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

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47   Running title: p.Ser1235Arg is not associated with CF disease

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

Introduction

Cystic Fibrosis (CF, MIM≠219700), one of the most frequent life-shortening recessively inherited diseases, is caused by alterations in the *CFTR* gene (*Cystic Fibrosis Transmembrane Conductance Regulator*; MIM≠602421), that result in loss or impairment of CFTR-mediated ion transport across epithelial cell membrane. The clinical expression and severity of CF are highly variable. Severe phenotypes are usually associated with high concentrations of sweat chloride, early onset of pancreatic insufficiency (PI), meconium ileus at birth and severe lung disease, whereas mild phenotypes are associated with lower sweat chloride concentrations, pancreatic sufficiency (PS) and variable lung disease. Mutations in the *CFTR* gene are also involved in atypical phenotypes[1] such as CBAVD (Congenital Bilateral Absence of the Vas Deferens; MIM # 277180), mild pulmonary diseases or ICP (Idiopathic Chronic Pancreatitis).

Since the cloning of the *CFTR* gene, a wide spectrum of molecular abnormalities (more than 1700 mutations, variants or polymorphisms) has already been reported to the Cystic Fibrosis Genetic Analysis Consortium (http://www.genet.sickkids.on.ca/cftr/). However, a clear statement on the pathogenicity of a mutation is difficult to obtain, in particular for missense mutations [2,3]. Moreover, the existence of at least two mutations or sequence variations on the same allele, named complex alleles, complicates genetic counseling [4-11]. p.Ser1235Arg (3837T>G or c.3705T>G), initially reported by Cuppens *et al.* [12] with a second mutation on the same allele, p.Gly628Arg, is located in a poorly conserved region in the second nucleotide binding fold (NBF2). Earlier reports failed to establish a clear impact of p.Ser1235Arg on the phenotype severity [13-16]. The aim of this 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

Molecular epidemiological study

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

Mutation Nomenclature

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

CFTR Genotype Analysis

Genomic DNA

Genomic DNA was prepared from peripherical blood leukocytes or from amniotic liquid according to standard protocols. *Patients:* The *CFTR* gene coding and flanking 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 extraction[17]. 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.fhcrc.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 1l2t) 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 *Xho*I/*Nhe*I 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 humans [24-26].

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   mutation [28]. 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 adults [29-31]. 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 points [33]. The analysis of its position on the 3D

300 structure of MJ0796 (Figure 1B) previously used as template for modeling the CFTR

301 NBDs [22], 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 tools [34], 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 pSer1235Arg 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 penetrance [36] 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   loci [13]. 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   proposed [39].

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

394

395   **Conflict of interest.**

396   The authors declare no conflict of interest.

397

398   **References**

- 17 -

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, Chazalette 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.   Rohlfs 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, Stuhrmann 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-Béroud G, Claustres M, Béroud 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, Begnini 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-Type[42]   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 synthetized. 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
		Suspicion CF/mild phenotype:	
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

604

605

606

607

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

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

IVS1(CA)-IVS8(CA)-M470V-IVS17b(CA)	IVS8(TG)m - IVS8(T)n	Mutation in *cis*	IVS17b(TA)	n	%
26-17-M-13	12-7	p.Arg785X	35	4	11.1
		875+1G>A (c.743+1C>A)	36	1	2.8
		None	33	1	2.8
			35	5	13.8
			36	9	25
			37	4	11.1
			38	1	2.8
			40	1	2.8
			44	1	2.8
			7	1	2.8
	13-5		33	8	22.2

- 30 -

Figure 1

A


```
                                          10              20              30
CFTR_NBD1/1-40   L L G T P V L K D I N F K I E R G Q L L A V A G S T G A G K T S L L M V I M G E
ABCC6_NBD1/1-40  Q E S P P C L H R I N L T V P Q G C L L A V V G P V G A G K S S L L S A L L G E
ABCC8_NBD1/1-40  P D G I P T L S N I T I R I P R G Q L T M I V G Q V G C G K S S L L L A A L G E
ABCA7_NBD1/1-40  G S P Q P A L R G L S L D F Y Q G H I T A F L G H N G A G K T T T L S I L S G L
MJ0796/1-40      E E I I Y A L K N V N L N I K E G E F V S I M G P S G S G K S T M L N I I G C L
ABCB9/1-40       R P H T Q V L Q N V S F S L S P G K V T A L V G P S G S G K S C V N I L E N F
CFTR_NBD2/1-40   E G G N A I L E N I S F S I S P G Q R V G L L G R T G S G K S T L L S A F L R L
ABCC6_NBD2/1-40  P E L P L A V Q G V S F K I H A G E K V G I V G R T G A G K S S L A S G L L R L
ABCC8_NBD2/1-40  S S L K P V L K H V N A L I S P G Q K I G I C G R T G S G K S S F S L A F F R M
ABCA7_NBD2/1-40  G Q R M P A V D R L C L G I P P G E C F G L L G V N G A G K T S T F R M V T G D
```

Conservation

B

NBD1   NBD2

S1235

C

Mock   CFTR-WT   CFTR-S1235R

Band C

Figure 2