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Heterogeneity for mutations in the *CFTR* gene and clinical correlations in patients with congenital absence of the vas deferens

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Congenital absence of the vas deferens (CAVD) is a heterogeneous disorder, largely due to mutations in the cystic fibrosis (*CFTR*) gene. Patients with unilateral absence of the vas deferens (CUAVD) and patients with CAVD in association with renal agenesis appear to have a different aetiology to those with isolated CAVD. We have studied 134 Spanish CAVD patients [110 congenital bilateral absence of the vas deferens (CBAVD) and 24 CUAVD], 16 of whom (six CBAVD, 10 CUAVD) had additional renal anomalies. Forty-two different *CFTR* mutations were identified, seven of them being novel. Some 45% of the *CFTR* mutations were specific to CAVD, and were not found in patients with cystic fibrosis or in the general Spanish population. *CFTR* mutations were detected in 85% of CBAVD patients and in 38% of those with CUAVD. Among those patients with renal anomalies, 31% carried one *CFTR* mutation. Anomalies in seminal vesicles and ejaculatory ducts were common in patients with CAVD. The prevalence of cryptorchidism and inguinal hernia appeared to be increased in CAVD patients, as well as nasal pathology and frequent respiratory infections. This study confirms the molecular heterogeneity of *CFTR* mutations in CAVD, and emphasizes the importance of an extensive *CFTR* analysis in these patients. In contrast with previous studies, this report suggests that *CFTR* might have a role in urogenital anomalies.

Key words: CAVD/cystic fibrosis/obstructive azoospermia/renal agenesis/vas deferens

Introduction

Cystic fibrosis (CF) is a common severe autosomal recessive disease that affects 1 in 2500 individuals in Caucasian populations. The disease is characterized by abnormal flux of chloride in the apical membrane of epithelial cells, leading to a wide variability in clinical presentation (pancreatic insufficiency, progressive lung disease, meconium ileus, elevated sweat electrolytes and male infertility) (Welsh *et al.*, 1995). CF is caused by mutations in the cystic fibrosis transmembrane regulator gene (*CFTR*) (Kerem *et al.*, 1989). More than 800 mutations in the *CFTR* gene have been reported (http://www.genet.sickkids.on.ca), which determine different phenotypes: CF (Welsh *et al.*, 1995); congenital absence of the vas deferens (CAVD) (Anguiano *et al.*, 1992; Chillón *et al.*, 1995); bronchiectasis (Pignatti *et al.*, 1995); and pancreatitis (Cohn *et al.*, 1998; Sharer *et al.*, 1998).

Congenital bilateral absence of the vas deferens (CBAVD) occurs in 1–2% of infertile men (Jequier *et al.*, 1985). Obstruction of the Wolffian duct results in the absence or atrophy of the vas deferens, epididymal body and tail, seminal vesicles and the ejaculatory ducts (Taussig *et al.*, 1972). Obstructive azoospermia is present in more than 95% of CF males. Different studies have shown a high frequency of *CFTR* mutations in CBAVD patients (Casals *et al.*, 1995; Costes *et al.*, 1995; Rave-Harel *et al.*, 1995; Dörk *et al.*, 1997; Mak *et al.*, 1999). The 5T allele in intron 8 of the *CFTR* gene leads to a higher proportion of mRNA transcripts lacking exon 9 than the two other alleles, 7T and 9T. Consequently, the 5T variant produces abnormally low levels of CFTR protein. The 5T variant is the most frequent mutation associated to the CBAVD phenotype (Chillón *et al.*, 1995).

A lower frequency of *CFTR* mutations has been detected in patients with unilateral absence of the vas deferens (CUAVD) (Casals *et al.*, 1995; Mickle *et al.*, 1995). Between 11% and 26% of patients with CAVD have renal agenesis in association (Schlegel *et al.*, 1996), and initial negative results in the analysis of *CFTR* mutations in these patients suggested that urogenital anomalies have a different aetiology to isolated CAVD (Augarten *et al.*, 1994; Casals *et al.*, 1995; Schlegel *et al.*, 1996).

We have performed an extensive *CFTR* gene analysis in 134 Spanish CAVD patients, 16 of whom had renal malformations, and have carried out a correlation with clinical features. The study confirms the high molecular heterogeneity for CAVD, emphasizes the importance of extensive *CFTR* screening, and also suggests that *CFTR* might have a role in urogenital anomalies.

Materials and methods

Patients and clinical evaluation

A total of 134 consecutive men with a diagnosis of CAVD were studied. These men had been referred by different centres within

Spain, and provided a good representation of all regions of the country. None of the men had been diagnosed with CF (Welsh *et al.*, 1995). In all cases, the initial evaluation included anamnesis, a scrotal examination and a semen analysis. Overall, CBAVD was found in 110 men, while 24 men had CUAVD which affected either the right (n = 11) or left (n = 13) side. All these individuals had consulted for couple infertility, except for two fertile men with CUAVD who were diagnosed during screening for vasectomy.

Although molecular analysis of *CFTR* was performed in the 134 patients with CAVD, 86 patients (64 CBAVD, 22 CUAVD) were studied clinically in detail as they had been referred from centres which had resident teams of experienced andrologists. In the remaining cases, the clinical information was scarce. Within this subset of 86 patients, complete clinical data concerning infertility were obtained and features of CF were excluded (Welsh *et al.*, 1995). The diagnosis of CAVD was based on physical examination, when one or both vasa deferentia were non-palpable in the scrotal portion. Semen analysis included volume, pH, sperm count and motility, in accordance with WHO guidelines (WHO, 1992). Concentrations of fructose and citrate in seminal plasma were measured with commercial kits (FructoScreen, CitricScreen, Bioscreen Inc., New York, USA). Alpha-glucosidase activity in seminal plasma (EpiScreen, Fertipro NV, Beernem, Belgium) was measured in those cases studied recently. Transrectal ultrasonography was performed using a Toshiba Sonolayer SSA-250-A, with a 7.5 MHz linear transducer (PVL 625-RT); this allowed studies to be made of the morphology and size of the seminal vesicles, prostate and ejaculatory ducts. Scrotal ultrasonography was performed if physical examination revealed testis atrophy, cystic masses or reflux of spermatic veins. Abdominal ultrasonography was performed in order to evaluate the pelvis and the upper urinary tract. Excretory urograms were also performed in some cases to confirm ultrasound findings. Vasograms were performed in selected cases of CUAVD (n = 6) to characterize the morphology of the preserved seminal tract when transurethral resection of ejaculatory ducts was foreseeable. Testicular biopsy was carried out under local anaesthesia by open incision, and the specimens fixed in Bouin's solution and processed for histological analysis. Sweat chloride analysis was performed in 59 individuals (Gibson and Cooke, 1959). Preliminary data of some patients (30 CBAVD, 10 CUAVD) were reported previously (Casals *et al.*, 1995).

CFTR gene analysis

Molecular analysis of the *CFTR* gene was performed in all 134 patients. Genomic DNA samples were isolated from peripheral blood lymphocytes using standard methods. Mutations ΔF508 and G542X (Kerem *et al.*, 1989, 1990) were analysed in all patients, as they are the most common mutations in Spanish CF patients, 53% and 8% respectively (Casals *et al.*, 1997). The haplotypes obtained with three *CFTR* microsatellites (IVS8CA, IVS17bTA and IVS17bCA) allowed us to identify other less common mutations (Morral *et al.*, 1996). Recently, direct analysis of 31 *CFTR* mutations (PCR/OLA Cystic Fibrosis Assay; Perkin Elmer, Foster City) was performed in 30 of these infertile men. An extensive *CFTR* screening was carried out in all samples by multiplex denaturing gradient gel electrophoresis (DGGE) (Costes *et al.*, 1993) for 15 exons and by single-strand conformation polymorphism analysis (SSCP) (Chillón *et al.*, 1994) (Multiphor; Amersham Pharmacia Biotech, Bucks, UK) for the other 12 exons, the combination of these techniques giving a mutation detection level of 97% (Casals *et al.*, 1997; also T.Casals, unpublished results). The DNA fragments were visualized by ethidium bromide staining in DGGE analysis, or by silver staining in SSCP gels. The abnormally migrating fragments were characterized by sequencing with the DyeDeoxy™ chain terminator method on an ABI 377 sequencer. The 5T variant in the polymorphic region IVS8-6(T) was analysed as described previously (Chillón *et al.*, 1995). The M470V polymorphism (Cuppens *et al.*, 1994) was analysed in 82 available samples. The same *CFTR* gene analysis was performed in 50 individuals from the general population (Lázaro *et al.*, 1999). In order to compare the frequencies of ΔF508, L997F, 3732delA and 5T mutations, a total of 200 control subjects was studied.

Statistical analysis

Differences between proportions were tested by the χ^2 statistic. A paired Pearson's coefficient was calculated to study correlation between continuous variables. Differences between groups of discrete variables were analysed by one-way analysis of variance (ANOVA) with the SSPS program (SPSS Hispanoportuguesa SL, Madrid, Spain) for personal computers (version 6).

Results

CFTR mutations in CAVD

Forty-two different *CFTR* mutations were identified in the 134 CAVD patients. Nineteen *CFTR* mutations (45%) were detected only in patients with CAVD, and were found neither in Spanish patients with CF (Casals *et al.*, 1997; T.Casals, unpublished results) nor in the general population (Lázaro *et al.*, 1999). A significant difference in the detection level was observed between CBAVD and CUAVD patients, with 38 mutations in CBAVD (accounting for 71% of alleles; 156/220), and six mutations in CUAVD (accounting for 29% of alleles; 14/48; $\chi^2 = 8.01$, $P = 0.004$). Twenty-nine of the 42 mutations were found only once, and seven novel mutations were detected (Table I). None of these novel mutations was found among the general population.

Among the mutations identified, the 5T variant was the most common in both groups of patients, accounting for 23% of CBAVD alleles (50/220) and 12% of those with CUAVD (6/48). ΔF508 and G542X were the most frequently identified CF mutations, but at lower frequencies than in CF patients (Casals *et al.*, 1997) [43% versus 53% ($\chi^2 = 156.44$, $P < 0.001$) and 6% versus 8% ($\chi^2 = 6.56$, $P < 0.02$) respectively]. In contrast, mutations L206W and R117H, each causing a mild CF phenotype (Dean *et al.*, 1990; Desgeorges *et al.*, 1995), were found with a higher frequency in CAVD than in CF patients [4.0% versus 0.6% ($\chi^2 = 20.09$, $P < 0.001$) and 3.6% versus 0.3% ($\chi^2 = 28.45$, $P < 0.001$) respectively].

Only 13 mutations were found more than once, accounting for a total of 83% of the mutated alleles, while 29 other mutations were detected in single patients (Table II). IVS8-6(5T), ΔF508, G542X, L206W and R117H are the most common mutations in CAVD, each with a frequency over 5% of the mutated alleles.

CFTR genotypes in CBAVD and CUAVD

The different genotypes found in the patients with CBAVD are shown in Table III. *CFTR* mutations were identified in 85% of these patients (56% with two *CFTR* mutations, and 29% with one) after extensive *CFTR* gene analysis. In four patients, two *CFTR* mutations were found in *cis*, being the 5T variant and another mutation (S50P, 2751+3A→G, A1006E

Table I. Description of the seven novel *CFTR* mutations and five polymorphisms in CAVD patients

Mutation	Location	Nucleotide changes	Amino acid change	Markers haplotype (T)n-8CA-17bTA-M470V
S50P	exon 2	280 T→C	Ser → Pro	5T/7T-16-31-ND
D110Y	exon 4	460 G→T	Asp acid → Tyr	7T-17-7-V470
L383S	exon 8	1280 T→C	Leu → Ser	7T-16-7-M470
H484Y	exon 10	1582 C→T	His → Tyr	no phase-M470
2751+3A→G	intron 14a	2751+3 A→G	–	5T-16-30-ND
Q890R	exon 15	2801 A→G	Glu → Arg	7T-16-7/29-V470
P1021S	exon 17a	3193 C→T	Pro → Ser	7T-17-7-M470
Polymorphisms				
104C/A	5′UTR		–	
296+128G/C	intron 3		–	
741C/T	exon 6a		Ile203 no change	
3195A/T	exon 17a		Pro1021 no change	
3212T/C	exon 17a		Ile1027 no change	

CAVD = congenital absence of the vas deferens; ND = not determined; 5′UTR = 5′ untranslated region.

Table II. Relative frequency of *CFTR* mutations in congenital absence of the vas deferens

Mutation (n = 42)	CBAVD 156 alleles (%)	CUAVD 14 alleles (%)	Total 170 alleles (%)
5T	50[a] (32)	6 (43)	56 (33)
ΔF508	40 (26)	3 (21)	43 (25)
G542X	10 (6)	1 (7)	11 (6)
L206W	9 (6)	0	9 (5)
R117H	8 (5)	0	8 (5)
2789+5G→A	4 (3)	0	4 (2)
D1270N+R74W	3 (2)	0	3 (2)
1949del84	2 (1)	0	2 (1)
V232D	2 (1)	0	2 (1)
3732delA	0	2 (14)	2 (1)
L383S	1	1 (7)	2 (1)
Others (27)	27 (17)	1 (7)	28 (17)

[a]S50P (n = 1), 2751+3A→G (n = 1), F1074L (n = 1), A1006E (n = 2). CBAVD = congenital bilateral absence of the vas deferens; CUAVD = congenital unilateral absence of the vas deferens.

and F1074L). Except for the S50P mutation, which is associated to 5T and 7T alleles in CAVD patients, the other three mutations were always found with the 5T allele in both CAVD and CF phenotypes. The most common genotype was the combination of any *CFTR* mutation and the 5T allele (30%). We detected only three homozygous patients (one for V232D and two for 5T). Table IV shows the CUAVD genotypes; *CFTR* mutations were identified in 38% patients (21% with two mutations, and 17% with one).

Among the patients of this study, 16 had renal agenesis associated (six CBAVD, 10 CUAVD). Five of these patients (31%) carried one *CFTR* mutation, three being ΔF508, L997F or 3732delA, and two the 5T variant. This frequency was significantly higher (χ^2 = 9.95, P = 0.001) than that expected in the general population for these *CFTR* mutations that cause either CF or CAVD (7.5%). The specific frequencies for each of these mutations in the general Spanish population (200 samples not CF) are ΔF508 2%, L997F 0.5%, 3732delA 0%, and 5T 5% (Chillón *et al.*, 1995; Casals *et al.*, 1997; Lázaro *et al.*, 1999).

CFTR polymorphisms

A total of 21 different polymorphisms were identified. The M470V variant in exon 10 was analysed in 82 patients (98 alleles M470 and 66 V470), (TTGA)n in intron 6a which presented the higher frequency of seven repeats (129/272 alleles), T854T in exon 14a (72/272 alleles), 4521G→A in exon 24 (62/272) and 3601-65C/A in intron 18 (48/272) were the most frequent. Six polymorphisms: 125G/C, 1525-61A/G, 1898+152T/A, 1716G/A, G576A and 875+40A/G presented frequencies of between 2.5% and 4.0%. Another four with frequencies of 1–2% were 1816G/A, 4404C/T, 1001+11C/T and R668C. Finally, six polymorphisms were found each in one patient: 104G/T, 296+128G/C, 741C/T, 3195A/T, 3212T/C and 4029A/G. The five new polymorphisms identified are described in Table I.

Clinical features

Two of the patients had relatives with known CAVD, and one patient had a sister with CF. Ten patients (five CUAVD, five CBAVD) had siblings who died during infancy of respiratory infections. Seven of these men had at least one *CFTR* mutation.

A number of renal anomalies were observed in the CAVD patients (Table V). Unilateral renal agenesis was diagnosed in 41% of CUAVD and in 5.4% of CBAVD patients (χ^2 = 12.4, $P < 0.001$). Renal agenesis predominated in men without *CFTR* mutations, but three patients with CUAVD and two with CBAVD showed co-existing renal agenesis and one *CFTR* mutation (three patients CF/–, two patients 5T/–). Frequencies of other clinical conditions, such as history of cryptorchidism, inguinal hernia, nasal polyps, rhinosinusitis and varicocele, are shown in Table V. Two men with CUAVD had unilateral cryptorchidism associated with ipsilateral inguinal hernia. In the remaining patients hernia was contralateral to the maldescended testis, or occurred in subjects with bilateral cryptorchidism. Nasal pathology was more frequent in CBAVD patients with mutations (36%) than in those without mutations (8.3%). None of the patients showed pulmonary or gastrointestinal symptoms of CF. However, repeated respiratory infections or bronchitis were common in both groups, often associated

Table III. *CFTR* genotypes in 110 patients with congenital bilateral absence of the vas deferens

Mutations	IVS8-6(T)	*n* (%)
Two *CFTR* mutations		62 (56)
ΔF508/–	5T/9T	17 (15)
G542X/–	5T/9T	6 (5)
ΔF508/L206W	9T/9T	6 (5)
ΔF508/D1270N+R74W	7T/9T	3 (3)
ΔF508/R117H	7T/7T	1
ΔF508/P1021S	7T/9T	1
ΔF508/M952T	7T/9T	1
ΔF508/D110Y	7T/9T	1
ΔF508/S50P	5T/9T	1
ΔF508/2751+3A→G	5T/9T	1
G542X/R117H	7T/9T	1
G542X/2789+5G→A	7T/9T	1
R117H/2789+5G→A	7T/7T	1
R117H/712-1G→T	7T/9T	1
R117H/ΔI507	7T/7T	1
L206W/–	5T/9T	1
L206W/3121-1G→A	7T/9T	1
L206W/1949del84	7T/9T	1
ΔE115/S50P	7T/7T	1
2869insG/R1070W	7T/7T	1
V232D/V232D	9T/9T	1
S945L/R258G	7T/7T	1
G551D/F1074L	5T/7T	1
A1006E/L383S	5T/7T	1
E92K/–	5T/7T	1
711+1G→T/–	5T/7T	1
R334W/–	5T/7T	1
S549R/–	5T/7T	1
1949del84/–	5T/7T	1
K1060T/–	5T/7T	1
R1162X/–	5T/7T	1
S1235R/–	5T/7T	1
A1006E/–	5T/5T	1
–/–	5T/5T	1
One *CFTR* mutation		32 (29)
–/–[a]	5T/7T	8 (7)
ΔF508/–	7T/9T	5 (4)
ΔF508/–	9T/9T	3 (3)
–/–	5T/9T	2 (2)
G542X/–	7T/9T	2 (2)
2789+5G→A/–	7T/7T	2 (2)
R117H/–	7T/7T	2 (2)
R117H/–	7T/9T	1
H484Y/–	7T/9T	1
G85E/–	7T/7T	1
2752-15C→G/–	7T/7T	1
L997F/–[a]	7T/7T	1
1677delTA/–	7T/7T	1
Y1014C/–	7T/9T	1
N1303K/–	7T/9T	1
Negative *CFTR* mutation		16 (15)
–/–	7T/7T	12 (11)
–/–	7T/9T	4 (4)

[a]Two carrier patients with renal agenesis.

Table IV. *CFTR* genotypes in 24 patients with congenital unilateral absence of the vas deferens

Mutations	IVS8-6(T)	*n* (%)
Two *CFTR* mutations		5 (21)
ΔF508/–	5T/9T	2 (8)
G542X/–	5T/9T	1
3732delA/–	5T/7T	1
L383S/–	5T/7T	1
One *CFTR* mutation		4 (17)
ΔF508/–[a]	7T/9T	1
3732delA/–[a]	7T/7T	1
Q890R/–	7T/7T	1
–/–[a]	5T/7T	1
Negative *CFTR* mutations		15 (62)
–/–	7T/7T	10 (42)
–/–	7T/9T	3 (12)
–/–	9T/9T	2 (8)

[a]Three carrier patients with renal agenesis.

with heavy smoking habit (>20 cigarettes per day). One patient with CUAVD and three with CBAVD (all with *CFTR* mutations) suffered from asthma.

Anomalies of seminal vesicles—including agenesis, hypoplasia and cystic dysplasia—were very common among CAVD individuals. Unilateral abnormalities (typically on the same side of the absent vas deferens) predominated in CUAVD, whereas bilateral dysplasia was more common in CBAVD (Table V). Total length of seminal vesicles measured by transrectal ultrasonography was significantly smaller in CBAVD than in CUAVD ($F = 8.1$, $P = 0.005$). Dilatation of ejaculatory ducts, often resembling utricular cysts, was demonstrable also in some men, all of whom were azoospermic (Figure 1). Vasograms performed in six patients with CUAVD not only confirmed ultrasonographic findings, but also showed additional abnormalities at various levels of the seminal tract. The volume and consistency of testes was normal, except in patients with other concomitant pathologies, such as cryptorchidism ($n = 6$), trauma ($n = 2$), orchitis ($n = 2$) or tumour ($n = 1$). The prostate gland showed normal size and morphology in all patients.

Semen volume, pH, sperm concentration and fructose were significantly different in CUAVD compared with CBAVD (Table VI). Azoospermia was more frequent in CUAVD patients with *CFTR* mutations (7/9) than in those without mutations (8/13), and sperm concentration was higher in the latter group. However, the differences were not statistically significant. Concentrations of citrate in seminal plasma showed no significant differences between groups. In a small group of patients, alpha-glucosidase activity was 18.5 ± 2.7 mU/ml in CUAVD ($n = 4$), and 26.7 ± 5.5 mU/ml in CBAVD ($n = 7$). After reclassification of the patients according to the presence of zero, one or two mutations, none of the variables showed significant differences either in CUAVD or CBAVD (not shown). As expected, fructose and citrate concentrations were highly correlated with seminal volume ($r = 0.70$, $r = 0.58$ respectively; $P < 0.001$). The length of seminal vesicles showed significant correlation with fructose in seminal plasma ($r = 0.52$, $P < 0.001$), semen pH ($r = 0.47$, $P < 0.001$) and with chloride in sweat ($r = -0\ 35$, $P = 0.02$).

Chloride concentrations in sweat were higher in CBAVD patients with *CFTR* mutations than in those without mutations ($F = 3.4$, $P = 0.07$), and approached statistical significance. Chloride concentration was higher in CUAVD men without mutations ($n = 5$) than in those with mutations ($n = 5$), but the number of cases tested was low and most likely not representative. Discrimination analysis showed that the single best variable to predict the presence of *CFTR* mutations was

Table V. Clinical features of patients with congenital absence of the vas deferens (CAVD)

Type of C AVD *CFTR* mutations	Unilateral			Bilateral		
	No (n = 13)	Yes (n = 9)	Total (n = 22)	No (n = 12)	Yes (n = 52)	Total (n = 64)
Renal agenesis	7/15* (47)	3 (33)	10/24* (41)[a]	4/18* (22)[b]	2/92* (2.1)[b]	6/110* (5.4)[a]
Cryptorchidism	3 (23)	1 (11)	4 (18)	0 (0)	4 (7.7)	4 (6.2)
Inguinal hernia	4 (31)	0 (0)	4 (18)	1 (8.3)	4 (7.7)	5 (7.8)
Varicocele	2 (15)	0 (0)	2 (9.1)	1 (8.3)	4 (7.7)	5 (7.8)
Bilateral dysplasia of seminal vesicles	3/10* (30)	2 (22)	5/19* (26)[c]	4 (33)	31 (59)	35 (54)[c]
Unilateral dysplasia of seminal vesicles	7/10* (70)	4 (44)	11/19* (58)[b]	1 (8.3)	3 (5.7)	4 (6.2)[b]
Dilatation of ejaculatory ducts	2 (15)	4 (44)	6 (27)	1 (8.3)	3 (5.7)	4 (6.2)
Respiratory infections, bronchitis	3 (23)	1 (11)	4 (18)	1 (8.3)	9 (17)	10 (15)
Nasal polyps, rhinosinusitis	1 (7.7)	2 (22)	3 (13)	1 (8.3)[c]	19 (36)[c]	20 (31)

Patients affected [/* patients evaluated, if different from that indicated in column heading] (percentage).
[a]$P < 0.001$; [b]$P < 0.001$; [c]$P < 0.05$ significance of proportion differences between labelled results in same row.

the type of CAVD, followed by the pH of semen and the sperm concentration. A canonical discriminatory function including all three variables was able to classify correctly in 80% of the cases.

Discussion

This report and previous studies (Chillón *et al.*, 1995; Costes *et al.*, 1995; Rave-Harel *et al.*, 1995; Dörk *et al.*, 1997; Mak *et al.*, 1999) highlight the heterogeneity that exists for mutations in the *CFTR* gene in patients with CAVD, and indicate that the spectrum of *CFTR* mutations is different in CAVD than in CF patients. Less than 60% of the mutations detected here are found in patients with CF (Casals *et al.*, 1997). Although only 11 *CFTR* mutations account for a total of 83% of the mutated alleles in CAVD, the presence of 31 other different mutations indicates a high heterogeneity in the *CFTR* gene in CAVD. This suggests that a complete characterization of the *CFTR* gene is needed in many patients with CAVD in order to identify one or two mutations that they might have. Thus, the OLA/PCR mutation detection system that covers 31 mutations in patients with CF, only allows the identification of 26% of the *CFTR* mutations in CAVD patients. Despite the fact that *CFTR* microsatellites facilitate mutation analysis in CF patients (Morral *et al.*, 1996), the heterogeneity and the different spectrum of mutations in CAVD patients indicate that microsatellite studies before mutation analysis are not helpful in identifying mutations in patients with CAVD.

Nineteen of the mutations detected in patients with CAVD were not previously identified in over 700 unrelated Spanish patients with CF (T.Casals, unpublished data). Most of these new mutations correspond to amino acid changes, in addition to some splice site mutations, and it is likely that they cause a mild *CFTR* dysfunction, as expected for an incomplete CF phenotype.

Genetic counselling is especially difficult in couples with infertility due to CAVD. The wide spectrum of *CFTR* mutations, some of them with unpredicted clinical consequences, suggest that the *CFTR* analysis should not only be focused on mutations common in CF patients. A complete characterization of the *CFTR* gene in these couples would be desirable, especially as some of the patients carry only one *CFTR* mutation, which is not present in those patients with CF.

One interesting finding was the strong association of the 5T allele with the valine at position 470 (71%) ($\chi^2 = 13.67$, $P < 0.001$). This result is in agreement with a previous report (De Meeus *et al.*, 1998), suggesting that the M470V locus could contribute to the variable expression of the 5T allele, with valine being involved in lower CFTR protein levels.

The high proportion of cryptorchidism (18% in CUAVD and 4.7% in CBAVD) confirms the increased prevalence of this alteration in CAVD described previously (Schlegel *et al.*, 1996), and suggests an association between CAVD and testicular descent. The prevalence of inguinal hernia was also significant in patients with CAVD, even after excluding the two cases with ipsilateral cryptorchidism. Both conditions can be observed at high rate in men with CF (Holsclaw *et al.*, 1971).

Anomalies of the seminal vesicles were very common in CAVD patients. Unilateral alterations predominated in CUAVD, whereas bilateral abnormalities were found more frequently in CBAVD. Overall, 84% of CUAVD and 60% of CBAVD patients showed some dysplasic seminal vesicles, with either agenesis, hypoplasia or cystic dysplasia. Additional explorations, such as vasography performed in some CUAVD patients, revealed new malformations in these men that were not detectable by transrectal ultrasonography. Previous reports found variable frequencies of changes in seminal vesicles, ranging from 36% to 92% in CBAVD (Goldstein and Schlossberg, 1988; Marmar *et al.*, 1993; Jarvi *et al.*, 1998; Taille *et al.*, 1998), and 85% in CUAVD (Mickle *et al.*, 1995; Schlegel *et al.*, 1996). Ultrasonographic measurement of seminal vesicles showed diminished length in CBAVD compared with CUAVD, and sweat chloride concentration showed a significant negative correlation with the size of seminal vesicles. These data suggest indirectly that variable phenotypic expression of *CFTR* could result in genital manifestations with corresponding degrees of severity.

Although none of the CAVD patients had symptoms of CF, nasal polyps and/or rhinosinusitis were associated with the presence of *CFTR* mutations, especially in patients with CBAVD. Frequent respiratory infections and bronchitis were also noted in CAVD individuals, though no clear relationship

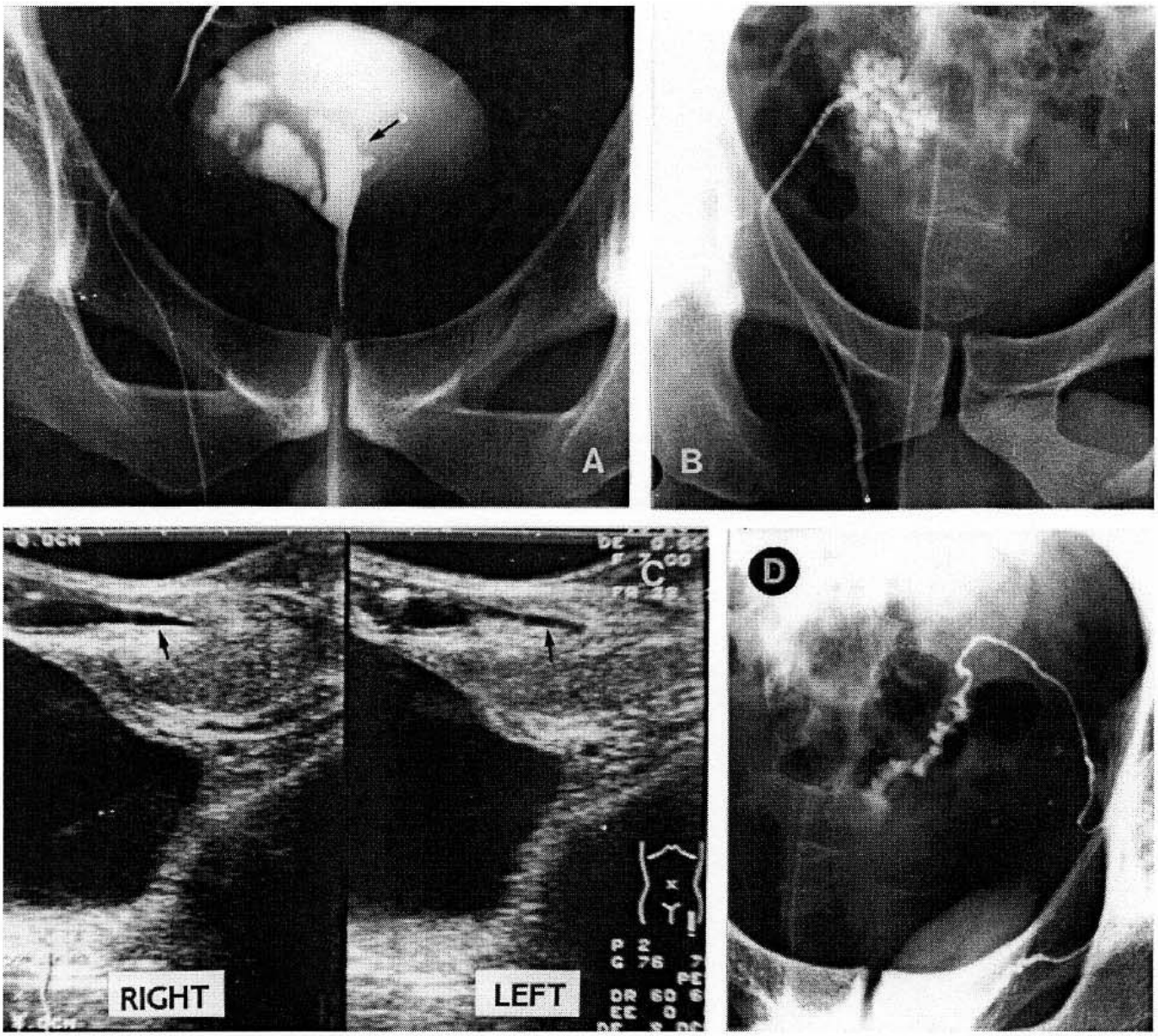

Figure 1. Imaging of the preserved seminal tract in some CUAVD patients. (**A**) Right vasogram of an azoospermic patient with left CAVD and normal *CFTR* genotype. The right vas deferens is patent, ending on a dilated ampoule with small diverticula (arrow). The seminal vesicle is dysplasic and the ejaculatory duct appears enlarged. Passage of contrast to urethra was difficult but possible. (**B**) Vasogram of a patient with azoospermia and left CUAVD, normal *CFTR* genotype. The lumen of the right vas deferens is dilated and irregular, connected directly to a dysplasic seminal vesicle, without communication with the distal seminal tract. (**C**) Transrectal ultrasonography of an azoospermic patient with right CUAVD and a *CFTR* genotype L383S/5T. Both ejaculatory ducts appear dilated, and the seminal vesicles are of normal size (arrow). (**D**) Left vasogram of this patient ends on a tortuous ampoule with no visible communication with seminal tract.

with genotype could be established, as other risk factors (e.g. heavy smoking habit) were likely to be implicated. Nevertheless, these observations suggest minor, often under-reported, clinical manifestations of some particularly sensitive epithelial tissues in our patients.

Seminal variables, such as volume, pH and fructose were more clearly affected in CBAVD than in CUAVD, and showed good correlation with the size of seminal vesicles. This is in keeping with the hypothesis that the different degrees of hypoplasia observed in CAVD exhibit progressive functional impairment. While all CBAVD subjects were azoospermic, 32% (7/22) of CUAVD patients had some spermatozoa in their semen. Three of these men (one of whom was fertile) had *CFTR* mutations. It was also indicated (Mickle *et al.*, 1995) that men with CUAVD, who had a patent contralateral seminal duct, showed no *CFTR* mutations; these workers concluded that two distinct subpopulations with different aetiologies could be established, based upon the mutational status of the *CFTR* gene. Our results add more complexity to this hypothesis, and suggest that some CUAVD patients with *CFTR* mutations have spermatozoa in their semen and may be fertile. The discrepancy may be due to the fact that our patients were not exclusively infertile, and *CFTR* analysis was more complete.

Measurement of neutral glucosidase has been proposed for diagnosis of obstructive lesions of the epididymis and the vas deferens (Guerin *et al.*, 1986). Previous studies have suggested that the different methods currently used for determination of glucosidase are suitable for clinical purposes in men

Table VI. Analytical and clinical variables in CAVD patients

Type of CAVD *CFTR* mutations	Unilateral			Bilateral		
	No (n = 13)	Yes (n = 9)	Total (n = 22)	No (n = 12)	Yes (n = 52)	Total (n = 64)
Semen analysis						
Volume (ml)	2.2 ± 0.3	1.6 ± 0.4*	2.0 ± 0.3[a]	0.8 ± 0.1	0.9 ± 0.1	0.9 ± 0.07[a]
pH	7.2 ± 0.1	7.0 ± 0.2	7.1 ± 0.1[a]	6.7 ± 0.2	6.5 ± 0.06	6.5 ± 0.06[a]
Sperm concentration ($\times 10^6$/ml)	15.6 ± 9.8	1.0 ± 1.0	9.6 ± 5.9[b]	0	0	0[b]
Fructose (µmol/ejaculate)	21.7 ± 6.4	20.0 ± 12.2	21.1 ± 5.6[a]	0.4 ± 0.3	6.0 ± 1.5	5.3 ± 1.3[a]
Citrate (µmol/ejaculate)	74.4 ± 13.3	99.9 ± 31.4	83.5 ± 13.8	39.0 ± 18.2	62.9 ± 9.3	59.5 ± 8.4
Transrectal ultrasonography						
Length of both seminal vesicles (cm)	3.2 ± 0.4	4.4 ± 0.6	3.7 ± 0.4[b]	3.1 ± 1.0	1.9 ± 0.3	2.1 ± 0.3[b]
Sweat chloride concn. (mEq/l)[†]	74.4 ± 10.4[c]	47.2 ± 4.4[c]	60.8 ± 7.0	56.4 ± 8.5	80.0 ± 6.1	75.2 ± 5.3

Values are mean ± SEM.
[a]$P < 0.001$; [b]$P < 0.01$; [c]$P < 0.05$ significance of differences between labelled results of same row.
*One patient was fertile and underwent vasectomy before the evaluation.
[†]Measured in 10 CUAVD and 49 CBAVD patients.

(Mahmoud *et al.*, 1998). Our results indicate that the EpiScreen method used in this study measures the activity of other acidic α-glucosidase isoenzymes, most likely an isoenzyme contained in the prostatic secretion, and probably leads to inaccurate results.

We have found that about one-third (5/16) of patients with CAVD and renal agenesis have mutations in *CFTR* (most of these patients being CUAVD). These results contrast with those reported previously of a low proportion of *CFTR* mutations in such patients (Anguiano *et al.*, 1992; Augarten *et al.*, 1994; Schlegel *et al.*, 1996; Taille *et al.*, 1998). We attribute this discrepancy to the reduced number of samples of these previous studies, which were focused on the most frequent *CFTR* mutations, without performing a complete analysis of the whole *CFTR* gene. Although the sample in the present study is small (16 patients), these findings should encourage other investigators to characterize fully those cases of CAVD and renal agenesis. These studies should help to define the proportion of cases of CAVD and renal agenesis that are due to mutations in *CFTR*.

It is still unclear how *CFTR* is involved in the development of CAVD. Moreover, the putative relationship between *CFTR* and renal agenesis (found here in 31% of cases) is even more intriguing. It has been proposed that when renal anomalies co-exist with CAVD, a defect in the Wolffian duct is produced at the time of, or before, formation of the ureteral bud, resulting in malformation of the entire Wolffian duct and subsequent vasal agenesis (Dumur *et al.*, 1995; Schlegel *et al.*, 1996). The involvement of *CFTR* in both CAVD and renal agenesis can be understood from a polygenic/multifactorial point of view. Mutations in *CFTR* in conjunction with variants in genes involved in renal formation could participate in a synergistic action in renal and vas deferens alterations. The identification of the genetic and environmental factors that participate in renal development will require the analysis of a larger number of cases and the use of genomic analysis approaches.

In summary, our results have confirmed the high molecular heterogeneity for CAVD and the different spectrum of *CFTR* mutations when compared with CF patients. The study has also highlighted the importance of an extensive *CFTR* molecular characterization of CAVD patients in order to provide a better understanding of the molecular basis of this disorder. Finally, our findings suggest that *CFTR* mutations might also have a role in urogenital anomalies.

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Different *CFTR* Mutational Spectrum in Alcoholic and Idiopathic Chronic Pancreatitis?

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Running title: "*CFTR* gene involvement in chronic pancreatitis"

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ABSTRACT

OBJECTIVE: *CFTR* mutations are responsible for cystic fibrosis (CF) disease and have been postulated as a predisposing risk factor to CP. However, controversial results demand additional support. We have therefore investigated the role of the *CFTR* gene in a cohort of 68 CP patients.

METHODS: We have performed the *CFTR* gene analysis using two screening techniques. Fragments showing abnormal migration patterns were characterized by sequencing. Patients were classified in alcoholic (n=37) and idiopathic (n=31). Clinical features of CP and CF were evaluated.

RESULTS: Sixteen mutations/variants were identified in 27 patients (40%), most of them (35%) presenting a single *CFTR* mutant gene. The 1716G/A variant showed the highest frequency accounting for 22% in ICP and 5% in ACP, in contrast with other more common mutations such as F508del found in 8% of ACP and the 5T variant identified in 7% of patients. Acute pancreatitis, abdominal pain, tobacco, pancreatic calcifications and pancreatic pseudocysts showed significant higher values in ACP than ICP. No significant differences were found between patients with and without mutations.

CONCLUSIONS: Apart from reinforcing previous studies our data highlight the increased susceptibility of *CFTR* heterozygous to developing CP. Heterozygosity combined with other factors puts these individuals at greater risk.

Key words: chronic pancreatitis, *CFTR* gene, mutational analysis, *CFTR*-related disease

INTRODUCTION

Chronic pancreatitis (CP) is a persistent inflammatory process of the pancreas characterized by irreversible destruction of pancreatic structure and loss of its function. The diagnosis of CP is currently based on clinical symptoms and on morphological and functional alterations. [1]

Whereas some environmental factors, mainly excessive alcohol consumption, are associated with it, little is known about other factors involved in the pathogenic mechanism of the disease. The abuse of alcohol consumption is related to a high proportion (65-80%) of CP. In contrast, only a minority of alcoholics (5-10%) develops CP,[2] suggesting that alcohol abuse is a predisposing factor but not a unique one. On the other hand, an important group of patients suffering CP (10-30%) has an unknown etiology, classified as idiopathic.

Mutations in the *CFTR* gene (MIM#602421) [3] are responsible for cystic fibrosis (CF) disease (MIM#219700), a hereditary recessive disorder affecting 1 in 2000-4000 newborns in the Caucasian population. The main characteristics of clinical CF features are chronic lung disease, pancreatic insufficiency and male infertility, associated with a high electrolyte concentration in sweat.[4] The CFTR protein [5] is a cAMP-regulated channel which controls chloride and water transport, as well as other ion channels across the apical membrane of the epithelial cells. In pancreatic duct cells, the CFTR protein regulates chloride, bicarbonate and sodium exchange. Consequently, CFTR dysfunction leads to an alteration of the pancreatic juice, which results in reduced alkaline concentration and dehydrated composition. The decreased enzyme secretion and the formation of protein plugs cause obstruction of the duct lumen and progressive exocrine pancreatic insufficiency. [6] Eighty-five per cent of CF patients show steatorrhea and need enzyme supplementation. Among CF patients the

prevalence of pancreatitis is low, it is estimated at around 2% and mainly defined as acute recurrent pancreatitis.[7]

Controversial results on *CFTR* mutations in chronic and acute pancreatitis were previously reported by several authors, [8-14] probably due to differences in the final diagnosis of patients as well as the fact that a greater part of the studies were focused in the common CF gene mutations.

In order to provide more data that could elucidate the role of the *CFTR* mutations in the pathogenesis of CP we have performed the *CFTR* gene analysis of all 27 coding regions and flanking intronic sequences in a cohort of 68 CP patients.

MATERALS AND METHODS

Subjects

Patients with the diagnosis of CP were recruited between October 2000 and October 2002 in two Spanish Hospitals, from Valencia (Hospital Clinic) and Barcelona (Hospital Sant Pau). The presence of at least one of the following criteria was taken into account in the diagnosis of CP: 1) chronic abdominal pain and/or pancreatic calcifications on abdominal x-ray, ultrasound (US) or computed tomography (CT). 2) abdominal pain and morphologic features of moderate or severe distortion in duct pancreatograms obtained by endoscopic retrograde cholangiopancreatography, magnetic resonance cholagiopancreatography, endoscopic US, US or CT, according to published criteria. [1,15] 3) persistent exocrine pancreatic insufficiency demonstrated by steatorrhea (Van de Kamer test, fecal fat > 7g/24h) or abnormal pancreatic function tests (fluorescein-dilaurate, fecal elastase-1 or secretin-CCK) with clinical, morphological or histopathological features in accordance with CP and in the absence of other causes of malabsorption. 4) histopathological changes consistent with pancreatitis in patients with clinical, morphological and functional changes suggestive of CP.

Under these criteria, 68 patients (54 males and 14 females) were enrolled to *CFTR* gene analysis. Furthermore, regarding the etiology, patients were classified in two groups: Group I comprised 37 patients diagnosed of alcoholic chronic pancreatitis (ACP). In this group alcohol consumption was considered to be the responsible etiology when ethanol intake exceeded 60g/day for at least 5 years before the onset of pancreatitis. All patients were males except one, the mean age was 51 (range 35-72) and the alcohol consumption ranged from 6 to 30 years. Group II comprised 31 patients with the diagnosis of idiopathic chronic pancreatitis (ICP). In this group

alcohol consumption was absolutely discarded and confirmed the absence of any other disease associated risk factors. In one patient with primary sclerosing cholangitis, CP was confirmed with secretin-CCK test and histopathological features. The patients were 18 males and 13 females with a mean age of 53 years (range 9-82). The wide age range was attributable to early-onset of symptoms (before 35 years old) found in 25% of patients.

All patients in this study were unrelated and without family history of CF or pancreatitis. The Ethical Committee of each Hospital approved the study.

Clinical study

Pancreatic disease study included, whenever possible to record, time evolved since the diagnosis, body mass index, alcohol and years of consumption, tobacco smoking, clinical, morphological and functional pancreatic features (acute pancreatitis, pain, pseudocysts, calcifications, diabetes mellitus, exocrine pancreatic insufficiency), enzyme replacement therapy and presence of concomitant hepatobiliary disease.

In addition, clinical features of CF were investigated in available patients. Lung function was evaluated by FEV1 (forced expiratory volume in 1 second) % predicted and FVC (forced vital capacity) % predicted. Sputum cultures, sweat test using the Wescor Macroduct System (Sweat check, Wescor Inc, USA) and questions relating to offspring were also recorded.

***CFTR* gene analysis**

The DNA was extracted from peripheral blood using standard protocols (Wizard® Genomic DNA Purification kit, Promega Corporation, Madison, USA). The complete 27 coding regions and exon/intron boundaries of the *CFTR* gene were analyzed in all patients using two screening techniques, multiplex denaturing gradient gel

electrophoresis (DGGE) [16] and single strand conformation polymorphism analysis (SSCP/Heteroduplex) (Genephor, Amersham Pharmacia Biotech, Buckinghamshire, England). The combination of these techniques has largely been used in our laboratory for CF diagnosis (750 families) giving a mutation detection level of 97% in the Spanish CF population [17] (T. Casals unpublished data). All fragments showing abnormal migration patterns were characterized by sequencing with the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, USA) on an ABI 377 sequencer. Analysis of exon 9 by DGGE has permitted the determination of the 5T, 7T and 9T alleles in the IVS8-6(T)n locus. When the 5T variant was identified, direct sequencing of the (TG)m locus and restriction enzyme analysis to the M470V polymorphism [18] were performed.

Additionally, the mutational spectrum found in chronic pancreatitis patients was compared with our series of CF patients, [17] congenital bilateral absence of the vas deferens (CBAVD) patients [19] and the general population (n = 41). [20]

Statistical analysis

Continuous variables from the groups studied and the statistical significance differences applying the Fisher's exact 2-tailed test to compare distributions of categorical variables were analyzed with the software package SPSS 10.0 Inc., Chicago, IL. *P* values < 0.05 were considered to indicate statistical significance.

RESULTS

Mutational *CFTR* analysis showed a similar high frequency of mutations in the two groups studied accounting for 40.5% in the ACP group (15 out of 37 patients) and 38.7% in the ICP group (12 out of 31 patients). As expected a high molecular heterogeneity has been observed and sixteen *CFTR* mutations/variants identified (Table 1), most of them found in a single patient. Twelve out of 16 changes observed (75%) were missense mutations/variants, the M281T a T>C substitution at nucleotide 974 is reported for the first time in this work. Differences in the mutational spectrum were noticed between the two groups (Table 1, Figure 1). The most common mutation in the *CFTR* gene, F508del, was found in 3 ACP patients (8%) but it was absent in the ICP group. Another common CF mutation, W1282X was identified in 1 ACP patient. In contrast, the 1716G/A variant displayed the highest frequency (13%) in this study as it was being detected in 22.6% of ICP patients. The 1716G/A variant modified the splice site consensus sequence determining the skipping of exon 10. The frequency in the ICP group was nearly twice that found in the general population (12%) and four times that detected in the ACP group (5.4%). However, no significant differences were found between the three groups (ACP, ICP and general population). Also, two missense mutations/variants, the R668C and L997F were detected in both CP groups. The R668C was found isolated in one ACP patient and as the complex allele D443Y+G576A+R668C (common in CBAVD) in one ICP patient.

The 5T variant was identified in 5 patients (7.3%) (3 ACP, 8% and 2 ICP, 6%), showing no significant difference from the general population (9.4%). The 5T-12TG-V470 haplotype, which results in a lower level of functional CFTR protein, was identified in one unique ACP patient (Table 1).

Three patients showed two changes. Although no familial studies were performed, taking into account our cohort of CF families and CBAVD patients, we assumed that the three patients (4%) would be compound heterozygous (Table 1, # 1, 2 and 16), whereas the remaining 24 patients (35%) carried only one *CFTR* mutant gene.

Clinical characteristics of 68 CP patients are shown in Table 2. When all patients were considered, we found that episodes of acute pancreatitis ($P = 0.005$), abdominal pain ($P = 0.02$), tobacco and pancreatic calcifications ($P < 0.001$) and pancreatic pseudocysts ($P = 0.002$) showed significantly higher values in ACP than ICP. However, within each group, no significant differences were found between patients with and without mutations in any of the parameters evaluated.

The clinical characteristics of CF disease were also evaluated. As a consequence of the chronic pancreatic damage, about 50% of the patients had developed pancreatic insufficiency and diabetes mellitus (Tables 1, 2). The sweat test was performed in available patients (23 ACP and 23 ICP), being considered normal when the qualitative conductivity test was < 95 mmol/L. The highest value observed was 85 mmol/L in one ACP patient (Table 1). Similar sweat test values were observed on patients with and without mutations. Recurrent lung infection was found in one 82-year-old man with ICP and in 3 ACP patients (a 50-year-old woman with bronchiectasis and two men, 36 and 72 respectively). One of these four patients was found compound heterozygous (Table 1, #2). Critical lung function was detected in one ICP patient (Table 1,#19), function being normal, for the age group, in 40 patients. Finally, infertility was unproven (single patients) or excluded in 42 out of 54 CP men.

DISCUSSION

We have analyzed the *CFTR* gene in a cohort of 68 clinically well defined CP patients in order to investigate the contribution of the *CFTR* mutations in this pancreatic disease.

Sixteen mutations/variants located along the gene were identified in 40% of ACP and 39% of ICP patients, most of them being rare mutations. Curiously, the mutational spectrum differs between ACP and ICP. Common CF mutations, F508del and W1282X, were found exclusively in ACP (11%). Taking into account the frequencies of these mutations in Spanish CF patients, 53% and 0.8% respectively [17] and assuming a 4% of CF carriers in the general population, we would expect around 2% of carriers for these CF mutations. Consequently, the carrier frequency for F508del and W1282X mutations was five times increased in ACP, although statistical significance was not found. On the other hand, the 1716G/A variant, which determines the skipping of exon 10, [21] proved to be the most frequent (22%) in ICP group. This variant was previously reported in recurrent acute pancreatitis [10] and CP.[12] Interestingly, the consequence of the 1716G/A variant (exon 10 skipping) could be compared to that of the 5T variant (exon 9 skipping) as both affect the NBF1 domain and produce incomplete CFTR protein. Our results suggest that the 1716G/A variant could be a predisposing factor to CP, as well as the L997F mutation, that was found associated with idiopathic pancreatitis and neonatal hypertrypsinemia. [22] In our cohort of patients, the L997F mutation was detected in both groups (3%).

Controversial data has been reported on ACP after the analysis of the most common CF mutations, [9,11,23,24] whereas Norton et al. suggested that *CFTR* mutations were not a predisposing factor to pancreatitis, other authors identified mutations in 8-18% of patients. The differences in the molecular analysis could explain our higher

frequency (40%). The 5T variant analysis led to their exclusion as a predisposing factor of ACP. [24,25] We have identified the 5T variant in 8% of ACP patients, that was a similar frequency to that found in our previous study (10.5%) [26] and in the general population (9.4%). Furthermore, the 5T-12TG-V470 haplotype that shows high specificity with the CBAVD phenotype [27] was identified in only one patient.

The involvement of *CFTR* mutations in ICP has been unanimously accepted. [8-14] Moreover, Noone et al. postulated that ICP and CBAVD phenotypes have similar *CFTR* gene characteristics. However, we found important molecular differences between these two phenotypes. First, the 5T variant frequency that ranges from 6% in ICP to 43% in CBAVD patients. [19] No patients with the 5T variant displayed another CFTR change when this genotype, *CFTR* / 5T, is the most frequent in CBAVD. Moreover, variable splice efficiency from the 5T variant had been observed in different tissues, indicating that its disease expression was more severe in vas deferens than others. [28, 29] The second discrepancy is due to the sensibility of the *CFTR* screening techniques. The size of the *CFTR* gene (230kb) and the fact that most mutations affect only one or a few nucleotides (point mutations) have led to the implementation of screening techniques, such as DGGE and SSCA that have a high sensibility to these changes (97% in CF Spanish patients) (unpublished data). Using this gene analysis strategy, we identified 16 mutations, mainly uncommon, in 39% of ICP patients, only one of them being compound heterozygous (3%). These frequencies are far from those reported in the CBAVD patients, in whom mutations were identified in 85% of CBAVD, 56% being compound heterozygous. [19]

Five of the clinical parameters analyzed showed differences with statistical significance between alcoholic and idiopathic groups. These differences could be due to an earlier age of onset and the longest evolution time of the CP in the alcoholic

group. No other statistical differences were found between the two groups, neither between patients with or without mutations. The lack of clinical differences is not rare as pancreatitis results from the combined effect of different predisposing risk factors masking the effect of each specific one.

It was seen that CFTR expression is not ubiquitous; higher CFTR levels have been shown in the pancreas and other gastrointestinal fetal tissues than elsewhere. [30] In fact, most of the CF patients present pancreatic insufficiency from an early age. These findings point to the fact that CFTR function must be essential to prevent gastrointestinal disease. Although we cannot exclude a mutation in another region that could increase the proportion of mutant genes, our data supports a higher carrier frequency (35%) among CP patients than expected. Predictably, the *CFTR* mutations could lead, by different mechanisms, to a decreasing level of CFTR function, the affected tissues (especially those with a high requirement) being more susceptible to the effect of other environmental and genetics factors. As was proposed by Truninger et al., [11] our data support the hypothesis that an asymptomatic *CFTR* mutant carrier would have an increased risk of developing CP after the consumption of drugs (alcohol, tobacco) that have an accumulative harmful effect on the tissues. This major susceptibility would be supported by the high frequency of CFTR mutant carriers (35%) among ACP patients and could explain, in part, why the pancreatitis frequency is low among alcoholic patients. Alternatively, the different *CFTR* mutational spectrum and the exclusion of alcohol in ICP group, suggest another mechanism of pancreatic dysfunction in these patients. Recent studies on *PRSS1*, *PSTI* and *CFTR* genes in ICP patients [12,14] suggest an accumulative mutational effect. However the number of patients in which mutations in two genes have been found is small,

indicating a more complex genetic background involved in the pathogenesis of the ICP.

In summary, we have found a high *CFTR* mutant frequency (40%) in alcoholic and idiopathic CP, supporting the involvement of *CFTR* gene in CP etiology and highlighting the major susceptibility of heterozygous individuals. Moreover, a different mutational spectrum was found in our series suggesting a different underlying mechanism that would need to be supported by further studies.

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TABLE 1. *Clinical Features and Genotype of 27 Chronic Pancreatitis Patients with the CFTR Mutations/Variants*

No	Sexe/Age	Evol. Time years	BMI	Alcohol	Alcohol time years	Tobacco	Pancreatic features	Hepatobiliary disease	*CFTR* genotype	Sweat Test mmol/L	FEV1 / FVC % predicted	Male fertility
	Alcoholic	*Chronic*	*Pancreatitis*		*(n = 15)*							
1	M / 52	15	24.5	110g/d	27	yes	AP, P, Ps, DM, PI	Chronic hepatitis[1]	F508del / S1235R	18	105 / 107	yes
2	M / 72	15	23.4	85g/d	22	yes	AP, P, C, PS	no	F508del /1716G/A	72	90 / 104	yes
3	M / 53	10	21.9	135g/d	20	yes	P, C, DM, PI	no	F508del / -	54	71 / 89	yes
4	M / 64	18	20.7	250g/d	27	yes	AP, P, C, Ps, DM, PI	cirrhosis, lithiasis	W1282X / -	68	71 / 78	unproved
5	M / 44	13	22.0	95g/d	6	yes	AP, P, C, Ps, DM, PI	lithiasis	R170C / -	16	105 / 111	yes
6	M / 62	12	22.1	> 60g/d	> 5	yes	AP, P, C, Ps, DM,PS	no	R258G / -	82	73 /82	yes
7	M / 38	9	18.0	210g/d	15	yes	AP, P, C, Ps, PS	no	M281T / -	62	132 / 126	yes
8	M / 40	11	-	> 60g/d	>5	yes	AP, P, C, Ps, PS	lithiasis	R297Q / -	46	103 / 99	yes
9	M / 42	2	21.4	150g/d	20	yes	AP, P, C, Ps, PS	no	1716G/A / -	19	93 / 102	yes
10	M / 44	3	22.2	95g/d	22	yes	AP, P, DM, PS	no	R668C / -	58	105 / 102	yes
11	M / 59	6	21.8	90g/d	18	yes	PS	lithiasis	L997F / -	85	69 / 84	nd
12	M / 72	16	-	> 60g/d	> 5	no	P, C, DM, PI	lithiasis	R1162L / -	-	-	yes
13	M / 35	8	21.0	90g/d	7	yes	AP, P, C, PS	no	5T-12TG-V470 / -	13	106 / 114	unproved
14	M / 60	14	28.0	80g/d	20	no	AP, P, C, Ps, DM, PI	no	5T-11TG /	28	80 / 77	yes
15	M / 65	12	24.4	100g/d	23	yes	AP, P, C, DM, PS	no	5T-11TG /	40	86 / 110	yes
	Idiopathic	*Chronic*	*Pancreatitis*		*(n = 12)*							
16	M / 21	5	-	no	-	yes	AP, P, PS	no	1716G/A / R170H	40	normal	yes
17	M / 59	4	24.2	no	-	no	PS	chronic hepatitis[2]	1716G/A / -	40	146 / 128	yes
18	M / 63	14	21.4	no	-	no	DM, PI	no	1716G/A / -	34	144 / 126	yes
19	M / 70	18	19.9	no	-	yes	AP, P, DM, PI	chronic hepatitis[1]	1716G/A / -	60	36 / 47	yes
20	M / 65	1	27.7	no	-	yes	P, Ps, DM, PI	no	1716G/A / -	38	79 / 78	yes
21	M / 76	8	24.1	no	-	no	AP, P, DM, PS	no	1716G/A / -	60	81 / 109	yes
22	M / 25	2	25.0	no	-	yes	AP, P, PS	no	1716G/A / -	48	94 / 86	nd
23	F / 42	10	22.6	no	-	yes	P, C, PS	lithiasis	P205S / -	72	111 / 109	-
24	F / 81	21	34.6	no	-	no	P, C, DM, PI	lithiasis	D443Y+G+R*/ -	42	121 / 108	-
25	F / 72	8	23.3	no	-	yes	AP, C, PS	no	L997F / -	40	100 / 93	-
26	M / 9	2	19.2	no	-	no	AP, P, PS	no	5T-11TG / -	30	101 / 110	nd
27	M / 63	6	-	no	-	no	C, DM, PI	cirrhosis	5T-11TG / -	-	-	yes

BMI: body mass index; FEV1: forced expiratory volume in one second; FVC: forced vital capacity

AP: acute pancreatitis; P: abdominal pain; Ps: pseudocysts; DM: diabetes mellitus; PI: pancreatic insufficiency; PS: pancreatic sufficiency; C: pancreatic calcifications

[1]: C virus hepatitis; [2]: assessed by histopathological study; *: the complex haplotype D443Y+G576A+R668C; nd: no documented

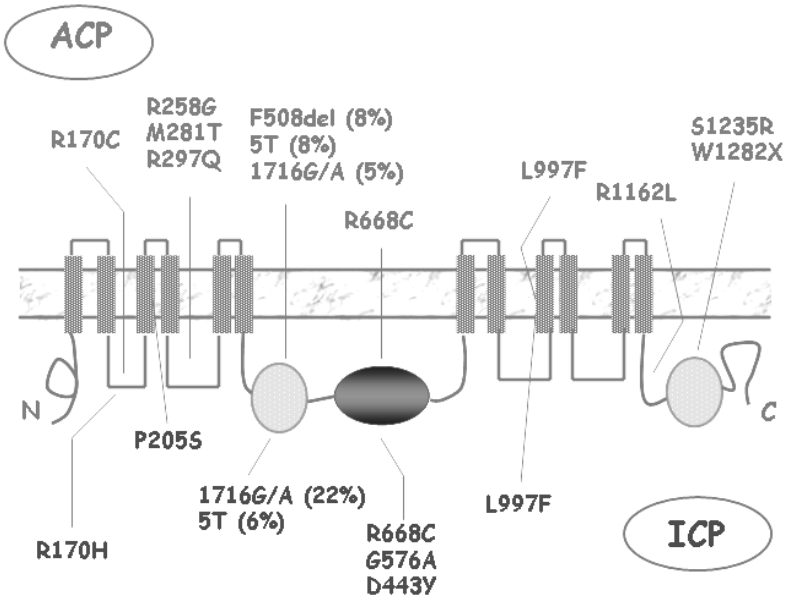
TABLE 2. *Clinical Characteristics of 68 Patients with Chronic Pancreatitis*

Features	Alcoholic Chronic Pancreatitis			Idiopathic Chronic Pancreatitis		
	Total n = 37	CFTR + n = 15	*CFTR* - n = 22	Total n = 31	*CFTR* + n = 12	*CFTR* - n = 19
Sex	36 M / 1 F	15 M / 0 F	21 M / 1 F	18 M / 13 F	9 M / 3 F	9 M / 10 F
Age (year, mean, range)	51.1 (35-72)	53.4 (35-72)	49.5 (35-63)	53.5 (9-82)	53.8 (9-81)	53.3 (12-82)
Age of onset (yrs, mean, range)	38.7 (25-57)	42.5 (27-57)	34.3 (25-46)	45.9 (7-72)	45.6 (7-68)	46.2 (8-72)
BMI (mean, range)	23.7 (18-34)	22.3 (18-28)	24.9 (18-34)	24.3 (16-35)	24.3 (19-35)	24.2 (16-34)
Evolution time (mean, range)	11.9 (2-25)	10.9 (2-18)	13.0 (2-20)	7.8 (1-21)	8.2 (1-21)	7.4 (1-20)
Acute pancreatitis #	23/28 (82%)	12/15 (80%)	11/13 (84%)	12/27 (44%)	6/12 (50%)	6/15 (40%)
Abdominal pain $	26/28 (93%)	14/15 (93%)	12/13 (92%)	18/27 (67%)	8/12 (67%)	10/13 (77%)
Tobacco &	26/28 (93%)	13/15 (86%)	13/13 (100%)	10/27 (37%)	6/12 (50%)	4/15 (27%)
Pancreatic calcifications &	32/37 (86%)	12/15 (80%)	20/22 (91%)	11/31 (35%)	4/12 (33%)	7/19 (37%)
Pancreatic pseudocysts *	18/28 (64%)	8/15 (53%)	10/13 (77%)	6/27 (22%)	1/12 (8%)	5/15 (33%)
Exocrine insufficiency	15/28 (53%)	6/15 (40%)	9/13 (69%)	13/27 (48%)	5/12 (42%)	8/15 (53%)
Diabetes mellitus	24/37 (65%)	9/15 (60%)	15/22 (68%)	17/31 (55%)	6/12 (50%)	11/19 (58%)
Chronic hepatitis	2/28 (7%)	1/15 (7%)	1/13 (8%)	3/27 (11%)	2/12 (16%)	1/15 (7%)
Liver cirrhosis	1/28 (3%)	1/15 (7%)	-	2/27 (7%)	1/12 (8%)	1/15 (7%)
Cholelithiasis	8/28 (28%)	5/15 (33%)	3/13 (23%)	11/27 (41%)	2/12 (17%)	9/15 (60%)

#:*P*= 0.005; $: *P*= 0.02; &: *P*< 0.001; *: *P*= 0.002, statistical significance referred to ACP (n=37) *vs* ICP (n=31)

FIGURE LEGEND

FIGURE 1: CFTR channel and location of the *CFTR* mutations identified in 27 patients with alcoholic (ACP) and idiopathic (ICP) chronic pancreatitis. Above, the mutations identified in 15 ACP patients. Below, those identified in 12 ICP patients.



Bronchiectasis in adult patients: an expression of heterozygosity for *CFTR* gene mutations?

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ABSTRACT

While all patients with cystic fibrosis (CF) have mutations in both *CFTR* alleles, often only one *CFTR* change is detected in patients with other lung disorders. The aim of this study was to investigate whether heterozygosity for *CFTR* mutations could be a determinant risk factor in the development of bronchiectasis in adult patients.

We have performed the *CFTR* gene analysis in a cohort of 55 bronchiectasis adult patients with unknown aetiology. The 5T variant, (TG)m and the M470V polymorphisms were also analyzed. A general population in which the same molecular analysis was previously performed was used as control group. The mutational spectrum of patients was also compared with that found in our CF population.

CFTR mutations/variants were found in 20 patients (36%), 14 with only one mutant gene (25%). All 6 patients colonized by *S aureus* presented at least one *CFTR* change (P=0.001). No statistical significance was observed between patients with and without mutations for other clinical features. The 5T variant was found in 4 patients. Additionally, 90% of patients with mutations had the more functional M470 allele (P<0.001).

These results reinforce the involvement of the *CFTR* gene in bronchiectasis of unknown aetiology in adult patients and support our hypothesis of haploinsufficiency.

Keywords: adult bronchiectasis, cystic fibrosis, *CFTR* mutations, CF-related diseases

INTRODUCTION

Bronchiectasis is defined as an abnormal irreversible dilatation of proximal subsegmental bronchi. It is not a disease *per se*, but it represents the end stage of a variety of pathological processes that cause the destruction of the bronchial wall and its supporting tissues. Although bronchiectasis can be focal, most cases are diffuse and are either associated with diseases predominantly affecting the lung, or they are the manifestation of a systemic disease. The spectrum of presumed causes and conditions associated with bronchiectasis is diverse leading investigation towards the intrinsic and extrinsic factors predisposing to bronchial damage. The cause of bronchiectasis in adult patients is only known in 40% of patients (1). However, the identification of that underlying cause could be vital for influencing treatment and preventing intervention.

Cystic fibrosis (CF; MIM#219700) is an autosomal recessive disorder that affects 1 in 2000-4000 newborns in the Caucasian population with a clinical phenotype that mainly includes chronic lung infection, gastrointestinal tract alterations and infertility in men (2). Mutations in the *CFTR* (*cystic fibrosis transmembrane conductance regulator*) gene (3) produce defective chloride ion transport, which causes dehydrated secretions leading to disease progression in the affected organs. More than 1000 mutations and 200 sequence variations have been identified in the *CFTR* gene (4), most of them in CF patients, but also in other related disorders. Thus, *CFTR* mutations have been found in several chronic pulmonary diseases (5-12). Additionally, previous studies support the role of polyvariant mutant *CFTR* alleles which, combined with the 5T variant, affect the *CFTR* expression level, contributing to the different *CFTR*-related phenotypes (13,14). In fact, the 5T variant

has shown a high specificity in congenital bilateral absence of vas deferens (CBAVD) (15).

We report here the complete analysis of the *CFTR* gene in a cohort of 55 adult patients with bronchiectasis of unknown aetiology. The aim of this study was to investigate whether *CFTR* mutations/variants, including the 5T-(TG)m-M470V polyvariant, are a determinant risk factor for the development of bronchiectasis in adult patients.

PATIENTS AND METHODS

Patients

Between 1994 and 2002, we recruited adult patients (≥ 18 years old) with bronchiectasis of unknown aetiology and normal sweat test for the *CFTR* gene study. The diagnosis of bronchiectasis was confirmed by high-resolution computed tomography (HRCT) as previously described (16). None of the patients fulfilled CF criteria. All patients had negative sweat tests (17) (normal being < 60 mEq/L chloride concentration or < 92 mEq/L sodium chloride conductance) and had normal pancreatic function (assessed by nutritional status, number and characteristics of stools, fecal fat or fecal elastase levels). None of the patients had previous history of pulmonary tuberculosis, aspiration or inhalation injury, lung abscess or severe pulmonary infection (Whooping cough, measles). Additionally, the following factors: primary or secondary hypogammaglobulinemia (IgG < 400 mg/dl in serum), absolute or partial IgA deficiency, α_1-antitrypsin deficiency, Young Syndrome, Kartagener's Syndrome (absence of dextrocardia), autoimmune systemic disorder or congenital anatomic defect, were excluded when considering the patients under study.

Personal history of pulmonary disease, bacteriologic sputum data, pulmonary function test [FVC (forced vital capacity) %predicted and FEV_1 (forced expiratory volume in one second) %predicted] and presence of allergic bronchopulmonary aspergillosis (ABPA) according to criteria diagnosis (18) were collected.

Fifty-five patients, 18 males (mean age 41.4 years, range 18-68) were included in this study. Two patients were sibs and another reported a second-degree relative with CF. All patients gave their informed written consent.

The *CFTR* mutations/variants found in this study were compared with those identified in the general population, partners of CF patients or carriers (n = 41) (12).

Additionally, we compared the frequencies of the common CF mutations in the bronchiectasis group with that found in CF Spanish patients (19).

CFTR gene analysis

Molecular analysis of the *CFTR* gene was performed in all 59 patients. Genomic DNA was isolated from peripheral blood lymphocytes using standard protocols (Promega, Wizard® Genomic DNA purification kit). After analysis of 31 common mutations in CF patients (PCR/OLA Applied Biosystems CF System), the whole coding region and intronic boundaries of the *CFTR* gene were analyzed using multiplex denaturing gradient gel electrophoresis (DGGE) (20) and single strand conformation polymorphism analysis (SSCP / Heteroduplex) (Genephor, Amersham Pharmacia Biotech, Buckinghamshire, England). The combination of these techniques gives a mutation detection level of 97% in the Spanish CF population (19; T Casals unpublished data). The fragments with an abnormal migration pattern were characterized by sequencing using the BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, USA) on an ABI 377 sequencer. The 5T variant was analyzed by DGGE, when it was identified, we performed direct sequencing of the (TG)m locus. The M470V polymorphism was determined by restriction enzyme analysis (21).

Statistical analysis

Statistical significance was evaluated by comparison between groups with the independent-samples T test and Levene's test for quantitative variables and the Fisher's exact 2-tailed test for qualitative traits, using the statistical analysis software package SPSS 8.0 (SPSS Inc., Chicago, IL). *P* values < 0.05 were considered to indicate statistical significance.

RESULTS

A total of fourteen mutations and two variants were identified in 20 patients (36%) with bronchiectasis (Table 1). There were no statistical differences in the clinical features as age, gender, bronchiectasis extension and pulmonary function test, between patients with and without *CFTR* mutations. However, presence of *Staphilococcus aureus* in sputum (P = 0.001) and ABPA were observed among patients with mutations and/or variants (Tables 1, 2).

F508del was the most common mutation, with a relative frequency of 25%. One stop mutation (G542X) and one splice site mutation (2789+5G>A) were detected in two other patients. Overall, these mutations were detected in 7 patients (12.7%). Taking into account their frequencies in Spanish CF patients (53.2%, 8.4% and 0.9%, respectively) (19) and assuming a 4% of CF carriers, the expected frequency of these mutations in the general population would be 2.5%, the frequency in bronchiectasis patients being significantly higher (P = 0.03).

Among the eleven missense mutations, the (G1237S) a G>A substitution at nucleotide 3841 is reported for the first time in this work and was detected in two sibs, the brother presenting oligozoospermia. In this patient, Young Syndrome was evaluated by means of scintigraphy to ciliary motility analysis and nasal mucus biopsy studied by electron microscopy. No alterations were found. Additionally, the patient was father of three children.

Moreover, two variants affecting normal CFTR synthesis (5T and 1716G/A) were found in 5 patients. The 1716G/A variant that changes the splice site consensus sequence leading to skipping of exon 10 (22) was identified in one patients (1.8%). Another four patients (7.2%) carried the 5T variant (one of them homozygous) a similar frequency to that found in the general population (9.4%). This variant

produces less splicing efficiency of exon 9, furthermore, a lower functional haplotype (5T-12TG-V470) was found in 3 out of 4 patients.

Eight patients had two *CFTR* changes, although it was not possible to determine if these changes corresponded to different alleles, we have found that they segregate independently in our CF families except the missense mutations G576A and R668C which occur on the same allele (26). So, we have found six patients (10%) with both *CFTR* genes mutated. The remaining 14 patients (25%) had only one *CFTR* mutant gene.

Finally, analysis of M470V polymorphism in exon 10 showed the M470 allele in 58% of patients, being not significantly different to the frequency in the general population (63%) (12). However, 90% of bronchiectasis patients with *CFTR* mutations had methionine (M) at this position, which is significantly different to the frequency observed in the general population (63%) ($P = 0.01$) or in bronchiectasis patients without *CFTR* mutations (40%) ($P < 0.001$).

DISCUSSION

We have performed clinical and molecular analysis in a series of 55 adult patients with bronchiectasis of unknown aetiology in order to investigate the role of the *CFTR* gene in this phenotype.

The mutational analysis performed led us to identify 20 patients (36%) with at least one *CFTR* mutation or variant. Previous reports (7-11), showed a frequency of *CFTR* mutations ranging from 8% to 48%. In order to obtain a homogeneous group, criteria for inclusion in our series were restricted to adult patients (>18 years old) suffering from bronchiectasis of unknown aetiology and without other features considered in the criteria for CF diagnosis (23). Moreover, all samples from patients and controls were submitted to a complete *CFTR* gene analysis using the two most common screening techniques (DGGE and SSCP/HD) followed by direct sequencing of the regions with abnormal patterns.

CFTR mutations have been described in patients with ABPA (5, 6) and oligoazoospermia (24), and it was not a surprise to find them in two and one, respectively, of our patients with *CFTR* changes. More interestingly, we found colonization for *Staphilococcus aureus*, which is frequent in CF patients (10) but rare in patients with bronquiectasis, (unless they posses a factor predisposing them to it) in six patients all of them with *CFTR* changes.

The mutational spectrum observed in adult patients with bronchiectasis differs considerably from that identified in the CF patients. Among the common mutations reported in Spanish CF families (19), only three were detected in the bronchiectasis group (F508del, G542X, 2789+5G>A). This different spectrum is probably due to the missense mutations, which show a higher frequency in bronchiectasis patients (69%) than in CF Spanish patients (37%) (T. Casals unpublished data).

One of the most extensively studied regions of *CFTR* is the poly-T tract of intron 8, which produces transcripts skipping exon 9 (25). Penetrance of the 5T variant depends on the (TG)m and M470V loci (13). Patients with the 5T variant presented two different haplotypes, 5T-12TG-V470 (the most frequent in CBAVD) and 5T-11TG-M470 or M/V (also identified on normal chromosomes). Controversial data on the role of the 5T variant in bronchiectasis patients have been reported. Pignatti and coworkers (7) after analyzing 16 bronchiectasis patients suggested that the 5T variant had a similar role to that described in the CBAVD phenotype. However, a second study did not detect a single case with the 5T variant out of 32 bronchiectasis patients studied (8). In our analysis of 55 patients we have found a slightly lower frequency (7.2%) of the 5T variant than in the general population (9.4%) (12) but significantly different ($P < 0.001$) from that reported in CBAVD patients (43%) (26). Consequently, we have discarded a predominant role of the 5T variant in bronchiectasis patients, however it was found in 4 patients.

We hypothesize a putative dominant effect of the *CFTR* mutations that could be associated with the presence of the M470 allele, which presents nearly twice the CFTR channel activity than the V470 allele (13) and was found over-represented (90%) among patients with *CFTR* changes. In fact, a similar frequency was found in a cohort of asthma (12) and rhinosinusitis (27) patients bearing *CFTR* mutations.

Little is known about most of missense mutations, especially that identified in normal chromosomes (4). However, a recent study (28) has showed that the G576A mutation, among others missense changes, lead to exon 12 skipping and the proportion of aberrant transcripts increase when R668C mutation in exon 13 is also present. The authors describe exonic regulatory sequences involved in the splicing process. This finding point out that missense changes must be accurately analyzed,

even if they have been found in normal chromosomes, because they could affect *CFTR* level expression.

In summary, our data suggest that heterozygosity (haploinsufficiency) for *CFTR* has pathogenic consequences, playing a role in the development of bronchiectasis in adult patients, probably with other genetic and epigenetic associated factors. Detailed epidemiological studies of subjects carrying *CFTR* mutations should be carried out in order to evaluate the importance of heterozygosity for *CFTR* mutations in *CFTR*-related phenotypes.

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Table 1. Comparision of clinical parameters between bronchiectasis patients with and without *CFTR* mutations/variants

Clinical data	**Total** (n = 55)	**Patients CFTR +** (n = 20)	**Patients CFTR -** (n = 35)
Gender - Male/Female	18 / 37	7 / 13	11 / 24
Age – years (mean ± SD)	40.6 ± 15.0	40.5 ± 14.2	40.6 ± 16.3
Age onset – years (mean ± SD)	15.9 ± 15.2	14.9 ± 14.1	16.5 ± 15.9
Sinusitis – n (%)	29 (52.7)	10 (50.0)	19 (54.2)
Pneumonia – n (%)	26 (47.2)	7 (35.0)	19 (54.2)
Bronchiectasis – n (%)			
unilateral	9 (16.3)	4 (20.0)	5 (14.2)
bilateral or disseminated	46 (83.6)	16 (80.0)	30 (85.7)
Sputum culture – n (%)	55	20	35
P. aeruginosa	21 (38.1)	5 (25.0)	16 (45.7)
*S. aureus**	6 (10.9)	6 (30.0	-
H. influenzae	15 (27.2)	3 (15.0)	12 (34.2)
FEV1 - mean %predicted	64.0	66.7	62.4
FVC - mean %predicted	75.7	76.2	75.4
ABPA – n (%)	2 (3.6)	2 (10.0)	-
Oligozoospermia – n (%)	2 (3.6)	1 (5.0)	1 (2.8)

*$P < 0.001$ (Fisher's exact two-tailed test).
FEV1: forced expiratory volume in one second (% of predicted value); FVC: forced vital capacity (% of predicted value); ABPA: allergic bronchopulmonary aspergillosis

Table 2. Clinical features and *CFTR* genotypes found in 20 adult patients with bronchiectasis

Sample	Sex/Age	Age onset years	FEV1/FVC %predicted	Bacterial colon.	Sweat test mEq/L	Lobes affected	Clinical features	First *CFTR* change	Second *CFTR* change	M470V
1	M / 41	5	20 / 43	*P*	30 [a]	>4	-	F508del	L997F	M / V
2	F / 23	17	85 / 89	*P, S*	46 [a]	>4	SN, ABPA, PN	F508del	-	M / M
3	F / 24	1	60 / 74	*P, S*	49 [a]	>4	SN, PN	F508del	-	M / V
4	M / 55	-	87 / 84	*S*	32 [a]	2	-	F508del	-	M / V
5#	F / 37	29	91 / 93	*S*	41 [a]	>4	PN	F508del	-	M / V
6	F / 33	32	86 / 84	no	51 [a]	2	-	G542X	-	M / M
7	F / 30	6	101/112	no	56 [a]	>4	-	2789+5G>A	5T-12TG	M / V
8	F / 38	15	106/104	no	29 [a]	2	Otitis	S1235R	-	M / V
9	F / 34	birth	75 / 100	*H*	20 [a]	>4	SN	V562L	5T-11TG	M / V
10*	F / 36	5	30 / 51	*P*	20 [a]	>4	SN, PN	G1237S	-	M / V
11*	M / 40	14	73 / 92	*H*	26 [a]	3	SN, PN, OZ	G1237S	-	M / V
12	F / 23	5	41 / 47	*S*	23 [a]	>4	Hemoptysis	R347H	R75Q	V / V
13	F / 68	5	48 / 52	no	34 [a]	>4	PN	Y1014C	5T-12TG	V / V
14	M / 64	30	88 / 84	*H*	39 [a]	2	-	R75Q	-	M / V
15	M / 40	childhood	56 / 79	no	33 [b]	>4	SN, asthma	V754M	-	M / M
16	M / 47	45	94 / 108	no	19 [a]	2	SN, PN	Q179K	-	M / V
17	M / 23	childhood	38 / 34	no	28 [a]	2	SN, PN	5T-12TG	5T-11TG	M / V
18	F / 69	50	68 / 89	*S*	52 [a]	4	Diabetes	G576A, R668C	-	M / V
19	F / 47	childhood	16 / 18	*P*	64 [b]	>4	-	G576A, R668C	-	M / V
20	F / 38	6	72 / 88	no	39 [b]	>4	SN, ABPA, asthma	1716G/A	-	M / M

#: a niece with CF disease; *: sibs; M: male; F: female

FEV1: forced expiratory volume in 1second (% of predicted value for height); FVC: forced vital capacity (% of predicted value for height)

P: *Pseudomonas aeruginosa*; *H*: *Haemophilus influenza*; *S*: *Staphylococcus aureus*

a: chloride concentration; b: sodium chloride conductance

SN: sinusitis; ABPA: allergic bronchopulmonary aspergillosis; PN: pneumonia; OZ: oligozoospermia