;(TG)13(T)5	p.Phe508del	respiratory symptoms	2
p.Ser1235Arg	406-6T>C (c.274-6T>C)	respiratory symptoms	1
p.Ser1235Arg	p.Tyr1092X	respiratory symptoms	1
p.Ser1235Arg	p.Glu831X	respiratory symptoms	1
p.Ser1235Arg	p.Gln493X	respiratory symptoms	1
p.Ser1235Arg	p.Ile507del	respiratory symptoms	1
p.Ser1235Arg	-	digestive symptoms	13
p.Ser1235Arg	p.Gly542X	digestive symptoms	1
p.Ser1235Arg	-	hyperechogenic fetal bowel	5
p.Ser1235Arg	p.Arg668Cys;p.Arg576Ala	hyperechogenic fetal bowel	1
p.Ser1235Arg	p.Val920Met	hyperechogenic fetal bowel	1
p.Ser1235Arg	p.Phe508del	hyperechogenic fetal bowel	1

599 ¹ NA: Not Available ; We could only test the mother and an healthy sister (the patient

600 was deceased and the father's DNA was not available)

601

602 Table 1B Genotype and familial history of asymptomatic individuals carrying the

603 p.Ser1235Arg mutation.

Case #	Genotype		Familial information
	allele 1	allele 2	
1	p.Ser1235Arg;(TG)13(T)5	p.Phe508del	brother of CUAVD (no parental project)
2	p.Ser1235Arg;(TG)13(T)5	p.Phe508del	sister of CUAVD
3	p.Ser1235Arg;(TG)13(T)5	p.Arg1070Trp	sister of CBAVD
4	p.Ser1235Arg	p.Gly542X	father of CF [p.Phe508del]+ [p.Gly542X] and healthy children [p.Ser1235Arg]+ [(TG)11(T)5]
5	p.Ser1235Arg	p.Gln493X	father of CF [p.Phe508del]+[p.Gln493X]
6	p.Ser1235Arg	p.Phe508del	uncle of CF (no parental project)
7	p.Ser1235Arg	p.Phe508del	mother of CF [p.Phe508del]+[1717-1G>A (c.1585-1G>A)]
8	p.Ser1235Arg	2347delG (c.2215delG)	mother of CF [p.Phe508del]+[2347delG]
9	p.Ser1235Arg	(TG)11(T)5	mother of non CF fetus with hyperechogenic fetal bowel [p.Phe508del]+[(TG)11(T)5]
10	p.Ser1235Arg	p.Phe508del	sister of CBAVD
11	p.Ser1235Arg	p.Phe508del	sister of CBAVD
12	p.Ser1235Arg	p.Glu831X	sister of CF-like patient
13	p.Ser1235Arg	p.Phe508del	first-cousin of CF-like patient
14	p.Ser1235Arg	p.Phe508del	18 month-old child with prenatal diagnostic based on familial history of CF

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608 Table 2: Comparison of the allelic frequencies of p.Ser1235Arg and p.Phe508del in the
609 general population, CF and CBAVD patients.

	Populations			Significance (p-values)	
	General population This study	CF patients ³⁰ n=7,420	CBAVD patients ³⁰ n=1,626	General population vs. CF	General population vs. CBAVD
p.Phe508del	0.82% (n=1950)	67.2%	21.6%	S (p< 0.05)	S (p< 0.05)
p.Ser1235Arg	0.76 % (n=4228)	0.11%	0.43%	S (p< 0.05)	NS (p= 0.17)
Significance (p-values) p.Phe508del vs p.Ser1235Arg	NS (p=0.91)	S (p< 0.05)	S (p< 0.05)		

610 n: number of unrelated tested chromosomes. S: Significant difference; NS: No

611 Significant difference. P-values significant at <0.05

612

613

614 Table 3: *CFTR* haplotypes for 36 fully haplotyped p.Ser1235Arg chromosomes from
 615 CF, CBAVD patients, ruled-out CF (including CF-like symptoms and Fetal echogenic
 616 bowel) and general population.

IVS1(CA)- IVS8(CA)-M470V- IVS17b(CA)	IVS8(TG)m - IVS8(T)n	Mutation in <i>cis</i>	IVS17b(TA)	n	%
26-17-M-13	12-7	p.Arg785X 875+1G>A (c.743+1C>A)	35	4	11.1
		None	36	1	2.8
			33	1	2.8
			35	5	13.9
			36	9	25.0
			37	4	11.1
			38	1	2.8
			40	1	2.8
			44	1	2.8
			7	1	2.8
					22.2
	13-5		33	8	24.2

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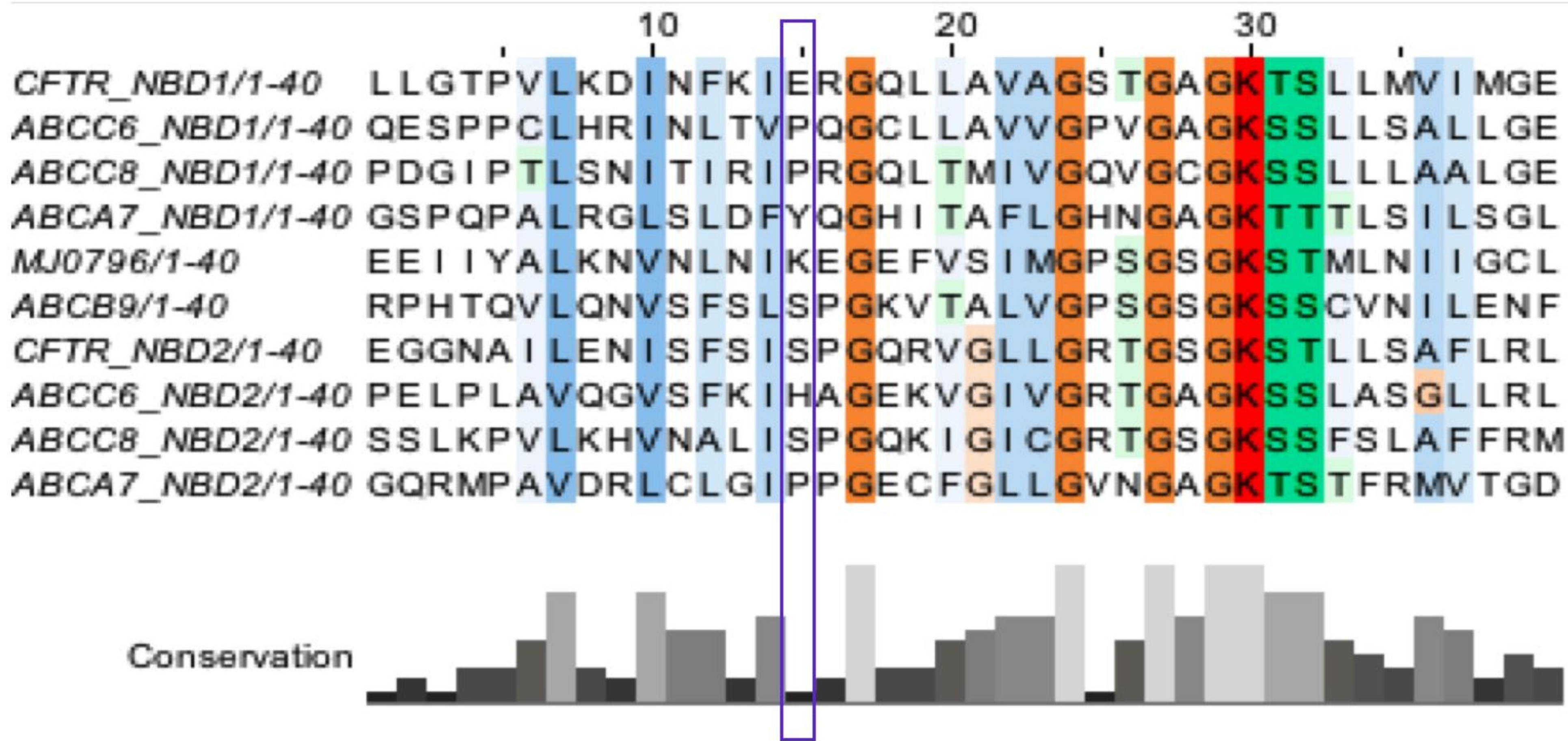
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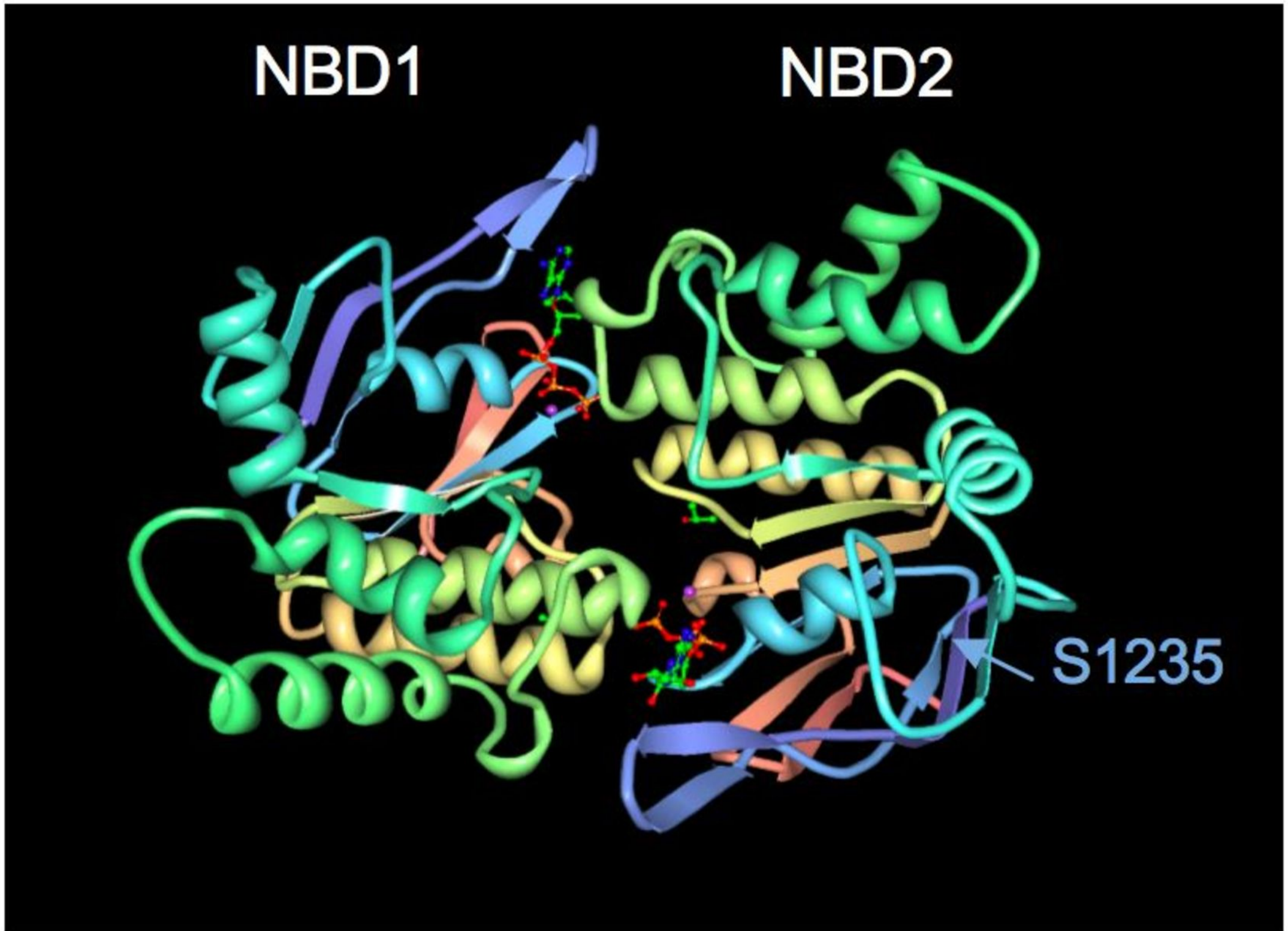
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Figure 1

A



B



C

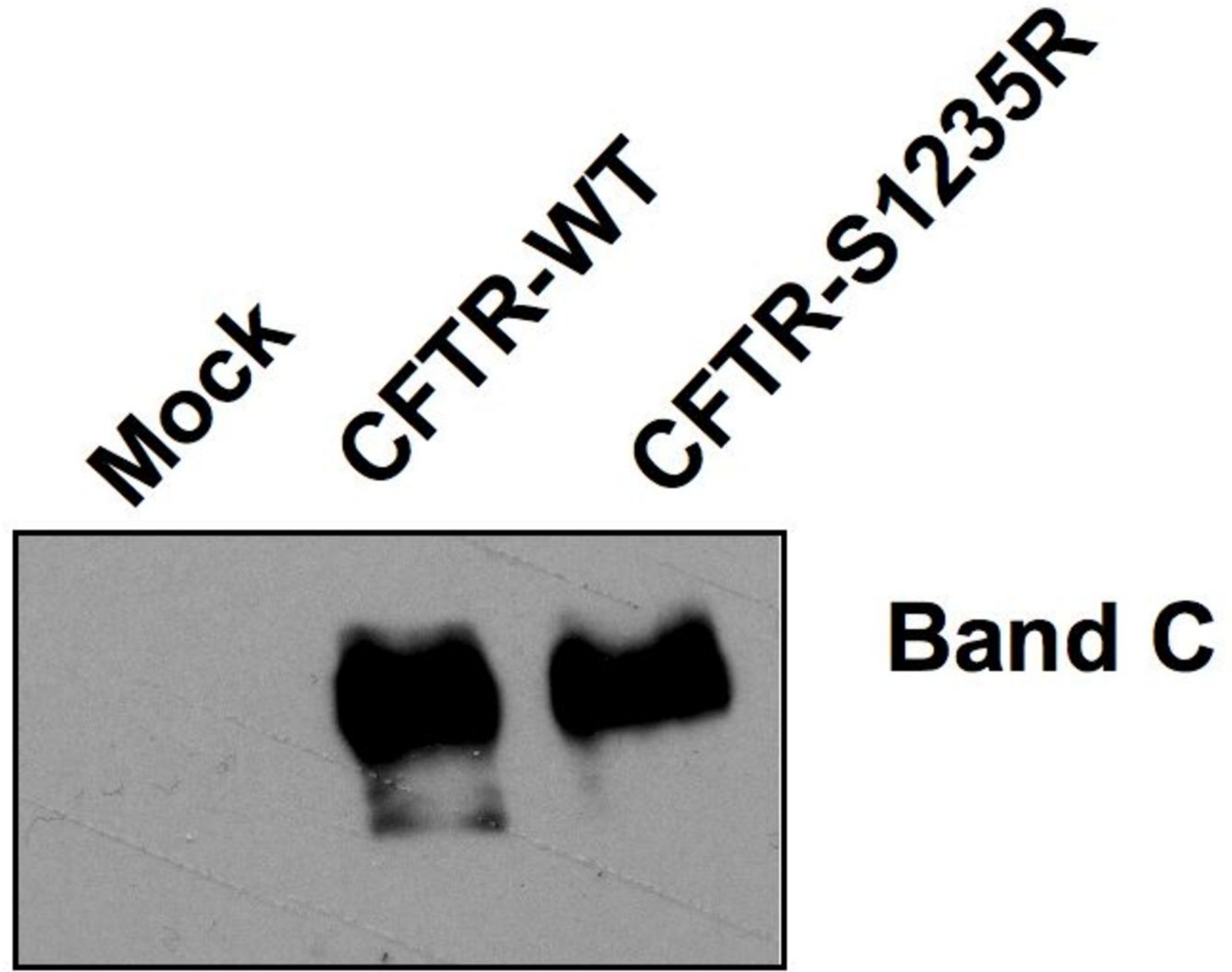


Figure 2

