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Congenital bilateral absence of the vas deferens and cystic fibrosis

A genetic commonality

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Summary. CF and CBAVD are really just ends of a clinical spectrum. The type and nature of the mutations in the CF gene probably determine the phenotypic expression of the patient. Perhaps all patients homozygous for ΔF508, for example, will have severe pulmonary and pancreatic disease as well as absent vasa, whereas those with other combinations, such as ΔF508/D1270N, will be unaffected in terms of pulmonary and pancreatic function but will have absent vasa. Besides contributing to a better understanding of the nature of CBAVD, this linkage of CF and CBAVD most importantly mandates genetic screening and counseling for appropriate family members and even the patient's spouse. In addition, a broader understanding of CF is now at hand, as this brings a whole new cohort of patients under the CF umbrella. Many of these will have at least one, if not two, rare or novel CF gene mutations. Once all of these mutations have been detected and defined, our knowledge of the CF gene, its mutations, and their implications will be dramatically expanded.

Congenital bilateral absence of the vas deferens (CBAVD) occurs in approximately 1.4% of the infertile male population [14]. The disorder is characterized by the bilateral absence of scrotal vasa. A strict definition of CBAVD needs to be adhered to and physical examination of the scrotal vasa remains the best way to make the diagnosis. If either one or both vasa are palpable from their origin at the lower pole of the epididymis all the way to their disappearance at the external ring, then CBAVD is *not* present. CBAVD, by definition, occurs only when both scrotal vasa are deficient, either partially or totally. This distinction is important and necessary at this point in time for the proper categorization of the various types of vasal agenesis and the subsequent study of the genetic aberrations and/or embryological mishaps underlying the development of each type. All patients have a physical examination but not all undergo maneuvers to detail the exact anatomy of the more distal extent of the vasa. As is discussed below, CBAVD is now known to be a primarily genital form of cystic fibrosis (CF). Genetic analysis searching for the mutations that cause CF and proper counseling about their implications is now an important part of the complete evaluation and management scheme for those patients discovered by the urologist to have CBAVD.

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CBAVD – clinical manifestations

Since the seminal vesicles are often anatomically and functionally abnormal in men with CBAVD, the patient presents with a low-volume (< 1 cc), acidic (pH < 7.0) ejaculate devoid of sperm. Kuligowska et al. [19] demonstrated in 36 patients with CBAVD who underwent transrectal ultrasonography that only a small percentage of the seminal vesicles imaged were completely normal when combinations of length, width, and echogenic pattern were evaluated. The seminal vesicles contribute up to 70% of the fluid found in a normal ejaculate. This fluid is rich in fructose and is highly alkaline. The prostate produces a counterbalancing acidic fluid that comprises up to 20% of the seminal volume. Since the prostate is normal in structure and function, the low-volume ejaculate produced by men with CBAVD is mostly, if not entirely, prostatic fluid and, therefore, acidic with an absence of fructose. Obviously, the azoospermia is "obstructive" in nature since no transport of testicular sperm is possible secondary to the absent vasa.

Testicular function, both androgenic and spermatogenic, is adequate as shown by testicular size and consistency, serum levels of follicle-stimulating hormone, and testicular biopsy [33]. The caput epididymis is always present, although frequently detectable are variable lengths of corpus and/or cauda. Therefore, when the azoospermic patient presents with a low-volume, acidic ejaculate; normal-size testicles with normal consistency; non-

palpable scrotal vasa; and firm and dilated epididymal remnants, the diagnosis is CBAVD and scrotal exploration is unwarranted. In this circumstance, transrectal ultrasonography will detail and confirm the coexistent seminal vesicle anomalies and cystic fibrosis mutation analysis will usually reveal that the patient has one or two mutations in the cystic fibrosis transmembrane conductance regulator protein gene (see below). These two tests will confirm the diagnosis of CBAVD if there is any doubt.

The typical CBAVD patient is otherwise healthy with no specific complaints referable to the pulmonary system or the gastrointestinal tract. There is a slightly higher than normal incidence of renal anomalies of position or number. Renal ultrasonography is best suited for noninvasive imaging of the upper genitourinrary tract [9, 19]. Until recently, vasal agenesis was considered untreatable and the diagnosis of CBAVD effectively terminated a man's pursuit of biological paternity. However, Silber et al. [31, 32] combined the direct microsurgical aspiration of sperm (MESA) from the proximal ductal system with the technique of in vitro fertilization and achieved pregnancies. Since their first report, many other groups comprised of urologists working in conjunction with gynecologists and reproductive endocrinologists have undertaken this complicated, coordinated effort with variable success (average 10% pregnancy rate per attempt) [23]. These initial successes have ignited a renewed interest in this entity and sparked further developments relating to the harvesting and processing of human epididymal sperm. In addition, much has been learned about the epididymal environment in the chronically obstructed system and about the maturation of human sperm and their acquisition of fertilizing ability. Most importantly, it has clearly been shown that CBAVD is a mild form of cystic fibrosis, whereby only the reproductive consequences of cystic fibrosis are manifested.

Cystic fibrosis – clinical manifestations

Cystic fibrosis (CF) is the most common, severe autosomal recessive affliction in Caucasians of Northern European descent, its carrier frequency being 1 in 25 [21]. The incidence of clinically recognized CF in that specific population ranges from 1:1600 to 1:2000 [29], whereas it is significantly lower in certain ethnic subgroups. The predominant, clinically relevant manifestations of the disease involve the respiratory and gastrointestinal systems [5, 29]. In its most aggressive form, chronic, obstructive pulmonary disease leads to significant morbidity and death. Bronchial secretions are thick and viscid, and difficulty in aeration can lead to patchy atelectasis, air trapping, and bronchiectasis. Superinfection with a multitude of opportunistic organisms (most often *Pseudomonas* species) is common and leads to a further, inexorable deterioration of pulmonary function. Present-day management schemes have reduced but not eliminated the consequent deleterious pulmonary effects of CF and have led to a prolongation of survival and an improved quality of life. Treatments include nonspecific measures such as intensive pulmonary physiotherapy and specific maneuvers involving targeted antibiotic therapy and inhalation of substances such as amiloride [18] aimed at potentially altering and counteracting the basic epithelial electrolyte defect present in CF.

Exocrine pancreatic function is significantly impaired in the majority of patients with CF, as the tenacious, mucoid secretions containing the digestive enzymes impede and occlude normal pancreatic ductal flow, resulting in obstruction and eventual dysfunction. These patients complain of fat intolerance and oily stools. However, oral pancreatic enzyme replacement has effectively combated the nutritional ravages of the disease, and the level of pulmonary disease determines for the most part the life expectancy of the patient. Chronic sinusitis, cholelithiasis, focal biliary cirrhosis, and intestinal obstruction are less frequently seen in CF. A family history is found in many patients, although the patient may certainly be the index case.

Finally, the vasa deferentia are palpably absent in approximately 80% – 97% of patients clinically diagnosed as having CF [11, 29]. After the initial report of Denning et al. [7] had documented low-volume azoospermia in men with CF, Kaplan et al. [15] discovered that the true cause was complete bilateral vasal agenesis. Their studies spawned an immediate outpouring of articles confirming these findings [20, 25, 35], demonstrating as well that the caput epididymis was invariably present and that the vasal aplasia existed at birth and was not an acquired lesion. However, no treatment existed and sterility was the rule, although MESA has recently been applied to a patient with CF [24]. It was even suggested by Stern et al. [34] that obstructive azoospermia should be used as a criterion for the diagnosis of atypical presentations of CF. However, at that time the latter authors could not be so bold as to propose that bilateral absence of the vas *as a sole finding* would also be a variant of classic CF. Holsclaw et al. [12] did state that CF should be entertained as a possibility in the infertile patient with obstructive azoospermia.

CF – clinical and genetic diagnosis

Until recently, the diagnosis of CF was predicated solely on the available clinical signs and symptoms. A combination of classic pulmonary and gastrointestinal findings along with elevated sweat chloride values established the diagnosis. The sweat test has been the gold standard in terms of laboratory assessment. If the sweat chloride value is >60 mEq/l, CF is highly likely, as there are only a few diseases of childhood that will cause this elevation and fewer still in the adult population. Today, genetic mutation analysis of the CF gene is coupled with the clinical signs and symptoms to complete the diagnostic workup.

The strategy for molecular isolation and recognition of the CF gene has required the concerted efforts of several major groups over the past several years [16, 26, 27] and has recently been reviewed [2]. The complete gene is approximately 250 kb in length and is located on chromosome 7. The sequence of the 27 exons and associated exon-intron boundaries is known [37]. Sequence analysis of the coding region predicts a polypeptide of 1,480 amino

acid residues encoded by a 6.5-kb mRNA. The residues of the 170-kDa protein are organized into two repeat motifs, each containing a membrane-spanning domain with six transmembrane regions and an intracytosolic adenosine 5′-triphosphate (ATP)-binding region. Bridging the two motifs is a large, globular "R domain" that is rich in charged residues and potential phosphorylation sites [13, 26]. Based on its predicted domain structure, the cystic fibrosis transmembrane conductance regulator (CFTR) protein is a member of a class of related proteins that are characteristically involved in pumping molecules into or out of cells. The clinical variability of CF suggested the possibility of allelic heterogeneity (multiple, different mutations occurring independently in a single gene). For CF, all mutations occur in the CFTR gene, but each different mutation disturbs the configuration and/or function of the eventual CFTR protein in a different way, thereby explaining the variability seen in the phenotypic (clinical) presentation of the patient.

The identification of the CF gene and the ability to directly detect the common CF alleles have opened up a new era for genetic counseling, carrier testing, and diagnosis for CF. Genomic DNA is routinely prepared from peripheral blood samples using standard methods of nuclear pellet isolation, lysis, protease digestion, and extraction with organic solvents or salt. Because the assays for direct detection of CF mutations utilize amplification of DNA via the polymerase chain reaction (PCR), crude DNA can be prepared to provide a more rapid result. Fetal DNA is prepared from either cultivated or noncultivated amniotic fluid or chorionic villous biopsy.

In the clinical setting, a number of strategies are used to detect different CF mutations. For example, the ΔF508 and ΔI507 deletions remove three base pairs from exon 10. Thus, the genotypes are visualized directly following amplification by electrophoretic size-fractionation on an 8% acrylamide gel. The assay that we use generates normal and mutant amplification products that are 83 and 80 bp long, respectively, as well as heteroduplex fragment that is always present in heterozygotes. The ΔF508 and ΔI507 mutations can be distinguished from one another on the basis of different mobilities of the heteroduplex fragment.

Many point mutations generate or remove a naturally occurring restriction endonuclease recognition site. Thus, as is the case for R117H, 621+1 G→T, 1717-1 G→A, S549N, G551D, R553X, R560T, and W1282X, digestion of the amplification product with the appropriate restriction enzyme and subsequent electrophoresis generates DNA fragments for the normal and mutant alleles that differ in size.

Another useful strategy, first developed by Haliassos et al. [10], detects mutations such as G542X and N1303K for which the base substitution does not alter a naturally occurring restriction endonuclease fine site [21]. This method exploits a modified primer directed a few bases upstream from the mutation that contains a single base pair mismatch such that amplification is achieved but an artificial recognition site is created. Thus, mismatch amplification across the G542X mutation allows electrophoretic resolution of the alleles by virtue of creation of a BstNI site for the normal allele but not for the mutant allele [22]. Similarly, mismatch amplification for N1303K generates an artificial BstNI site for the N1303K mutant allele. For efficiency, some mutations can be coamplified in multiplex fashion in the same reaction tube. A more general strategy can be used to analyze these mutations by hybridization to allele-specific oligomeric probes. The amplified DNA is applied in duplicate to two hybridization membranes, one of which is probed with a DNA sequence that matches the normal allele and the other, with a mutant oligomer. However, the utility of this general method is limited by the length of time required for the procedure and the requirement of radioactivity.

In theory, the most complete method for detecting rare mutations in CF patients is determining the complete base sequence of the CFTR gene. In practice, however, this method is not feasible because of the large size of the gene and the amount of labor involved. Thus, methods have evolved for efficient screening of exons for potential changes prior to sequence determination. The method that we have most frequently used for CBAVD patients is assay of single-stranded conformation polymorphisms (SSCPs). This newly described technology allows the detection of insertions, deletions, and point mutations in short stretches of DNA by denaturating the DNA and resolving it on nondenaturing acrylamide gels. Each strand of the DNA fragment forms a unique conformation in the gel, and any mutation(s) within that segment can potentially affect its mobility. This technique has been used to identify many CFTR mutations in classic CF patients [6, 36]. This technique was also used to detect the G576A and D1270N mutations in men with CBAVD [1].

Once a potential new mutation has been identified on the basis of exon screening and direct sequencing, we develop a routine assay so as to screen a large collection of known CF chromosomes. We also test the parents of the CBAVD-afflicted man in whom the mutation has been detected so as to determine the parental origin of the mutation. If both CF mutations of the CBAVD patient have been defined by these analyses of common, rare, and novel mutations, we determine whether the two mutations occur in different parental CF genes. Where possible, we compare the genotype of the CBAVD patient with that of his siblings.

We designate an alteration detected by our screening assays as a true mutation only if the sequence variant has potential significance on the CFTR protein and is not found on normal chromosomes and if the two CF mutations in the CBAVD patient are derived from different parents. If, however, the alteration represents a benign polymorphism, we would expect to see this change on normal chromosomes. Moreover, we would expect a benign alteration to appear on the same parental chromosome as another CF mutation.

The major mutation in the CF gene is ΔF508, a 3-bp deletion in exon 10 that removes a phenylalanine residue at position 508 in one of the ATP-binding domains [16]. ΔF508 is found on 68% of CF chromosomes worldwide and on 76% of CF cromosomes in the Northern European population [3]. Intensive efforts by the CF International Genetic Analysis Consortium, which includes

more than 80 laboratories, have now identified over 250 additional CF mutations. The majority of CF mutations are point mutations that result in amino acid substitution, introduction of a stop codon, or splicing errors. Frameshift due to deletion or insertion, as well as potential regulatory mutants, have also been detected in CF patients. The majority of CF mutations are private, occurring only in individual families.

Among Northern Europeans, 11 additional mutations (R117H, 621+1 G→T, ΔI507, 1717-1, G→A, S549D, G542X, G551D, R553X, R560T, W1282X, and N1303K) are found at a combined frequency of 12.8% [22]. Thus, based on 12 common mutations, the rate of mutation ascertainment for this population approaches 90%. The frequency of ΔF508 is lower in other ethnic subgroups. Within the Jewish population, for example, ΔF508 is seen in only approximately 25% of CF chromosomes; G542X occurs on about 5%, and G551D and R553X have not been observed with any significant frequency [22]. Shoshani et al. [30] report that W1282X occurs on 58% of Ashkenazi Jewish CF chromosomes in Israel; thus, mutation detection in this population approaches 90%.

CBAVD and CF – genetic commonality

With access to a large population of patients with well-characterized CBAVD, the question arose in 1990 as to whether this disorder was related in any fashion to CF, since the reproductive ductal deficiencies were so similar. Except for the obstructive azoospermia, the clinical presentation of the patient with CBAVD is dramatically different from that of the man with classic CF. Until this time, CBAVD had been considered its own separate entity and had been catalogued as such. The inheritance pattern of CBAVD had been assumed to be autosomal recessive [4], although different modes of inheritance such as X-linked recessive and autosomal dominant had been proposed [17]. It was unclear, therefore, whether any solid connection between CF and CBAVD would or could be discovered.

With the newfound ability to assay for an detect the various mutations in the CFTR gene that result in CF, as described above, it was a beautiful synthesis of clinical medicine (the recognition that CBAVD and CF may indeed be similar and related) and molecular genetics (the expertise to perform the genetic analyses and interpret the results) that allowed us to embark on our study of the genetics of CBAVD in 1990 [1]. Our results confirmed and substantially expanded upon the preliminary work published in a letter by Dumar et al. [8] that suggested a genetic commonality between CBAVD and CF. Of 25 patients in our initial study cohort with confirmed CBAVD (no patient had pulmonary or gastrointestinal signs or symptoms consistent with CF), 16 (64%) had at least 1 detectable CF mutation. This is 16 times the frequency expected in a randomly selected population (4% carrier rate). Three of these men were determined to be compound heterozygotes (a mutation was detected in both maternal and paternal CFTR genes). In all, 13 of these men carried ΔF508 and no patient was ΔF508 homozygous. One patient had a completely new mutation (D1270N). Our population of patients continues to expand, and most live regionally. Our latest results are detailed in Tables 1 and 2 and Fig. 1[1], which list the different mutations found and their overall incidence in our CBAVD cohort.

Genetic implications

The consequences of this newly found information are quite profound. A man with CBAVD should have genetic screening at a reputable laboratory that can carry out assays to detect not only the common mutations but also the rarer mutations seen in this population. Appropriate counseling is then in order with a clinician who appreciates the nature of this disorder and the implications of the diagnosis of CF, albeit a "mild" form. A careful search for pulmonary and pancreatic clinical signs and symptoms should be undertaken. Any subtle abnormality present should be pursued, as it may be a harbinger of more serious clinical disease to come. For example, mild pulmonary disease as detected by pulmonary function testing may be an indication that symptomatic disease will arise in the future and that a prophylactic and/or cautious approach is appropriate to forestall the more serious consequences of CF that may develop. Although it cannot be assumed a priori that mutations in CF genes will necessarily lead to pulmonary dysfunction, this is the safest approach for the patient at present, and, therefore, baseline testing is warranted.

If the patient is pursuing biological fatherhood via microsurgical sperm aspiration coupled with the advanced reproductive techniques, then his partner should be genetically assessed for accurate evaluation of their risks as a couple of passing along CBAVD or CF. The baseline risk that the patient's wife, who is unrelated and has no family history of CF, is a carrier is 1 in 25 (4%). If this couple conceives as a result of microsurgical aspiration, the risk of CF to the fetus is as high as 1 in 50 (2%). This is a 50-fold increase in risk relative to the general population. However, 90% of CF mutations are detected by assay of the wife's DNA for the 12 common mutations listed above. If she is negative for each of these 12 mutations, her carrier risk is decreased 10-fold to 0.4% and the risk of CF to the fetus is similarly reduced to 0.2%. Alternatively, if the wife is found to be a carrier, the risk of CF/CBAVD transmission of the fetus is as high as 50%. In this unusual circumstance, preconception genetic counseling would outline three possibilities for the couple if they desired pregnancy and fertilization occurred during aspiration/in vitro fertilization (IVF). The couple could choose to continue the pregnancy without regard for the genetic status of the conceptus and with full knowledge of the possibility that the child could be born with CF. Alternatively, the couple could opt for spontaneous termination if the fetus were shown to be at risk for CF/CBAVD as determined by genetic analysis on samples

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Table 1. CFTR gene status in 41 patients with CBAVD

Patient number	R75Q	R117H	R347P	ΔF508	Q493X	G551D	G576A	Y1092X	D1270N	Wild
1										×, ×
2				×			×			
3				×						×
4				×			×			
5				×						×
6				×						×
7					×					×
8				×						×
9										×, ×
10	×									×
11										×, ×
12		×		×						
13										×, ×
14				×						×
15			×							×
16				×						×
17		×				×				
18				×						×
19		×		×						
20				×						×
21		×		×						
22	×									×
23				×						×
24						×				×
25										×, ×
26				×						×
27				×						×
28				×					×	
29								×		×
30										×, ×
31				×						×
32				×						×
33										×, ×
34										×, ×
35				×						
36				×						×
37				×						×
38										×, ×
39				×						×
40				×						×
41				×						×

All subjects are patients of R.D.O. *Wild*, No mutation presently identified

Table 2. Summary of mutations: number of patients with specific combinations

ΔF508/ W	ΔF508/ G576A	ΔF508/ R117H	ΔF508/ D1270N	Δ508/ R75Q	R117H/ G551D	Q493X/ W	R75Q/ W	R347P/ W	G551D/ W	Y1092X/ W	W/ W
18	2	3	1	1	1	1	2	1	1	1	9

Total = 41 patients. Patients wtih Δ508 = 25 (61%); patients with R117H = 4 (9.7%); patients with R75Q = 3 (7.3%); patients with G551D = 2 (4.8%); patients with G576A = 2 (4.8%); patients with at least 1 mutation identified = 32 (78%); patients with 2 mutations identified = 8 (19.5%), compound heterozygotes); patients with no mutation identified = 9 (22%)

provided by chorionic villous sampling (performed during weeks 9–12 of gestation) or amniocentesis (performed during weeks 15–16 of gestation). Finally, since the technique of IVF is necessarily employed, preimplantation genetic screening via blastomere biopsy could be performed on all resultant embryos, and only those deemed not at risk for CF/CBAVD could be transferred to the uterus (IVF) or fallopian tubes (TET, tubal embryo transfer).

Family members of the patient should also be offered CF mutation analysis. These mutations, like all others, are heritable and are likely to occur in other family members. Parental testing will help determine which other members of the extended family should be screened. As an example, if the paternal mutation is ΔF508, all of the father's siblings (the patient's paternal aunts and uncles) will have a chance of being carriers for ΔF508, as will their children as well (the patient's first cousins on his fa-

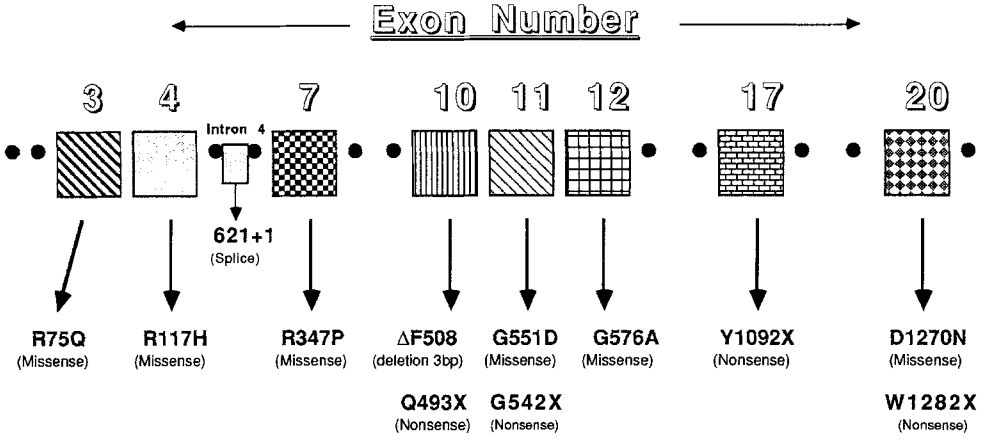

Fig. 1. CF gene mutations detected in patients with (CBAVD) *Nonsense mutation:* transcribed into a stop codon – truncated protein; *missense mutation:* transcribed into a different codon – altered a.a.

ther's side). The patient's siblings have an even greater risk of inheriting one or possibly two CF mutations. If the patient is ΔF508/G576A heterozygous (assuming that ΔF508 has been inherited from the father and G576A, from the mother), then each sibling has a 25% chance of inheriting both mutations, a 50% chance of being a carrier with one mutation only, and a 25% chance of having no mutation at all (Fig. 2).

If a male sibling has inherited both mutations, he will in all likelihood express a CBAVD phenotype but may be affected to a greater or lesser degree than his brother in terms of pulmonary and pancreatic function. At this time there is no strict correlation between the genotype found in men and the ultimate phenotype expressed. This situation of more than one brother being afflicted with CBAVD is probably not rare, as the frequency of this happening should approach 25% if there is an identified index male sibling in the family. Sporadic reports have documented the existence of such brothers [28].

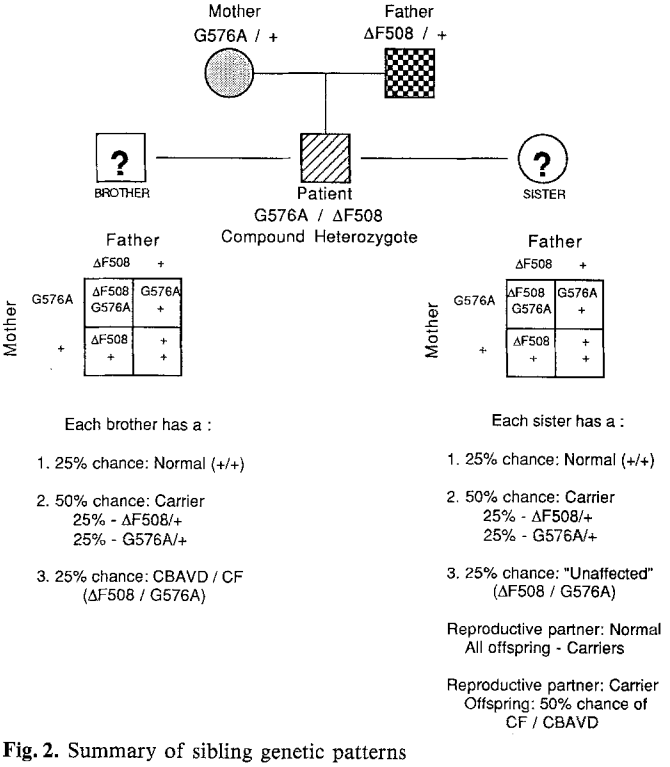

Fig. 2. Summary of sibling genetic patterns

If a sister has inherited both mutations, she may not have any sign or symptom of CF, as the fallopian tubes (the counterpart of the internal male ductal system) are not anatomically affected in CF and fertility is not compromised. However, all of her children will be at least obligate CF carriers. If she reproduces with a man who is a CF carrier (a 4% – 5% chance), each of her offspring will have a 50% chance of having two CF mutations and manifesting either CF or CBAVD, depending on the exact combination.

If the patient's brother or sister is only a carrier and their partner is a carrier as well, the frequency of that couple's child having two mutations and potentially expressing CF or CBAVD is 25%. As can be seen, it is extremely important to screen all of the patient's siblings so as to estimate and revise their risk of passing along CF/CBAVD or even to diagnose the disorder if that sibling is a brother.

Therefore, we have conclusively shown that CF and CBAVD share a common genetic background. The phenotypic spectrum of disease is wide, with a general correlation existing between genotype and phenotype that is based on the exact combination of specific mutations inherited.

CBAVD/CF: vasal embryology

It is unclear why there is such a devastating effect on the development of the reproductive portion of the mesonephric duct in men who are heterozygous for two CF mutations. The timing of the insult must, however, be after the 7th week of gestation, by which time the mesonephric duct has split into its reproductive and ureteral divisions. If the insult manifested itself before this time and the entire, more primitive mesonephric duct were affected, a high incidence of renal agenesis or ectopy would be expected. The actual incidence is quite low in those patients who have been diagnosed as having CBAVD and in whom renal ultrasound has been performed. It is more than likely, albeit certainly not proved, that the CFTR protein and the effects of its mutation alter the intraluminal environment of the developing vasal structures, whereby their morphogenesis is grossly impaired. There is some precedent for this in that gallbladder atresia is known to occur in CF patients. How the defect in

epithelial cell Cl^- transport would affect vasal development is unknown. Hypothetically, for the lumen of the embryonic vas to achieve patency during its earliest stages and to remain that way during the later stages of gestation, the luminal epithelial cells may need to secrete a fluid whose normalcy is dependent on proper transmembrane Cl^- transport. Studies looking at the fetal vas and its transmembrane ionic transport and intraluminal fluid composition may begin to help answer this fundamental question.

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