

## Cystic fibrosis transmembrane conductance regulator (*CFTR*) gene abnormalities in Indian males with congenital bilateral absence of vas deferens & renal anomalies

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**Background & objectives:** The role of cystic fibrosis transmembrane conductance regulator (*CFTR*) gene mutations in congenital bilateral absence of vas deferens and unilateral renal agenesis (CBAVD-URA) has been controversial. Here, we report the cases of five Indian males with CBAVD-URA. The objective was to evaluate the presence or absence of *CFTR* gene mutations and variants in CBAVD-URA. The female partners of these males were also screened for cystic fibrosis (CF) carrier status.

**Methods:** Direct DNA sequencing of *CFTR* gene was carried out in five Indian infertile males having CBAVD-URA. Female partners (n=5) and healthy controls (n=32) were also screened.

**Results:** Three potential regulatory *CFTR* gene variants (c.1540A>G, c.2694T>G and c.4521G>A) were detected along with IVS8-5T mutation in three infertile males with CBAVD-URA. Five novel *CFTR* gene variants (c.621+91A>G, c.2752+106A>T, c.2751+85\_88delTA, c.3120+529InsC and c.4375-69C>T), four potential regulatory *CFTR* gene variants (M470V, T854T, P1290P, Q1463Q) and seven previously reported *CFTR* gene variants (c.196+12T>C, c.875+40A>G, c.3041-71G>C, c.3271+42A>T, c.3272-93T>C, c.3500-140A>C and c.3601-65C>A) were detected in infertile men having CBAVD and renal anomalies

**Interpretation & conclusions:** Based on our findings, we speculate that CBAVD-URA may also be attributed to *CFTR* gene mutations and can be considered as CFTR-related disorder (CFTR-RD). The *CFTR* gene mutation screening may be offered to CBAVD-URA men and their female partners undergoing ICSI. Further studies need to be done in a large sample to confirm the findings.

**Key words** Congenital bilateral absence of vas deferens - congenital absence of seminal vesicles - cystic fibrosis transmembrane conductance regulator gene - ectopic kidney - unilateral renal agenesis

The incidence of cystic fibrosis (CF) is considered to be low in Asian population as compared to Caucasians; however, the incidence of congenital bilateral absence of vas deferens (CBAVD) does not differ between these populations<sup>1</sup>. The CBAVD occurs in 2-6 per cent of infertile but otherwise healthy men and is responsible for as much as 25 per cent of obstructive azoospermia<sup>2</sup>. Isolated CBAVD is transmitted as autosomal recessive trait and is frequently associated with mutations in cystic fibrosis transmembrane conductance regulator (*CFTR*) gene<sup>3</sup>.

Geographical or ethnic differences have been demonstrated in *CFTR* gene mutations in males with CBAVD with a high frequency of F508del mutation in Caucasians but rarely observed in Japanese, Taiwanese and Chinese populations<sup>1,4,5</sup>. However, the IVS8-5T allele is reported to be higher or of same frequency in non-Caucasian populations<sup>1,6</sup>. CBAVD is known to be associated with renal anomalies in 12-21 per cent of cases<sup>3</sup>. The exact mechanism of pathogenesis of CBAVD contributed by *CFTR* gene mutations and variants is still poorly understood. The role of *CFTR* gene mutations in patients with CBAVD having unilateral renal agenesis (CBAVD-URA) is controversial as in majority of cases with CBAVD-URA reported in literature; *CFTR* gene mutations were not detected<sup>3,7,8</sup>. In view of these observations, such cases of CBAVD-URA are proposed as a distinct entity arising of genetic causes other than *CFTR* gene mutations probably due to paucity of such data<sup>3</sup>. Information about the frequency of *CFTR* gene mutations in Indian patients with CBAVD is limited. Further, there are no guidelines for *CFTR* gene mutations screening in India for males with CBAVD.

We describe here the detection of *CFTR* gene mutations and variants in five infertile males with CBAVD-URA. The female partners were also screened to assess the CF carrier status.

### Material & Methods

The study was conducted at the National Institute for Research in Reproductive Health, (NIRRH) Mumbai, India, during 2011-2014. Written informed consent was obtained from cases, their female partners and healthy controls included in our study. The study was approved by the institutional ethics committee. None of the patients with CBAVD-URA reported in our study had associated clinical manifestations (pulmonary or gastrointestinal) or a family history suggestive of CF. The diagnosis of CBAVD was based

on azoospermia, low seminal volume (<1.5 ml), absent/low fructose in seminal fluid and absence of bilateral vas deferens on scrotal examination by the andrologist. Testicular volume was evaluated using a Prader orchidometer (Holtain, UK). Ultrasonography (USG) of the abdomen and pelvis was done to detect renal anomalies. Transrectal USG (TRUS) was carried out to detect anomalies of seminal vesicles and ejaculatory ducts. Proven fertile men from general population attending Family Welfare Clinics of NIRRH, Mumbai, willing to participate in this study were included as controls (n=32). The inclusion criteria of controls were as follows: (i) Proven fertile males having normal semen analysis<sup>9</sup> (ii) The vas deferens was bilaterally palpable; and (iii) No signs and symptoms of CF and any CF related disorders.

Peripheral venous blood (5 ml) was collected into EDTA tubes and genomic DNA was isolated with commercial blood genomic DNA purification kit (GenElute™ Blood Genomic DNA Kit, Sigma, USA). The essential promoter, entire coding regions and splice sites of 27 exons of the *CFTR* gene were amplified by polymerase chain reaction (PCR) using specific primers (designed manually, specificity checked using *in silico* PCR; <https://genome.ucsc.edu/cgi-bin/hgPcr>). PCR cycling conditions were as follows: initial denaturation at 94° C for four min, 35 cycles of denaturation at 94° C for one min, annealing at 56-58° C for one min (depending on the primer sequence), extension at 72° C for one min and final extension step at 72° C for five min. PCR products were sequenced on ABI 3730xl DNA Analyzer automated sequencer (Applied Biosystems, Foster City, USA). Female partners were also screened for *CFTR* gene mutations to assess their CF carrier status. The sequencing results were compared with the wild type *CFTR* gene sequence published in the Gen Bank (NM\_000492.3). The original *CFTR* nucleotide numbering was used, *i.e.* from the 1<sup>st</sup> nucleotide of exon 1.

*Statistical analysis:* The data were evaluated with Fisher's exact test using SPSS software version 19 (SPSS, South Asia Pvt. Ltd, Bengaluru, India).

### Results

*Case 1:* A 37 yr old male from Maharashtra, married since 10 years presented with a history of infertility for evaluation in andrology clinic at NIRRH, Mumbai, in January 2011. There was no history of consanguineous marriage. The detailed semen parameters, reproductive hormones, scrotal examination, renal assessment are

**Table I.** Demographic characteristics, semen and hormonal parameters in infertile males with congenital bilateral absence of vas deferens and unilateral renal agenesis (CBAVD-URA)

CBAVD-URA case no.	Place of origin	Age (yr)	Duration of infertility (yr)	Semen analysis		Reproductive hormones	
				Volume (ml)	Sperm count	FSH Reference range (1.7-12.0 mIU/ml)	LH (1.5-9.3 mIU/ml)
1	Maharashtra	37	10	0.5	Azoospermia	5.55	ND
2	Gujarat	28	2	0.3	Azoospermia	1.81	2.99
3	Gujarat	28	1.5	1	Azoospermia	2.80	2.32
4	Tamil Nadu	39	10	0.2	Azoospermia	6.1	ND
5	Maharashtra	32	2	1	Azoospermia	6.2	4.26

FSH, follicle stimulating hormone; LH, luteinizing hormone; ND, not done

**Table II.** Clinical characteristics of infertile males having CBAVD-URA

Case no.	Testis size (ml)		Epididymal status		Renal status		Variants of <i>CFTR</i> gene
	Left	Right	Left	Right	Left	Right	
1	20	20	Caput	Caput	Absent	Normal	TG12-5T/TG10-9T, c.1540G>A, c.3041-71G>C, c.3272-93T>C, c.3120+529InsC, c.4002A>G, c.4521G>A
2	20	25	Caput	Caput	Absent	Compensatory hypertrophy	TG11-5T/TG10-7T, c.196+12T>C, c.875+40A>G, c.1540G>A, c.2694T>G, c.3271+42A>T, c.3120+529InsC, c.3500-140A>C, c.3601-65C>A
3	20	20	Caput	Caput	Ectopic kidney in pelvis with compensatory hypertrophy	Absent	TG11-7T/TG12-5T, c.1540G>A, c.2694T>G, c.2902-92G>A, c.3500-140A>C, c.3601-65C>A, c.4375-69C>T, c.4521G>A
4	20	20	Caput	Caput	Absent	Normal	TG13-5T/TG12-7T, c.1540G>A, c.2694T>G, c.2751+85_88delTA, c.2752+106A>T, c.4521G>A
5	18	18	Caput	Caput	Normal	Absent	TG11-7T/TG12-7T, c.621+91A>G, c.1540G>A, c.3120+529InsC

presented in Tables I and II. The testicular volume was within normal range (>15 ml). On scrotal examination, only head of the epididymis was palpable and bilateral vas deferens was found to be absent on both sides. Scrotal USG showed bilateral dilated tubules in head of epididymis suggestive of obstruction at the level of epididymis. Abdominal USG showed absence of left kidney. Testicular biopsy showed normal spermatogenesis. The sequence analysis of *CFTR* gene resulted in identification of one novel intronic variant

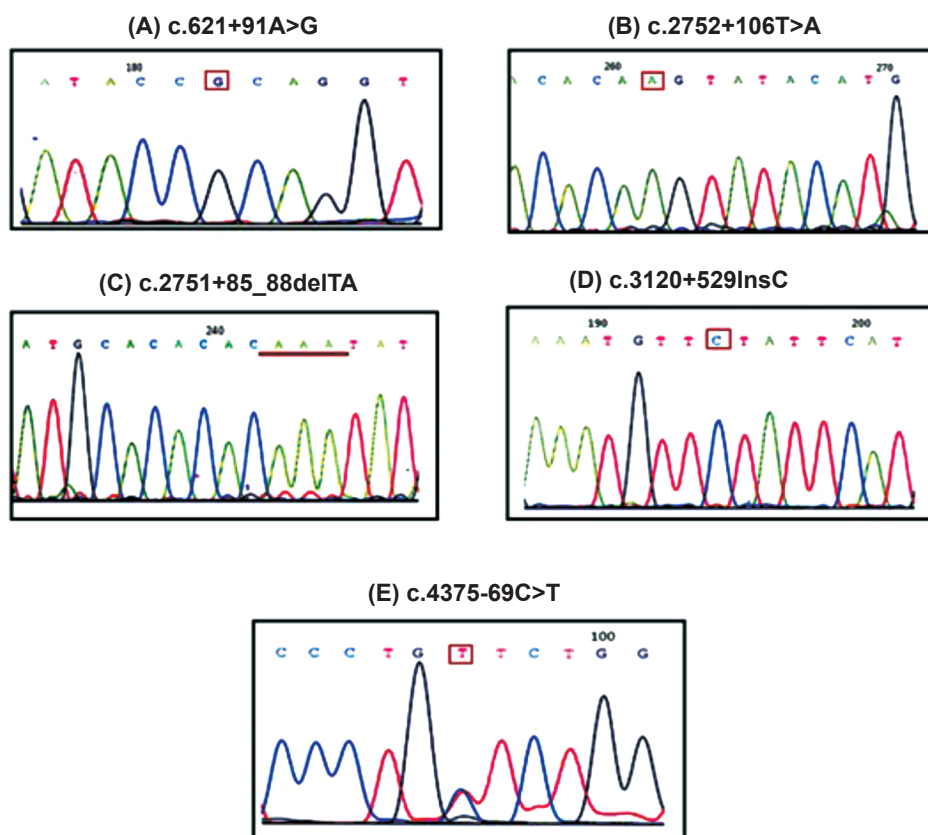
c.3120+529InsC (KJ159564; homozygous) (Figure D), three previously reported potential regulatory coding *CFTR* gene variants (AGA haplotype) c.1540G>A M470V (homozygous), c.4002A>G P1290P (homozygous), c.4521G>A Q1463Q (homozygous)<sup>10</sup> and two previously reported intronic variants c.3041-71G>C (heterozygous), c.3272-93T>C (homozygous)<sup>10</sup>. Intron 8 splice site variant [TG(n)-5T] analysis identified TG12-5T/TG10-9T (heterozygous) in intron 8. The female partner was not found to be CF carrier.

**Case 2:** A 28 yr old male from Gujarat, married since last two years with non-consanguineous marriage presented with a history of infertility in May 2011. The semen parameters, reproductive hormones status, scrotal examination, renal assessments are represented in Tables I and II. The testicular volume was within normal range (>15 ml). On scrotal examination, only head of the epididymis was palpable and vas deferens was absent on both sides. Scrotal USG showed bilateral varicocele and an anechoic tubular lesion in the head of right epididymis due to dilated efferent tubules. Abdominal USG showed absent left kidney and there was compensatory hypertrophy of right kidney. Bilateral testicular biopsy showed normal spermatogenesis in 70 per cent of seminiferous tubules.

TG11-5T/TG10-7T (heterozygous) mutation, two previously reported potential regulatory coding *CFTR* gene variants c.1540G>A V470M (heterozygous), c.2694T>G T854T (homozygous) and five previously reported intronic variants c.196+12T>C (heterozygous)<sup>11</sup>, c.875+40A>G (heterozygous)<sup>12</sup>,

c.3271+42A>T (heterozygous)<sup>12</sup>, c.3500-140A>C (heterozygous)<sup>13</sup>, c.3601-65C>A (heterozygous)<sup>14</sup>, one novel variant c.3120+529InsC (homozygous) (Figure D) were observed in this patient (Table III). His female partner was 26 years old whose *CFTR* gene sequencing analysis revealed that she was not a CF carrier.

**Case 3:** A 28 yr old male from Gujarat, married since one and half years in a non-consanguineous marriage was referred for andrology evaluation in June 2011. His 26 years old female partner was also evaluated for infertility. The semen parameters, reproductive hormones, scrotal examination, renal assessments are presented in Tables I and II. Semen analysis on two separate occasions showed azoospermia with absent fructose, low volume of  $\leq 1$  ml and low pH of 6.0. The testicular volume was within normal range (>15 ml). On palpation, only head of the epididymis was palpable and bilateral vas deferens was found to be absent. Scrotal USG showed a normal testicular structure and the epididymal heads of the right and left epididymis with the defect in epididymal bodies and



**Figure.** Identification of novel variants in infertile males with CBAVD and renal anomalies. (A) c.621+91A>G (intron 4); (B) c.2752+106T>A (intron 14A); (C) c.2751+85\_88 delTA (intron 14A); (D) c.3120+529 InsC (intron 19); (E) c.4375-69C>T (intron 23).

**Table III.** Identified *CFTR* gene variants in five cases having CBAVD-URA

CBAVD-URA case nos.	Novel variants	Polymorphisms
1	c.3120+529InsC	V470M, P1290P, Q1463Q
2	c.3120+529InsC	V470M, T854T
3	c.4375-69C>T	V470M, T854T, Q1463Q
4	c.2751+85_88delTA, c.2752+106A>T	V470M, T854T, Q1463Q
5	c.621+91A>G, c.3120+529InsC	V470M

tails. Scrotal USG also showed bilateral gross dilatation of epididymal tubules suggestive of distal obstruction. Doppler study of scrotum showed bilateral dilated and tortuous veins of pampiniform plexus suggestive of bilateral varicocele. TRUS revealed absence of bilateral seminal vesicles, bilateral absences of vas deferens and normal prostate. Transabdominal and pelvic USG showed absent right kidney and left was ectopic kidney in pelvis on left side of urinary bladder with mild compensatory hypertrophy. Serum creatinine level was normal.

The *CFTR* gene mutation analysis identified one novel intronic variant c.4375-69C>T (KJ159563; heterozygous) (Figure E), three previously reported potential regulatory coding *CFTR* gene variants (AGA haplotype) c.1540G>A V470M (heterozygous), c.2694T>G T854T (heterozygous), c.4521G>A Q1463Q (homozygous)<sup>10</sup> and three previously reported intronic variants c.2909-92G>A (heterozygous)<sup>11</sup>, c.3500-140A>C (heterozygous)<sup>13</sup>, c.3601-65C>A (heterozygous)<sup>13</sup> and TG12-5T/TG11-7T (heterozygous) (Table III). The female partner was not found to be a potential CF carrier. Percutaneous epididymal sperm aspiration (PESA) revealed good quality motile sperm. The couple had undergone one cycle of intra-cytoplasmic sperm injection (ICSI), however, there was no pregnancy.

**Case 4:** A 39 yr old male from Tamil Nadu, married since 10 years in non-consanguineous marriage presented with a history of infertility in May 2013. The semen parameters, reproductive hormones, scrotal examination, renal assessment are presented in Tables I and II. Abdominal USG showed normal right kidney and absent left kidney. The sequencing analysis resulted

in identification of TG13-5T/TG12-7T (heterozygous) mutation, three previously reported potential regulatory coding *CFTR* gene variants (AGA haplotype) c.1540G>A M470V (homozygous), c.2694T>G T854T (homozygous), c.4521G>A Q1463Q (heterozygous)<sup>10</sup> and two novel intronic variants c.2751+85\_88delTA (KJ159561; homozygous) and c.2752+106A>T (KJ159562; homozygous). (Figure B, C) (Table III). No previously known intronic variants were identified in this patient. The female partner was not found to be a potential CF carrier.

**Case 5:** A 32 yr old Indian male from Maharashtra, married since two years with no consanguinity presented with history of infertility in February 2014. The semen parameters, reproductive hormones, scrotal examination, renal assessment are presented in Tables I and II. Abdominal USG showed absent right kidney and normal left kidney. The sequencing analysis resulted in identification of two novel intronic variants c.621+91A>G (heterozygous) and c.3120+529InsC (homozygous) (Figure A, D), and two of the previously reported potential regulatory coding *CFTR* gene variants (AGA haplotype) c.1540G>A M470V (homozygous)<sup>10</sup> and TG11-7T/TG11-7T (homozygous) (Table III). No previously known intronic variants were identified in this patient. The female partner was not found to be a potential CF carrier.

***CFTR* gene screening in controls:** A total of 64 chromosomes from 32 control subjects were screened for detection of *CFTR* gene mutation. None of the 32 controls were carriers of F508del or other *CFTR* gene mutations. However, the most common *CFTR* gene variant IVS8-5T was detected in 18.75 per cent (6 of 32). V470M variant was found in 46.87 per cent (15 of 32) controls. c.4521G>A Q1463Q SNP was found in 28.12 per cent (9 of 32) controls.

The sequencing results were compared with the sequence of the wild type *CFTR* gene published in the *CFTR* mutation databases such as Cystic Fibrosis Mutation Database ([www.genet.sickkids.on.ca/](http://www.genet.sickkids.on.ca/)), Universal Mutation Database ([www.umd.be/CFTR/](http://www.umd.be/CFTR/)) and recently developed CFTR-2 database (<http://www.cftr2.org/>).

***In-silico* analysis:** Five novel and seven previously reported intronic variants in the *CFTR* gene were detected in five males with CBAVD-URA and their female partner. To better understand the splicing defects



due to the presence of intronic variants in these males, we analysed the novel and previously known intronic variants using Human Splicing Finder 3.0 (<http://www.umd.be/HSF3/>). *In-silico* splice site analysis using this software predicted four novel intronic variants (c.621+91A>G, c.2751+85\_88delTA, c.4095+61T>A and c.4375-69C>T) to have potential to alter splicing in the *CFTR* gene (Table IV) and one novel intronic variant (c.3120+529InsC) was found to have no significant splicing motif alteration in the *CFTR* gene. However, two previously known intronic variants (c.875+40A>G, and c.3271+42A>T) were predicted to have potential to alter splicing in the *CFTR* gene and five previously known intronic variants (c.196+12T>C, c.3041-71G>C, c.3272-93T>C, c.3500-140A>C and c.3601-65C>A) were found to have no significant splicing motif alteration in the *CFTR* gene (Table IV).

### Discussion

Role of *CFTR* gene mutations in males with CBAVD-URA has been controversial, as the previously reported studies did not detect any *CFTR* gene mutations<sup>14-16</sup>. In the present study, we found *CFTR* gene variants in five cases of CBAVD having renal malformations. Congenital bilateral absence of seminal vesicles (CASV) and CBAVD are uncommon anomalies and such patients usually have normal kidneys<sup>17</sup>. However, in the present study, case 3 was

found to have bilateral absence of seminal vesicles and left ectopic kidney; which is a rare observation. Case 5 showed presence of hypoplastic seminal vesicles and absence of right kidney. Normally, in addition to prostatic secretions, seminal vesicular secretions also contribute to the alkalinity of the ejaculate and make up approximately 90 per cent of fluid in ejaculate. Thus, patients having CASV and CBAVD present with history of infertility and usually patients with URA remain undiagnosed until there is some pathology in the contralateral kidney. We detected IVS8-5T mutation in three out five CBAVD-URA patients, all the CBAVD-URA males had V470M all CBAVD-URA males harboured intronic variants and other SNPs T854T, P1290P and Q1463Q.

In this study, five novel *CFTR* gene variants (c.621+91A>G, c.2752+106A>T, c.2751+85\_88delTA, c.3120+529InsC and c.4375-69C>T), other coding SNPs such as T854T, P1290P, Q1463Q and seven previously reported *CFTR* gene variants were identified in patients with CBAVD-URA. Two CBAVD-URA patients in our study were found to have longer IVS8-TG repeats (TG12 or TG13) in *cis* with 5T and M470V polymorphism. Previous studies reported that M470V along with short poly-T (5T) and long TG-repeat tracks (TG12, TG13) may contribute to CBAVD risk<sup>2,18</sup>. This genotype was not detected in normal male

**Table IV.** Splice site analysis of *CFTR* gene intronic variants in infertile males with CBAVD-URA

Sr. no.	Nucleotide change	Intron	Position	Interpretation
1	c.196+12T>C	2	Deep intronic	No change in splice site
2	c.621+91A>G*	4	Deep intronic	Activation of an intronic cryptic acceptor site Creation of an intronic ESE site
3	c.875+40A>G	6A	Deep intronic	Alteration of an intronic ESS site
4	c.2752+106T>A*	14A	Deep intronic	Alteration of an intronic cryptic acceptor site Creation of an intronic ESE site
5	c.2751+85_88delTA*	14A	Deep intronic	Alteration of an intronic ESS site Creation of an intronic ESE site
6	c.3041-71G>C	16	Deep intronic	No significant splicing motif alteration
7	c.3271+42A>T	17A	Late exonic	Alteration of an exonic ESE site
8	c.3272-93T>C	17B	Deep intronic	No significant splicing motif alteration
9	c.3500-140A>C	17B	Deep intronic	No significant splicing motif alteration
10	c.3601-65C>A	18	Deep intronic	No significant splicing motif alteration
11	c.3120+529InsC*	19	Deep intronic	No significant splicing motif alteration
12	c.4375-69C>T*	23	Deep intronic	Creation of an intronic ESE site

\*Novel intronic variants, ESE, exonic splicing enhancer. ESS, exonic splicing silencer

participants suggesting that longer TG-short T repeats in association with M470V and other variants might be responsible for CBAVD-URA phenotype. It is reported that IVS8-5T allele is found in higher frequencies in non-Caucasian populations such as Indians and is involved in many cases of CBAVD even in populations where CF is rare<sup>3</sup>.

It has been reported that three common *CFTR* coding SNPs (c.1540A>G, c.2694T>G and c.4521G>A) might contribute to reduced levels of *CFTR* transcripts<sup>10</sup>. They also reported AGA haplotype (c.1540A>G, c.2694T>G and c.4521G>A) in association with CF in individual having chronic pancreatitis and this haplotype was found to have independent contribution to the phenotype irrespective of CF mutations<sup>10</sup>. In our study, three potential regulatory *CFTR* gene variants (c.1540A>G, c.2694T>G and c.4521G>A) were detected in cases 1, 3 and 4. Based on the information available in the literature, we can speculate that these common coding single nucleotide polymorphism (cSNPs) in the *CFTR* gene along with IVS-5T might be leading to decreased levels of normal transcript contributing to the variable phenotype as observed in these patients. It has been reported that patients with c.1540G>A M470V, c.2694T>G T854T and c.4521G>A Q1463Q haplotypes could have lower *CFTR* transcript levels than that expected from otherwise normal haplotypes. This might lead to a major and/or earlier clinical expression of cystic fibrosis or cystic fibrosis-related disorders (CFTR-RD)<sup>10</sup>. Also, a combination of IVS8-5T mutation and M470V has been known to influence the efficiency of exon 9<sup>19</sup>.

It is important to note that due to the large size of Indian population, the total burden of CF and *CFTR* gene carrier could be much larger than in many European countries. Thus, *CFTR* gene analysis should be offered to CBAVD-URA patients and their female partners if the couple is opting for ICSI. Further studies are in progress to investigate the complete spectrum of *CFTR* gene mutations in Indian CBAVD males.

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**Conflicts of Interest:** None.

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