DOI:10.14753/SE.2014.1863

Potential role of cystic fibrosis genetic modifier factors in congenital bilateral absence of the vas deferens

PhD Dissertation

Viktória Havasi M.D.

Semmelweis University

Doctoral School of Basic Medicine

Supervisor: Ákos Zsembery, Ph.D., associate professor

Official reviewers: Miklós Garami, Ph.D., associate professor
András Váradi, Ph.D.,D.Sc., professor

Head of the examination committee: Mária Sasvári, Ph.D., D.Sc., professor
Members of the examination committee: László Csanády, Ph.D., associate professor
László Fodor Ph.D., researcher

Budapest
2013

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Abbreviations

ABC: ATP binding cassette

ABPA: allergic bronchopulmonary aspergillosis

ACMG: American College of Medical Genetics

ACP: alcoholic chronic pancreatitis

ART: assisted reproduction techniques

ASL: airway surface liquid

ATP: adenosine-5'-triphosphate

BALF: bronchoalveolar lavage fluid

BMI: body mass index

BMP: bone morphogenetic protein

cAMP: cyclic adenosine-monophosphate

CBAVD: congenital bilateral absence of the vas deferens

CF: cystic fibrosis

CFTR: cystic fibrosis conductance regulator gene

CFTR: cystic fibrosis conductance regulator protein

CFTR-RD: CFTR-related disorder

CREB: cAMP responsive element binding protein

CUAVD: congenital unilateral absence of the vas deferens

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

EDN-1: endothelin 1 gene

EDN-1: endothelin 1 protein

EDN-2: endothelin 2 protein

EDN-3: endothelin 3 gene

EDN-3: endothelin 3 protein

EDNRA: endothelin receptor type A gene

EDNRA: endothelin receptor type A protein

EDNRB: endothelin receptor type B gene

EDNRB: endothelin receptor type B protein

ENaC: epithelial sodium channel protein

ER: endoplasmic reticulum

FDA: Food and Drug Administration

FOX: forkhead box

gDNA: genomic DNA

GWAS: genome wide association studies

HPLC: high performance liquid chromatography

ICM: intestinal current measurement

ICP: idiopathic chronic pancreatitis

IGF: insulin-like growth factor

IRT: immunoreactive trypsin

IVS8: intervening sequence (intron) 8

IVS9: intervening sequence (intron) 9

MDR1: multidrug resistance gene 1

MI: meconium ileus

mRNA: messenger ribonucleic acid

MSD: membrane spanning domain

mTTGE: modified temporal temperature gradient analysis

NCBI: National Center for Biotechnology Information

NBD: nucleotide binding domain

NPD: nasal potential difference

OMIM: Online Mendelian Inheritance in Men

OR: odds ratio

PCR: polymerase chain reaction

PI: pancreatic insufficient

PHA-1: pseudohypoaldosteronism type 1

PKA: protein-kinase A

PKC: protein-kinase C

PKG: protein-kinase G

PS: pancreatic sufficient

PTC: premature termination codon

shRNA: short hairpin ribonucleic acid

SMAD: small+ mothers against decapentaplegic pathway

SNP: single nucleotide polymorphism

SPSS: Statistical Package for the Social Sciences

SSCP: single strand conformational polymorphism

TFGB1: transforming growth factor β-1 gene

TGFβ-1: transforming growth factor β-1 protein

TG_m: polyTG tract in intron 8 of the *CFTR* gene

T_n or polyT: polythymidine tract in intron 8 of the *CFTR* gene

UPR: ubiquitin proteasome system

UTR: untranslated region

UV: ultraviolet

Wnt: wingless+integration 1 pathway

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1. Introduction

1.1. Cystic fibrosis

1.1.1. Definition of cystic fibrosis

Cystic fibrosis (CF) (OMIM # 219700) is the most common life-shortening autosomal recessive disorder in Caucasians with an incidence of 1:3,000-1:3,500 live births in the United States and Europe (Farrell, et al. 2008, Lucarelli M, et al. 2012, Scotet, et al. 2012). CF is characterized by recurrent pulmonary infections, elevated sweat chloride, pancreatic and hepatic insufficiency, intestinal abnormalities, failure to thrive, diabetes, meconium ileus (MI) and other glandular defects (Rowe, et al. 2005, Stalvey and Flotte 2009, Rogan, et al. 2011, Lucarelli M, et al. 2012). Approximately 98% of CF males are infertile and exhibit Wolffian duct abnormalities such as congenital bilateral absence of the vas deferens (CBAVD) or the lack of epididymis, seminal vesicles and ejaculatory ducts (Taussig, et al. 1972).

Cystic fibrosis is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (OMIM # 602421) that encodes an epithelial cyclic adenosine-monophosphate (cAMP) regulated sodium-chloride-bicarbonate channel (Kerem, et al. 1989, Riordan, et al. 1989, Rommens, et al. 1989). The gene is located on the long arm of chromosome 7 (7q31.2) and consists of 27 exons (Zielenski, et al. 1991). More than 1,900 *CFTR* gene mutations and polymorphisms have been reported in the Cystic Fibrosis Mutation Database to date (http://www.genet.sickkids.on.ca/app). The *CFTR* gene is under the control of a housekeeping-type promoter, and its transcription can be controlled by alternative starting sites and/or alternative splicing (Vankeerberghen, et al. 2002). The most common *CFTR* gene transcript consists of 6128 nucleotides and is translated into a 1480 amino acids long protein.

The CFTR protein is widely expressed in the epithelium of various parts of the human body including airways, sweat glands, pancreas, intestinal tissues and the reproductive tract (Quinton 2007). Functions of the protein include Cl^- secretion in the airways and the colon, and Cl^- reabsorption in the sweat glands (Vankeerberghen, et al. 2002, Riordan 2008). Diminished Cl^- secretion together with elevated Na^+ absorption -

caused by the amiloride sensitive epithelial Na^+ channel (ENaC) hyperactivity - leads to reduced airway surface liquid (ASL) in CF subjects (Lucarelli M, et al. 2012). Dehydration in the airway epithelium results in sticky, tenacious mucus and impaired mucociliary clearance in cystic fibrosis lungs. Bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* colonize the abnormally thick mucus and cause inflammation leading to bronchial obstruction, airway destruction and eventually pulmonary failure (Rowe, et al. 2005).

Cystic fibrosis patients exhibit a wide spectrum of disease severity and progression ranging from a very mild, adult-onset form to extremely severe cases manifesting with meconium ileus in newborns. These distinct clinical manifestations are most likely attributable to not only the diversity of mutations in the *CFTR* gene but other genetic and environmental modifier factors as well (WHO/ICF(M)A/ECFTN 2002). With advances in disease therapy, now CF patients in the US live well into their mid-thirties. This is a significant improvement in life expectancy compared to the 1950s when cystic fibrosis patients were not expected to live long enough to attend school (http://www.cff.org/AboutCF/).

1.1.2. CFTR protein structure, functions and its maturation process

The CFTR protein belongs to the adenosine-5'-triphosphate-binding cassette (ABC) transporters superfamily and consists of two nucleotide-binding domains (NBD) that hydrolyze adenosine-5'-triphophate (ATP), two membrane-spanning domains (MSD) and a regulatory domain (R). (See Figure 1. below.) Most ABC transporters regulate small molecule transports through biological membranes while CFTR functions as an ion-channel, and unlike other ABC transporters, it also contains a unique R-domain that harbors protein-kinase-A (PKA), protein-kinase C (PKC) and protein-kinase G (PKG) phosphorylation sites (Lucarelli M, et al. 2012). The C-terminus of the protein binds to PDZ receptors (PDZ domains are part of cell membrane associated proteins and intracellular signaling molecules) that brings the protein close to other ion channels, receptors and the cytoskeleton (Rowe, et al. 2005). The CFTR protein also interacts with and regulates the activity of ENaC in the lung epithelia, although the exact details of this interaction are not well understood, and controversial data has been published in

this topic. It is generally accepted, however, that CFTR normally inhibits ENaC function in the airways, and *CFTR* mutations disturbs this interchange and generate ENaC hyperactivity in CF patients (Azad, et al. 2009, Lucarelli M, et al. 2012).

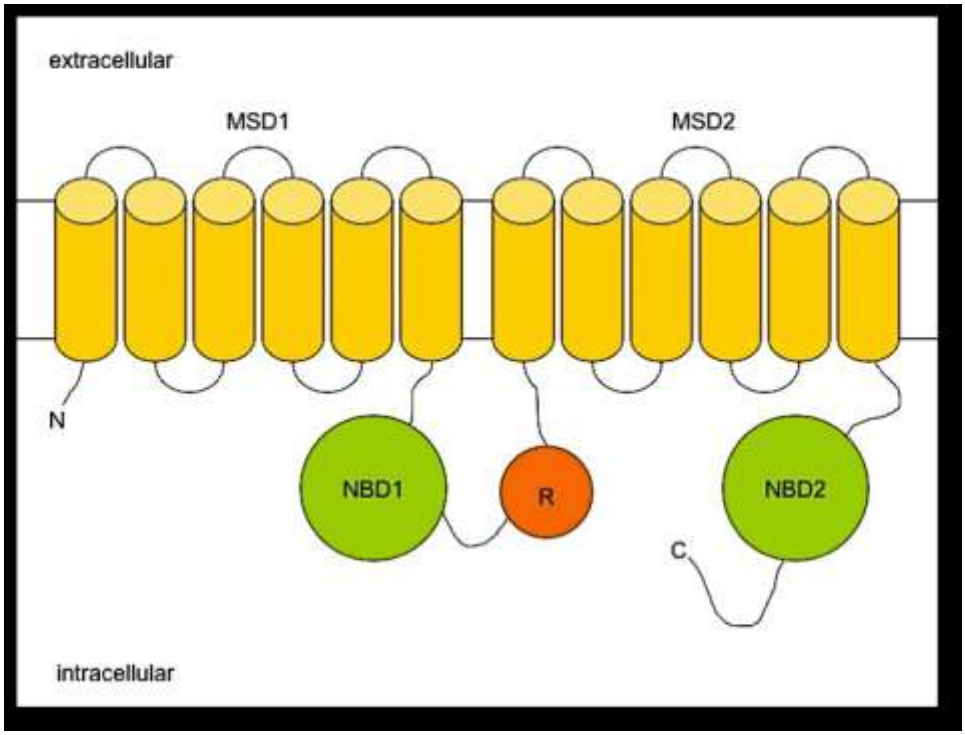


Figure 1. CFTR domain configuration
MSD1: Membrane-Spanning Domain 1, MSD2: Membrane-Spanning Domain 2, NBD1: Nucleotide Binding Domain 1, NBD2: Nucleotide Binding Domain 2, R: Regulatory Domain, C: C-terminus, N: N-terminus

During its complex maturation process, the CFTR protein interacts with several cellular chaperones, some of which have not been identified thus far (Rogan, et al. 2011, Lucarelli M, et al. 2012). After the nascent CFTR polypeptide has been synthesized in the endoplasmic reticulum (ER), the protein goes through co-translational transport and an initial N-glycosylation. This core-glycosylated form of the CFTR protein (150 kDa, "Band B" on Western Blot) undergoes a complex folding process in the ER before being transported into the Golgi apparatus. CFTR folding efficiency is extremely low (app. 25%), and most newly synthesized not correctly folded (misfolded) protein molecules - as well as mutants with aberrant structure - are recognized by the ER quality control, and degraded by the ubiquitin proteasome system (UPR). After further glycosylation in the Golgi, the protein becomes mature (170-180 kDa, "Band C" on Western Blot). From the Golgi apparatus, mature CFTR is

transported onto the plasma membrane by clathrin-coated vesicles, where it has a half-life of 12-24 hours. CFTR is also recycled through clathrin-coated vesicles from the cell surface, and is finally degraded in lysosomes.

Several details of the CFTR channel functioning are not completely understood and represent an area of intensive study. It is already known that CFTR channel opening requires R-domain phosphorylation by PKA that is followed by ATP binding to NBD1 and NBD2. ATP binding to NBD1 initiates channel activity while ATP binding to NBD2 leads to a formation of a tight interaction between NBD1 and NBD2, and also promotes channel opening. ATPase activity results in dissociation of the NBD-s and the channel returns to closed configuration (Rogan, et al. 2011, Lucarelli M, et al. 2012). Despite of continuous efforts, there are no full-length high-resolution structures available for the whole CFTR or any other single chain ABC transporter proteins thus far. NBD1 is the only part of the CFTR molecule of which detailed structure is available, and most of our understanding of the CFTR structure is based on structural similarities to previously characterized bacterial proteins (Atwell, et al. 2010).

1.1.3. CFTR mutation classes

CFTR gene mutations can be subdivided into six different classes based on their molecular mechanism of action (Rowe, et al. 2005). (See Figure 2.) Class I mutations are predominantly represented by premature termination codons (PTC) (e.g. G542X, W1282X) or large deletions (CFTRdele2,3) that completely abrogate CFTR synthesis. Class II defects (e.g. F508del that is a deletion of phenylalanine at amino acid position 508 within NBD1) result in impaired protein maturation and premature proteasomal degeneration, thus CFTR molecules with these mutations are not capable of reaching the cell surface. CFTR proteins with Class III abnormalities (e.g. G551D) reach the cell surface but cause disordered CFTR-ATP or CFTR-cAMP regulation. Class IV mutants (e.g. R117H) are expressed on the cell surface but show signs of aberrant chloride conductance. Class V alterations reduce the number of CFTR transcripts due to splicing abnormalities, and Class VI defects accelerate protein turnover from the cell surface. Both Class V and VI mutations result in reduction of functional CFTR proteins, and therefore certain studies do not differentiate between these two mutational classes

(Stalvey and Flotte 2009, Rogan, et al. 2011). In addition to these, some *CFTR* mutations meet the criteria of more than one mutation classes. While the F508del mutation is usually considered as a Class II defect, a small amount of protein with this mutation reaches the cell surface and behaves as a Class III mutation with reduced ATP-binding capacity. Since the F508del-CFTR protein also has reduced surface stability and accelerated turnover, the mutation could also qualify as a Class VI abnormality. (Lucarelli M, et al. 2012). Therefore, when a patient harbors F508del in homozygous form, the mutation has multiple consequences that make mutation-specific, personalized therapy very challenging.

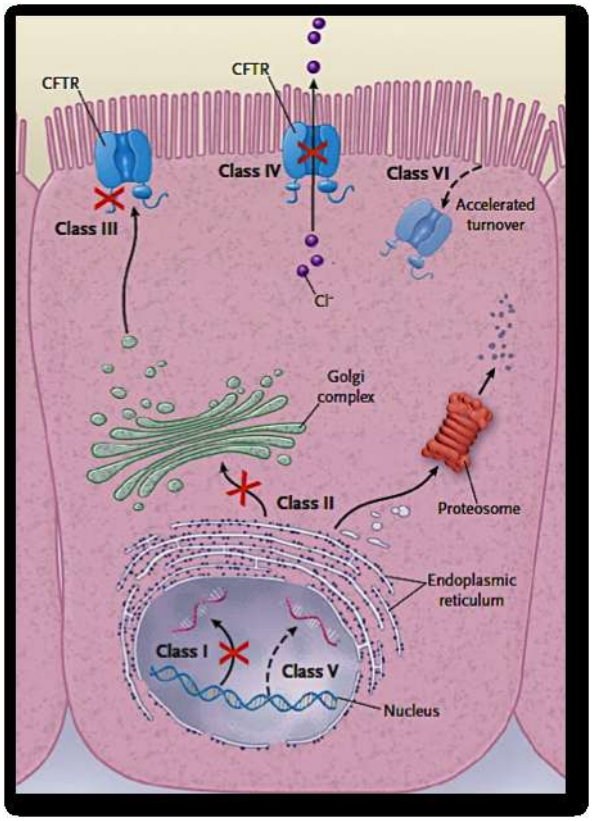


Figure 2. CFTR mutation classes (Rowe, et al. 2005).
Class I defects: abrogated protein synthesis; Class II mutations: trafficking defects and premature protein degeneration; Class III abnormalities: defective ATP or cAMP binding capacity; Class IV: aberrant Cl^- conductance; Class V mutations: reduced number of CFTR proteins due to splicing abnormalities; Class VI defects: accelerated protein turnover.

CFTR mutations are often referred as "mild" or "severe" based on their effects regarding pancreatic symptoms in CF subjects. *CFTR* mutations are considered "severe" if they cause pancreatic insufficiency (PI) in CF patients (Classes I-III), and "mild"

(Classes IV-VI) if they result in pancreatic sufficient (PS) phenotype (Kristidis, et al. 1992).

1.1.4. CFTR mutation screening

Since the *CFTR* gene sequence has been determined in 1989, more than 1,900 sequence alterations have been published in the CF Mutation Database (http://www.genet.sickkids.on.ca/app). The F508del mutation represent 60-70% of all CF alleles, and can be found up to 90% of CF patients in some populations (Quinton 1999, Watson, et al. 2004, Quinton 2007, Rogan, et al. 2011). However, besides the F508del only a very few mutations reach more than 1% incidence in CF patients. The four most frequent mutations include G542X (2.4%), G551D (1.6%), N1303K (1.3%) and W1282X (1.2%). The disease prevalence is highly variable between different ethnicities: app. 1 in 3,000-1:3,500 in Europeans and White Americans; 1 in 17,000 in African Americans and close to 1 in 30,000 in Asian Americans. Cystic fibrosis is extremely rare in Africa and Asia with the reported disease frequency being as low as 1 in 350,000 in Japan (Farrell, et al. 2008, Lucarelli M, et al. 2012, Scotet, et al. 2012). Interestingly, in certain ethnic groups a single mutation - besides the F508del - can be accounted for the majority of the *CFTR* alleles, e.g. 46% of the Ashkenazi Jewish population carries the W1282X CFTR mutation (Watson, et al. 2004).

Because of the high number of *CFTR* sequence alterations, the pathological effects of most changes are not well understood, and this makes CF genetic diagnosis very challenging. The American College of Medical Genetics (ACMG) created a list of the 23 most frequent mutations (frequency >0.1%) that are recommended for cystic fibrosis genetic screening (Watson, et al. 2004). (See Table 1. for the list of mutations.) A widely used approach of *CFTR* mutation screening includes multiple diagnostic steps (Level I-III). The first step requires entry-level tests probing for the ACMG mutation panel. Several *CFTR* mutation detection tests incorporating these 23 and additional locally frequent sequence alterations are commercially available (e.g. CFTR InPlex® from Hologic, Tag-It™ Cystic Fibrosis Kit from Tm Bioscience Corporation). Rare mutations that are not included in the ACMG panel can be detected by Level II scanning methods (e.g. single-strand conformation polymorphisms (SSCP), re-

sequencing, denaturing high performance liquid chromatography (HPLC)). However, even after completing both steps, only 97% of the sequence changes can be identified. Therefore, a small number of mutations remain hidden and require further analysis. As the third step, search for large deletions, insertions and sequence alterations in the promoter, 5' untranslated region (UTR) or introns needs to be applied (Lucarelli M, et al. 2012).

Table 1. CFTR mutation screening panel based on the ACMG recommendations (Watson, et al. 2004).
Mutation nomenclature is based on the updated Cystic Fibrosis Mutation Database (http://www.genet.sickkids.on.ca/app).

F508del	R553X	R1162X	c.2052delA	c.2988+1G>A
I507del	G542X	G551D	W1282X	N1303K
c.489+1G>T	R117H	c.1585-1G>A	A455E	R560T
G85E	R334W	R347P	c.579+1G>T	c.1766+1G>A
c.3717+12191C>T	c.2657+5G>A	c.3528delC		

Making CF genetic screening even more complicated is the existence of complex *CFTR* alleles. Most genetic tests do not include testing for additional *in cis* (located on the same parental allele) sequence abnormalities when one mutation is detected in each chromosome. Therefore, patients with various phenotypes could appear carrying identical *CFTR* mutations when *in cis* sequence alterations (that might be responsible for the diverse symptoms) escaped detection. Thus far, only few complex alleles (e.g. F508C-S1251N, R117H-TG_mT_n) have been identified and their prevalence is mostly unknown (Lucarelli M, et al. 2012).

1.1.5. Functional CFTR tests in CF diagnosis

Nasal potential difference (NPD) measurement, intestinal current measurement (ICM) and sweat chloride test are useful tools to assess the level of functional CFTR proteins in human subjects.

As of today, pilocarpine iontophoresis based sweat chloride measurement is still the most reliable ("gold standard") functional method for establishing cystic fibrosis diagnosis. Since defective CFTR activity leads to reduction in chloride reabsorption of

the sweat glands, an elevated sweat chloride level measurement is most likely due to cystic fibrosis. Sweat chloride test results are interpreted as follows: abnormal >60 mmol/L, intermediate (borderline) 40-60 mmol/L and normal <40 mmol/L. (http://www.hopkinscf.org/main/whatiscf/diag_testsweat.html)

Nasal potential difference (NPD) can be employed to measure *in vivo* ion-transport aberrations caused by defective CFTR functions, and to determine therapeutic effects on CFTR activity. The concept behind this method is that nasal epithelial function reflects lower airway abnormalities. Respiratory epithelium forms a tight monolayer with enough resistance to create a measurable potential difference between its two sides. In non-CF individuals, balanced sodium absorption and active chloride transport maintains this potential difference. NPD measurement records the electric potential difference between a subcutaneous reference (zero) electrode and an exploring electrode that is attached to a nasal catheter (Rowe, et al. 2011). A hyperpolarization of more than -5 mV demonstrates wild-type CFTR function (Bombieri, et al. 2011). In CF subjects there is a more negative baseline, a larger inhibition of NPD after the addition of amiloride caused by ENaC hyperactivity in CF; and little or no change in nasal potential difference after the addition of chloride-free isoproterenol solution (defective or absent CFTR function) (http://www.hopkinscf.org/main/whatiscf/diag_testnasal.html). A good correlation between NPD measurements and CF disease phenotype exist, however, despite current efforts, there is not a generally accepted standardized NPD protocol in use for diagnosis or research trials (Solomon, et al. 2010).

Intestinal current measurement (ICM) is an important tool for studying the function of CFTR and other alternative ion channels *ex vivo*. The CFTR protein is widely expressed in colonic epithelia where it leads Cl^- and HCO_3^- out of epithelial cells in a cAMP-dependent manner (De Boeck, et al. 2011). Micro Ussing chambers are used to measure transepithelial short circuit current (I_{sc}) or transepithelial voltage (V_{te}) in human rectal biopsy samples. ICM is a safe, minimally invasive technique that could be applied in newborns to assess CFTR function. However, ICM has been mainly employed in research thus far, and should only be used as a diagnostic tool when sweat test and additional *CFTR* mutation analysis do not result in a clear CF diagnosis (Bombieri, et al. 2011).

1.1.6. Diagnostic challenges in cystic fibrosis

The diagnostic criteria for cystic fibrosis are 1) one or more phenotypic features of the disease or 2) CF in a sibling or 3) positive immunoreactive trypsin (IRT) test together with at least one other feature (Rosenstein and Cutting 1998). Additional features include positive sweat tests on two separate occasions, CF-causing mutations in each *CFTR* genes or an abnormal nasal potential difference measurement (Castellani, et al. 2008, Farrell, et al. 2008). Elevated sweat chloride level and two proven pathologic *CFTR* mutations present in a patient typically establish the diagnosis of the classical form of the disease (Rosenstein and Cutting 1998, Farrell, et al. 2008). However, these strict diagnostic criteria cannot be applied in all cystic fibrosis patients. "Atypical" cases usually do not fit into these categories, and getting a negative genetic screening result based on the ACMG recommended 23 mutations panel, should not exclude the diagnosis of cystic fibrosis (Watson, et al. 2004). While this panel covers the most frequent CFTR-disease associated sequence abnormalities, the detection rate could be as low as 64.5% in African Americans, 71.9% in Hispanic Caucasians and 48.9% in Asian Americans when probing for only these mutations (Watson, et al. 2004). Therefore, a multiple step mutation screening approach (described earlier) should be applied in cases when no mutations were found during the initial screen.

The diagnosis of cystic fibrosis can be established by the presence of two disease causing gene mutations or the existence of epithelial electrolyte transport disorder caused by abnormal or absent CFTR protein function. However, because of the wide variety of cystic fibrosis phenotypic manifestations and disease progression rates, the diagnosis remains mainly a clinical decision, combining results of clinical, genetic and functional tests, and it cannot be based solely on one test result (WHO/ICF(M)A/ECFTN 2002, Bombieri, et al. 2011). In the majority of patients, based on the presence of recurrent pulmonary infections and elevated sweat chloride levels, the disease is straightforward to diagnose. Although, some subjects exhibit milder symptoms and/or later onset, the "gold standard" sweat chloride test and *CFTR* mutation screening help solve diagnostic dilemmas. Nevertheless, a small subset of patients (app. 5%) exhibits atypical phenotypes: borderline or normal sweat chloride levels with two *CFTR* mutations, or one or no detectable *CFTR* mutation together with

typical CF pulmonary symptoms (WHO/ICF(M)A/ECFTN 2002). These “atypical” cases are the most difficult to diagnose.

In all fifty states of the US and most parts of Europe, cystic fibrosis neonatal screening programs (IRT and/or genetic analysis) are employed (Castellani, et al. 2009) (http://www.cff.org/AboutCF/Testing/NewbornScreening/ScreeningforCF/Neonatal). The main advantage of neonatal screening programs is identifying cystic fibrosis patients at a very early age and starting combined therapies that could potentially prevent lung infection, malnutrition and improve life expectancy and quality of life. However, while neonatal screening programs could distinguish at risk individuals, it should be taken into consideration that these screenings often yield false positive results. Therefore, newborns with positive test results must be followed up, and the definite diagnosis of cystic fibrosis can only be established after positive sweat chloride test or additional tests examining CFTR function (e.g. ICM, NPD) (Bombieri, et al. 2011, Lucarelli M, et al. 2012).

1.1.7. Mutation specific therapies in cystic fibrosis

Majority of the therapeutic agents (e.g. antibiotics, pancreas enzymes, nutritional support, vitamin supplementation) administered in CF patients are aided to alleviate existing symptoms, improve quality of life and do not target the molecular defects leading to the disease. However, the numerous research studies in cystic fibrosis molecular biology since the discovery of the gene in 1989, significantly improved our understanding of the CFTR protein synthesis, maturation process and functions. These scientific improvements made it possible that personalized, mutation-specific therapies have become a reality to cystic fibrosis patients last year.

As described previously, *CFTR* mutations can be classified into 6 different groups. Class I mutations include mostly PTCs (nonsense mutations) that account for approximately 10% of all *CFTR* mutations. It has been previously reported that aminoglycoside antibiotics (e.g. Gentamicin) enhance read-through of PTCs in bacteria. However, side effects (nephro- and ototoxicity) and unsuccessful human trials with Gentamicin lead to a search for molecules with similar effects but reduced toxicity. PTC Therapeutics has developed a drug, Ataluren (PTC124) which does not have antibiotic

activity but promotes read-through, and was found to be generally safe in Phase III clinical trials in human cystic fibrosis subjects (Rogan, et al. 2011).

F508del, the most frequent *CFTR* mutation has multiple consequences, it results in premature degradation of the majority of newly synthesized proteins (Class II) and the small percentage of molecules that reach the cell surface, exhibit decreased channel activity (Class III), reduced half-life and accelerated turnover (Class VI). Molecules that rescue Class II mutations (including F508del), and help these defective proteins to reach the cell surface, are called "correctors". "Corrector" molecules are likely to effect protein trafficking, interact with molecular chaperones or bypass ER quality controls steps. The most promising "corrector" type drug currently investigated is VX-809 from Vertex Pharmaceuticals. VX-809 was well tolerated in clinical trials, resulted in significant improvement in sweat-chloride levels but no improvements of CFTR function on NPD measurements (Rogan, et al. 2011). "Potentiator" molecules enhance activity and half-life of cell-surface located defective CFTR molecules (Class III mutants). Approximately 1.6% of CF patients (4% of the US CF population) carries a Class III mutation G551D, and therefore could benefit from "potentiator" therapy. In early 2012, VX-770 (Kalydeco™, ivacaftor) has been approved by the US Food and Drug Administration (FDA) and the European Commission for treatment of CF patients with the CFTR-G551D allele. Kalydeco™ has become the first drug in CF therapy that corrects the underlying cause of the disease. Since "corrector" molecules promote the transport of F508del-CFTR to the surface where it acts as a Class III mutation, combined "corrector" and "potentiator" therapies have been tried in CF patients carrying this frequent defect. Homozygous F508del patients treated with a "potentiator" (VX-770) and "corrector" (VX-809) exhibited significantly improved lung function compared to placebo controls (http://www.cff.org/treatments/Therapies/Kalydeco/).

It has been estimated that as low as 5-15% of wild-type CFTR function could be sufficient to maintain epithelial chloride transport (Rogan, et al. 2011). Therefore, even a slight improvement in CFTR activity by "potentiator" or "corrector" therapy could lead to significant reduction in CF symptoms. Results from mutation-based therapy trials are promising, although long-term safety and efficacy of these drugs need to be determined in the future.

1.2. CFTR-related diseases and CBAVD

1.2.1. Definition and forms of CFTR-related diseases

Relating *CFTR* genotype to CF phenotype is a challenging task. In addition to the large number of *CFTR* mutations and CF cases with atypical phenotypes, the fact that subjects heterozygous for CF disease-causing mutations are also predisposed to a diverse group of disorders besides cystic fibrosis, makes establishing diagnosis even more complex. *CFTR* mutations have been implicated in a variety of pathologic conditions such as disseminated bronchiectasis, allergic bronchopulmonary aspergillosis, acute recurrent or chronic idiopathic pancreatitis, alcoholic chronic pancreatitis (ACP), and congenital bilateral absence of the vas deferens (WHO/ICF(M)A/ECFTN 2002, Kerem 2006, Castellani, et al. 2008, Bombieri, et al. 2011). Based upon possible etiologic connection with *CFTR* mutations, these disorders are considered CFTR-related disorders (CFTR-RD). The latest consensus report defines CFTR-RD as "a clinical entity associated with CFTR dysfunction that does not fulfill the diagnostic criteria for CF" (Bombieri, et al. 2011). The causative connection between *CFTR* mutations and certain diseases is still debated, mainly because some studies have been performed in small group of patients, and in several CFTR-RD cases only one *CFTR* mutation has been detected (Lucarelli M, et al. 2012). Moreover, these disorders are likely influenced by a spectrum of additional genetic and environmental factors (Lukowski, et al. 2011).

Allergic bronchopulmonary aspergillosis (ABPA) is an immune bronchial disease in children and adults with chronic lung diseases. This pulmonary disease is caused by allergic responses to multiple antigens expressed by the *Aspergillus fumigatus* fungus (Chetty 2003). In a previous study, Miller and colleagues sequenced the whole *CFTR* gene in a group of eleven carefully selected ABPA patients (strict diagnostic criteria, normal sweat chloride level). ABPA subjects of this study exhibited an elevated F508del mutation frequency compared to 53 control Caucasian subjects (Miller, et al. 1996). Marchand *et al.* examined the frequency of 13 *CFTR* mutations in ABPA patients. This research group found an elevated incidence of *CFTR* gene abnormalities (28.5%) in the patient group compared to control asthma patients with no *Aspergillus*

allergy (4.6%) and subjects seeking genetic counseling for diseases other than cystic fibrosis (4.2%). They concluded that *CFTR* mutations together with additional genetic and environmental factors might play a role in the pathogenesis of this disorder (Marchand, et al. 2001).

The CFTR protein is expressed in exocrine pancreatic ducts where it alkalinizes and dilutes acinar secretion therefore preventing the formation of protein plugs (Chen and Ferec 2009). Approximately 30% of patients with idiopathic pancreatitis (ICP) or recurrent acute pancreatitis carry *CFTR* mutations. No particular mutation has been associated with ICP so far, however "mild" mutations are usually found in these patients (Bombieri, et al. 2011).

A previous study compared *CFTR* intron 8 (IVS8) polymorphisms frequency in patients with ACP, chronic alcoholics without pancreatitis and healthy controls. (da Costa, et al. 2009). daCosta *et al.* found that IVS8-T5/T7 allele was significantly more frequent in the ACP group than the other two study groups. Based on these, they suggested that individuals with *CFTR*-IVS8-T5/T7 genotype have an elevated risk of developing chronic pancreatitis if they become chronic alcoholics (da Costa, et al. 2009).

Bronchiectasis is characterized by localized (focal) or generalized (involving both lungs) abnormal and irreversible dilatation of thick-walled bronchi. In approximately half of the bronchiectasis cases the underlying cause of the condition cannot be determined (idiopathic bronchiectasis) (Pasteur, et al. 2000). Ten to fifty percent of patients with bronchiectasis carry at least one *CFTR* mutation, and 5-20% of all bronchiectasis subjects carry two defective *CFTR* alleles. A high incidence of IVS8-5T allele has been reported in these individuals but no specific *CFTR* mutations have been shown to directly associate with bronchiectasis (Casals, et al. 2004, Ziedalski, et al. 2006). Because of similar pulmonary symptoms, bronchiectasis patients carrying *CFTR* mutation(s) should be closely monitored in a specialized CF center since they might have undiagnosed cystic fibrosis and not bronchiectasis (Dequeker, et al. 2009).

Since CFTR dysfunction is a diagnostic criterion for CFTR-RD, previous attempts were made to apply functional methods such ICM and nasal potential difference measurements in CFTR-RD diagnosis. However, both NPD and ICM measurements are available only from smaller group of patients with limited experience and confounding

results to date. Therefore, further studies are required to clarify the importance of these assays in establishing disease diagnosis in CFTR-RD individuals (Bombieri, et al. 2011).

1.2.2. Definition and development of CBAVD

Congenital bilateral absence of the vas deferens (CBAVD) (OMIM # 277180) is a rare condition associated with normal spermatogenesis and obstructive azoospermia (lack of live spermatozoa in the semen) due to lack of ducts that connect the epididymides to the urethra. (Anguiano, et al. 1992). CBAVD is responsible for 2-3% of all male infertility cases (Claustres 2005, Bombieri, et al. 2011). The human male internal genitalia develop from the Wolffian ducts during intrauterine life. The ducts that differentiate between 9-13 weeks of gestation are important not just for the development of the male genital tract, but in kidney development in both male and female embryos (Hannema and Hughes 2007). The proximal portion of the Wolffian ducts forms the epididymis while the distal segment forms the vas deferens. The seminal vesicles develop from the caudal part of vas deferens later during intrauterine life. In CBAVD subjects, the vas deferens together with the body and tail of the epididymis and the seminal vesicles are missing. The head of the epididymis is present in all cases and appears to have normal function. (Hannema and Hughes 2007). Figure 3. describes location and embryonic development of vas deferens in male genital tract.

Congenital bilateral absence of the vas deferens results in no particular phenotype other than infertility (Grangeia, et al. 2004). CBAVD is usually discovered at adulthood during medical investigations for causes of clinical infertility in otherwise asymptomatic males. CBAVD is easy to diagnose by a urologist based on impalpable vas deferens and scrotal examination, but still takes an average of 4.3 years before the correct diagnosis is established (Weiske, et al. 2000). In addition to physical examination and scrotal ultrasonography, transrectal ultrasonography also needs to be performed to evaluate the anatomy of prostate, seminal vesicles and ejaculatory ducts. Semen analysis shows azoospermia, low seminal volume (<1 ml), low or absent fructose and low semen pH (<6.8) in most cases (Weiske, et al. 2000). Abdominal ultrasonography is used in CBAVD subjects to diagnose potential concomitant morphological abnormalities of the upper urinary tract. The etiology of CBAVD is not

fully understood; however a well-established connection between CBAVD, cystic fibrosis and *CFTR* gene mutations exists.

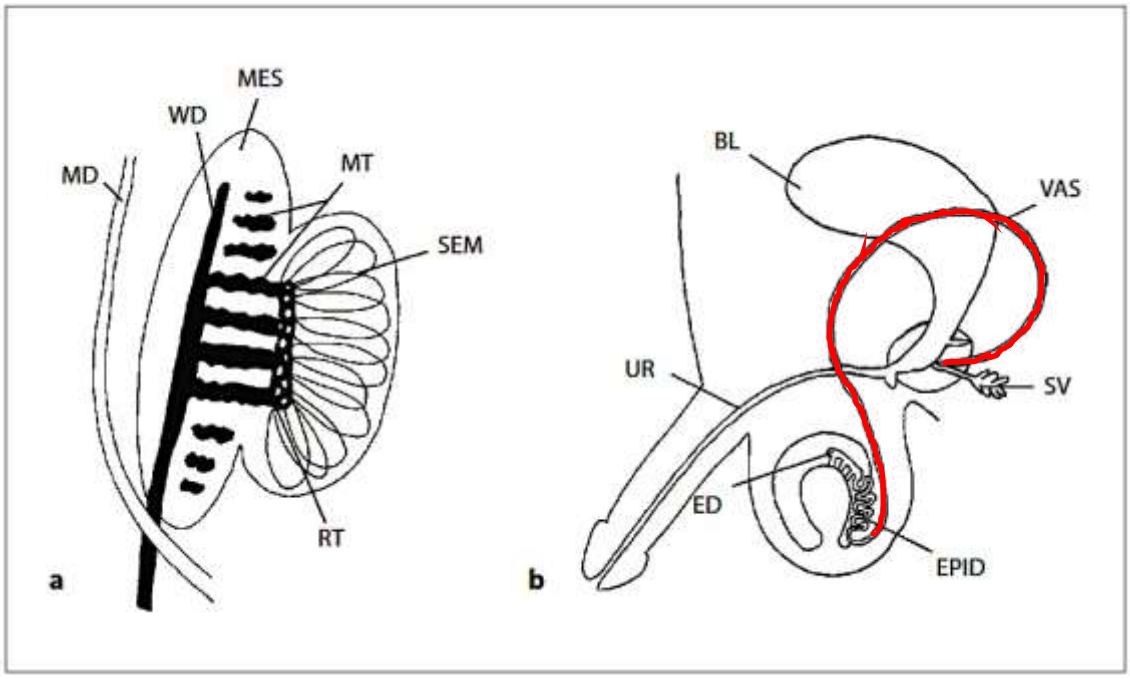


Figure 3. Schematic drawing of the development of human male genitalia
1. a: Depicts the excretory system of the gonad and the mesonephros (MES) at 8 weeks of gestation. The seminiferous cords (SEM) anastomose to form the rete testis (RT). The rete testis is connected to the Müllerian duct (MD) and joins the Wolffian duct (WD). Mesonephric tubules (MT) which are not connected to the testis degenerate. **1. b:** Newborn male urogenital tract. The mesonephric tubules and the Wolffian ducts develop into efferent ducts (ED), epididymis (EPID), vas deferens (VAS: highlighted in red) and seminal vesicle (SV). BL: bladder, UR: urethra (Hannema and Hughes 2007).

1.2.3. CFTR mutation distribution in CBAVD

As described above, CBAVD belongs to the group of CFTR-related disorders. A clear connection between *CFTR* mutations and CBAVD exist, however, the spectrum of *CFTR* mutations in CBAVD differs substantially from classical CF. Two clinically "severe" *CFTR* mutations are found in most cystic fibrosis individuals (88%), while one "severe" and one "mild" allele are present in only 12% of CF patients. In comparison, CBAVD subjects typically carry a "severe" and a "mild" allele (88%) or two "mild" alleles (12%). Moreover, no CBAVD patients without cystic fibrosis carry two "severe" mutations. The two most common CBAVD genotypes in Europe are the F508del *in*

trans (located in two different chromosomes) with IVS8-5T (28%) and F508del *in trans* with R117H (6%). (Chillon, et al. 1995, Cuppens and Cassiman 2004, Danziger, et al. 2004, Bombieri, et al. 2011). A small subset of males with CBAVD exhibits this disease phenotype without known *CFTR* defects. However, 80-97% of CBAVD subjects possess at least one defective *CFTR* allele, and 50-93% of individuals with CBAVD carry two variants, including Class IV or V *CFTR* abnormalities (Casals, et al. 2000, Cuppens and Cassiman 2004, Grangeia, et al. 2007, Ratbi, et al. 2007, Taulan, et al. 2007).

While *CFTR* mutation prevalence in CF patients show a significant variability between different ethnic groups, the IVS8-5T variant seems to be present at the same or very similar frequency in CBAVD individuals from Asia, Africa and Europe. The *CFTR*-IVS8-5T allele has been detected in 25% in Indian, 44% in Egyptian, 44% in Taiwanese; and 30% in Japanese CBAVD subjects. It is plausible to conclude that *CFTR*-IVS8-5T plays a role in the pathogenesis of CBAVD even in these populations where cystic fibrosis is extremely rare (Bombieri, et al. 2011). Unlike CF, most CBAVD sequence alterations are essentially point mutations, and large deletions or insertions are responsible for less than 1% of CBAVD cases (Bombieri, et al. 2011).

1.2.4. Polyvariant and complex mutant CFTR alleles in CBAVD

The best characterized CBAVD specific *CFTR* mutation is the polymorphic polythymidine tract (T_n or polyT) in intron 8 (IVS8-T_n) for which length is inversely correlated with the degree of exon 9 skipping during messenger ribonucleic acid (mRNA) splicing. The number of thymidines in this tract generally varies between 5 and 9, but extremely short alleles (with 2 or 3 thymidines) have also been described in CBAVD subjects (Disset, et al. 2005, Radpour, et al. 2009). Lower numbers of thymidine nucleotides (5T) in the tract predict an increasing proportion of nonfunctional *CFTR* (i.e. lacking exon 9), and are more often observed in CBAVD patients (Cuppens and Cassiman 2004). The effects of different length polyT alleles on the extent of exon 9 skipping, is shown in Figure 4. below.

DOI:10.14753/SE.2014.1863

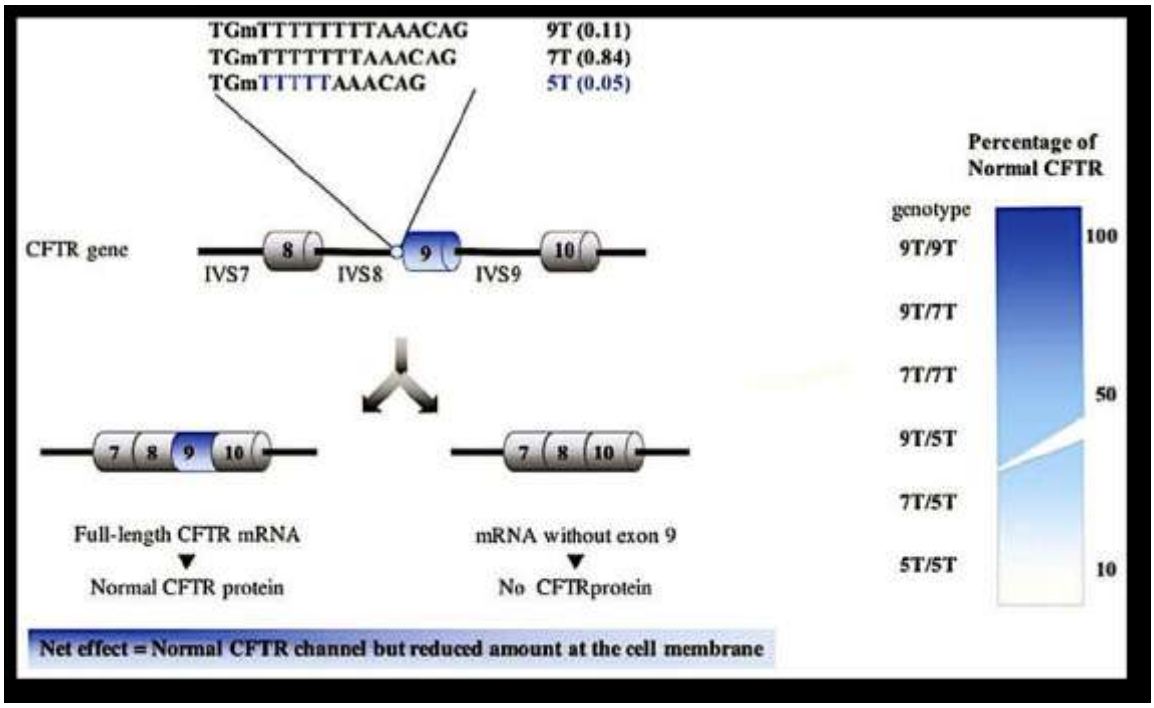


Figure 4. Effects of the variable length intron 8 polyT tract on exon 9 skipping. Healthy individuals produce normal CFTR (with exon 9) or abnormal CFTR mRNA (without exon 9) depending on the length of their polyT tract. The shorter the polyT tract, the more incomplete (exon 9 lacking) CFTR mRNA is synthesized. Homozygous 5T alleles result in 75%-90% of mRNA missing exon 9, while homozygous 7T and 9T alleles yield only 25% and 15% of abnormal mRNA, respectively. Other allelic combinations generate intermediate levels of normal mRNA molecules (Claustres 2005).

Five percent of the general population carry the 5T variant while the 7T and 9T alleles can be found in 84% and 11% of healthy individuals, respectively (Claustres 2005). However, the frequency of the *CFTR*-IVS8-5T allele is approximately 5-8 fold higher in CBAVD subjects compared to healthy individuals (25-40% versus 5%) (Chillon, et al. 1995, Bombieri, et al. 2011). The IVS8-5T variant is also found in 2-3% of non-CF alleles of fathers of CF patients and in pancreatic sufficient CF subjects. The inheritance of the IVS8-5T allele *in trans* with a "severe" *CFTR* mutation does not always result in CBAVD. The degree of penetrance of the 5T variant as a CBAVD-causing allele (in conjunction with another CFTR mutation), is estimated to be 56%. This polyT allele also acts as a genetic modifier when located *in cis* with the R117H mutation. The R117-5T allele is mostly detected in pancreatic sufficient CF patients while the R117H-7T is usually present in CBAVD subjects, and the R117H-9T variant

is found in healthy individuals. Therefore, the IVS8-5T allele is considered a CBAVD-specific mutation with incomplete penetrance (Kiesewetter, et al. 1993, Bombieri, et al. 2011).

Another polymorphic locus consisting of variable numbers (between 9-13) of TG repeats (TG_m) is located adjacent to the polyT tract in *CFTR* intron 8. It was proposed by Cuppens *et al.* that longer versions of these TG repeats can reduce the amount of normally spliced *CFTR* mRNA (Cuppens, et al. 1998). Because of their close physical proximity to the 3' splice site in IVS8, both the T_n and the TG_m loci are believed to affect the proportion of normally and abnormally spliced *CFTR* mRNA molecules. In CBAVD patients, the 5T allele is often present together with longer (12-13) TG repeats, while the 5T-TG_{11} combination is found primarily in unaffected individuals (Groman, et al. 2004). The effect of the complex IVS8 T_n-TG_m locus on the amount of synthesized functional *CFTR* mRNA is described in Figure 5. below.

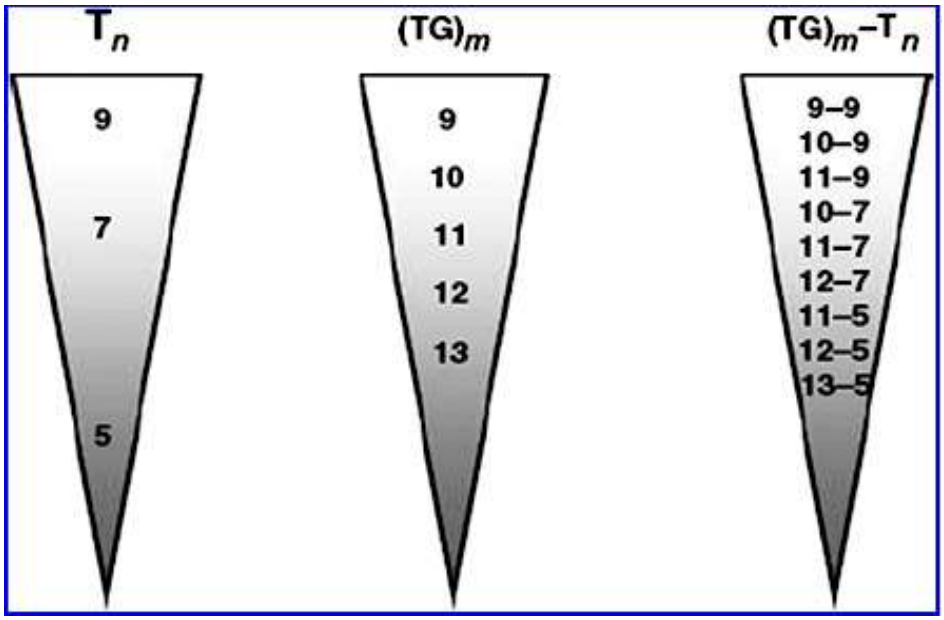


Figure 5. The effects of polyvariant mutant alleles on the amount of synthesized functional CFTR. The effect of each combined haplotype (T_n-TG_m) is shown. Decreasing amount of functional CFTR is obtained from top to bottom (Radpour, et al. 2008).

Another polymorphism, the M470V amino acid change (resulting in reduced CFTR activity) is strongly associated with the IVS8-5T allele in CBAVD patients (Bombieri, et al. 2011). Cuppens and colleagues recommended designating these alleles "polyvariant mutant *CFTR* genes" (Cuppens, et al. 1998). Besides these "polyvariant

mutant genes", other complex alleles e.g. D443Y-G576A-R668C and S1235R-IVS8-5T can also be found in CBAVD patients whose deoxyribonucleic acid (DNA) is scanned or sequenced across the whole *CFTR* coding sequence and exon-intron boundaries (Bombieri, et al. 2011).

1.2.5. Role of tissue-specific CFTR expression in CBAVD

Mak *et al.* (Mak, et al. 1997) demonstrated that a CBAVD patient with the common F508del mutation and an IVS8-5T variant produced 32% of the normal level of CFTR in the lung (exon 9 intact; a level of expression sufficient to maintain a normal pulmonary phenotype), but insufficient full-length CFTR (26% in reproductive tissues) to allow proper structural development of the vas deferens. The amount of functional CFTR (with exon 9) in the reproductive tract of a CF F508del/IVS8-7T carrier male (38%) was suggested to be sufficient for normal function and vas deferens development (Mak, et al. 1997). Rave-Harel *et al.* examined epithelial tissues from CBAVD subjects and showed that levels of normal CFTR transcripts were higher in the nasal epithelium than in the epididymal epithelium (Rave-Harel, et al. 1997). Therefore it has been suggested that amounts of CFTR protein required for normal function vary between different tissues. In general, the vas deferens has been viewed as a tissue among the most sensitive to reduced CFTR activity (Claustres 2005).

1.2.6. Pathogenesis of CBAVD

The pathogenesis of CBAVD and the mechanism by which abnormal CFTR function causes infertility in CF males are not fully understood. CFTR is expressed in vas deferens and epididymis during intrauterine and postnatal life, indicating that CFTR might play a role in the development of these organs (Chen, et al. 2012). Various distinct hypotheses of disease development exist. According to one model, the vas deferens begins developing normally but the duct and the epididymis become obstructed and are destroyed later in intrauterine life due to abnormally viscous intraluminal secretions (Valman and France 1969). This model assumes that vas deferens and epididymis destruction occurs the same fashion as in the exocrine pancreas of CF patients. According to a study by Patrizio and Zielenski, the obstruction of vas deferens

might also lead to disrupted development of other Wolffian duct derived structures as a result of insufficient paracrine effects (Patrizio and Zielenski 1996). Another possible explanation for the absent vas deferens in CBAVD and CF patients is that CFTR is a key component for the normal development of the Wolffian duct, and reduced CFTR function leads to vas deferens "agenesis". This model, however, does not explain how abnormal CFTR results in disrupted Wolffian duct development (Brugman SM 1987).

Two previously published reports have described the development of vas deferens in human fetuses. Gaillard compared aborted male CF fetuses to controls. They observed no signs of vas deferens obstruction in their samples from fetuses up to 18 weeks of gestation (Gaillard, et al. 1997). In a more recent study, Marcorelles *et al.* examined microscopic morphology of the male genital tract in aborted cystic fibrosis fetuses. They found no excretory duct obstruction or agenesis at up to 22 weeks of gestation. However, focal inflammation and mucinous plugs were seen in the oldest (26 weeks old) CF subject (Marcorelles, et al. 2012). Both studies seem to support the "atresia" and not the "agenesis" hypothesis: the male excretory system is present during fetal life in cystic fibrosis and CBAVD subjects, and the progressive obstruction occurs subsequently (perhaps contributed by inflammatory events).

1.2.7. Genetic diagnosis in CBAVD

In addition to clinical evaluation, DNA analysis is performed to identify *CFTR* mutations in CBAVD males if they exhibit symptoms of the disease and seek genetic counseling because of infertility problems. The application of assisted reproduction techniques (ART) makes it possible for these individuals to father their own biological children, and successful pregnancies have been previously described in the literature (Bombieri, et al. 2011). However, because of the high risk of transmitting *CFTR* mutation(s) to the offspring, it is mandatory to offer genetic counseling to both partners before ART. CBAVD males should be screened for the most common *CFTR* mutations and the IVS8-5T allele first, and if no mutation found, the whole *CFTR* gene should be investigated for sequence abnormalities. Most CBAVD mutations are unique and commercially available *CFTR* mutation detection kits are not designed to identify these defects (except the IVS8-5T). It is also important to screen for the most prevalent *CFTR*

mutations in the female partner of CBAVD patients (especially in females with Caucasian origins since this population has the highest CF carrier frequency) (Bombieri, et al. 2011).

1.2.8. Additional forms of male infertility associated with CFTR mutations

The potential pathogenic role of *CFTR* abnormalities has been implicated in other forms of male infertility besides CBAVD. A high-prevalence of *CFTR* mutations (e.g. F508del, R117H, W1282X, G542X and IVS8-5T) has been described in non-obstructive azoospermia (no viable sperm in semen), oligozoospermia (low sperm concentration in semen), oligoasthenozoospermia (low sperm count with reduced sperm motility in semen) and oligoasthenoteratozoopsermia (low sperm count with reduced motility and abnormal sperm morphology in semen) cases in previous reports. (Stuppia, et al. 2005, Schulz, et al. 2006). Some studies reported elevated incidence of IVS8-5T allele in non-CBAVD azoospermia cases compared to fertile males or healthy subjects which indicates that IVS8-5T carrier status might be associated with increased risk of these forms of infertility (Stuppia, et al. 2005, Mocanu, et al. 2010, Chen, et al. 2012). However, not all reports found a significant increase in *CFTR* mutation frequency in non-obstructive infertility cases compared to fertile men (Ravnik-Glavac, et al. 2001). Moreover, *CFTR* mutations have been detected in congenital unilateral absence of the vas deferens (CUAVD) subjects, although the extremely small number of cases makes it difficult to draw any conclusions according to the causative role of *CFTR* defects in this disorder (Kolettis and Sandlow 2002, Manno, et al. 2003). It is also possible that since CUAVD cases without *CFTR* mutations associate with ipsilateral renal agenesis, CUAVD represent a different disorder with a pathogenesis distinct from CBAVD.

1.3. Role of genetic modifier factors in CF and CFTR-related disorders

1.3.1. ENaC mutations contributing to CF-like and CFTR-related disorder phenotype

Since *CFTR* mutations cannot be detected even with extensive mutation scanning techniques in a small percentage of CF patients and a larger portion of CFTR-RD

subjects, it has been postulated that mutations in genes other than *CFTR* play a role in the pathogenesis of these disorders. It is known that ENaC hyperactivity contribute to the symptoms of CF lung disease, therefore ENaC mutations and polymorphisms have been investigated in both CF and CFTR-RD subjects in previous studies with somewhat conflicting results.

The human ENaC is a heterotrimeric protein that consists of 3 different subunits α, β and γ. These three subunits are encoded by the sodium channel nonvoltage-gated 1-alpha (SCNN1A); sodium channel nonvoltage-gated 1-beta (SCNN1B); and sodium channel nonvoltage-gated 1-gamma (SCNN1G) genes, respectively. The ENaC protein is a highly selective Na^+ channel with low conductance and amiloride sensitivity (Azad, et al. 2009, Lucarelli M, et al. 2012). The molecular structure of the ENaC heterotrimer, and the exact mechanism by which CFTR regulates ENaC function in epithelial tissues, is still debated and not completely understood. Loss of function mutations in the ENaC genes cause pseudohypoaldosteronism type I (PHA-1) with severe renal salt wasting and arterial hypotension (Chang, et al. 1996). Gain of function mutations in SCNN1B or SCNN1G genes result in Liddle-syndrome: a severe form of arterial hypertension (Shimkets, et al. 1994). Furthermore, some PHA-1 patients were found to have CF-like lung symptoms without *CFTR* mutations (Hanukoglu, et al. 1994). In addition to these, the β-ENaC (SCNN1B) overexpressing transgenic mice exhibit increased airway epithelial Na^+ absorption and show signs of CF-like lung disease (Mall, et al. 2004).

Azad *et al.* compared mutation frequencies of the three ENaC subunit-coding genes in a group of 76 cystic fibrosis patients (either one or no *CFTR* mutation detected) to 234 healthy controls. A total of 30 sequence variants in SCNN1A, SCNN1B and SCNN1G genes have been detected in this patient population. The authors found a significantly increased frequency of ENaC genes abnormalities in patients compared to controls (15.3% versus 8.9%). In addition to this, a hyperactive SCNN1A variant, W493R, exhibited a more than two-fold higher incidence in patients compared to controls (8% versus 3%) (Azad, et al. 2009). Amato *et al.* investigated the role of ENaC mutations in CFTR-RD subjects including 59 CBAVD, 21 recurrent pancreatitis and 19 disseminated bronchiectasis subjects. Three, two and seven known variants in the SCNN1A, SCNN1B and SCNN1G genes were observed, respectively. However, none of these variants had a significantly higher allelic frequency in the patient group

compared to controls. The authors therefore concluded that ENaC mutations did not play a role in CFTR-RD pathogenesis including CBAVD (Amato, et al. 2012). In contrast to these, Fajac and colleagues found ENaC mutations in 10 subjects (18%) of a group of 55 idiopathic bronchiectasis cases. Six of these patients carried either the F508del or the IVS8-5T *CFTR* mutation in addition to an ENaC defect. The authors have calculated that approximately 1 in 334 individuals (0.3%) are expected to carry both a *CFTR* sequence abnormality and a missense ENaC mutation. Interestingly, Fajac *et al.* detected an unexpectedly high proportion of transheterozygosity (5%) for a *CFTR* mutation/variant and an ENaC mutation in this study. These results suggested that carrying both an ENaC and a *CFTR* mutations/variant could lead to abnormal epithelial ion transport in the lung and result in bronchiectasis (Fajac, et al. 2009).

1.3.2. Cystic fibrosis modifier genes

The observation that monozygotic twins have a more concordant cystic fibrosis phenotype than dizygotic twins indicates that other genetic factors in addition to the *CFTR* genotype influence disease severity (Mekus, et al. 2000). Moreover, patients with identical CF mutations can exhibit substantial differences in disease manifestation (Kerem, et al. 1990). Some but not all aspects of the disease can be linked to *CFTR* genotype, and there is only a loose connection between *CFTR* mutations and the severity of pulmonary symptoms. A strong correlation, however, exists between pancreatic function and *CFTR* mutations; “mild” (Class IV-VI) mutations result in pancreatic sufficiency while “severe” (Class I-III) mutations lead to pancreatic insufficient disease phenotype. Lung function, meconium ileus, CF-related diabetes and anthropometric measures e.g. body mass index (BMI) of cystic fibrosis patients were shown to correlate better with genes other than *CFTR* (Cutting 2010). Therefore, genetic modifiers of the CF phenotype represent an area of intensive study.

Most CF modifier studies use the candidate gene approach and examine effects of genes that are known to interact with *CFTR*; affect the inflammation process or have influence on viral and bacterial infection. A statistically significant association between a single gene variant and CF phenotype can easily be detected even in a small group of subjects, thus it is desirable for modifier gene studies to be repeated multiple times in

different patient populations. Several candidate genes have been evaluated for cystic fibrosis modifier status, however only a small portion of these results have been confirmed by subsequent studies (Cutting 2010). The probable CF modifier genes that have been investigated by at least two independent studies in a total of more than 500 patients are listed in Table 2. Because genetic modifiers influence CF disease progression, these factors could be useful tools in prognosis, and products of these genes might serve as potential therapeutic targets.

Table 2. Cystic fibrosis modifier genes (http://www.genecards.org/) (Cutting 2010)

Gene	Gene function	Possible effect on CF phenotype
ADIPOR2: adiponectin receptor 2	Adiponectin receptor 2; expressed in muscle and adipose tissue, antidiabetic effect	MI/intestinal obstruction
EDNRA: endothelin receptor type A	Vasocontrictor, vascular smooth cell proliferation	Pulmonary function
IFRD1: interferon related developmental regulator gene 1	Growth and differentiation regulation during embryonic development	Pulmonary function
IL8: interleukin 8	Neutrophil chemotaxis mediator	Pulmonary function
MBL2: mannose binding lectin 2	MBL deficiency predispose to viral and bacterial infections	Pulmonary function
MSRA: methionine sulfoxide reductase	Reduces oxidized methionine residues in proteins	MI/intestinal obstruction
SERPINA1: α-1 antitrypsin	Serine protease inhibitor	Liver disease
TCF7L2: transcription factor 7-like 2	Transcription factor in Wnt (wingless+integration 1) pathway	CF-related diabetes
TGFB-1: transforming growth factor β-1	Inflammation regulation/tissue remodeling	Pulmonary function

1.3.3. Genetic modifier factors in the pathogenesis of CBAVD

Unraveling the genetic factors responsible for complex disorders such as CFTR-related diseases including CBAVD, represent a major challenge in molecular biology. It is becoming more and more apparent that the array of mutations which determine complex traits includes a) missense mutations that cause subtle changes in protein function and b) sequence alterations in non-coding, regulatory sequences (e.g. promoters, intronic

regulatory elements and 5' and 3'UTRs) (Lukowski, et al. 2011). In addition to these, a finding of a single *CFTR* mutation is a poor predictor for involution of the vas deferens. Although a large percentage of patients with CBAVD carry mutations in *CFTR*, approximately one in 29 Caucasian males in the United States carries one *CFTR* variant but does not develop CBAVD (Baskin, et al. 2002). Other genetic and/or environmental factors must modify penetrance of CBAVD, as is the case for CF, however these genetic modifiers are not yet known.

1.3.4. F508del-CFTR and F508C-CFTR

The F508del-*CFTR* mutation is the best described and most frequent "severe" *CFTR* mutation and is present in more than half of the CF population (Lucarelli M, et al. 2012). Several hypotheses have been proposed to explain the high incidence of cystic fibrosis and the F508del-*CFTR* carrier status in particular. The most likely explanation is that F508del heterozygosity protects against cholera and other dehydrating diseases, and this evolutionary advantage allowed the mutation to survive app. 50,000 years in the population (Bertranpetit and Calafell 1996). The F508del mutation is very frequent not only in CF but also in CBAVD, and is often present in conjunction with a "mild" mutation (or no known second allelic defect) in CBAVD subjects. Another *CFTR* mutation at the same residue - F508C - has also been described. F508C is a phenylalanine-to-cysteine replacement at amino acid position 508 resulting from a T>G change at nucleotide position 1523 (previously 1655) of the *CFTR* gene. This F508C variant has traditionally been considered to represent a clinically silent polymorphism that does not contribute to CF or CBAVD. Since this defect is located at the same position where the most frequent *CFTR* mutation is, its functional and phenotypic consequences are particularly interesting.

The F508del mutation dramatically decreases the efficiency of protein folding, results in premature CFTR degradation from the endoplasmic reticulum and the small portion of F508del-CFTR molecules that reach the cell surface, exhibit reduced ion-channel activity and decreased protein half-life. (Kopito 1999, Rogan, et al. 2011). In contrast to this, the F508C is permissive for CFTR maturation. When Du *et al.* replaced F508 with a cysteine in vitro; the protein matured, reached the cell surface and exhibited

measurable chloride channel function. However, these investigators also found that while F508C-CFTR had a close to normal half-life, this defect reduced CFTR expression levels to 35% of wild-type and decreased folding efficiency from 40% (wild-type) to 20-25%. Although F508C did not affect NBD1 folding, results of Du *et al*. suggested that side-chain interactions of the 508 residue are substantial for uninterrupted folding of NBD2 and the whole CFTR protein (Du, et al. 2005). Cui *et al.* also introduced a cysteine at position 508 but conducted a more detailed study to examine modifications of CFTR chloride channel activity. In these experiments, F508C did not affect channel mean open time, ATP-binding or the rate of ATP hydrolysis of the mature CFTR protein, however it significantly increased channel mean closed time compared with wild-type CFTR. Therefore, the authors concluded that the phenylalanine at position 508 play an important role in channel gating (Cui, et al. 2006).

The F508C variant was first described in 1990 (Kobayashi, et al. 1990), and has been viewed primarily as a diagnostic confounder. Since F508C is located at the same amino acid position as F508del, it can confer a nearly identical result in the setting of some heteroduplex *CFTR* genotyping methods, necessitating direct sequencing to distinguish F508del from F508C. The Cystic Fibrosis Mutation Database (http://www.genet.sickkids.on.ca/app) and the National Center for Biotechnology Information (NCBI) Single Nucleotide Polymorphisms (SNP) database (http://www.ncbi.nlm.nih.gov/projects/SNP/) did not relate F508C (rs74571530 previously rs1800093) to any particular phenotype, or ascribe clinical significance to this polymorphism at the time our study was conducted. Figures 6. and 7. describe the cystic fibrosis phenotype associated with the *F508C-CFTR* variant by the CF Mutation Database and the NCBI SNP in 2008. Both of these databases are useful tools to determine the consequences of *CFTR* sequence abnormalities and reliable sources that are commonly applied in the establishment of genetic diagnosis of cystic fibrosis.

DOI:10.14753/SE.2014.1863

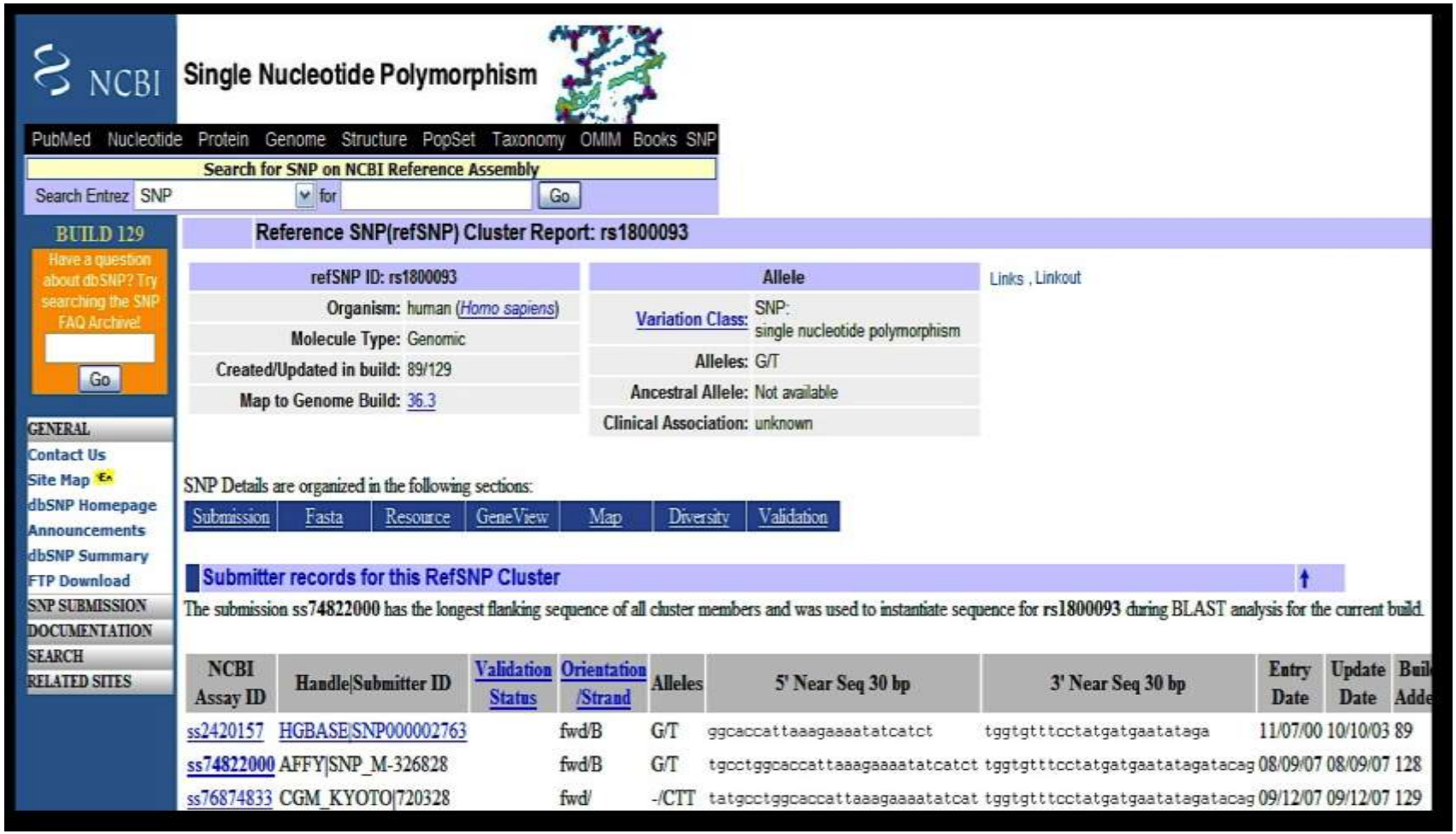


Figure 6. NCBI SNP database describing the F508C amino acid change. (From 2008.) (http://www.ncbi.nlm.nih.gov/projects/SNP/)

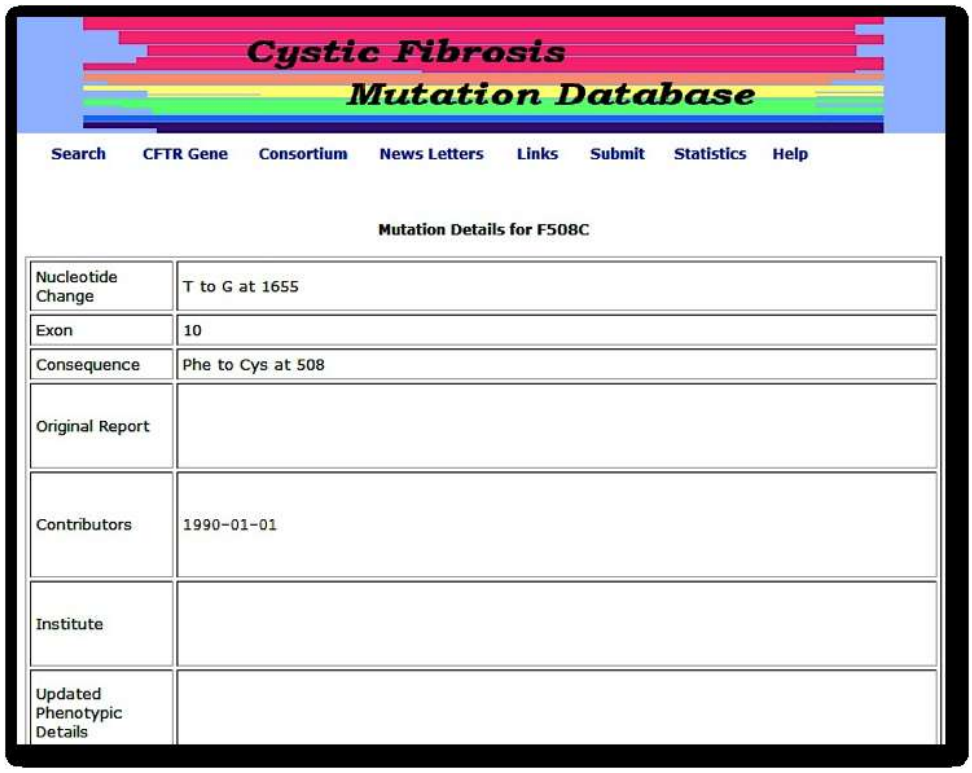


Figure 7. The Cystic Fibrosis Mutation Database describing the phenotype associated with the F508C amino acid change. (From 2008.) (http://www.genet.sickkids.on.ca/app)

1.3.5. Tissue growth factor B-1 as a CF modifier

Tissue growth factor β-1 (TGFβ-1) is a multifunctional cytokine that takes part in cell differentiation and proliferation, pro-inflammatory and anti-inflammatory events, and extracellular matrix metabolism. The TGFβ-1 protein has a potential role in angiogenesis, pathologic fibrosis, developmental defects and other clinical phenotypes (Blobe, et al. 2000). TGFβ-1 also contributes to the pathogenesis of lung fibrosis and asthma (Corrin, et al. 1994, Awad, et al. 1998). Drumm *et al.* showed that TGFB-1 gene codon 10 CC genotype (rs1800470 previously rs1982073) is associated with severe lung disease among individuals homozygous for *CFTR* mutations. This study included 808 F508del homozygous patients, and represents one of the largest CF modifier studies to date (Drumm, et al. 2005). Thus far, at least six studies (a total of more than 2,500 subjects) described an association between TGFB-1 genotype and CF lung phenotype (Arkwright, et al. 2000, Drumm, et al. 2005, Bremer, et al. 2008, Corvol, et al. 2008, Dorfman, et al. 2008, Faria, et al. 2009). One TGFB-1 modifier study of 118 CF

patients from Europe could not replicate the results of Drumm *et al.* (Brazova, et al. 2006), while another study demonstrated association with the opposite (GG) TGFB-1 allele (Arkwright, et al. 2000). The TGFB-1 gene codon 10 CC allele is linked to elevated TGFB-1 gene expression and higher circulating levels of TGFβ-1 protein in human subjects (Yamada, et al. 1998, Suthanthiran, et al. 2000, Dunning, et al. 2003). A second TGFB-1 single nucleotide polymorphism in codon 25 (rs1800471) may also influence TGFβ-1 protein levels, and has been implicated as a contributor to CF lung disease progression in some but not all studies (Awad, et al. 1998, Arkwright, et al. 2000). Awad *et al.* conducted a large study including 23 CF patients who underwent lung transplantation, and detected TGFB-1 codon 25 GG genotype in all of these subjects. (Awad, et al. 1998). In contrast to these results, Arkwright *et al.* could not prove any association between TGFB-1 codon 25 GG genotype and lung function when compared 171 CF patients to 107 healthy controls (Arkwright, et al. 2000).

Harris *et al.* examined TGFβ-1 protein levels in bronchoalveloar lavage fluid (BALF) samples from pediatric CF patients. The authors have found that BALF TGFβ-1 level was elevated in CF subjects compared to non-CF controls, and increased BALF TGFβ-1 was also associated with neutrophilic inflammation, decreased lung function and recent hospitalization (Harris, et al. 2009). In a subsequent study, Harris and colleagues also demonstrated that elevated plasma TGFβ-1 in CF subjects is associated with *Pseudomonas* infection and lung disease, and is reduced in response to therapy (Harris, et al. 2011). TGFβ-1 gene polymorphisms were not investigated in the studies of Harris *et al*. (Harris, et al. 2009, Harris, et al. 2011).

1.3.6. Role of TGFβ in the development of male genitalia

A rat gene expression-array suggested that during Wolffian duct formation, androgens indirectly modify insulin-like growth factor (IGF) and TGFβ signaling pathways, both of which play an important role during epithelial-mesenchymal interactions and normal development of the vas deferens (Hannema, et al. 2006, Hannema and Hughes 2007). Gene interactions that are substantial during Wolffian duct development are shown in Figure 8.

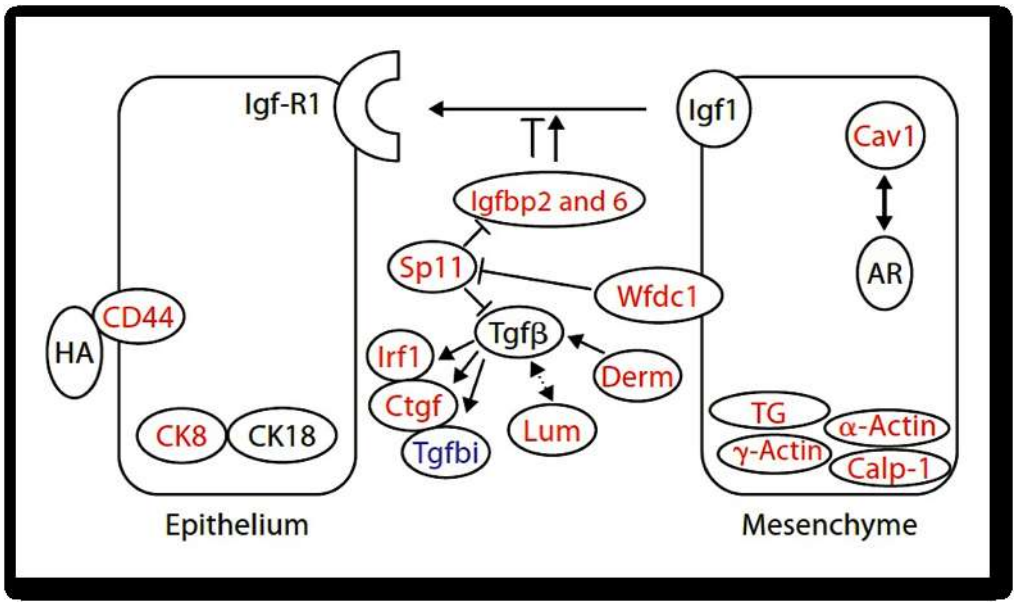


Figure 8. Gene interactions during Wolffian duct development in rats. Genes previously shown to be upregulated (red) and downregulated (blue) between embryonic days 17.5 and 20.5. AR: Androgen receptor; Calp1: Calponin-1; Cav1: Caveolin-1; CK8: Cytokeratin-8; CK18: Cytokeratin-18; Ctgf: connective tissue growth factor; Derm: Dermatopontin; HA: Hyaluronan; Igf1: Insulin-like growth factor-1, Igfbp-2: insulin-like growth factor binding protein-2; Igf-R1: insulin-like growth factor receptor-1; Irf1: Interferon regulatory factor-1; Lum: Lumican; Sp11: Serine protease 11; TG: Transgelin; Tgfbi: Tgfβ-induced; Wfdc1: WAP four disulfide core domain-1 (Hannema and Hughes 2007)

The TGFβ-bone morphogenetic protein (BMP)-small+mothers against decapentaplegic (SMADs) pathway has also been implicated as playing an important role during normal development of male genitalia. In mammals, precursors of both the female (Müllerian-duct) and the male (Wolffian duct) genitalia develop in the beginning of intrauterine life. For normal sexual development to occur, one of the tracts needs to differentiate into a corresponding organ while the other must regress. The anti-Müllerian hormone responsible for involution of the Müllerian-duct is part of the TGFβ superfamily, and it has been shown to act through type I TGFβ receptors and the BMP-SMADs pathway in mice. Disruption of this pathway causes segments of the Müllerian-duct to remain present and confers infertility in virilized males (Orvis, et al. 2008).

1.3.7. Endothelin receptor type A as a CF modifier

In addition to an emerging understanding of TGFB-1 gene as a modifier of cystic

fibrosis severity, Darrah *et al.* found a strong correlation between lung phenotype in cystic fibrosis and polymorphisms in the endothelin receptor type A (*EDNRA*) gene (Darrah 2007.). Three endothelin isomers have been described in humans: endothelin 1 (EDN-1), endothelin 2 (EDN-2) and endothelin 3 (EDN-3) (Ishikawa, et al. 2005). Endothelins are produced by endothelial cells, vascular smooth cells, neurons and astrocytes (Henry 1999, Schinelli 2006). Endothelin receptors include types A and B (EDNRB) which are found in many internal organs (e.g. heart, adrenals, kidneys, central nervous system and lungs) (Goraca 2002). Endothelin receptor type A is located mainly in vascular smooth muscle cells and responds to EDN-1 and EDN-3 (Ishikawa, et al. 2005). In the study from Darrah and colleagues, the *EDNRA* genotype AA at position -231 from AUG (rs1801708) and genotype CC at nucleotide 211 in exon eight (rs5335) were associated with more severe lung disease in CF females (Darrah 2007.). With reference to these results, McKone *et al.* (McKone, et al. 2007) examined 21 tag SNPs in the *EDN-1*, *EDN-3*, *EDNRA*, and *EDNRB* genes. This latter study confirmed a significant association between an *EDNRA* haplotype including SNP rs5335 and CF lung disease, but no association with tag SNPs in other candidate genes (McKone, et al. 2007). Moreover, polymorphism rs5335 of the *EDNRA* gene has been investigated in a total of 1,577 CF patients from North America and Ireland, and it has been shown that subjects with the CC genotype had poorer lung functions than CF individuals carrying the GG genotype (Darrah, et al. 2010). This latter study also demonstrated that in primary smooth muscle cell cultures, the rs5335 CC genotype result in elevated *EDNRA* mRNA level and higher cell proliferation rate compared to cells with rs5335 GG allele. Darrah *et al.* proposed that EDNRA variants in CF act through smooth muscle (Darrah, et al. 2010).

Interestingly, both TGFβ and endothelin play a role in extracellular matrix formation (Lawrence 1996, Goraca 2002), wound healing (Lawrence 1996, Goraca 2002) and lung fibrosis (Corrin, et al. 1994, Awad, et al. 1998, Blobe, et al. 2000, Hocher, et al. 2000). Moreover, Jain *et al.* observed a connection between TGFβ and the endothelin-EDNRA system in idiopathic lung fibrosis and demonstrated that EDN-1 influences TGFβ-1 production through EDNRA (Jain, et al. 2007).

DOI:10.14753/SE.2014.1863

2. Objectives

Besides the well-established connection between *CFTR* mutations and CBAVD, the role of additional genetic factors behind this disorder has not been studied extensively before. Since not all males that carry a *CFTR* mutation develop CBAVD, and *CFTR* mutations could not be detected in a portion of CBAVD patients, other genetic or non-genetic mechanisms most certainly play a role in disease pathogenesis. Moreover, because of the extremely large number of *CFTR* sequence alterations, the exact role of most mutations and/or polymorphisms in CFTR-related disorders including CBAVD has not been determined thus far.

The central hypotheses including these projects are 1) a very "mild" *CFTR* mutation at position 508 can contribute to the CBAVD phenotype and 2) cystic fibrosis genetic modifiers influence not only the disease expression of CF but also impact upon the manifestation of a CFTR-related disease: CBAVD. This project provides an insight into the question of why some males with a *CFTR* mutation develop CBAVD while others remain fertile despite carrying similar *CFTR* gene defects.

Our aims were:

- **Aim 1.** Investigate the incidence and study the potential pathogenic role of *F508C-CFTR* amino acid change in large CF carrier, cystic fibrosis and CBAVD cohorts.

- **Aim 2.** Examine frequencies of polymorphisms rs1800470, rs1800471 (TGFB-1) and rs1801708, rs5335 (*EDNRA*) in CBAVD subjects.

- **Aim 3.** Study the hypothesis that cystic fibrosis modifier genes influence CBAVD penetrance.

The F508C mutation has been selected based on its critical location, early evidence of subtle defects in F508C-*CFTR* Cl^- channel gating and recent insights regarding the genetic basis of diseases with complex phenotypes. We compared F508C-*CFTR* mutation frequencies in patients with CBAVD to individuals screened either for CF carrier status or cystic fibrosis as a means of investigating whether any clinical

significance might be associated with this variant. This study is the largest to date to examine the role of F508C-*CFTR* in cystic fibrosis and CBAVD subjects.

We also hypothesized that functional polymorphisms - rs1800470, rs1800471, rs5353, rs1801708 - in TGFB-1 and *EDNRA* genes, may play a role in the penetrance of not just cystic fibrosis but also CBAVD. Therefore, we designed an additional study to test whether codon 10 and codon 25 TGFB-1 polymorphisms, or either of the two *EDNRA* gene SNPs might also contribute as genetic modifiers to CBAVD. The TGFB-1 and *EDNRA* SNPs were examined in CBAVD pathogenesis because of their well-established role in CF lung disease pathogenesis and the association between cystic fibrosis and CBAVD.

DOI:10.14753/SE.2014.1863

3. Materials and Methods

3.1. Study populations

3.1.1. F508C study subjects

Subjects in the F508C-*CFTR* study included men who had undergone full *CFTR* gene sequence analysis with the Ambry Test™:CF at Ambry Genetics (Aliso Viejo, CA, USA) between January 2002 and June 2007. This group was comprised of 6,970 male patients, 850 of whom were analyzed for CF carrier screening purposes. All remaining specimens were submitted for diagnostic testing on suspicion of CF (5,938) or with a clinical diagnosis of CBAVD (182). The referral base for CBAVD typically includes male subjects with physical examination indicating absence of the vas deferens. The genetic test is sought as a means to determine associated *CFTR* mutation(s) or for assessment of *CFTR* status prior to *in vitro* fertilization. Individuals undergoing cystic fibrosis screening typically include subjects with a suspected CF phenotype (for whom genetic confirmation is sought) and those with an atypical presentation for whom a genetic test can establish the underlying diagnosis of cystic fibrosis. Each individual tested for CF carrier status or having either clinical CF or CBAVD had given written consent to have a DNA sample available for future research studies.

3.1.2. Modifier gene study subjects

In our CBAVD modifier gene study, we analyzed genomic DNA samples from 80 CBAVD individuals and 51 healthy male control subjects from Europe. This included 19 patient samples and 20 (non-CBAVD) controls from Spain (Medical and Molecular Genetics Center-IDIBELL, Barcelona), 31 CBAVD subjects and 31 controls from Turkey (Department of Medical Biology, Hacettepe University, Ankara), and 30 individuals with CBAVD from Portugal (Department of Genetics, Faculty of Medicine and Laboratory of Cell Biology; Institute Biomedical Sciences Abel Salazar of University of Porto, Porto). The study therefore represents one of the largest genetic analyses to date of CBAVD (a disease for which large patient populations are not

readily available). Criteria for inclusion as a study subject required known *CFTR* variants. Controls were defined as healthy sperm donors or other unrelated individuals with an intact vas deferens. Over 40 different *CFTR* polymorphisms of varying severity were represented. Because more than 1,900 *CFTR* sequence alterations have been described, it is very likely that other mutations in certain subjects were present but not detected by the genotyping methods used here. The study subjects have given permission to have their samples used for research studies, and the protocol was approved by the Institutional Review Board of Human Use at the University of Alabama at Birmingham and by local Portuguese, Spanish, and Turkish ethical committees.

3.2. Genotyping methods

3.2.1. F508C-CFTR analysis

Samples for the F508C study were processed in Ambry Genetics (Aliso Viejo, CA, USA). Genomic DNA (gDNA) was isolated from blood leukocytes according to standard procedures. gDNA was either isolated using the GFX genomic blood DNA purification kit (GE Healthcare, Chalfont St. Giles, UK) or using a Corbett X-Tractor automated system (Corbett Robotics, Sydney, Australia). DNA was assessed for quality and quantity by agarose gel electrophoresis. All subjects in this study were analyzed with the Ambry Test^{TM}:CF which evaluates the *CFTR* gene by modified temporal temperature gradient electrophoresis analysis (mTTGE) followed by dye terminator DNA sequencing of suspect regions. The test covers all exons, at least 20 bases 5' and 3' into each intervening sequence, and select deep intronic mutations. Briefly, all exons as well as relevant intronic regions were amplified using polymerase chain reaction (PCR). Standard PCR amplification was performed using HotStarTaq Master Mix (Qiagen, Valencia, CA, USA) with 100-150 ng input gDNA per reaction. Typical PCR conditions were 1 cycle: 95 °C/15 minutes, 35 cycles: 94°C/30 s, X°C/30 s, 72°C/30 s, 1 cycle: 72°C/10 minutes (annealing temperature (X) was adjusted based on the particular primer pair). Prior to gel analysis, PCR products were denatured and slowly cooled to allow for maximal heteroduplex formation. For a subset of *CFTR* regions, DNA was mixed with

known wild-type DNA samples to facilitate detection of homozygous mutations. PCR products were then processed for mTTGE on DCode gels (BioRad, Hercules, CA, USA). Polyacrylamide gels were analyzed for the presence of mutations following staining in ethidium-bromide and image capture under UV light using a GelDoc system (BioRad, Hercules, CA, USA). Gel analysis fragments were scored against known controls. By this protocol, and by incorporating overlapping mTTGE designs into the analysis, the method has been found to have roughly equivalent sensitivity and specificity to direct dye terminator sequencing. Regions indicating the presence of a mutation by mTTGE were processed for dye terminator sequencing. Exons that appeared to be affected were first amplified with a unique primer set using Taq PCR Master Mix (Qiagen, Valencia, CA, USA). Typical PCR conditions were 1 cycle: 95°C/5 minutes, 35 cycles: 94°C/30 s, X°C (see above)/ 30 s, 72°C/30 s, 1 cycle: 72°C/10 minutes. PCR products were analyzed via agarose gel electrophoresis followed by treatment with ExoSAP-It (USB, Cleveland, OH, USA) according to manufacturer recommendations. Standard dye terminator cycle sequencing (Beckman Coulter, Fullerton, CA, USA) was followed by analysis on a CEQ8000 capillary electrophoresis sequencer. Exons were sequenced in both sense and antisense orientations to identify the precise nucleotide variation. All reported variations follow the nomenclature based on GenBank entry NM__000492 and the public database for *CFTR* (http://www.genet.sickkids.on.ca/app).

3.2.2. TGFB-1 and EDNRA SNP genotyping

For the TGFB-1 and *EDNRA* SNP detection methods, our primers were designed based on sequences available from the National Center for Biotechnology Information (GenBank) and the European Bioinformatics Institute (Ensembl) databases. A 453 bp region of the 5' end of TGFB1 gene (GenBank accession number: NT_011109) was amplified using 5'GAGGACCTCAGCTTTCCCTC3' (forward) and 5'CTCCTTGGCGTAGTAGTCGG3' (reverse) primers. This region includes both rs1800470 and rs1800471 TGFB-1 SNPs. PCR conditions were as follows: predenaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 45 s, and a final extension at 72°C for 5 minutes. A 480 bases long region of the promoter region of *EDNRA* gene

(Ensembl Gene ID: ENSG00000151617), including SNP rs1801708, was amplified using primers 5'GTGGAAGGTCTGGAGCTTTG3' and 5'TTCCCAGCTCTCGTCTTCTC3'. Conditions for PCR were: 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 40 s. The final extension step was 72°C for 7 minutes. For detection of the exon 8 SNP of the *EDNRA* gene (rs5335), we used primers: 5'CTGCTGCTGTTACCAGTCCA3' and 5'TGACCAGTTCCCATTGAACA3' (95°C for 5 minutes, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 45 s, with a final extension step of 72°C for 7 minutes). Primer sequences used for amplification of the particular TGFB-1 and *EDNRA* regions are listed in Table 3.

Table 3. Primers used for TGFB-1 and *EDNRA* SNP genotyping

TGFB-1 SNPs rs1800470 and rs1800471 primers	5'GAGGACCTCAGCTTTCCCTC3' 5'CTCCTTGGCGTAGTAGTCGG3'
EDNRA SNP rs1801708 primers	5'GTGGAAGGTCTGGAGCTTTG3' 5'TTCCCAGCTCTCGTCTTCTC3'
EDNRA SNP rs5335 primers	5'CTGCTGCTGTTACCAGTCCA3' 5'TGACCAGTTCCCATTGAACA3'

Platinum^{R} Blue PCR Supermix (Invitrogen, Carlsbad, CA, USA), Apex^{TM} RED Taq DNA Polymerase Mastermix (Genesee Scientific, San Diego, CA, USA), or RedTaq^{R} DNA polymerase with 10x RedTaq^{R} PCR Reaction Buffer (Sigma-Aldrich, Saint Louis, MI, USA) were used for amplifications. Because the *EDNRA* promoter constitutes a guanin-citozin (GC) rich region, dimethyl sulfoxide (DMSO, 10%) was added to the PCR mixture to increase efficiency of this particular PCR. QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) was used prior to sequence analyses with BigDye Terminator v3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA).

The sequencing products were run according to standard protocols on an Applied Biosystems 3730 Genetic Analyzer with POP-7 polymer (Genomics Core Facility of the Howell and Elizabeth Heflin Center for Human Genetics, University of Alabama at Birmingham, AL, USA). Genomic locations of all four SNPs examined in this study are

summarized in Figure 9. Sequence analyses and comparisons were conducted using Chromas Lite (http://technelysium.com.au/) and Clustal W Multiple Sequence Alignment softwares (Thompson J.D. 1994.).

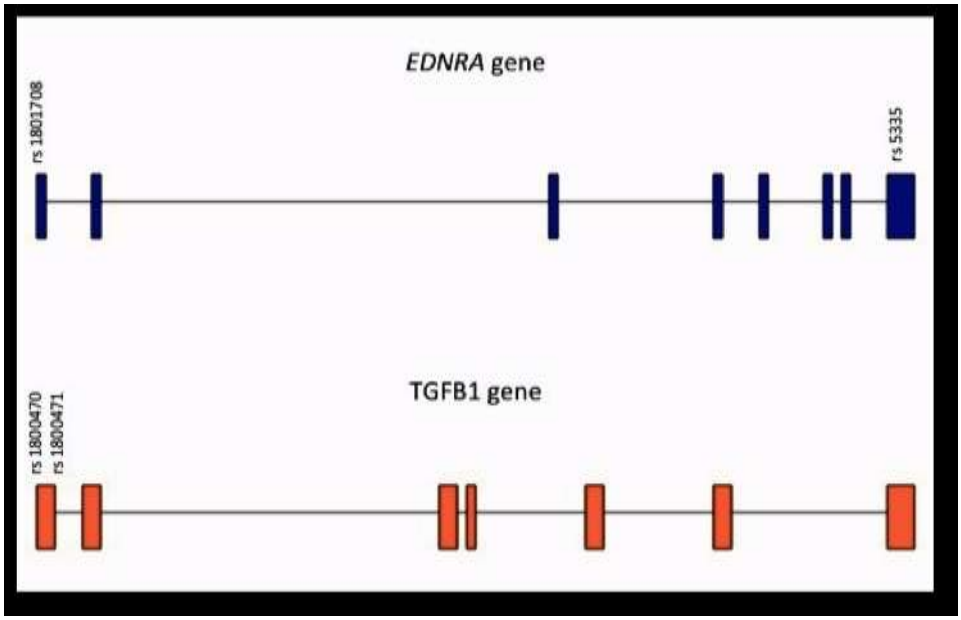


Figure 9. Genomic locations of the *EDNRA* and TGFB-1 SNPs evaluated in this study. The rectangles represent exonic sequences.

3.3. Statistical methods

3.1.1. F508C frequency analysis

A 3-way Chi-Square contingency test was performed to ascertain the significance of F508C frequency differences among the three populations examined in our first study: 1) patients submitted for diagnostic testing on suspicion of cystic fibrosis, 2) individuals screened for CF carrier status and 3) individuals with a clinical diagnosis of CBAVD. The nature of the data collection and analysis preclude conclusions to be drawn regarding ethnic background, although no large differences in ethnic composition between the groups would be expected. Statistical analyses were performed using the VassarStats web tool operated by the Vassar College Statistics Department (http://www.vassarstats.net/). Since F508C was present in fewer than 5 subjects in two of the three study groups, a Monte Carlo simulation of a three categorical sampling distribution was run for an additional 10,000 samples. While a computational analysis

of 10,000 samples requires time, the advantages of the simulation include unbiased estimates and easier application than analytical methods. Pairwise comparisons between the subdivided groups, as well as odds ratios (OR), were also calculated during this simulation.

3.1.2. Genetic modifier study statistics

For each TGFB-1 and *EDNRA* SNP, an assessment was performed assuming both a dominant and non-dominant genetic relationship with the CBAVD phenotype, as the precise relationships between the SNP genotype and TGFB-1 or EDNRA activity were not known. Differences in the distribution of SNP genotypes were compared using χ^2 analysis. In addition, a two-sample proportion test to monitor differences in overall allelic frequencies was conducted between groups. Comparisons were performed between all cases and controls collected in the study and subdivided by ethnicity to evaluate for population specific differences. Only historical controls were available for the Portuguese subjects, therefore these were not included in the statistical analyses. Due to the selective nature of the candidate genes being explored, no corrections were made for multiple comparisons. All statistic analyses were done using Statistical Package for the Social Sciences (SPSS) software package.

DOI:10.14753/SE.2014.1863

4. Results

4.1. Results of the F508C CBAVD study

Genotype data for F508C was obtained from 850 subjects undergoing complete *CFTR* sequence analysis strictly for CF carrier screening, as well as a group of 182 subjects with a clinical diagnosis of congenital bilateral absence of the vas deferens. In addition, data from 5,938 patients submitted for diagnostic testing on suspicion of having CF were also analyzed. Among the 850 individuals referred solely for CF carrier screening, 3 (0.35%) subjects had the F508C variant without a second mutation. As expected for a carrier screen, none carried F508C with a second variant. When *CFTR* from 182 individuals with a clinical diagnosis of CBAVD was analyzed, a total of 3 (1.65%) individuals were identified as carriers of F508C in addition to another mutation. Detailed genotype information and clinical history from these three CBAVD patients are shown in Table 4.

Table 4. Genotypes of CBAVD patients with F508C.

Patient #	Mutation 1	Mutation 2	Clinical History	Age
1	F508del	F508C	CBAVD	39
2	F508del	F508C	CBAVD, Sinusitis, Asthma	34
3[a]	L206W	F508C	CBAVD	40

Footnotes: [a]Family studies were not performed to determine whether these variants are in position *cis* or *trans*.

Further analysis was also conducted to determine the incidence of F508C among the 5,938 male individuals submitted for diagnostic testing on suspicion of cystic fibrosis. In 18 specimens submitted for diagnostic testing on suspicion of CF, F508C was the only detected variant (18/5,938=0.303%). In 5 individuals, the F508C variant was present along with a second mutation (5/5,938=0.084%). See Table 5. for genotypes and clinical history regarding these CF subjects. Comparatively, the allelic frequency of

F508C in the CBAVD population (3/364 alleles) was 4.68 times higher than the CF carrier screening population (3/1700 alleles), conferring an OR of 4.70. Detailed F508C frequency data from all three study groups are presented in Table 6. below.

Table 5. Genotypes of patients with F508C submitted for diagnostic testing on suspicion of CF.

Patient #	Mutation 1	Mutation 2	Clinical History	Age
1	G551D	F508C	Positive newborn screen	>1 mo
2	V754M	F508C	Clinical suspicion of CF	1 mo
3	F508del	F508C	Clinical suspicion of CF	24 yrs
4	F508del	F508C	Clinical suspicion of CF	11 yrs
5	F508del	F508C	Clinical suspicion of CF	32 yrs

Three subjects exhibited the S1251N-F508C genotype (with and without an additional third variant) and were excluded from the analysis since the CF associated variant S1251N is known to occur *in cis* with F508C. When comparing allelic frequencies of F508C in the group of patients with CBAVD (3/364 alleles) to those CF patients for whom F508C was observed with a second *CFTR* mutation (5/11,876 alleles), F508C among CBAVD patients was 19.6 times more frequent. Two-way χ^2 tests revealed that the frequency of F508C was significantly higher in the CBAVD group than any other group (see Table 6.). Specifically, frequency of the F508C allele in the CBAVD group was significantly increased compared with the entire group of patients submitted for diagnostic testing on suspicion of having CF (23/11,876 alleles) χ^2=4.01 (p=0.038). This was also the case when this group was subdivided to reflect those carrying a second, known CF-causing mutation (5/11876 alleles) χ^2=21.94 (p=0.002) and those who only carried F508C (18/11876 alleles) χ^2=5.87 (p=0.023). Direct comparison of the CBAVD group to the CF carrier screen group (3/1700 alleles) yielded a χ^2 of 2.38 (corrected p=0.073).

DOI:10.14753/SE.2014.1863

Table 6. Frequencies of F508C among individuals referred for 1) CF carrier screening (n=850), 2) clinical diagnosis of CBAVD (n=182), and 3) submitted for diagnostic testing on suspicion of CF (n=5,938).

Chi square and odds ratios are shown for the group with a diagnosis of CBAVD compared to the CF carrier screening, patients submitted for diagnostic testing on suspicion of CF, or all other pooled groups in pairwise comparisons. P values shown are corrected for 10,000 samples simulation. Statistically significant p values, odd ratios and F508C allelic frequency in CBAVD patients are highlighted in red color.

	Patients diagnosed with CBAVD (F508C plus another mutation)	**CF carrier screening (F508C only)**	**Patients submitted for diagnostic testing on suspicion of having CF**		**Total**
			(F508C plus another mutation)	**(F508C only)**	
Patients with F508C	3	3	5[b]	18	29
Total patients (Alleles)	182 (364)	850 (1,700)	5,938 (11,876)	5,938 (11,876)	6,970 (13,940)
F508C allele frequency	**0.824%**	0.176%	0.042%	0.151%	0.208%
			0.193%		
χ^2, (p)		2.38 (0.073)	21.94 **(0.002)**	5.87 **(0.023)**	
			4.01 **(0.038)**		
Odds ratio		**4.701**	**4.283**		

Footnotes: [b]Determined to carry one disease-associated *CFTR* mutation in addition to F508C.

This nominal p value is a result of the low F508C frequency in the general population; a comparison of the F508C frequencies between the CF carrier screen group and the group submitted for diagnostic testing on suspicion of CF showed no detectable difference (χ^2=0, p=1). In a more robust three-way comparison, the frequency of F508C in individuals with CBAVD was significantly increased relative to both the CF carrier screen group (3/1,700) and the group submitted for diagnostic testing on suspicion of CF (23/11,876) (χ^2=6.95, p=0.031). The Monte Carlo simulation for 10,000 replications yielded a cumulative probability of 0.0486.

4.2. Results of the CBAVD modifier genes study

4.2.1. CFTR mutation distribution in the CBAVD samples

CFTR mutation and polymorphism data for all CBAVD modifier study groups are provided in Table 7. below. In Portuguese CBAVD patients, the IVS8-5T allele was the most frequent mutation (37%) followed by the F508del (23%) and R334W (8%) (seen in 14 and 5 alleles, respectively). The majority of Spanish subjects carried the F508del mutation (29%), while the IVS8-5T (24%) variant was detected in 9 alleles and the D443Y-G576A-R668C complex genotype in 3 (8%). The IVS8-5T variant was noted in 20 Turkish alleles (32%), while mutations D1152H and D110H were detected in 14 and 3 alleles (22% and 5%), respectively. Interestingly, the F508del mutation was present in only two Turkish alleles (3%). TGFB-1 and *EDNRA* SNPs were analyzed by automated sequencing. Representative sequences from a heterozygous form of each of the four SNPs examined in our study are shown in Figure 10.

DOI:10.14753/SE.2014.1863

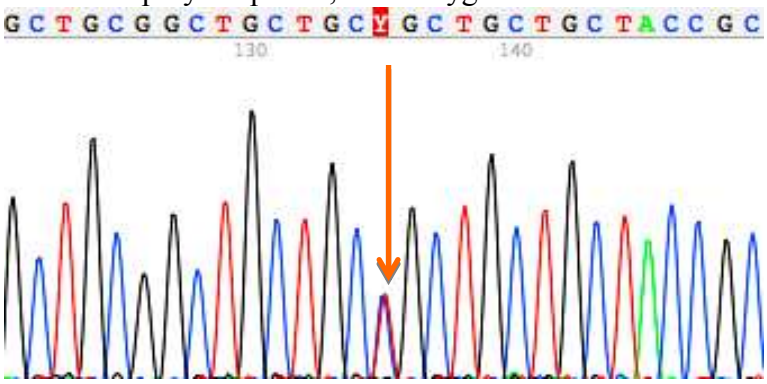
Table 7. ***CFTR*** **mutation distribution among CBAVD samples.**

Portuguese	CFTR alleles	Spanish	CFTR alleles	Turkish	CFTR alleles
IVS8-5T	22	F508del	11	IVS8-5T	20
F508del	14	IVS8-5T	9	D1152H	14
R334W	5	D443Y[c]	3	D110H	3
R117H	3	G576A[c]	3	F508del	2
S1235R	3	R668C[c]	3	c.2909-11_2909-5del[d]	2
N1303K	2	G542X	2	c.1635_1640del[d]	2
P205S	2	R117H	2	c.2657+5G>A[d]	2
D614G	2	V232D	2	CFTRdele2(ins186)	2
G542X	1	L997F	1	c.2988+1G>A[d]	1
L206W	1	H609R	1	G1130A	1
V562I	1	N1303H	1	M952I	1
I507del	1	L206W	1	c.233insT[d]	1
c.3140-26A>G[d]	1	c.3140-26A>G[d]	1	E585X	1
c.2657+5G>A[d]	1	L15P	1	c.2620-15 C>G[d]	1
G576A[c]	1	R347H	1	R334Q	1
R668C[c]	1	c.2557insG[d]	1	R347H	1
CFTRdele2,3	1	R1070W	1	E831X	1
L1227S	1	I1027T	1	R1070W	1
E831X	1			c.3140-26A>G[d]	1
				L997F	1
				I853F	1
				A349V	1
				IVS8-6T	1

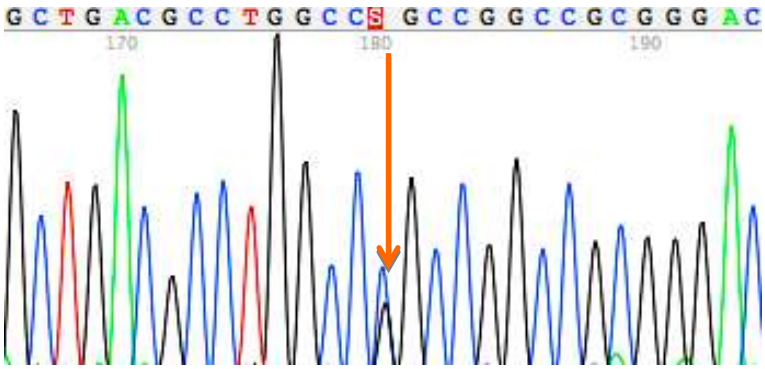
Footnotes: [c]in *cis.* This complex allele is usually found in CBAVD patients whose DNA sample is sequenced across all *CFTR* exons (Bombieri, et al. 2011).
[d]Mutation nomenclature is based on upgraded data from the Cystic Fibrosis Mutation Database (http://www.genet.sickkids.on.ca/app) and is different from the original publication.

DOI:10.14753/SE.2014.1863

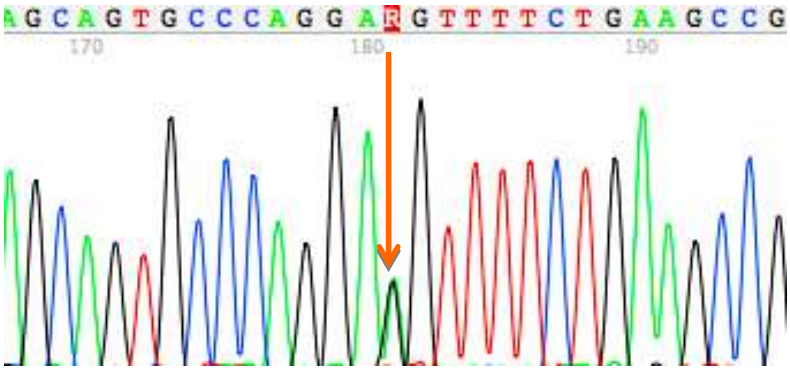
A. TGFB-1 codon 10 polymorphism, Heterozygous C/T



B. TGFB-1 codon 25 polymorphism, Heterozygous G/C



C. *EDNRA* promoter SNP heterozygous A/G



D. *EDNRA* exon 8 SNP heterozygous C/G

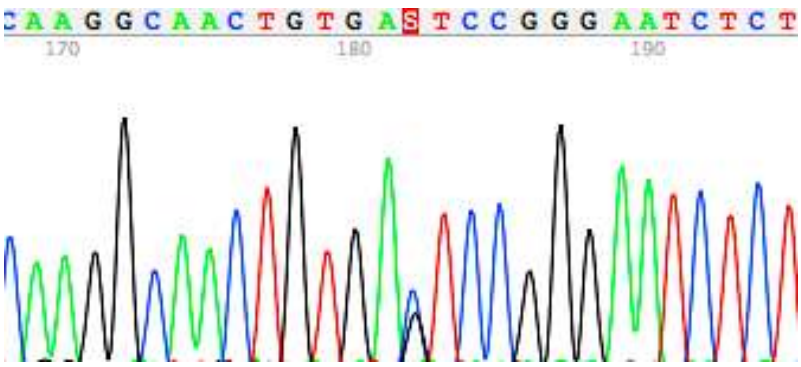


Figure 10. Representative images of TGFB-1 and *EDNRA* gene sequences

4.2.2. EDNRA genotype data

Both the AA genotype of SNP rs1801708 (*EDNRA*) and the CC allele of rs5335 (*EDNRA*) were reported to occur more frequently among CF individuals with severe lung symptoms. In a large cohort of CBAVD subjects and controls, we observed a notable increase of the CC allele at SNP rs5335 in association with CBAVD (shown in Table 8). The *EDNRA* exon 8 CC allelic frequency was significantly greater in the largest matched study cohort (i.e. Turkish patients vs. controls 45.2% vs. 19.4%, $p<0.05$ by χ^2-analysis), and between all cases vs. controls (36% vs. 15.7%, $p<0.05$). The *EDNRA* promoter SNP (rs1801708) did not appear to influence the penetrance of CBAVD ($p=0.22$) for either Turkish or Spanish cases vs. controls (Table 8).

Table 8. ***EDNRA*** **genotype distributions in CBAVD patients and controls**

EDNRA **polymorphisms**			**Patients**		**Controls**	
Exon 8 (rs5335)	*Turkish*	CC[e]	14	**45.2%**	6	**19.4%**
		CG	12	38.7%	20	64.5%
		GG	5	16.1%	5	16.1%
	Spanish	CC	4	21.1%	2	10%
		CG	11	57.8%	13	65%
		GG	4	21.1%	5	25%
	Total[f]	**50**			**51**	
Promoter (rs1801708)	*Turkish*	AA	5	16.1%	8	25.8%
		AG	18	58.1%	10	32.3%
		GG	8	25.8%	13	41.9%
	Spanish	AA	0	0%	2	10%
		AG	7	36.8%	8	40%
		GG	12	63.2%	10	50%
	Total	**50**			**51**	

Footnotes: [e]$p<0.05$ for Turkish population (χ^2 analysis) and [f]$p<0.05$ for all subjects shown (χ^2 analysis). Statistically significant different genotype distributions are highlighted in red color.

4.2.3. TGFB-1 genotype data

Studies of the rs1800470 (TGFB-1 codon 10) SNP indicated a trend towards increased T allelic frequency in all CBAVD subjects compared to controls (55% vs. 45%), although neither the subgroup analyses for polymorphism distribution by ethnicity nor genotype frequency yielded a significant association with CBAVD penetrance. With regard to TGFB-1 codon 25 SNP (rs1800471), there was no association with CBAVD for any of the analyses performed. Detailed TGFB1 genotype distribution data are listed in Table 9. Allelic frequency data of all *EDNRA* and TGF-1 SNPs are described in Table 10. below.

Table 9. TGFB-1 genotype distribution in CBAVD patients and controls

TGFB-1 polymorphisms			**Patients**		**Controls**	
Codon 10 (rs1800470)	*Turkish*	CC	8	25.8%	13	41.9%
		CT	12	38.7%	8	25.8%
		TT	11	35.5%	10	32.3%
	Spanish	CC	4	21.1%	6	30%
		CT	6	31.6%	10	50%
		TT	9	47.4%	4	20%
	Total	**50**			**51**	
Codon 25 (rs1800471)	*Turkish*	GG	26	83.9%	28	90.3%
		GC	5	16.1%	3	9.7%
		CC	0	0%	0	0%
	Spanish	GG	19	100%	17	85%
		GC	0	0%	3	15%
		CC	0	0%	0	0%
	Total	**50**			**51**	

DOI:10.14753/SE.2014.1863

Table 10. Allelic frequencies of *EDNRA* and TGFB-1 SNPs

EDNRA exon 8 SNP (rs5335)

C allelic frequency	Ethnicity	Patients	Controls
	Turkish	64.5%	51.6%
	Spanish	50%	42.5%
	Portuguese	33.3%	ND [g]

EDNRA promoter SNP (rs1801708)

A allelic frequency	Ethnicity	Patients	Controls
	Turkish	45.2%	30%
	Spanish	18.4%	41.9%
	Portuguese	26.8%	ND[g]

TGFB-1 codon 10 SNP (rs1800470)

T allelic frequency	Ethnicity	Patients	Controls
	Turkish	**54.8%**	**45.1%**
	Spanish	63.2%	45%
	Portuguese[h]	55%	44.4%

TGFB-1 codon 25 SNP (rs1800471)

G allelic frequency	Ethnicity	Patients	Controls
	Turkish	91.9%	95.2%
	Spanish	100%	92.5%
	Portuguese[h]	95%	92.5%

Footnotes: [g] ND = not done

[h] Control data from Alves H, Histocompatibility Center, University of Porto, Porto, Portugal.

DOI:10.14753/SE.2014.1863

5. Discussion

There is a well-established association between *CFTR* gene mutations and congenital bilateral absence of the vas deferens. However, while most CBAVD male carry one or two *CFTR* defects, sequence abnormalities in the *CFTR* gene cannot be accounted for the disease in all cases. In addition to this, since the large number of sequence abnormalities in the *CFTR* gene (over 1,900 to date in the Cystic Fibrosis Mutation Database) (http://www.genet.sickkids.on.ca/app), the effects on CFTR-related disorders for most of these defects are not known. Furthermore, with the exception of a handful of *CFTR* sequence abnormalities (IVS8-5T, IVS8-TG_m, R117H) and ENaC variants; additional genetic factors in CBAVD disease pathogenesis have not been investigated thus far. Therefore, in our research studies, we examined the role of F508C-*CFTR*, codon 10 and 25 TGFB-1 SNPs, and two additional *EDNRA* SNPs - both of these latter genes have been previously indicated as CF lung disease modifiers - in CBAVD disease pathogenesis.

The deletion of phenylalanine in CFTR position 508 is the most studied and most frequent cystic fibrosis mutation. It has been also demonstrated that phenylalanine at position 508 plays an important role in CFTR channel gating and protein folding efficiency (Du, et al. 2005, Cui, et al. 2006). Based on these data, we assumed that sequence alterations that lead to subtle functional changes in the 508 side-chain of the CFTR protein could play a role in the development of CBAVD. The CFTR 508 phenylalanine-cysteine amino acid change was originally observed in two healthy persons by Kobayashi *et al.* (Kobayashi, et al. 1990) and has subsequently been reported in the literature in at least 16 other individuals. Four of these were described as normal, healthy subjects without CF symptoms (Macek, et al. 1992, Desgeorges, et al. 1994, Dufourcq, et al. 1994, Warren, et al. 1997), seven were CF patients (Kerem, et al. 1990, Kalin, et al. 1992, Traystman, et al. 1993, Pompei, et al. 2006) four were CBAVD subjects (Meschede, et al. 1993, Dork, et al. 1997, Pallares-Ruiz, et al. 1999) and one subject had severe oligoasthenoteratozoospermia (Pallares-Ruiz, et al. 1999). Among CF patients carrying F508C, other *CFTR* alterations (i.e. non F508C) have sometimes been invoked to explain a CF phenotype. Overall, F508C has been thought of as a benign variant.

In 1993, Meschede and colleagues first described the presence of F508C in a surgically documented case of CBAVD (Meschede, et al. 1993). Except for childhood pneumonia, that individual had no typical CF symptoms and exhibited sweat electrolytes in the normal range. Later, Dörk *et al.* (Dork, et al. 1997) examined a cohort of 106 German CBAVD subjects and identified F508C in three individuals (Dork, et al. 1997) (including one of the three patients that had previously been described by Meschede *et al.*). F508C was not detected in 94 control subjects or in 500 CF subjects in the same study. These findings suggested that F508C might act as a CBAVD specific "mild" mutation with reduced penetrance, although Dörk *et al.* recommended further investigation to understand the pathogenic role of the defect (Dork, et al. 1997).

In our first study, we examined the frequencies of F508C in a large group of DNA samples from a total population of 6,970 men, which on submission to the clinical laboratory had either been classified 1) as possible CF carriers, 2) clinical diagnosis of CBAVD, or 3) submitted for diagnostic testing on suspicion of CF. As noted above, CBAVD is a rare condition that effects approximately 2-3% of infertile men (Grangeia, et al. 2004, Claustres 2005, Bombieri, et al. 2011). Large-scale data set analyses such as the one described here can be helpful for identifying rare or "mild" *CFTR* mutations in the setting of a clinical condition such as CBAVD. We observed F508C in 3 of the 182 CBAVD patients, all of whom carried an additional *CFTR* mutation. One of the additional mutations represents a "mild" allele (L206W, Table 4.) We noted F508C in 3 of the 850 CF carrier screens, none of which carried F508C in conjunction with another disease causing mutation as expected from a carrier screening. In addition to these cases, F508C was observed in 23 of the 5,938 individuals submitted for diagnostic testing on suspicion of CF. The F508C mutation frequency was significantly higher in the CBAVD subjects in comparison with either the CF carrier screening group or the entire group of patients submitted for genetic diagnosis on suspicion on having CF. Moreover, when comparing allelic frequencies of F508C in the CBAVD group (0.82%) to CF patients who carried F508C with a second *CFTR* mutation (0.04%), F508C among CBAVD patients was approximately 20 times more frequent.

The F508C mutation was found to be extremely rare in healthy individuals (0-0.08%), (Traystman, et al. 1993, Dork, et al. 1997, Pallares-Ruiz, et al. 1999, Pompei, et al. 2006), however, higher frequencies in CF subjects (0.2-0.7%) (Dork, et al. 1997,

Pallares-Ruiz, et al. 1999) and those with CBAVD (1-1.4%) were noted (Traystman, et al. 1993, Dork, et al. 1997). The present findings represent the largest study to date of the F508C-*CFTR* mutation and provide additional evidence for increased frequency of this allele among subjects with CBAVD. The extremely low frequency of F508C observed among normal individuals, during CF carrier screening, and among subjects genotyped for suspicion of cystic fibrosis, together with the previously demonstrated mild functional defects (Du, et al. 2005), suggest that F508C is a "mild" pathogenic mutation that can result in CBAVD. Since F508C together with another disease causing mutation could lead to both CF and CBAVD, it is possible that the effects of F508C on CBAVD disease phenotype are modified by other *CFTR* polymorphisms *in cis* (similarly to the IVS8-5T allele) or additional modifier genes. However, these theories have not been examined here and would require further investigation.

Five years after our study was published, the Cystic Fibrosis Mutation Database still has not assigned any clinical significance to the F508C mutation (http://www.genet.sickkids.on.ca/app). On the other hand, the NCBI SNP Database has updated information regarding the significance of the F508C amino acid change (Figure 11.). This latter database currently lists 99.9% allelic frequency for the wild-type (1523T) allele and 0.01% for the F508C (1523G) mutant allele, and also identifies F508C (rs74571530 previously rs1800093) as a pathological *CFTR* mutation (http://www.ncbi.nlm.nih.gov/projects/SNP/). Taken these together, F508C should be viewed as a potential disease causing allele in genetic screening for either cystic fibrosis, CBAVD or other CFTR-related disorders when it is in conjunction with another *CFTR* mutation.

DOI:10.14753/SE.2014.1863

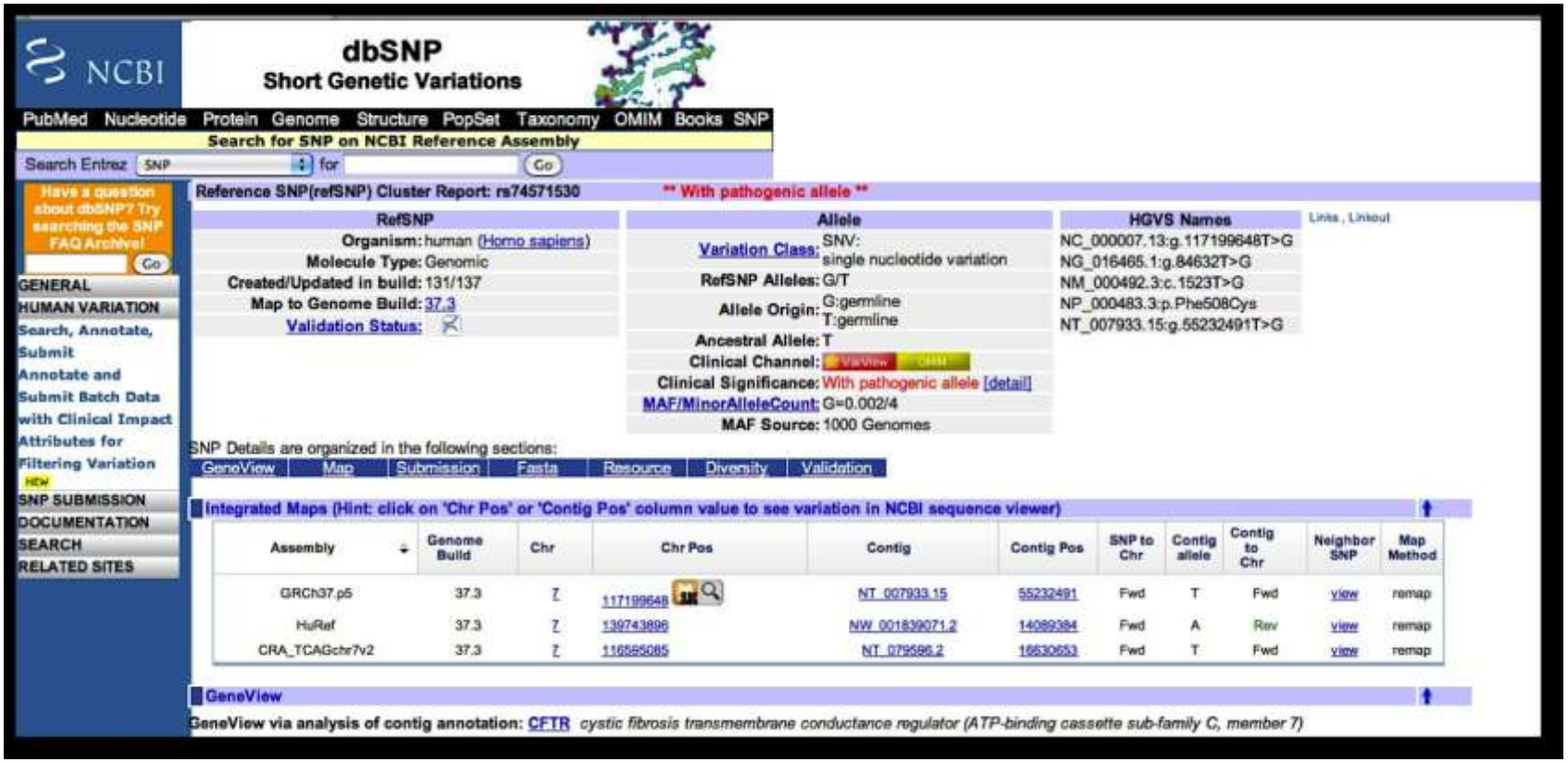


Figure 11. The NCBI SNP Database describing the clinical significance of the F508C CFTR mutation. (2012. November) (http://www.ncbi.nlm.nih.gov/projects/SNP/)

Besides examining F508C-*CFTR* mutation frequency in a large group of CF individuals, CBAVD patients and potential CF carrier males, we designed a study to pursue additional genetic factors influencing the disease. We investigated whether previously established cystic fibrosis modifier genes play a role in the development of CBAVD. In this study, we examined CBAVD patients and healthy controls from Spain, Turkey and Portugal. Only CBAVD cases with at least one detected *CFTR* mutation were included as study subjects. *CFTR* mutation frequencies between patient groups from different countries were also compared in this study. As described before, *CFTR* gene mutation distribution in CBAVD differs from mutations detected in CF subjects, however the majority of *CFTR* mutations have been identified in CBAVD patients. Yu and colleagues performed a systemic review and meta-analysis using articles published between 1992-2011 describing *CFTR* mutation frequency in CBAVD patients. They found that IVS8-5T is the most frequent mutation in CBAVD subjects (allelic frequency of 25%), followed by 17% for F508del and 3% for R117H, respectively (Yu, et al. 2012). In two of our patients groups, we report allelic frequencies similar magnitudes to those described by Yu *et al*. The Portuguese patients exhibited 37% allelic frequency for the intron 8-5T allele and 23% for the F508del mutation. The Spanish group showed 24% allelic frequency for the 5T variant and 29% for the F508del mutation. The 5T allele was the most frequent in our Turkish group (32% allelic frequency), however the F508del mutation was observed only in 2 Turkish alleles. The low allelic frequency of F508del in this latter group could be attributable to the different genetic origin and the extensive *CFTR* allelic heterogeneity of the Turkish population (Onay, et al. 2001, Kilinc, et al. 2002). Cystic fibrosis is less frequent in Turkey compared to Europe or North America (1:10,000) (Dayangac, et al. 2004), and a previous study investigating *CFTR* mutations in Turkish CBAVD patients detected F508del in only 6% of all cases (Samli, et al. 2006). Altogether, the CBAVD specific IVS8-5T variant was the most frequent *CFTR* mutation in the Portugal and the Turkish patient groups, and the second most frequent defect in the Spanish CBAVD population.

Eighty CBAVD subjects (Table 7.) and 51 controls from various regions of Europe were investigated for candidate polymorphisms in TGFB-1 or *EDNRA* in this genetic modifier analysis. Darrah *et al.* (Darrah 2007.) previously described two polymorphisms (rs1801708, rs5335) in the *EDNRA* gene associated with a more severe lung phenotype

among CF subjects. Transforming growth factor B-1 gene is one of the most characterized modifiers of the CF pulmonary phenotype. With reference to the present study; the human vas deferens, epididymis, and seminal vesicle develop from the Wolffian ducts, and it is well established that TGFβ and related signaling pathways are crucial during normal Wolffian duct development and differentiation (Sanford, et al. 1997, Stenvers, et al. 2003, Hannema, et al. 2006, Orvis, et al. 2008). Although TGFβ and associated signaling pathways have been shown to serve a crucial role in the normal vas deferens and clearly contribute to CFTR-related pathology in tissues such as lung, the significance of this pathway in atypical CFTR-related diseases such as CBAVD has not been studied previously.

In our experiments, we found that TGFB-1 polymorphisms rs1800470 and rs1800471 do not impact the CBAVD clinical phenotype. The TGFB-1 codon 10 SNP indicated a trend towards elevated T allelic frequency in all CBAVD subjects compared to all controls (55% versus 45%), however neither of the statistical analyses performed proved to be significant. None of the subgroup analyses for polymorphism distribution by ethnicity or genotype frequency for codon 25 TGFB-1 SNP showed any association with CBAVD penetrance. These result suggests important differences in pathogenesis attributable to altered CFTR expression in CBAVD versus pulmonary CF. For example, CF lung manifestations, including polymorphonuclear cell infiltration and cytokine release, are known to exacerbate CF lung injury. TGFβ-1 (a known inflammatory modulator) might influence the extent of pulmonary inflammation due to chronic infection. Such mechanisms may not be relevant to vas deferens development *in utero* and therefore cannot be invoked to account for CBAVD in the setting of CFTR deficiency. It is also possible that cytokines other than TGFβ-1 may play a more important role in the inflammation seen in normally developing embryonic vas deferens as suggested by the study from Marcorelles and colleagues (Marcorelles, et al. 2012).

On the other hand, results from this initial survey indicate that at least one known genetic modifier of CF lung disease (*EDNRA*) does appear to be associated with CBAVD. Endothelin receptor type A (or a close homolog) has been implicated previously as important during normal formation of the mammalian nervous system, the anorectum, and cranio-facial structures such as the mandible (Pla and Larue 2003, Ruest, et al. 2004, Stanchina, et al. 2006, Moore and Zaahl 2007). In addition to these,

McKone *et al.* demonstrated that primary smooth muscle cells with the CC genotype of the *EDNRA* rs5335 SNP exhibit a two-fold higher mRNA level and elevated proliferation rate compared to cells homozygous for the GG genotype (McKone, et al. 2007). Since the exact mechanisms of CBAVD disease pathogenesis is still not completely understood, based on our results one might speculate that elevated vas deferens smooth muscle cell proliferation caused by the *EDNRA* rs5335 CC genotype could contribute to the development of vas deferens obstruction. The vas deferens is composed of mostly smooth muscle cells that might be expressing endothelin receptor type A molecules. The EDNRA protein has been localized in interstitial and Sertoli cells in testis of certain mammalian species (Okada, et al. 2004) but it has not been investigated whether the receptor is expressed in human vas deferens tissues. Therefore, this theory needs to be further explored in future experiments. Altogether, our results point to *EDNRA* as playing a potential role during either the development of the vas deferens or its obstruction, and indicate that the gene product may contribute to loss of the vas deferens in the setting of CFTR insufficiency.

Besides our research, several studies have been published recently describing the role of additional genetic factors such as classically “silent” polymorphisms and changes in regulatory sequences in the pathology of cystic fibrosis and CFTR-related diseases. Bartosewszki *et al.* (Bartoszewski, et al. 2010) have described a silent *CFTR* polymorphism that affects *CFTR* mRNA secondary structure. The F508del mutation, (CTT deletion between nucleotide positions 1521-1523) also creates a synonymous change in codon 507Ile, causing a switch from ATC to ATT (both encoding isoleucine). The authors compared predicted mRNA structures of Ile507ATC-F508del, Ile507ATT-F508del and wild-type CFTR molecules and found the secondary structure of the Ile507ATC-F508del mRNA to be more similar to wild-type than the structure of the Ile507ATT-F508del variant. Translational rate and cellular expression of the Ile507ATC-F508del allele also proved to be higher compared to the Ile507ATT-F508del-CFTR. These indicated that reduced translational rates were caused by differences in mRNA secondary structure, and the authors proposed that mRNA "misfolding" contributed to not only F508del-CFTR protein misfolding, but also to the severity of the human F508del phenotype (Bartoszewski, et al. 2010).

In addition to synonymous *CFTR* polymorphisms, sequence changes in regulatory elements have been also suggested to be associated with CFTR-related disorders in some studies. Lukowski *et al.* found a patient with disseminated bronchiectasis heterozygous for a single nucleotide substitution in the 5' UTR (c. -34 C>T) of *CFTR*. This mutation creates a new upstream AUG start codon and an upstream open reading frame that is out-of-frame but also overlaps with the CFTR protein coding sequence. The c. -34 C>T change decreased gene expression by 85-99% as judged by a luciferase reporter system leading to reduced mRNA stability and translational efficiency (Lukowski, et al. 2011).

Moreover, CBAVD patients have also been reported to carry *CFTR* mutations exclusively in regulatory sequences. Lopez and colleagues identified two CBAVD subjects carrying previously unpublished $(TAAA)_6$ and $(TAAA)_8$ repeats in intron 9 (IVS9) of the *CFTR* gene (Lopez, et al. 2011). One of these patients had an additional CBAVD-specific mutation (5T-TG_{12} in intron 8) while the other subject carried a G>A change in position -33 in the *CFTR* promoter region. Internet based prediction tools showed that this G-A transition disrupts a Forkhead box 1 (FOX1) transcription factor binding site. Luciferase reporter gene assays in vas deferens cells showed that the -33A variant, which alters the FOX1 binding site, significantly reduced *CFTR* promoter activity. These assays also showed that IVS9 $(TAAA)_n$ repeats influence *CFTR* mRNA expression since deletion of one or three TAAA repeats (found in the two patients) modified the putative binding site for FOX1 transcription factors. The FOX1 binding factor was previously reported to be a mediator of male fertility (Blomqvist, et al. 2006).

Recently, it has been suggested that CFTR participates in multiple cellular mechanisms besides ion transport, and CFTR regulated ion transport abnormalities influence epithelial wound repair. Schiller and colleagues showed that CFTR is involved in bronchial epithelial reapposition in disrupted cellular monolayers. (Schiller, et al. 2010). They used human bronchial epithelial and Calu-3 (bronchial adenocarcinoma) cell lines to study wound repair. CFTR inhibition with specific $CFTR_{inh}$-172 or short hairpin RNA (shRNA) resulted in a significant delay in wound repair. Morphologic analysis of migrating cells indicated that CFTR inhibition or CFTR silencing decreased lamellipodia protrusion (Schiller, et al. 2010).

Welsh and colleagues developed a *CFTR* knockout porcine model that shares strong similarity to human cystic fibrosis pulmonary disease in contrast to CF mouse models (Welsh, et al. 2009). This group demonstrated that in addition to typical CF symptoms, newborn CF piglets exhibit trachea developmental abnormalities (Meyerholz, et al. 2010). In particular, trachea and mainstem bronchi from CF pigs were smaller caliber, and tracheal cross-sections were less circular in *CFTR* knockout newborn animals compared to wild-type controls. Altered bundle orientation of trachealis smooth muscle, and hypoplastic submucosal glands were also observed in CF piglets. Moreover, Meyerholz *et al.* examined CT scans from children under 2 years of age and found that CF trachea were less circular in cross-section compared to healthy controls. Analysis of previously published data also showed reduced trachea luminal area in CF neonates. The findings indicated that these defects originated before birth, and airflow obstruction, an early clinical finding in CF patients, may be caused by inborn tracheal defects (Meyerholz, et al. 2010).

Pierucci-Alves *et al.* examined the reproductive tract phenotype in knockout (*CFTR* -/-) and F508del-*CFTR* homozygous newborn pigs (Welsh, et al. 2009, Ostedgaard, et al. 2011, Pierucci-Alves, et al. 2011). Reproductive tracts from both the knockout and the homozygous F508del piglets completely or partially lacked the vas deferens and/or the epididymis. Ion transport measurements in vas deferens cells isolated from these newborn animals revealed impaired CFTR-dependent Cl^- and/or HCO_3^- transport. Histopathologic analysis revealed abnormalities in tubular organization, however increased mucus synthesis or inflammation in swine male excurrent ducts was not observed. These data suggest that abnormal or absent CFTR expression in male genital tract disrupts normal duct development and/or induces pathological mechanisms during intrauterine life resulting in CBAVD (Pierucci-Alves, et al. 2011).

A recent manuscript from Chen *et al.* summarized data from previous studies examining possible connections between CFTR function and male fertility (Chen, et al. 2012). According to their review, increased *CFTR* mutation frequencies were found not only in CBAVD but also in association with oligospermia, non-obstructive azoospermia, asthenospermia and teratospermia cases. CFTR has been shown to regulate the cAMP-PKA-cAMP responsive element binding protein (CREB) pathway in human Sertoli cells, and is also involved in maintenance of the blood-testis barrier.

Therefore, CFTR appears to serve as a key component during spermatogenesis. CFTR has been shown to be expressed in the epithelium lining of the male excurrent duct system. The CFTR protein also participates in oviductal and endometrial HCO_3^- secretion that is necessary for sperm capacitation. Chen and colleagues reported that CFTR is a key contributor to male fertility, and recommended CFTR as a potential target for male contraceptive agents (Chen, et al. 2012).

With the discovery of the *CFTR* gene in 1989 (Kerem, et al. 1989, Riordan, et al. 1989, Rommens, et al. 1989), opportunities for a simplified method of cystic fibrosis diagnosis were sought. Experts assumed that because CF is a monogenic disorder, merely analyzing the *CFTR* gene sequence for mutations that change amino acid sequence would result in a clear diagnosis. Since then, a handful of diseases have been linked to *CFTR* mutations (the term CFTR-related disorders has been created for this group) and multiple genes other than *CFTR* (e.g. ENaC, TGFB-1, *EDNRA*, MBL2) have been identified to influence cystic fibrosis phenotype. It is evident that simply probing for mutations in the *CFTR* gene is not sufficient for an understanding of cystic fibrosis or CFTR-related diseases phenotype. Furthermore, an increasing number of *CFTR* polymorphisms previously considered benign - including sequence changes in non-coding elements - have been shown to be disease-associated. Interpretation of sequencing results and the search for truly pathogenic *CFTR* mutations have become increasingly challenging. Moreover, CFTR proved to be a multifunctional protein responsible not only for ion transport and regulation of ENaC, but also for maintenance of the blood testis barrier, male fertility, normal intrauterine development of multiple organs and epithelial tissue repair. Cystic fibrosis and CFTR-related diseases are no longer considered “monogenic” disorders: environmental factors and genes other than *CFTR* clearly participate in disease development and influence phenotypic manifestations.

The results from our research studies suggest that F508C-*CFTR* is a CBAVD specific mutation with reduced penetrance and *EDNRA* is a potential modifier gene not just in cystic fibrosis but CBAVD. Having said this, CBAVD is most likely a multifactorial condition, and a number of other modifying factors in addition to those we studied here almost certainly influence disease penetrance. Further investigations of

DOI:10.14753/SE.2014.1863

larger patient cohorts, as well as genome-wide association studies (GWAS), will be necessary to determine the major effectors that influence the penetrance of CBAVD.

DOI:10.14753/SE.2014.1863

6. Conclusions

Firstly, the F508C-*CFTR* mutation frequency was significantly elevated among subjects with CBAVD compared to either CF carriers or CF patients. Based on our results and functional data from earlier studies, F508C is causative for both CBAVD and CF when present with a second *CFTR* mutation. However, because of the significantly elevated frequency of the mutation among CBAVD subjects, we can conclude that F508C (together with an additional *CFTR* gene defect) is more likely to lead to CBAVD than CF.

Secondly, TGFB-1 polymorphisms rs1800470 and rs1800471 do not affect CBAVD penetrance in our subject groups. Thus, TGFB-1 - a known genetic modifier of cystic fibrosis lung disease - does not function as a CBAVD specific modifier gene.

Thirdly, the *EDNRA* exon 8 CC allele was significantly more prevalent among matched study samples from individuals with CBAVD (i.e. Turkish patients vs. controls 45.2% vs. 19.4%, $p<0.05$ by χ^2-analysis) and between all cases vs. controls (36% vs. 15.7%, $p<0.05$). However, the *EDNRA* promoter SNP (rs1801708) did not influence the CBAVD phenotype in our study groups. Based on these data, we can state that endothelin receptor type A gene serves as a CBAVD specific genetic modifier.

These studies therefore provide important new information regarding the genetic variants that contribute to male infertility, normal development of the vas deferens and in particular, CBAVD. Large, multicenter studies will be needed to clarify the role of other, classically non-disease-associated *CFTR* mutations in CBAVD, and to examine the effects of additional putative CBAVD modifier genes.

DOI:10.14753/SE.2014.1863

7. Summary

Congenital bilateral absence of the vas deferens (CBAVD) is a pathologic condition associated with normal spermatogenesis, azoospermia, and lack of both vasa deferentia. Pathogenesis of CBAVD is not fully understood, however an association between mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene among men with CBAVD has been established. The objectives of our studies were to determine whether the F508C variant in the *CFTR* gene has a significant effect on CBAVD prevalence when present in conjunction with a second *CFTR* disease causing mutation, and whether genetic modifiers of cystic fibrosis (CF) lung disease predispose individuals to CBAVD. First, we compared the frequency of F508C in male subjects undergoing diagnostic testing on suspicion of cystic fibrosis or during CF carrier screening, to men with a clinical diagnosis of CBAVD. Although frequencies of F508C did not vary significantly between 850 individuals undergoing CF carrier screening and those submitted for diagnostic testing on suspicion of CF, the frequency of F508C in the CBAVD population was significantly higher than expected (χ^2=6.95, corrected P=0.049). In addition to this, we tested the hypothesis that polymorphisms of transforming growth factor B1 (TGFB-1) (rs1800470, rs1800471) and endothelin receptor type A (*EDNRA*) (rs5335, rs1801708) were associated with CBAVD phenotype. TGFB-1 and *EDNRA* SNP frequencies in DNA samples from 80 CBAVD subjects and 51 healthy male controls from various regions of Europe were examined. For *EDNRA* SNP rs5335, we found increased frequency of the CC genotype among CBAVD subjects. The difference was significant among Turkish patients vs. controls (45.2% vs. 19.4%, $p<0.05$) and between all cases vs. controls (36% vs. 15.7%, $p<0.05$). No associations between CBAVD penetrance and polymorphisms rs1800470, rs1800471 or rs1801708 were observed. We conclude that the F508C variant in *CFTR* represents a pathogenic defect and more likely lead to CBAVD than CF when combined with a second *CFTR* mutation. Our findings also establish that rs5335, an *EDNRA* polymorphism, is associated with CBAVD penetrance. To our knowledge, these are the largest studies to date that examine F508C-*CFTR* or other genetic modifiers in CBAVD subjects. Our findings provide new and important information regarding genetic contributors to male infertility and integrity of the vas deferens at CBAVD.

DOI:10.14753/SE.2014.1863

8. Összefoglalás

A kongenitalis bilateralis vas deferens hiány (CBAVD) olyan normális spermatogenesissel, azospermiával és kétoldali vas deferens hiánnyal járó pathológiás állapot, amely az esetek többségében egy vagy két cisztás fibrózis transzmembrán konduktancia regulátor (*CFTR*) génmutációval társul. Kutatásunk során az F508C-*CFTR* génvariáns, valamint két bizonyítottan cisztás fibrózis (CF) módosító gén CBAVD pathogenezisében betöltött szerepét vizsgáltuk meg. Elsőként az F508C aminosavcsere előfordulási gyakoriságát hasonlítottuk össze CF gyanús (n=5938 fő), CF hordozó (n=850) és klinikailag igazolt CBAVD-s (n=182) férfiakban. Míg számottevő különbség a CF hordozó és CF gyanús betegcsoportok között nem volt igazolható, az F508C variáns szignifikánsan gyakrabban fordult elő a CBAVD-s betegcsoportban (χ^2=6,95; korrigált P=0,049). Kísérleteink során a transzformáló növekedési faktor B1 (TGFB-1) rs1800470, rs1800471 valamint az endothelin receptor A típus (*EDNRA*) rs5335, rs1801708 polimorfizmusainak CBAVD-ben játszott szerepét is meghatároztuk. A módosító gén-vizsgálatunk során 80 klinikailag igazolt CBAVD-s és 50 egészséges kontroll spanyol, portugál és török férfi DNS mintáját analizáltuk. Az *EDNRA* rs5335 polimorfizmus CC genotípusának emelkedett gyakoriságát találtuk CBAVD betegcsoportunkban. Ez a különbség szignifikánsnak bizonyult a török beteg- és török kontrollcsoport (45,2% és 19,4%; p=<0,05), valamint az összes beteg és az összes kontroll esetek összehasonlításakor (36% és 15,7%; p<0,05). Az rs1800470, az rs1800471 valamint az rs1801708 polimorfizmusok gyakorisága és a CBAVD penetrancia között nem találtunk összefüggést. Vizsgálataink azt mutatják, hogy az F508C „variáns" CBAVD és CF kórokozó mutáció egyben, amely nagyobb valószínűséggel eredményez CBAVD-t mint CF-t abban az esetben ha egy második *CFTR* mutációval társul. Az adataink alapján azt mondhatjuk, hogy az *EDNRA* gén rs5335 polimorfizmusa szerepet játszik a CBAVD pathogenezisében. Tudomásunk szerint a F508C-*CFTR* gyakoriságot vizsgáló tanulmányunk az eddigi legnagyobb esetszámmal bíró ilyen irányú analízis, és a mi csoportunk volt az első kutatócsoport, amely *CFTR* módosító gének CBAVD-ben betöltött szerepét vizsgálta.

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DOI:10.14753/SE.2014.1863

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DOI:10.14753/SE.2014.1863

11. Acknowledgements

I am grateful (from the bottom of my heart) to the following individuals:

My consultant, Dr. Ákos Zsembery who was kind enough to accept me as his individually preparing PhD student, and who has shown me kindness and support.

My former boss, Prof. Dr. Eric J. Sorscher who is the most supporting and helpful mentor anyone can have.

My previous mentors: Prof. Dr. Miklós Kellermayer Sr. and Prof. Dr. Attila Miseta. I am especially grateful to Prof. Kellermayer for all his support and encouragement throughout my PhD and residency years.

All of my former coworkers at the Department of Medical Genetics and Child Development from the University of Pécs and at the Gregory Fleming James Cystic Fibrosis Research Center in the University of Alabama at Birmingham.

I will always be grateful to Judit Bene for teaching me almost everything I know of molecular biology. I would not have been able to write this dissertation if I had not benefitted knowledge and technical training from her.

I am grateful for the assistance provided by the Genomics Core Facility of the Howell and Elizabeth Heflin Center for Human Genetics at UAB.

I also thank the staff of Ambry Genetics for expert technical help in processing 6,970 specimens.

The CBAVD genetic modifier project was partially supported by grants PI050804 from FIS/FEDER (ISCiii), Spain and by FCT (POCI/SAU-MMO/59997/60555/60709/2004; UMIB), NIH P30 DK 54781 and RDP 464-CR02 (USA).

DOI:10.14753/SE.2014.1863

Special thanks go to my parents, my sister and to all of my friends and family members who supported me during this long journey towards PhD throughout the years.

I am grateful to Michelle McClure Ph.D. and Prof. Bryan K. Fair for editing and proofreading my dissertation.

Last but not least, I am thankful to all the CF patients, CBAVD subjects and healthy controls that generously participated in our studies.