Universidade de Lisboa
Faculdade de Ciências
Departamento de Química e Bioquímica

Correction of the ion transport defect in Cystic Fibrosis by small molecules

Marisa Isabel Lopes de Sousa

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Aos meus pais
e também aos meus avós

Marisa Isabel Lopes de Sousa foi bolseira de doutoramento da
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De acordo com o disposto no artigo 40º do Regulamento de Estudos Pós Graduados da Universidade de Lisboa, Deliberação nº 961/2003, publicada no Diário da República – III Série nº 153 – 5 de Julho de 2003, foram utilizados nesta dissertação resultados dos seguintes artigos:

Sousa M, Servidoni MF, Vinagre AM, Bonadia LC, Ramalho AS, Felício V, Ribeiro MA, Uliyakina I, Marson FA, Kmit A, Cardoso SR, Ribeiro JD, Bertuzzo C, Kunzelmann K, Ribeiro AF, Amaral MD. CFTR-mediated Cl^- Secretion in Human Rectal Biopsies as a Robust Biomarker for Cystic Fibrosis Diagnosis and Prognosis (2012). (Submitted to *PLoS one*).

Servidoni MF, Sousa M, Vinagre AM, Cardoso SR, Ribeiro MA, Meirelles L, Carvalho R, Kunzelmann K, Ribeiro AF, Ribeiro JD, Amaral MD. Rectal Biopsies Procedure in Cystic Fibrosis: Technical Aspects and Patients Perspective for Clinical Trials Feasibility (2012). (Submitted to *J Gastroenterology*).

Sousa M, Vinagre AM, Servidoni MF, Uliyakina I, Lopes AI, Malaquias J, Castelo HB, Ribeiro AF, Barreto C, Kunzelmann K, Amaral MD. Pre-clinical evaluation of small molecule compounds efficacy to restore mutant-CFTR activity in native tissues (2012). (Manuscript in preparation).

No cumprimento do disposto da referida deliberação, a autora esclarece serem da sua responsabilidade, excepto quando referido em contrário, a execução das experiências que permitiram a elaboração dos resultados apresentados, assim como a interpretação e discussão dos mesmos. Os resultados obtidos por outros autores foram incluídos com autorização dos mesmos para facilitar a compreensão dos trabalhos e estão assinalados nas respectivas metodologias.

Outros artigos publicados em revistas internacionais contendo resultados obtidos durante o doutoramento:

Da Paula AC, Sousa M, Xu Z, Dawson ES, Boyd AC, Sheppard DN and Amaral MD. Folding and Rescue of a Cystic Fibrosis Transmembrane Conductance Regulator Trafficking Mutant Identified Using Human-Murine Chimeric Proteins. *J Biol Chem* 285: 27033–044 (2010).

Luz S, Kongsuphol P, Mendes AI, Romeiras F, Sousa M, Schreiber R, Matos P, Jordan P, Mehta A, Amaral MD, Kunzelmann K and Farinha CM. The Contribution of Casein Kinase 2 and Spleen Tyrosine Kinase to CFTR Trafficking and Protein Kinase A-Induced Activity. *Mol Cell Biol* 31: 5517-11 (2011).

Tian Y, Schreiber R, Kongsuphol P, Sousa M, Uliyakina I, Palma M, Faria D, Traynor-Kaplan AE, Fragata JI, Amaral MD and Kunzelmann K. Control of TMEM16A by INO-4995 and other inositolphosphates (2012). (*British J Pharmacology* – in review).

Almaça J, Faria D, Sousa M, Conrad C, Huber W, Schreiber R, Kunzelmann K, Pepperkok R and Amaral MD. High-content RNAi Screens reveal Phosphoinositides Cycle as a Portential Target for ENaC Inhibition and Treatment of Cystic Fibrosis (2012). (Submitted to *Cell*).

Moniz S, Sousa M, Moraes B, Mendes AI, Palma M, Barreto C, Fragata José I, Jordan P, Amaral MD and Matos P. HGF treatment enhances apical anchoring and retention of chemically corrected F508del-CFTR via Rac1 signaling (2012). (Submitted to *PNAS*).

Farinha CM, King-Underwood J, Sousa M, Roxo-Rosa M, Da Paula AC, Correia AR, Williams J, Gomes C, Hirst S and Amaral MD. Folding, traffic and kinetic defects account for F508del-CFTR retention in the endoplasmic reticulum (2012). (Manuscript in preparation).

Ramalho AS, Sousa M and Amaral MD. An unsual splicing pattern resulting from a CFTR missense mutation. (2012). (Manuscript in preparation).

Preface

"Woe to that child which when kissed on the forehead tastes salty. He is bewitched and soon must die." *This adage is an early reference from Northen European folklore to the disease today known as Cystic Fibrosis.*

Cystic fibrosis is the most common lethal inherited disorder in the Caucasian population and is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. Because CFTR encodes a chloride transporter, the manifestations of this disease are due to abnormalities in salt and water transport in the epithelia of many organs. It causes the body to produce thick, sticky mucus that clogs the lungs, leading to bacterial infections, and blocks the pancreas, stopping digestive enzymes from reaching the intestines. Also most male patients are infertile due to the early blockage of the spermatic duct. Abnormally high salt content in sweat is characteristic of cystic fibrosis and also used to diagnose the disease.

For years ***"Sixty-Five Roses"*** *has been used by children to name their disease because the words are much easier for them to pronounce. It started in 1965, when a mother discovered her three little boys had the disease. She became a volunteer for the Cystic Fibrosis Foundation and her duty was to call and raise money in supporting research for a cure. Her four-year-old son overheard his mother making many phone calls and told his mom, "I know what you are working for". She was surprised because she had not told her children what she was doing, nor that they had cystic fibrosis. "What am I working for, Ricky?", she asked. "You are working for Sixty-Five Roses", he answered so innocently. She hugged her son tightly so he could not see the tears streaming down her cheeks, "Yes Ricky, I'm working for Sixty-Five Roses" [http://www.65roses.com].*

The survival of cystic fibrosis patients has increased constantly during past decades due to improvements in the healthcare and the average life span is now over 25 years. Until very recently the only treatment for a great majority of the patients was symptomatic: daily physiotherapy and mucolytic agents to remove the viscous mucus from the airways, frequent hospitalisation to treat repeated bacterial infections, dietary regulations and pancreatic enzyme supplementation. But in the beginning of the present year the first drug to tackle the basic defect this disease, arising from the joint efforts of both Cystic Fibrosis

Foundation and a pharmaceutical company (Vertex), was released for a minority of cystic fibrosis patients, showing very promising clinical results.

There is currently great demand for a good cystic fibrosis biomarker, since extended newborn screening programs identify increasing numbers of asymptomatic babies as having the disease, which creates major clinical challenges for clinicians to establish a definite cystic fibrosis diagnosis. In the following pages I will show that ex vivo measurements of colonic CFTR function (as chloride channel) can be used as a sensitive biomarker for diagnosis and prognosis of the cystic fibrosis disease. And that, with this same method, we can probably develop a personalized-medicine approach involving those new promising CFTR-modulators.

*The **rose** has become a symbol of hope for those living with cystic fibrosis and my hope is that basic research continues to enlarge our knowledge and keeps leading to innovative therapies that will improve and extend lives.*

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Summary

Cystic fibrosis (CF) is the most common life-shortening genetic disorder amoung Caucasians, affecting about 1 in 2500-6000 live births and with a carrier frequency of 1 in 25 individuals. More than 1900 CF mutations have already been reported on the *Cystic Fibrosis Transmembrane Conductance Regulator* (*CFTR*) gene, but one single mutation, named F508del, is responsible for 70% of CF chromosomes worldwide.

CFTR is expressed at the apical membrane of epithelial cells to control salt and water transport. Clinically, CF is characterized by multiple manifestations in different organs, but is dominated by the respiratory disease, the main cause of morbidity and mortality. Bacterial infections, especially by *Pseudomonas aeruginosa*, cause inefficient mucociliary clearance, originating thick mucus, and recurrent infections give rise to an exacerbated inflammatory response contributing to impairment of respiratory function and ultimately to death. Other CF classical symptoms include exocrine pancreatic insufficiency (85%), intestinal obstruction (meconium ileus in 15-20% of CF newborns and/or distal intestinal obstruction syndrome at a later age), elevated sweat electrolytes and male infertility (95%). Besides this classic picture of the CF disease, there is a minority of patients presenting non-classic phenotypes and being diagnosed from child to adulthood. Moreover the increasing numbers of asymptomatic patients identified in recent newborn CF screens have posed major challenges to clinicians for the establishment of CF diagnoses, prognosis and adequacy of therapeutics.

High-throughput screens have identified several novel small molecules with potential to treat the basic defect in CF and some are starting hit the clinical setting. These include correctors (like VX-661 and VX-809-Lumacaftor, both in Phase IIb clinical trial) that partially rescue the trafficking defect of F508del-CFTR, and a potentiator (like VX-770-, Ivacaftor (FDA-approved drug) that corrects the gating defect of G551D-CFTR, which underwent pre-clinical validation in CF airway primary cultures. However, to accelerate the entry of novel compounds into the clinic, the respective mechanism of action should be established, which usually requires studies in cellular systems with heterologous expression. Comparative efficacy assessment between heterologous expression systems and airway primary cutures/native human

tissues is thus of the ultimate importance before CFTR modulators reach the clinical setting.

In the first part of this work, we aimed to further establish measurement of CFTR function as a sensitive and robust biomarker for diagnosis and prognosis of CF. To this end, we assessed cholinergic and cAMP-CFTR-mediated Cl^- secretion in 524 freshly excised rectal biopsies from 118 individuals, including patients with confirmed CF clinical diagnosis (n=51), individuals with clinical CF suspicion (n=49) and age-matched non-CF controls (n=18). Conclusive measurements were obtained for 96% of cases. Patients with "Classic CF", presenting earlier onset of symptoms, pancreatic insufficiency, severe lung disease and were found to lack CFTR-mediated Cl^- secretion (≤5%). Individuals with milder CF disease presented residual CFTR-mediated Cl^- secretion (10-57%), and non-CF controls show CFTR-mediated Cl^- secretion above 30-35%. Moreover, this data was well correlated with various clinical parameters and proved to be the best discriminator among Classic/Non-Classic CF and non-CF groups. Determination of CFTR-mediated Cl^- secretion in rectal biopsies is demonstrated here to be a sensitive, reproducible and robust predictive biomarker for the diagnosis and prognosis of CF.

In the second part, our goals were: first, to evaluate technical aspects of this procedure regarding the viability of the rectal specimens for *ex vivo* bioelectrical and biochemical laboratory analyses; and secondly, to evaluate the overall assessment (comfort, invasiveness, pain, sedation requirement, etc) of the rectal biopsy procedure from the patients perspective, in order to assess its feasibility as an outcome measure for (pre-)clinical trials. Regarding the technical aspects of biopsing, we compared three solutions for bowel preparation (NaCl 0.9%, glycerol 12% and oral mannitol), and two biopsy forceps (standard and jumbo) in 580 rectal specimens. As to the assessment of the overall rectal biopsy procedure, telephone surveys were applied to 75 individuals. Our data shows that disruption and friability of the specimens obtained correlate with their transepithelial resistance and are also influenced by the solution used for bowel preparation and biopsy forceps, with NaCl 0.9% proving to be the most compatible with the analysis and jumbo the best forceps. The great majority of the individuals (76%) did not report high levels of discomfort due to the short time (max 15 min) and relatively painless procedure (79%). Moreover, this procedure was shown to be well tolerated by

patients with or without sedation: most (53%) accept repeating it four more times, demonstrating the feasibility of the current approach as an outcome measure for (pre-)clinical trials.

Finally, in the last chapter of this thesis we proposed to evaluate the impact of different small molecules correctors and potentiators on CFTR-protein maturation and Cl^- channel activity in heterologous cellular systems, so as to better understand their mechanism of action; and also to comparatively determine efficacy of those compounds in modulating CFTR activity in those systems and directly in primary cultures of human airway cells/and in native human tissues *ex vivo*. Functional data for F508del-CFTR rescue by iodide efflux on heterologous systems (BHK cells) shows that VRT-325, C4a, VRT-640 and VRT-532 are all able to rescue CFTR function to at varying levels (26, 33, 19 and 35%, respectively of wt-CFTR function), with both VRT-325 and C4a having additive effects to the low temperature. On polarized F508del-transduced CFBE cells, VRT-325 and C4a treatment resulted 3.32 and 8.5% in CFTR function, respectively, relative to wt-CFTR monolayer cells. In contrast, preliminary data resulting from the functional analysis conducted in primary airway cells indicates that the efficiency of the correctors VRT-325 and C4a is reduced (4.18% and 3.16% of wt-CFTR function, respectively). Remarkably, VX-809 treatment resulted in a ~5.7-fold increase in CFTR-mediated Cl^- secretion, which represents a recovery of ~16% of the CFTR function observed in primary non-CF HBE monolayers cells. Preliminary results in biopsies from F508del/F508del and F508del/G542X patients did not provide evidence for a clear effect for VRT-325 and nor C4a correctors, but showed a modest effect for a novel corrector compound (TS-01-02-D8) which we recently identified. All together, these results suggest that the effects of a given compound in rescuing F508del-CFTR activity in heterologous systems are poorly predictive of its rescuing capacity in native tissues, which indicates that further pre-clinical validation in native tissues *ex vivo* is highly recommended.

Keywords: CFTR function; CF biomarkers; outcome measures innovative therapeutics; diagnosis-algorithm prediction; patient comfort; CFTR modulators; rectal biopsies; cellular systems.

Resumo

A Fibrose Quística (FQ) é a doença autossómica recessiva letal mais comum na população Caucasiana, afectando cerca de 1 em 2500-6000 nados vivos, dependendo da região geográfica, e com uma frequência de portadores de 1 em cada 25 indivíduos. Esta doença é causada por mutações no *gene* CFTR (do inglês *Cystic Fibrosis Transmembrane Conductance Regulator*) localizado no cromossoma 7. Até à data, foram já descritas mais de 1900 mutações neste gene. No entanto, uma única mutação denominada por F508del (representando a deleção do aminoácido fenilalanina na posição 508 da proteína) é responsável por 70% dos cromossomas FQ mundiais. A proteína CFTR é expressa na membrana apical das células epiteliais onde funciona como um canal de cloreto (Cl^-), regulando o transporte de sais e de água.

Clinicamente, a FQ é caracterizada por múltiplas manifestações em diferentes órgãos, sendo contudo a doença a nível pulmonar a principal causa de morbilidade e mortalidade. A desidratação das vias respiratórias e a produção de secreções brônquicas extremamente viscosas devido a infecções bacterianas de repetição e persistentes, levam a um transporte/limpeza muco-ciliar ineficiente o que resulta na obstrução das glândulas exócrinas e, consequentemente, das vias respiratórias. Estas infecções bacterianas contribuem ainda para uma resposta inflamatória exacerbada que, por sua vez, origina o agravamento da insuficiência respiratória e, finalmente, à morte. Outros sintomas clássicos da FQ incluem insuficiência pancreática exócrina (85%), obstrução intestinal (íleo meconial em 15-20% dos recém-nascidos e/ou síndrome de obstrução intestinal distal na idade adulta), infertilidade masculina (95%) e concentração elevada de electrólitos no suor (característica utilizada no principal teste de diagnóstico).

A apresentação dum quadro clínico grave, em conjunto com a demonstração de que a proteína CFTR é disfuncional, no geral, através de dois testes do suor alterados ou da detecção de duas mutações no gene CFTR, permitem o diagnóstico da maioria dos doentes ainda na primeira infância. No entanto, nem todos os doentes apresentam este quadro clássico da doença, existindo um grupo de doentes que apresenta fenótipos não-clássicos, resultando no seu diagnóstico tardio, o que pode prejudicar o seu tratamento. Mais recentemente, os números crescentes de doentes FQ assintomáticos, identificados através dos programas de rastreio neonatal, têm colocado

grandes desafios clínicos no que toca ao estabelecimento do diagnóstico FQ definitivo, prognóstico e adequadas estratégias terapêuticas. É crucial implantar um método que permita discriminar estes dois grupos de doentes através do seu diagnóstico diferencial.

A FQ apesar de todos os avanços clínicos e científicos é actualmente ainda uma doença letal, sendo a esperança média de vida acima dos 25 anos. Felizmente, nos últimos anos têm vindo a ser identificadas, em screens de alto rendimento, diversas pequenas moléculas com potencial terapêutico para corrigir o defeito básico na FQ estando já algumas aprovadas para uso clínico. Como exemplo, existem: i) os compostos "correctores" como o VX-661 e o VX-809-Lumacaftor (ambos em ensaio clínicos de fase IIb) que visam corrigir os defeitos básicos de folding e tráfego da proteína mutada, F508del-CFTR; e b) os compostos "potencializadores" (como o VX-770-Ivacaftor (recentemente aprovado pela Food and Drug Administration (FDA), nos Estados Unidos da América) que pretendem restaurar o defeito de gating do canal de Cl^- mutado, G551D-CFTR. Para poderem entrar em ensaios clínicos, todos estes compostos sofreram uma pré-validação clínica em culturas primárias de células epiteliais brônquicas humanas. No entanto, para acelerar a entrada de novos compostos em ensaios clínicos, os respectivos mecanismos de acção destas moléculas deverão ser determinados, o que, regra geral, exige estudos em sistemas celulares de expressão heteróloga. Deste modo, estudos que pretendam comparar a avaliação da eficácia destes compostos em sistemas de expressão heteróloga e em culturas primárias ou em tecidos nativos humanos são da máxima importância.

A primeira parte deste trabalho procura consolidar a medição da função do canal CFTR como um marcador biológico sensível e robusto para o diagnóstico e prognóstico da doença FQ. Para este fim, avaliaram-se as secreções de Cl^- através da CFTR com estimulação colinérgica e mediada pelo AMP cíclico (cAMP) em 524 biópsias rectais de 118 indivíduos. Este estudo incluiu pacientes com diagnóstico clínico de FQ (n = 51), indivíduos com suspeita clínica de FQ (n = 49) e indivíduos controlos não-FQ (n = 18). No decorrer do estudo, foram obtidos resultados bioeléctricos conclusivos para 96% dos casos. Nos doentes clinicamente caracterizados com a forma clássica da doença, ou seja, apresentando um início mais precoce dos sintomas clínicos, insuficiência pancreática e doença pulmonar grave, observou-se uma ausência de secreção de Cl^- mediada pela CFTR ou apenas existência de níveis

muito baixos (≤ 5%). Para os indivíduos que apresentavam uma doença mais suave (não-clássica) e um diagnóstico mais tardio, foi possível a detecção de uma secreção de Cl^- residual mediada pela CFTR (10-57%) e, no que diz respeito aos indivíduos controlo não-FQ, a secreção de Cl^- mediada pela CFTR situou-se acima dos 30-35%. Mais ainda, estes dados bioeléctricos evidenciaram boas correlações com os diversos parâmetros clínicos estudados, demonstrando constituír o melhor discriminador entre as formas clássica e não clássica da doença e estes e os indivíduos não-FQ. Assim, podemos concluir que a determinação da secreção de Cl^- mediada pela CFTR em biópsias rectais é um marcador biológico sensível, reprodutível e robusto, e ainda preditivo prognóstico da FQ.

O foco da segunda parte deste trabalho doutoral foi, por um lado, avaliar os aspectos técnicos deste procedimento em relação à viabilidade das amostras rectais para a realização das análises laboratoriais bioeléctricas e bioquímicas *ex vivo*; e, por outro lado, avaliar globalmente o procedimento de sigmoidoscopia para recolha da biópsia rectal (conforto, grau de invasão, dor, requisito de sedação, etc) sob a perspectiva do paciente, com o objectivo último de estimar a sua viabilidade como forma para avaliar a eficácia terapêutica de novas pequenas moléculas em ensaios (pré-)clínicos.

Quanto aos aspectos técnicos deste procedimento, foram comparadas três soluções para a preparação intestinal (cloreto de sódio 0,9%, glicerol 12% e manitol oral) e duas pinças de biópsia (padrão e jumbo) em 580 biópsias rectais. Os dados recolhidos mostram que a integridade e friabilidade dos tecidos obtidos está correlacionada com a sua resistência transepitelial, ou seja, com a sua viabilidade, sendo que quanto maior a integridade e menor friabilidade da biópsia, maior a resistência observada. Estas características dos tecidos colhidos são influenciadas pela solução utilizada durante a preparação intestinal e pinça de biópsia, sendo a preparação com cloreto de sódio e a pinça jumbo as que permitem uma maior viabilidade do tecido, logo mais compatíveis com a análise bioeléctrica .

Quanto à avaliação global do procedimento de colheita da biópsia rectal, foram feitas entrevistas telefónicas a 75 indivíduos que tinham sido submetidos ao procedimento de sigmoidoscopia e colheita de biópsia. A grande maioria dos indivíduos (76%) não relatou níveis elevados de desconforto devido ao curto período de tempo requerido (máximo 15 minutos) e ao facto deste procedimento ser praticamente indolor (79%). Além disso, este exame mostra

ser bem tolerado pelos pacientes com ou sem sedação visto que maioria dos entrevistados (53%) aceitam repeti-lo mais quatro vezes, demonstrando a viabilidade desta abordagem como uma ferramenta para medir em ensaios (pré-)clínicos, a eficácia de novas moléculas com potencial terapêutico para a FQ.

Finalmente, no último capítulo desta tese, avaliou-se o impacto de diferentes pequenas moléculas correctoras e potencializadoras da maturação e função da proteína CFTR mutada em sistemas celulares de expressão heteróloga, de modo a compreender melhor seu mecanismo de acção. Os dados funcionais resultantes da técnica de efluxo de iodeto em células BHK (sistema de expressão heteróloga) que expressam estavelmente a proteína mutante, F508del-CFTR, mostram que os compostos VRT-325, C4a, VRT-640 e VRT-532 são capazes de resgatar a função CFTR em níveis diferentes: 26, 33, 19 e 35% da função observada em wt-CFTR, respectivamente. Verificou-se ainda que tanto o VRT-325 como o C4a têm efeitos aditivos ao tratamento com baixa temperatura. Fez-se também uma análise comparativa da eficácia de tais compostos na actividade moduladora da CFTR em sistemas heterológos, em culturas primárias de células brônquicas humanas e em tecidos nativos (biópsias rectais). Em células CFBE transduzidas com um vector viral que expressa o mutante F508del-CFTR e polarizadas, o tratamento com os compostos VRT-325 e C4a resultou na correcção de 3.32 e 8,5% da função observada em wt-CFTR, respectivamente. Em contraste, os dados preliminares resultantes da análise funcional em células primárias do epitélio brônquico indicam que a eficiência dos correctores VRT-325 e C4a é reduzida neste sistema (4.18% e 3.16% da função observada em wt-CFTR, respectivamente). Notavelmente, o tratamento com o corrector VX-809-Lumacaftor resultou num aumento de cerca de 6 vezes da secreção de Cl^- mediada pela CFTR relativamente a células CFBE F508del-CFTR controlo, este valor representa uma recuperação de ~16% da função da proteína CFTR observada em células primárias brônquicas não-FQ. No que diz respeito às medições da secreção de Cl^- mediada pela CFTR em biópsias rectais de pacientes F508del/F508del e F508del/G542X, os resultados preliminares desta pesquisa não evidenciam um efeito claro dos correctores VRT-325 e C4a, mas mostram um efeito modesto para um outro composto corrector (TS-01-02-D8), recentemente identificado noutros estudos realizados no nosso laboratório. Os resultados apresentados para os compostos aqui estudados sugerem que a análise da

correcção da actividade da proteína mutante F508del-CFTR como canal de Cl^- em sistemas heterólogos se revela pouco preditiva da sua capacidade real de restaurar esta função em culturas primárias e em tecidos nativos. Indicando, assim que a validação pré-clínica dos moduladores do canal CFTR em tecidos nativos (*ex vivo*) é de extrema importância para a avaliação correta do seu potencial interesse terapêutico.

No global, o trabalho realizado nesta tese doutoral permitiu validar técnica de análise funcional da proteína CFTR como canal de cloreto em biópsias rectais com meio de diagnóstico discriminante e correcto prognóstico da doença FQ. Esta técnica revela-se ainda de grande interesse para a validação pré-clínica de terapias com novas moléculas com potencial para corrigir ou potencializar a função da CFTR em doentes FQ. Em conjunto, estes resultados poderão no futuro permitir um diagnóstico correcto da FQ e antecipar a validação de novas formas eficazes de terapia, o que tem uma importância crucial para a escolha da terapêutica mais eficaz e, consequentemente, para a melhoria de vida dos doentes com FQ.

Palavras-chave: função da CFTR; marcadores biológicos na FQ; outcomes da eficácia terapêutica; ferramentas preditivas do diagnóstico FQ; conforto do paciente; moduladores da CFTR; biópsias rectais; sistemas celulares.

Abbreviations

1-EBIO	1-ethyl-2-benzimidazolone
aa	Amino acid
ABC	ATP-binding cassette
ABPA	Allergic bronchopulmonary aspergillosis
ACTV	Amphotericin, ceftazidime, tobramycin, and vancomycin (antibiotics cocktail)
ALI	Air liquid interface
ASL	Airway surface liquid
ATP	Adenosine 5'Triphosphate
BHK	Baby hamster kidney cell line
BMI	Body mass index
C4a	N-[2-(5-Chloro-2-methoxy-phenylamino)-4'-methyl-[4,5']bithiazolyl-2'-yl]-benazmide
Ca^{2+}	Calcium ion
CaCC	Calcium-activated Chloride Channel
cAMP	cyclic Adenosine 5'Monophosphate
CBAVD	Congenital bilateral absence of the vas deferens
CCH	Carbachol
CF	Cystic fibrosis
CFBE41o-	Cystic fibrosis human bronchial cell line
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CFTR	Gene encoding CFTR
CFTR-RD	CFTR-Related disorders
CF-RDM	CF-related diabetes mellitus
Cl^-	Chloride ion
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DIOS	Distal intestinal obstruction syndrome
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
ENaC	Epithelium Sodium Channel
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ERQC	Endoplasmic reticulum quality control
FDA	Food and Drug Administration
FEE	Fecal Elastase E1
FEV_1	Forced expiratory volume in first second

FRT	Fisher rat thyroid cell line
Fsk	Forskolin
FVC	Forced vital capacity
GI	Glucose intolerance
GSH	Glutathione
H^+	Hydrogen ion
HBE	Human Bronchial Epithelial cells
HCO_3^-	Bicarbonate
Hdj	Human DnaJ homologue
HE	Heamatoxilin-Eosin
HEPES	N-(2-hydroxyethyl)-piperazine-N-(2-etanesulphonic acid)
HPOA	Hypertrophic pulmonary osteoarthritis
Hsp	Heat shock protein
HTS	High Throughput Screening
IBMX	3-isobutyl-1-methylxanthine
IRT	Immunoreactive trypsinogen
I_{sc}	Short-circuit current
K^+	Potassium ion
LT	Low temperature (26°C)
MI	Meconium ileus
MoA	Mechanism of Action
MSD	Membrane-spanning domain
Na^+	Sodium ion
NBD	Nucleotide binding domain
NH3	Na^+/H^+ exchanger
NHERF1	Na^+/H^+ exchanger regulatory factor isoform-1
NKCC1	Na^+-K^+-$2Cl^-$ cotransporter
NMD	Non-sense mediated decay
NPD	Nasal Potential Difference
ORCC	Outwardly Rectifying Chloride Channel
PCL	Periciliary layer
PBS	Phosphate buffered saline
PDE	Phosphodiesterases
PGE	Prostaglandins
PK	Protein kinase
PI	Pancreatic Insufficiency
PS	Pancreatic Sufficiency
PTC	Premature termination codon
PTC124	(3-[5-(2-fluorophenyl)-[1,2,4]oxadiazol-3-yl]-benzoic acid
RD	Regulatory domain

ROMK	Renal outer medullary K^+ channel
RT	Room Temperature
R_{te}	Transepithelial resistance
SCFA	Short-chain fatty acids
SDS	Sodium Dodecyl Sulfate
SLC26	Solute carrier 26 family
SK (score)	Shwachman-Kulczycki (score)
SNARE	Soluble NSF Attachment Protein Receptor
SOP	Standardized operating procedure
SPD	Sweat potential difference
TMAO	Trimethylamine-N-oxide
Tris-HCl	Tris(hydroxymethyl)aminomethane- hydrochloric acid
Ubc	Ubiquitin-conjugating enzymes
UPP	Ubiquitin Proteasome Pathway
UTP	Uridine 5'Triphosphate
VRT-325	4-cyclohexyloxy-2-{1-[4-(4-methoxy-benzenesulfonyl) Piperazin-1-yl]ethyl}quinazoline
VRT-532	4-methyl-2-(5-phenyl-1H-pyrazol-3-yl)-phenol
VRT-640	2-{1-[4-(4-chloro-benzensulfonyl)-piperazin-1-yl]-ethyl} -4-piperidin-1-yl-quinazoline
V_{te}	Transepithelial voltage
% v/v	Percentage expressed in volume/volume
WB	Western blot
wt-CFTR	Wild-type CFTR
% w/v	Percentage expressed in weight/volume

I. General Introduction

1 Cystic Fibrosis

1.1 Historic Introduction

Cystic fibrosis (CF) is the most common life-shortening genetic disorder amoung Caucasians, affecting about 1 in 2500-6000 live births and with a carrier frequency of 1 in 25 individuals (Collins, 1992).

Although first medical reports of CF symptoms (e.g. *meconium ileus*) date back to the mid 17th century, CF as a unique disorder probably remained unrecognized in the late 1930's. In 1936, Fanconi (Switzerland) described the disease as *"muskoviszidose"* (German term that means "thickened mucus") and in 1938 Dorothy Anderson (US) gave the first clear pathophysiological description of CF and named it as "cystic fibrosis of the pancreas" (Anderson 1938).

Almost a decade later, while Anderson and Hodges deduced that CF should be an autosomal recessive disease (Anderson & Hodges, 1946), Sydney Faber observed that "a generalised state of thickened mucus" was the main cause of CF morbidity and mortality (Farber, 1943).

During the 1950's, Paul Di Sant'Agnese reported a major clinical finding: the identification of an increased salt content in the sweat of CF patients; which allowed the establishment of the sweat chloride test as a diagnostic tool that is still used today (Di Sant'Agnese et al. 1953).

During the 1980's, Quinton and Bijman developed work focused on the ducts of sweat glands that explained the Cl^- impermeability in these epithelial cells (Quinton & Bijman, 1983). It was also shown that the basic electrolytic defect was localised in the apical membrane of CF epithelial cells with diminished chloride (Cl^-) and increased sodium (Na^+) transport in CF airways (Knowles et al. 1981, 1986; Boucher et al. 1986; Frizzell et al. 1986). Finally in 1989 by the joint efforts of three laboratories, the gene was cloned and the protein encoded named as Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989), later shown to function as a Cl^- channel (Bear et al., 1992; Welsh & Smith, 1993).

The high frequency of CF and F508del mutation (responsible for 70% of CF chromosomes worldwide) in the Caucasian population suggests that mutant CF alleles confer a selective advantage. There is a hypothesis that

F508del heterozygotes may be protected against CFTR-mediated secretory diarrhoea induced by the cholera toxin, typhoid fever or other enterotoxins, which is supported by experimental results (Gabriel et al. 1994; Pier et al., 1998).

1.2 Clinical Phenotypes

The CF pathogenesis cascade starts by the presence of two defective *CFTR* genes previously associated with the CF phenotype (Figure I.1.1). At the cellular level, the CF phenotype most commonly identified is the lack or reduction of cAMP-mediated Cl^- secretion: the cells from CF patients do not have the ability to transport Cl^- to the same extent as normal cells (Amaral & Kunzelmann, 2007). In view of the emerging functions of CFTR, testing for the CF phenotype should also include testing of these new features.

CFTR is expressed at the apical membrane of epithelial cells to control salt and water transport (Rich et al., 1990). Due to the fact that water follows the direction of salt transport across the epithelia, the CF defect is also associated with enhanced water reabsorption, which according to some authors leads to increased salt concentration in the airway surface liquid (ASL) (Puchelle, et al. 2002).

Clinically, CF is characterized by multiple manifestations in different organs, but is dominated by the respiratory disease, the main cause of morbidity and mortality (Collins, 1992). Bacterial infections, especially by *Pseudomonas aeruginosa*, cause mucociliary clearance to be inefficient, originating thick mucus, and recurrent infections originate an exacerbate inflammatory response contributing to impairment of respiratory function and ultimately to death (Figure I.1.1) (Amaral & Kunzelmann, 2007).

Other CF symptoms include exocrine pancreatic insufficiency (PI, 85%), intestinal obstruction (meconium ileus-MI in 15-20% of CF newborns and/or distal intestinal obstruction syndrome-DIOS at a later age), elevated sweat electrolytes and male infertility (95%) (Collins, 1992; Jarvi et al., 1995; Welsh & Smith, 1995; Zielenski & Tsui, 1995). Other manifestations include hepatobiliary disease (7%) and diabetes mellitus (Welsh & Smith, 1995).

Despite the fact that CF is a monogenic disease, the genotype-phenotype correlation is very complex. Homozygosity for the common mutation F508del causes the classic phenotype: progressive obstructive lung disease, pancreatic insufficiency, male infertility and elevated sweat Cl^- concentrations. Each

organ affected in CF requires a different level of CFTR function. Decreasing levels of CFTR function are associated with progressive involvement of more organs or systems and with a more severe phenotype, in the following sequence of sensitivity: vas deferens > lungs > sweat duct > pancreas > intestine.

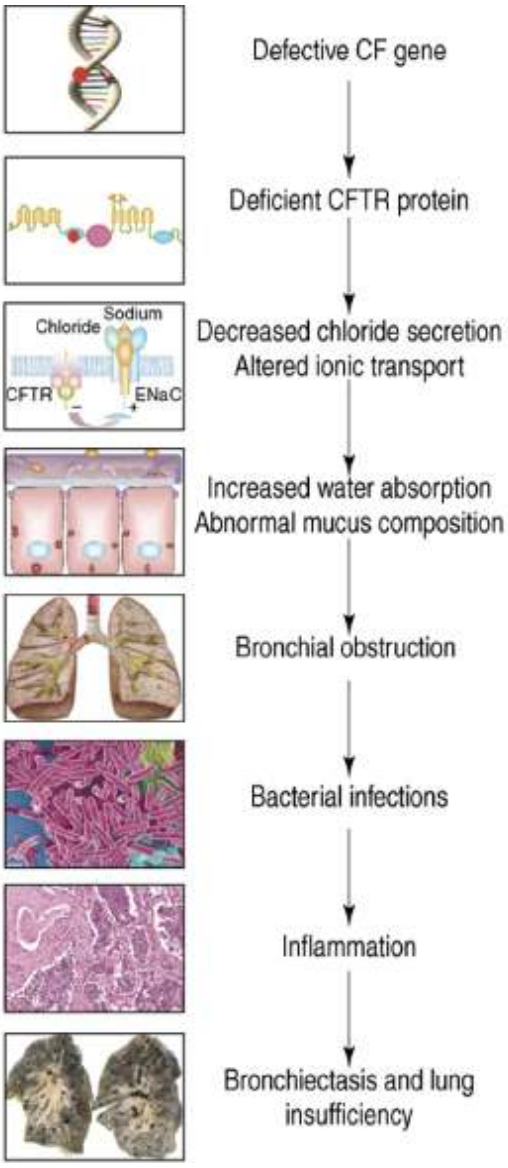


Figure I.1.1 – CF Pathogenesis Cascade: From primary gene defect to lung disease. Reproduced from Amaral and Kunzelmann 2007.

1.3 Diagnosis & Prognosis

For the vast majority of patients, the diagnosis of classic forms of CF is established early in life and suggested by one or more characteristic clinical features, a history of CF in a sibling or, more recently, by a positive newborn

screening result (Rosenstein & Cutting 1998; Farrell et al. 2008; Moskowitz et al. 2008) (Table I.1.1).

Table I.1.1 – Cystic Fibrosis diagnosis criteria established by presence of CF characteristic features and evidence of a CFTR abnormality (adapted from Moskowitz et al. 2008).

Diagnosis criteria for Cystic Fibrosis		
One or more characteristic phenotypic features	**and**	*Evidence of CFTR dysfucntion*
Phenotypic features:		**CFTR dysfunction:**
Chronic sinopulmonary disease		Presence of two *CFTR*-disease causing mutations
Obstructive azoospermia		
Gastrointestinal/nutritional abnormalities (MI, DIOS, PI with malabsorption, steatorrhea)		Two abnormal quantitative pilocarpine iontophoresis sweat-Cl^- values (> 60mmol/L)
Salt-loss syndrome (with or without metabolic alkalosis)		NPD measurements characteristic of CF
Failure to thrive		
Recurrent pancreatitis		
Hepatobiliary disease		
Hypoproteinemia		
Fat-soluble vitamin deficiencies		
A history of CF in siblings		
A positive newborn screening test		

Such diagnosis is usually supported by evidence of CFTR dysfunction through identification of two CF-disease causing mutations, two abnormal sweat-Cl^- tests (≥60mEq/L), and/or distinctive transepithelial nasal potential difference (NPD) measurements (Rosenstein & Cutting 1998; Farrell et al. 2008) (Table I.1.1).

However, depending on the ethnic background of the populations tested (Bobadilla et al. 2002), there is a portion of patients being diagnosed from infancy to adulthood that present "non-classical" symptoms, or a milder disease (Figure I.1.2), representing 2% to 10% (Boyle, 2003) of all CF diagnoses. Most non-classic CF patients meet the same diagnostic criteria as

classic CF patients; however they may have a later presentation or have involvement of only one or two organ systems (Boyle, 2003). The majority of patients with nonclassic CF carry one severe and one mild or two mild CFTR mutations, with mild mutations categorized as those allowing varying degrees of residual CFTR function (Groman et al. 2005; Noone & Knowles 2001).

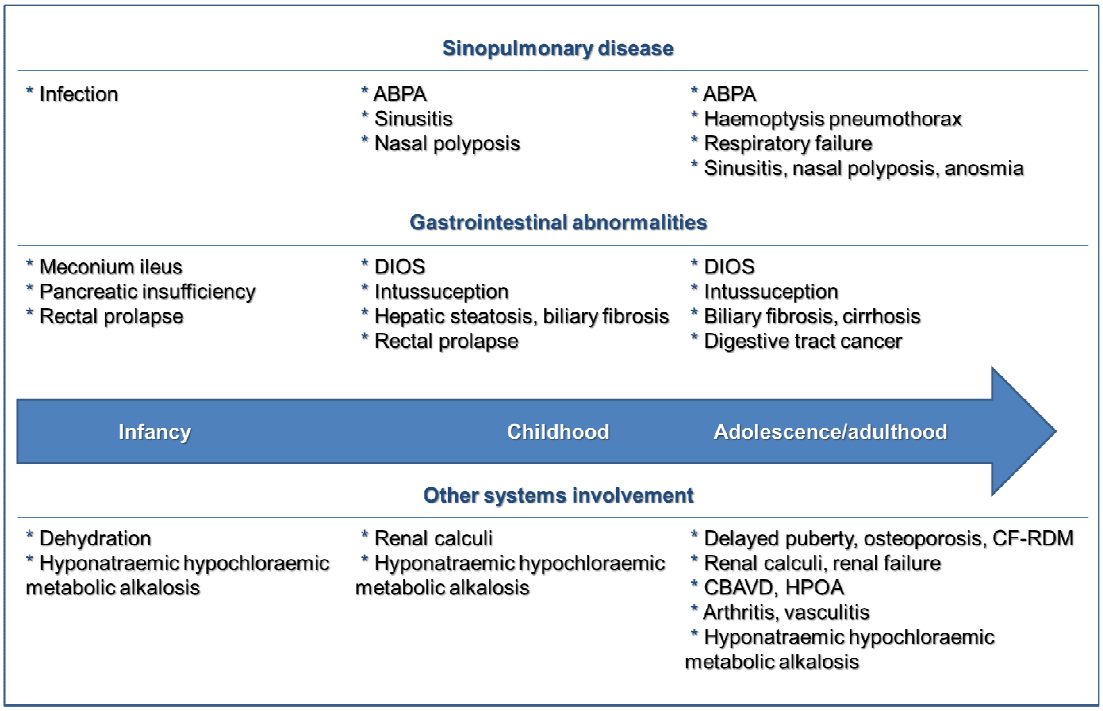


Figure I.1.2 - Approximate age of onset of clinical manifestations of cystic fibrosis. ABPA, allergic bronchopulmonary aspergillosis. CBAVD, congenital bilateral absence of the vas deferens. CF-RDM, cystic fibrosis-related diabetes mellitus. DIOS, distal intestinal obstruction syndrome. HPOA, hypertrophic pulmonary osteoarthritis. Adapted from O'Sullivan and Freedman 2009.

In addition, there are also some individuals showing conflicting results from the diagnostic tools available, such as inconclusive genetic testing, borderline sweat tests or NPD measurements with both non-CF and CF features (De Boeck et al. 2006; Boyle 2003). For such individuals with clinical phenotypes not fully meeting the CF diagnostic criteria it is also difficult to exclude CF. Some of these patients have described as having "CFTR-opathies" (Noone & Knowles 2001) or CFTR-related disorders (CFTR-RD) (Bombieri et al., 2011; Paranjape & Zeitlin, 2008).

Moreover, the recently implemented extensive newborn screening programs identify increasing numbers of asymptomatic CF patients merely identified by elevated serum concentrations of immunoreactive trypsinogen (IRT), posing new challenges to the CF diagnosis paradigm (Farrell et al. 2008;

Parad and Comeau 2005; Taylor et al. 2009), especially when associated with borderline sweat [Cl-] and/or inconclusive CFTR genotypes (Boyle 2003; Parad and Comeau 2005; Taylor et al. 2009; De Boeck et al. 2006).

As referred above, CF is associated with defective cAMP-mediated (CFTR) Cl^- secretion and an accelerated rate of basal Na^+ transport across epithelial membranes resulting in a change in transepithelial potential difference (PD) that can be measured in both airway and intestinal epithelia (Taylor et al. 2009).

Thus, besides NPD, investigators have also been using intestinal current or voltage measurements to demonstrate CFTR dysfunction and establish a diagnosis of CF in patients with atypical presentations (Taylor et al. 2009; de Jonge et al. 2004; Mall et al. 2004a; Hirtz et al. 2004). This subject will be further discussed below.

Despite impressive advances in our understanding of the molecular basis of CF, life expectancy and quality of life for CF patients are still limited (Rowe et al. 2005).

1.3.1 Sweat-test

Since Gibson and Cooke (1959), measurement of sweat electrolyte concentrations have been the gold standard test for CF diagnosis. In the normal sweat gland adrenergic stimulation induces an isosmolar flow of sweat into the sweat duct where activation of CFTR and Epithelium Na^+ Channel (ENaC) leads to a reabsorption of Na^+, Cl^- and water resulting in the flow of a small volume of sweat containing, on average, 16 mmol/l of Cl^- and 23 mmol/L of Na^+ (Di Sant'Agnese et al., 1953; Di Sant'Agnese & Powell, 1962). On the other hand, mean sweat Cl^- concentrations in CF patients with known genotypes ranged from 97.6–100 mmol/L (Farrell and Koscik 1996). It was then established that Cl^- concentrations above 60 mmol/L are abnormal and that values between 40mmol/L and 60mmolL are borderline, requiring further evidence of CFTR dysfunction (Taylor et al. 2009).

Although the ability to test for CFTR gene mutations gave a new dimension to diagnosing CF, the sweat Cl^- test remains the most accessible and reliable test for CF, if performed by qualified personnel and if samples are carefully handled to avoid contamination or evaporation. False positive sweat Cl^- results may be associated with other conditions, most notably mucopolysaccharidosis type 1 (Hurler syndrome) (Blackman et al., 2006).

False negative sweat chloride results may occur in the setting of acute CF-related salt losses (Bosco et al. 1999).

1.3.2 Genetic Testing

Genotyping usually starts with the search for F508del mutation – the most common mutation in the CFTR gene. In addition, if a family member has been previously diagnosed to have CF and the respective *CFTR* mutations identified, the child can also be screened for these specific mutations. If those mutations are not found or found in just one allele, genetic screening should be carried out for the most common CF mutations (mutation panels, Watson et al., 2004) in the patient's ethnic group (Girodon-Boulandet et al. 2000).

Nevertheless, establishment of a definite *CFTR* genotype can be difficult if it involves very rare *CFTR* mutations. For such cases, some laboratories already offer direct sequencing of the entire *CFTR* gene coding sequences, in spite of the costs involved. Notwithstanding, there is still the possibility of missing large gene deletions and mutations in *CFTR* introns, that are not sequenced because of their extensive length.

1.3.3 Nasal Potential Difference

Active transport of ions across the nasal epithelium results in a potential difference (PD), which can be measured by a voltmeter between two electrodes: 1) an exploring electrode placed on the surface of the nasal epithelium and 2) a reference electrode (generally subcutaneous), which represents the internal surface (Knowles et al. 1981).

Basal values for NPD in non-CF individuals are usually situated between -15 and -25 mV (Knowles et al. 1981). These negative values reflect transepithelial absorption of Na^+, which is the dominant ion movement in the airway.

NPD is often assessed using a perfusion protocol which allows examination of different components of airway ion transport. Thus, addition of amiloride will block ENaC mediated Na^+ absorption and result in a reduction in the magnitude of negative PD. Subsequent removal of Cl^- (low Cl^- solution) from the perfusate will provide a driving force for Cl^- secretion (mainly through CFTR) resulting in hyperpolarisation. Addition of a β-adrenergic agonist (such as isoprenaline), that raises intracellular cAMP, further increases CFTR

chloride secretion. Other stimulators, like ATP, can also be used to activate alternative Cl^- conductances (such as Calcium-Ca^{2+}-activated Cl^- channels-CaCC) (Boucher et al. 1991).

CF patients usually present an increased magnitude of baseline PD (reflecting Na^+ hyperabsorption), a dramatic response to amiloride, a transient or blunted response to low Cl^- solution and isoprenaline and an exaggerated response to ATP (reflecting Cl- secretion through CaCC) (Taylor et al. 2009).

1.3.4 Intestinal Current / Voltage Measurements

Bioelectrical measurements on rectal epithelia can be applied as a functional diagnostic tool to aid in establishing a diagnosis of CF, if sweat test results are equivocal and/or if CFTR-disease causing mutations are not readily identified by genetic testing. In the past 20 years, several research groups developed modified Ussing chambers to study the CF ion transport defect in freshly excised native tissues (Hardcastle et al. 1991; Veeze et al. 1991; Mall et al. 1998a; Taylor et al. 1987). CFTR is present in abundance in the intestinal epithelium where it is localised at the luminal membrane of the enterocytes (Crawford et al. 1991; Mendes et al. 2004a).

In the CF intestine, Cl^- secretion is impaired (Taylor et al. 1987; Taylor et al. 1988) while Na^+ and Na^+-linked nutrient absorption are enhanced (Mall et al. 1999). As the mechanism of Cl^- secretion is electrogenic, its activity can be monitored by measuring transintestinal electrical activity in response to secretagogues. Moreover, in the CF intestine, this activity is not subject to the confounding effects of inflammation, haemorrhage or infection, seen in the airway.

Measurements can be carried out on suction or forceps biopsy tissue obtained from either the small intestinal or colonic mucosa. Biopsy tissue is mounted in an Ussing chamber and the transepithelial electrical activity can be monitored by either short-circuit current measurements (Rotterdam protocol, de Jonge et al. 2004) or transepithelial voltage measurements, with further calculation of the equivalent short-circuit currents (Freiburg protocol, Mall et al. 2004a).

Studies from Mall and collaborators (Mall et al. 1998b; Mall et al. 2000) demonstrated that cAMP-mediated and Ca^{2+}-dependent agonists act co-operatively to determine the magnitude of colonic Cl^- secretion. Agonists increasing cytosolic cAMP induce large and sustained Cl^- secretory responses

in non-CF tissues, while cAMP-dependent Cl^- secretion is typically defective in CF tissues. Cholinergic co-activation increases the driving force for CFTR-mediated Cl^- secretion and may be of value in determining low levels of residual function of mutant CFTR (Mall et al. 1998a; Mall et al. 1998b; Mall et al. 2004a).

In CF rectal epithelia, the readouts from those approaches include enhanced amiloride-sensitive Na^+ transport, defective cAMP-dependent Cl^- secretion and defective cholinergic Cl^- secretion. This subject will be further discussed in *2.4.3 Gastrointestinal Tract* and in *Chapters 1 and 2* of the **III. Results & Discussion** section.

1.3.5 Neonatal Screening

Newborn screening using immunoreactive trypsinogen (IRT) assays performed on blood spots was long been suggested (Audrezet et al., 1993). In CF, the trypsinogen release into the circulation appears to be enhanced by abnormal pancreatic duct secretions. Thus, IRT levels found to be elevated in CF and determination of newborns IRT levels constitutes the CF neonatal screening (Audrezet et al., 1993). IRT values tend to remain raised for several months in newborns with CF, whereas in false positives they usually return to normal within the first weeks of life.

Abnormal IRT results are therefore evaluated through sweat testing and/or molecular genetic testing of CFTR and/or assessment of pancreatic function (Boyne et al. 2000; Castellani et al., 2009; Gregg et al., 1997; Massie et al., 2000).

This approach has demonstrated such long-term benefits as early nutritional treatment, reduced hospitalizations (due to pulmonary exacerbations) and improved survival (Grosse et al., 2004; Sontag et al. 2005).

1.4 CFTR-related Disorders

A CFTR-related Disorder (CFTR-RD) is defined as: "*a clinical entity associated with CFTR dysfunction that does not fulfil the diagnostic criteria for CF"* (Bombieri et al., 2011).

Three main clinical entities illustrate these phenotypes when associated with CFTR dysfunction: CBAVD (congenital bilateral absence of the vas deferens), acute recurrent or chronic pancreatitis and disseminated bronchiectasis. Careful attention should be paid to exclude other known aetiologies, to the degree of screening for CFTR mutations and to the evaluation of CFTR function in these patients (Bombieri et al. 2011; Noone & Knowles 2001; Paranjape & Zeitlin 2008).

2 Molecular Basis of Cystic Fibrosis

2.1 CFTR Gene

The CFTR gene or ABCC7 is located on the long (q) arm of chromosome 7, band 31 (7q31-7q32), covering 189 kb. It contains 27 exons that after splicing result in a 6.5 kb mRNA, translated into a protein with 1480 aa residues (J R Riordan et al., 1989) (Figure I.2.1).

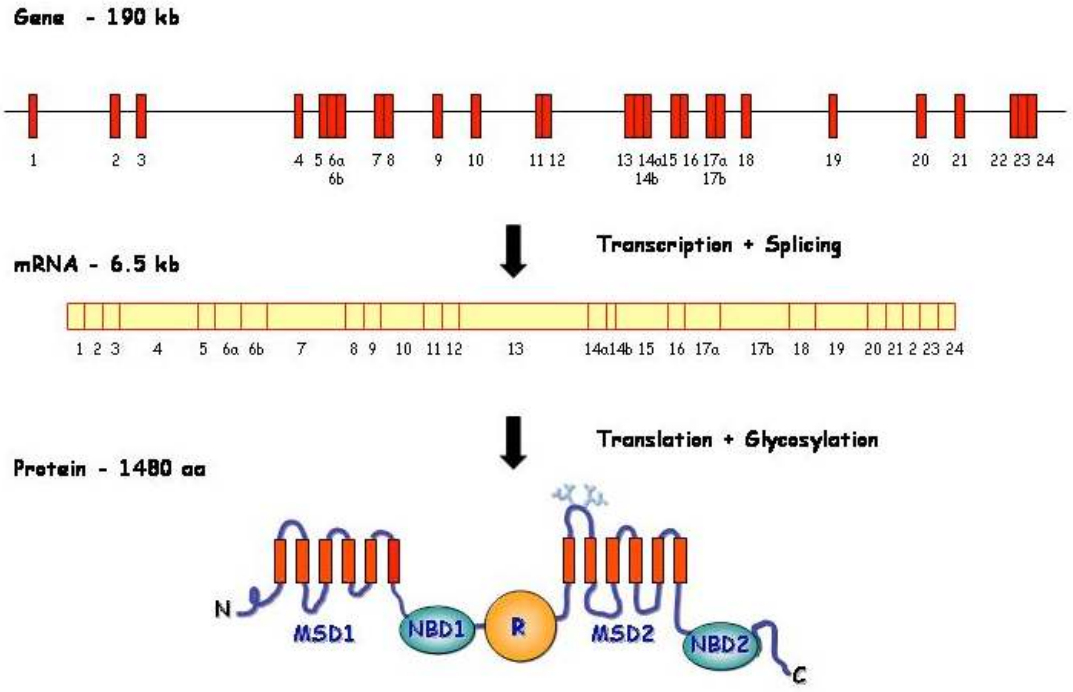


Figure I.2.1 – Scheme representing the *CFTR* gene, mRNA and protein. N – N-terminus; MSD – membrane-spanning domain; NBD – nucleotide-binding domain; R – regulatory domain; C – C-terminus (more details in section 2.2.1). Adapted from Zielenski and Tsui 1995.

2.1.1 Frequencies and Distribution of CFTR Mutations

CF birth prevalence varies from country to country, and also with ethnic background (O'Sullivan & Freedman, 2009). CF is most common in populations of northern European descent with a prevalence of 1 in 3,000 births; the same for white Americans. For Latin Americans and African Americans the disease presents lower birth prevalences: 1 in 4,000-10,000 and 1 in 15,000-20,000, respectively (O'Sullivan & Freedman, 2009). In the Brazilian population, ethnic background varies from north to south, as does

CF prevalence: from 1 in aproximately 10,000 in the north to 1 in 3,000 in the south (Raskin et al. 1999; 2007). For Africans and Asians the CF disease is very uncommon, with a reported frequency of 1 in 350 000 in Japan (Yamashiro et al., 1997); with 3120+1G>A being a very common mutation among African populations (Dork et al., 1998; Macek Jr. et al., 1997). In Europe the F508del mutation predominates in the northwest, and decreases in frequency towards the southeast; the most common mutation in Israel is W1282X (O'Sullivan & Freedman, 2009).

2.1.2 Functional Classes of CFTR Mutations

More than 1900 CF mutations have already been reported on the CFTR Mutation Database (http://www.genet.sickkids.on.ca/cftr/), thus making it difficult to ascertain how they affect processing and/or function of CFTR protein and how to repair each single defect.

Therefore, Tsui (Tsui, 1992), subsequently Welsh and Smith (Welsh & Smith, 1993) and later Lukacs (Haardt et al. 1999) proposed six mechanisms by which mutations disrupt CFTR Cl^- channel function (Figure I.2.2).

This classification is important for the development of therapeutic strategies, as mutations in the same class will likely need the same approach aimed at correcting their basic defects.

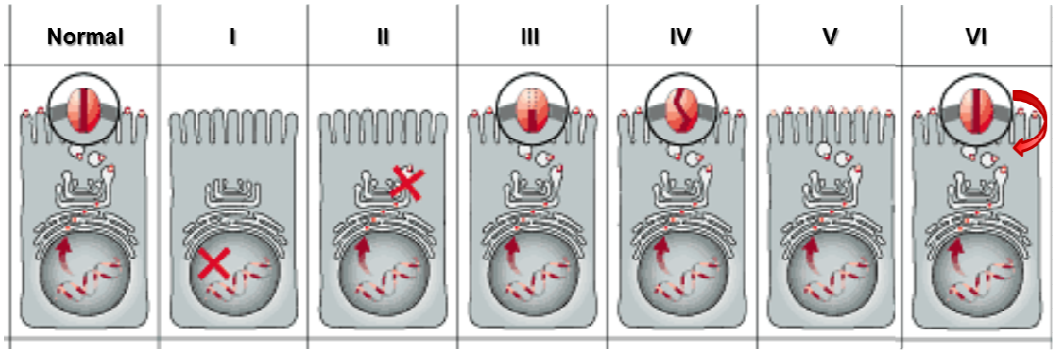


Figure I.2.2 – Classes of CFTR mutations. (Normal) CFTR protein in the plamas membrane of cells functioning as a Cl- channel; (I) class I mutation: prevent translation; (II) class II: defective processing; (III) class III: defective regulation; (IV) class IV: defective conductance; (V) class V: reduced synthesis; (VI) class VI: decreased stability. Adapted from Zielenski and Tsui 1995.

Class I mutations cause premature termination of CFTR mRNA translation in the nucleus, resulting in severely decreased or absent CFTR protein production. A number of class I mutants are due to nonsense,

frameshift or aberrant splicing. As examples of mutations belonging to this class we have G542X and W1282X.

Class II mutations lead to degradation of the protein within the endoplasmic reticulum (ER) and little or no functional protein is transferred to the cell membrane. Mutants in this class include F508del and A561E. The disease severity in this class correlates with the amount of correctly processed protein that is able to reach the apical membrane.

Class III mutants, such as G551D or S549R, are located correctly at the cell membrane but are unable to function as a cAMP-activated Cl^- channel.

Class IV mutants are also properly located but have altered conductance or gating properties causing a reduced Cl^- efflux rate through the CFTR channel, like R334W or P205S, associated with a milder CF disease.

Class V mutants cause a reduction in the levels of functional CFTR often due to alternative splicing or recycling. In this case, disease severity will also correlate with the levels of normal transcripts – as an example we have 3272-26A>G and G576A, associated with milder CF phenotypes.

Class VI mutants lead to reduced stability of CFTR at the cell surface, caused by C-terminal truncations. Whereas the biosynthesis, processing, and macroscopic chloride channel function of truncated CFTR protein are essentially normal, the degradation rate of the mature, complex-glycosylated form is 5-6-fold faster than the wild-type (wt) CFTR. C-terminal truncations, like 4326delTC, are thus associated with severe CF phenotypes.

Interestingly, little correlation between mutation classes and severity of airway disease has been described. This indicates that the progression of CF lung disease may be significantly influenced by environmental factors, modifier genes or immunological events that are not influenced directly by the specific CFTR genotype. In patients who are compound heterozygotes for CF, the "milder" mutation acts as dominant; this resembles the no-disease condition of carriers of a CF-disease causing mutation.

2.2 CFTR Protein

2.2.1 Structure

As mentioned above, CFTR is a multidomain protein containing 1480 aa residues and functioning as a cAMP-activated and phosphorylation-regulated chloride (Cl^-) channel at the apical membrane of epithelial cells. CFTR is a

member of the ATP-binding cassette (ABC) transporter superfamily, being also named ABCC7. Members of the ABC transporter superfamily, one of the largest in the human genome, are involved in active transport of various metabolites, peptides or ions across cell membranes of Prokaryotes and Eukaryotes. The mechanism of transport is usually ATP driven and each ABC transporter is usually specific for a given substrate, such as aminoacids, proteins, sugars, lipids and drugs (Higgins, 1992; Schneider & Hunke, 1998).

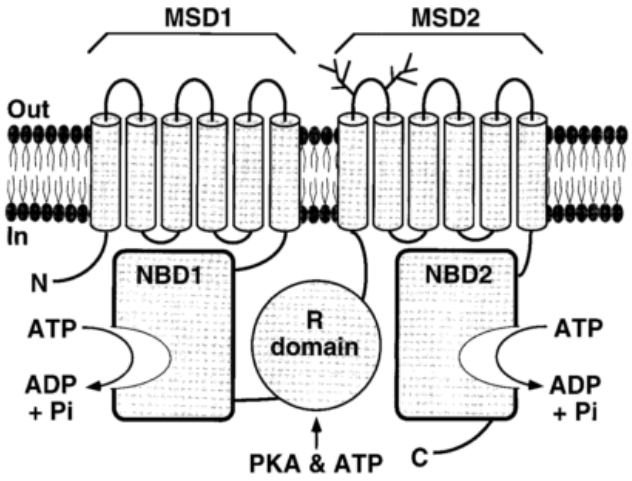


Figure I.2.3 – Model for CFTR domain structure at the plasma membrane. MSD, membrane-spanning domain; NDB, nucleotide-binding domais; R, regulatory domain; PKA, cAMP-dependent protein kinase A. Reproduced from Sheppard and Welsh 1999.

As shown in Figure I.2.3, CFTR structure resembles that of a typical ABC transporter with two membrane-spanning domains (MSDs), composed of six spanning α-helices, and two nucleotide-binding domains (NBD1 and NBD2), which bind and hydrolyse ATP. Uniquely, CFTR also contains a large regulatory domain (RD) where multiple phosphorylation occurs (Riordan et al., 1989; Sheppard & Welsh, 1999).

2.2.2 Biosynthesis, Degradation and Localization

Like most membrane proteins entering the secretory pathway, CFTR assembly begins with synthesis and folding in the ER where it is core-glycosylated (Cheng et al., 1990). This CFTR immature form, termed band B on Western blots, has a molecular mass of about 140 kDa (figure I.2.4). Once checked for its correct folding, the core-glycosylated form of wt-CFTR migrates to the Golgi complex where it undergoes further glycosylation and gradually reaches its mature form, which on Western blots is known as band C (170-180 kDa, figure I.2.4) (Cheng et al., 1990).

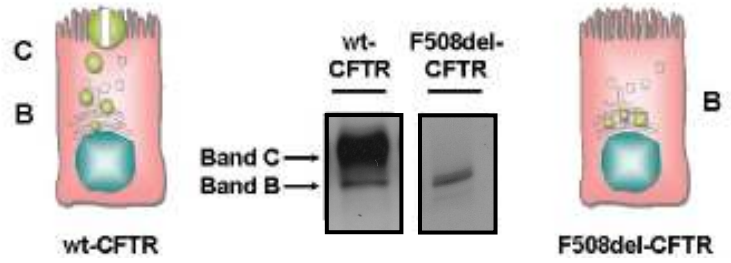


Figure I.2.4 – Western blot CFBE41o- cells expressing wt- and F508del-CFTR. Cartoons with permission, Amaral M.D., unpublished; own blot images.

On the other hand, the misfolded mutant protein, F508del-CFTR, is almost completely retained at the ER (Cheng et al., 1990) from where it is degraded via the ubiquitin-proteasome pathway (figure I.2.5) (Jensen et al. 1995; Ward et al. 1995). The assembly and co-translational insertion of the nascent polypeptide chain into the ER-membrane is mediated by specialized cellular machinery that assists folding and prevents aggregation of folding intermediates (Skach 2000). CFTR protein thus passes through several checkpoints taking place by ER quality control (ERQC) mechanisms that are capable of discriminating normally folded from abnormally folded proteins, and ensuring that only correctly folded proteins exit the ER and undergo Golgi maturation (Farinha & Amaral 2005).

Further progress of CFTR along the secretory pathway is accomplished by its incorporation into coated protein II (COPII) vesicles and transport to the Golgi, for which ER retention motifs of the protein, namely the arginine-framed tripeptide, need to be masked (Wang et al. 2004; Roxo-Rosa et al. 2006). Transport of CFTR protein from the trans-Golgi to the plasma membrane can occur indirectly via transcytosis from the basolateral membrane from recycling

endosomes, or directly to the apical membrane by Golgi-derived vesicles undergoing anterograde traffic (figure I.2.5) (Kleizen et al. 2000).

When CFTR finally reaches the plasma membrane, it can be recycled and removed from the cell surface by clathrin-mediated endocytosis through trafficking signals embedded in its primary sequence. Furthermore, the protein can be recycled back to the plasma membrane directly, or through recycling endosomes (figure I.2.5) (Ameen et al. 2007; Riordan 1999).

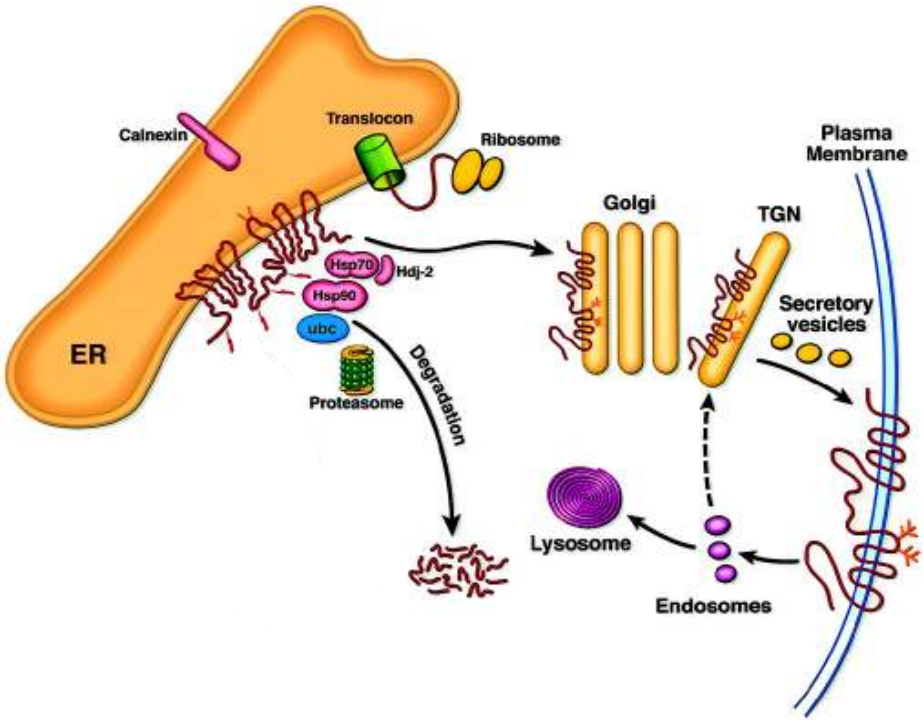


Figure I.2.5 - Biosynthesis and degradation of CFTR. As the primary structure of CFTR is formed, the polypeptide is incorporated into the ER membrane. Core oligosaccharide chains are attached, to which calnexin binds. In addition, the cytosolic chaperones Hsp70, Hdj-2, and Hsp90 bind, and ubiquitination may occur. The export from the ER is accompanied by dissociation of calnexin and cytosolic chaperones. Fully folded CFTR is protected from degradation, but misfolded proteins are substrates of ubiquitin-conjugating enzymes (ubc) and are degraded by the proteasome. The export competent population travels from the ER to the Golgi apparatus, where complex oligosaccharide chains are completed. Vesicles then carry mature CFTR from the trans-Golgi network (TGN) to the membrane. Endocytic recycling of this population and degradation of internalized protein by lysosomal proteases accounts for the turnover of surface CFTR (adapted from Riordan, 1999).

CFTR localization to the apical membrane is also dependent on interactions with PDZ domains containing proteins (Short et al., 1998; Wang et al. 1998). PDZ binding proteins can inhibit CFTR endocytosis from the plasma membrane, as well as facilitate recycling of internalised CFTR from early endosomes (Ameen et al. 2007). The amount of CFTR protein at the cell

surface is thus finely regulated and the damaged protein is targeted for lysosomal degradation (figure 2.5) (Ameen et al. 2007; Riordan 1999).

Different types of approaches have been utilized to determine the subcellular localization of CFTR. These include immunocytochemistry, immunoelectron microscopy, subcellular fractionation followed by functional assays or immunoblot and finally, recombinant strategies involving tagged CFTR. The studies of CFTR localization have been performed both in unpolarized and polarized cultured cells and tissue sections. However, due to the low level of CFTR endogenous expression, the obtention of a detectable CFTR signal often requires the overexpression in heterologous systems (for review see Bradbury 1999; Bertrand & Frizzell 2003). Nevertheless, contradictory findings in CFTR localization between endogenously expressing and overexpressing cells highlight the importance of studying CFTR in epithelial cells and tissues that natively express the protein (further discussed under the **III. Results & Discussion** section).

2.3 CFTR Function as a Chloride Channel

Proteins belonging to the family of ABC transporters are responsible for active transport of substrates across cell membranes, where ATP hydrolysis serves as the source of energy to drive the transport. However, CFTR is functionally distinct from other ABC transporters as it enables bidirectional permeation of anions, rather than vectorial transport of solutes (Riordan, 2005).

Several hypotheses were raised regarding the function of CFTR protein. The first postulated that CFTR was an ATP-driven transporter, due to its overall resemblance to many transporter proteins. A second hypothesis held that CFTR functioned as a regulator of a separate channel molecule, and finally a third hypothesis proposed that CFTR was itself a regulated Cl^- channel. Ultimately, compelling proof that CFTR exhibited regulated Cl^- channel activity came with the reconstitution of CFTR in a planar lipid bilayer (Bear et al., 1992), as referred to above in *1.1. Historic Introduction*.

In the model currently accepted for the mechanism of channel function and regulation (figure I.2.6), phosphorylation of the RD by cAMP-dependent protein kinase A (PKA, and also PKC), possibly allows ATP binding to the NBDs, which then dimerize (Hwang & Sheppard, 2009; Sheppard & Welsh, 1999). Consequently, the MSDs will alter their conformation, allowing the opening of

the channel pore and passive flow of ions. It has also been shown that intramolecular interaction between the R domain (RD) and either the N-terminal or NBD1 are required to regulate CFTR function (Baker et al., 2007).

Although the mechanism of the CFTR channel gating is not fully understood, opening and closing of this Cl^- channel is tightly regulated by the cellular balance of kinase and phosphatase activity and by ATP levels. Furthermore, the open probability (Po) of the channel is controlled by the extent of RD phosphorylation at multiple sites (Hwang & Sheppard, 2009).

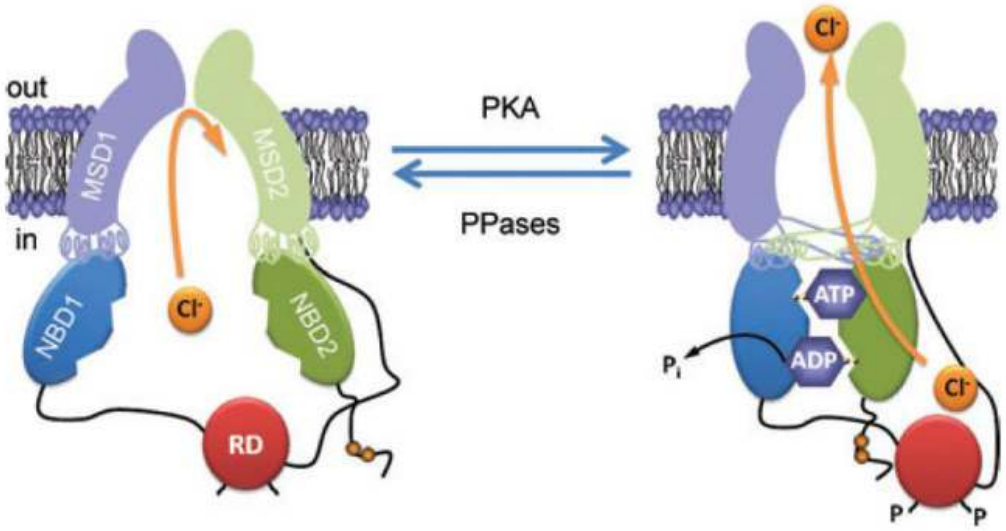


Figure I.2.6 – Conformational changes of the CFTR Cl^- channel during channel gating. The simplified model shows a CFTR Cl^- channel under quiescent (left) and activated (right) conditions. Communication between the NBDs and MSDs via the intracellular loops is either or orthogonal (e.g. NBD1–MSD2) according to the most recent CFTR structural models based on Sav168834.
P (phosphorylation of the RD); Pi (inorganic phosphate); PKA (cAMP-dependent protein kinase); PPase (protein phosphatase). In and Out denote the intra- and extracellular sides of the membrane, respectively. Reproduced from Hwang and Sheppard 2009.

The function and cooperation of the various domains of CFTR confer to the channel several distinguishing characteristics: i) a small single-channel conductance (6-10 pS) (Welsh et al., 1992); ii) a linear current-voltage (I-V) relationship; iii) selectivity for anions over cations; iv) anion permeability sequence of $Br^- > Cl^- > I^-$; v) time- and voltage-independent gating behaviour; vi) activity regulated by cAMP-dependent phosphorylation and by intracellular nucleotides (Sheppard & Welsh, 1999).

2.4 CFTR as Regulator of Epithelial Ion Transport

Besides the function of CFTR as Cl^- channel, CFTR has also been shown to regulate several channels and transporters, thus regulating ion transport in many epithelia. CFTR is found to be expressed in luminal membranes of both secretory and absorptive epithelia, playing a predominant role in both cAMP- and Ca^{2+}-activated secretion of electrolytes (see figure I.2.7).

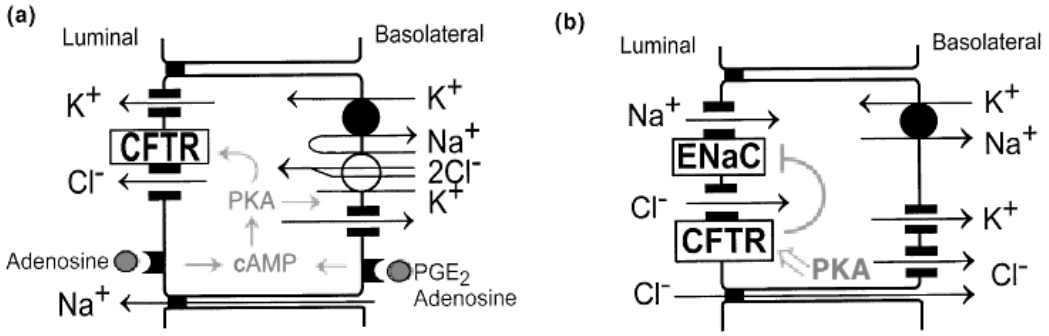


Figure I.2.7 - Cell models of the mechanism of electrolyte secretion and electrolyte absorption in the airway and intestinal epithelia. (a) In secretory cells, Cl^- is taken up from the basolateral (blood) side by the Na^+-K^+-$2Cl^-$ (NKCC1) cotransporter. K^+ is recycled via basolateral K^+ channels, and Na^+ is pumped out of the cell by the Na^+-K^+-ATPase. Cl^- leaves the cell via luminal (apical) CFTR Cl^- channels, and Na^+ is secreted via the paracellular shunt following the electrical driving force generated by the lumen negative transepithelial voltage. K^+ is also secreted to the luminal side via luminal K^+ channels. Depending on the tissue, intracellular cAMP is increased and secretion is activated by adenosine (airways) or prostaglandin E_2 (PGE_2, intestine). **(b)** In absorptive epithelial cells, Na^+ is taken up by ENaC. Cl^- is transported via the basolateral shunt and probably via CFTR Cl^- channels. Na^+ is pumped out of the cell by the basolateral Na^+-K^+-ATPase, whereas Cl^- and K^+ leave the cell via Cl^- and K^+ channels, respectively. In cells that coexpress CFTR and ENaC, CFTR stimulation and/or expression leads to inhibition of ENaC. Thus, cAMP-mediated stimulation may shift the epithelium from absorption towards secretion of NaCl. Reproduced from Kunzelmann and Mall 2001.

In secretory epithelia, Cl^- is absorbed on the basolateral side by the Na^+-K^+-$2Cl^-$ (NKCC1) co-transporter, accumulating Cl^- inside the cell in preparation for secretion through CFTR when it receives the appropriate stimulus at the apical membrane (figure I.2.7a). Apart from luminal Cl^- secretion, CFTR also regulates reabsorption of electrolytes by controlling the activity of the amiloride-sensitive epithelial Na^+ channel (ENaC; figure I.2.7b). When activated via the PKA-dependent pathway, it is believed that CFTR inhibits ENaC, thus reducing Na^+ absorption (Kunzelmann et al. 2001). In CF epithelia, both the secretion and absorption of electrolytes are found to be impaired.

2.4.1 Sweat Gland

In absorptive epithelia, like sweat glands, the transport direction is reverted, i.e. both Cl^- and Na^+ are absorbed by the apical surface of epithelia (see above Figure I.2.7b), building up inside the epithelial cell to be secreted basolaterally upon appropriate stimuli. Here, CFTR is actually required for upregulation of ENaC and cAMP-dependent activation of Na^+ absorption, and thus causing a decreased Na^+ conductance in CF sweat ducts (Reddy et al. 1999).

2.4.2 Airways

As referred to above, CFTR can function either as an absorptive or a secretory pathway for Cl^- ions in both respiratory and intestinal epithelia.

The airway surface liquid (ASL) protecting the epithelium lining the airways is generally described as a biphasic layer composed of a periciliary layer (PCL) where the cilia beat, and a more superficial gel layer that constitutes an efficient barrier against micro-organisms (mucus). The regulation of ASL in the respiratory epithelia is ensured by both electrolyte absorption, occurring at the surface epithelium, and secretion, that takes place in the submucosal glands (see figure I.2.8) (Kunzelmann & Mall 2001).

Cl^- secretion by the submucosal glands creates a lumen-negative transepithelial voltage, forcing Na^+ and water to exit towards the airway lumen, ensuring its proper hydration. The accompanying absorptive movement of Cl^- and water occurs both through cellular and paracellular pathways (figure I.2.8).

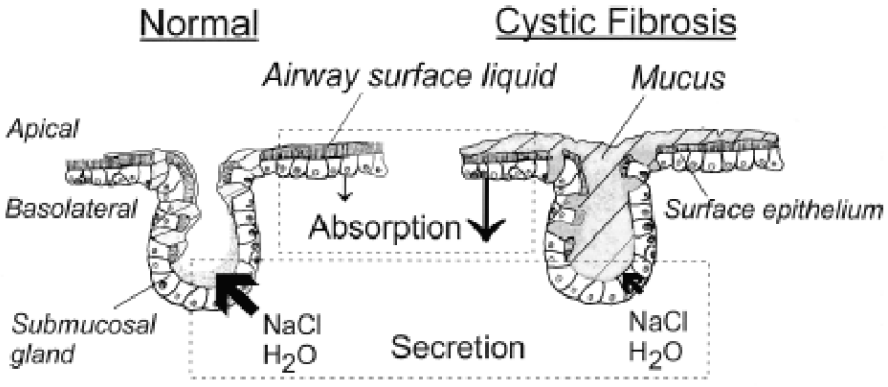


Figure I.2.8 – Model of airway epithelium, consisting of an absorptive surface epithelium and secretory in the submucosal glands. Normal (left panel) and cystic fibrosis (right panel) epithelium models are shown. Adapted from (Kunzelmann and Mall 2001).

In this regard, different authors have proposed two competing theories to explain the abnormalities in CF airway epithelial functions, namely: the "High Salt" model, where dysfunction of CFTR leads to diminished absorption of counter-ions, favouring the accumulation of salt in the ASL; and the "Low Volume" model, where dysfunction of CFTR leads to hyperabsorption of mainly Na^+, decreasing osmotic pressure and consequent deydration of ASL (Rowe et al. 2005).

Nevertheless, both secretory and absorptive processes are defective in CF respiratory tissues, since net absorption of electrolytes is enhanced in the CF surface epithelium and secretion is inhibited, further dehydrating the epithelia (Kunzelmann & Mall 2001).

Glutathione Transporter

CFTR has also been implicated in the control of oxidative stress in the airways. The peptide glutathione (GSH) is the main antioxidant secreted in response to inflammation in the lung (Kogan et al., 2003), and it was found to be reduced in ASL of CF patients (Roum et al. 1993). It was then shown that CFTR is capable of mediating transport of GSH in its reduced form, possibly justifying the augmented basal state of inflammation found in CF lungs (Kogan et al., 2003; Linsdell & Hanrahan, 1998). Recently CFTR was also implicated in the control of the intracellular reactive oxidative species balance, possibly also related to some CFTR-dependent modulation of GSH concentration (l' Hoste et al., 2010).

2.4.3 Gastrointestinal Tract

As outlined before, the colonic epithelium has both absorptive and secretory functions. Epithelial transport in the colon is characterized by a net absorption of NaCl, short-chain fatty acids (SCFA), and water, allowing very little water and salt content in faeces. In addition, colonic cells also secret mucus, bicarbonate, and KCl. CFTR may have a dual function in the mammalian colon (Kunzelmann & Mall 2002).

It has been shown that both secretory and absorptive functions are present in both surface epithelium and crypts of Lieberkühn (Figure I.2.9, Köckerling & Fromm 1993), and that colonocytes thus have the ability to

switch from absorption to secretion when stimulated by secretagogues (Kunzelmann & Mall 2002).

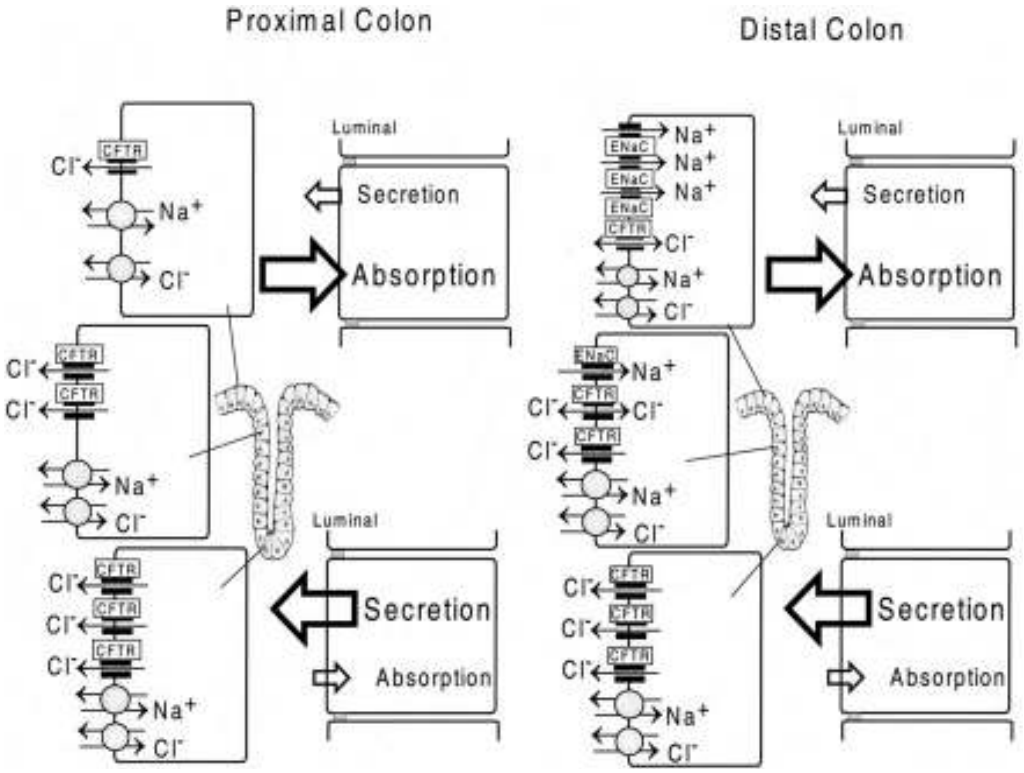


Figure I.2.9 - Models for electrolyte transport in proximal and distal colonic epithelium and expression of different ion transporters along the crypt axis. Electroneutral NaCl absorption (parallel Na^+/H^+ and Cl^-/HCO_3^- exchange) dominates in the surface epithelium and is also present in the crypts. Electrogenic Na^+ absorption through ENaC takes place in the surface epithelium and upper crypts of the distal colon. CFTR is expressed throughout the colonic epithelium and dominates in the crypts. Reproduced from Kunzelmann and Mall 2002.

CFTR seems to be essential for Cl^- secretion in the lower crypts, while in the upper crypts, and particularly at the surface epithelium, it may regulate other transport proteins such as ENaC and Na^+/H^+ exchanger NHE3, playing a role in absorption (see figure I.2.9). Absorption can be electrogenic via the ENaC or electroneutral via parallel Na^+/H^+ and Cl^-/HCO_3^- exchangers. Electrogenic absorption via luminal ENaC is confined to the surface epithelium of the distal colon (figure I.2.9) (Kunzelmann & Mall 2002).

As outlined above under *1.3.4 Intestinal Current / Voltage* Measurements, studies by Mall and colleagues (Mall et al. 1998a; Mall et al. 1998b) evidenced a clear correlation between expression of wt-CFTR and stimulation of colonic Cl^- secretion by increasing both intracellular cAMP and Ca^{2+}. In these Ussing chamber studies, activation of a Cl^- secretion, demonstrated by a negative

voltage deflection, was detected in the non-CF rectal epithelia. On the other hand, tissues from patients homozygous for F508del-CFTR demonstrated only a K^+ secretory response, as indicated by a positive voltage deflection (Figure I.2.10). Patients, however, carrying a non-severe mutation, like S108F, showed both K^+ secretion and a residual Cl^- secretion. (Figure I.2.10, right trace).

Impaired cAMP-regulated Cl^- secretion and excessive absorption (water and Na^+) leads to intestinal dehydration and obstruction presenting with meconium ileus at birth, and DIOS and chronic constipation with rectal prolapse in adult CF patients (Eggermont, 1996). Altered ion transport properties in jejunal and rectal biopsies of CF patients have been proposed to assist in the diagnosis of CF, which will be further discussed in *Chapters 1 and 2* of the **III. Results & Discussion** section.

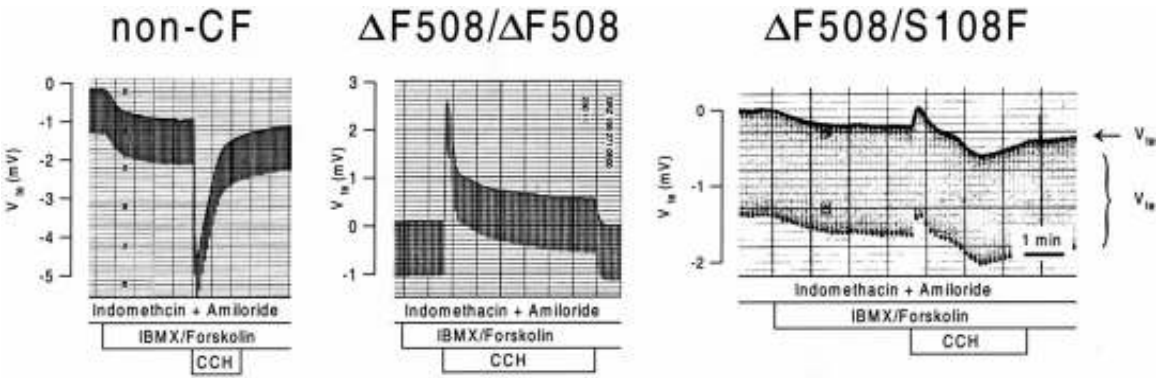


Figure I.2.10 - Assessment of CFTR function and diagnosis of CF in Ussing chamber recordings of the transepithelial voltage (V_{te}) and resistance (R_{te}) in human rectal biopsies. In these experiments, indomethacin and amiloride have been included to block generation of the main endogenous secretagogue PGE2 and to abolish the influence of Na^+ absorption. In non-CF subjects, increases in intracellular cAMP by 3-isobutyl-1-methylxanthine (IBMX) and forskolin induce a sustained lumen-negative response caused by CFTR-mediated Cl^- secretion. Stimulation with carbachol (CCH) acts cooperatively and increases Cl^- secretion even further. In CF rectal biopsies from patients homozygous for ΔF508-CFTR, cAMP-dependent Cl^- secretion is defective and CCH induces an inverse lumen-positive response, corresponding to K^+ secretion. In rectal biopsies from a CF patient compound heterozygous for the CFTR mutations ΔF508 and S108F, cAMP-dependent stimulation induces an attenuated Cl^- secretory response, which is further increased during the plateau phase of the biphasic CCH response. This indicates residual CFTR function of the mutant S108F in native tissue.

Bicarbonate Transport

Besides transporting Cl^-, CFTR has also been implicated in a number of other functions, namely in HCO_3^- transport in the lungs, gastrointestinal tract and pancreas (Hug et al. 2003; Park et al. 2010; Clarke et al. 2001). Although

CFTR functions primarily as a Cl^- ion channel and exhibits a low permeability to HCO_3^- ions (Poulsen et al. 1994), this permeation has important implications in CF pathophysiology. HCO_3^- is involved in several cellular functions, including acting as a pH buffer and thus enhancing the solubility of many proteins.

The importance of HCO_3^- transport by CFTR was further evidenced by the correlation of HCO_3^- transport and pancreatic function (Lee et al., 1999). Indeed, CFTR mutants that do not support HCO_3^- transport, but show normal or only slightly reduced Cl- permeability, are associated with pancreatic insufficiency (Choi et al., 2001).

It was also hypothesized that CFTR permeability to HCO_3^- can affect the pH of luminal electrolyte and consequently affect mucus swelling and hydration during its exocytosis, like for instance in the respiratory epithelia. Additionally, it has been suggested that mucins show a tendency to remain compact due to lowered pH in the extracellular fluid matrix in CF tissues (Kreda et al., 2007), thereby augmenting the viscosity of the ASL.

2.5 Other CFTR Functions

In addition to its function as a Cl^- channel, CFTR has been implicated in the regulation of a number of other ion channels and of other molecular entities. In this regard, a role for CFTR as an "anchoring platform" at the cell membrane has also been proposed. Specialized "microdomains" anchored to CFTR may group together a number of proteins that are dynamically regulated by molecular switches, e.g. PDZ-domain proteins (Guggino & Stanton, 2006). These include signalling molecules, kinases, transport proteins, PDZ-domain-containing proteins, myosin motors, Rab GTPases and SNAREs.

Several reports show that a large dynamic signalling complex consisting of PDZ domain proteins and kinases is involved in the interaction between these channels. In this context, the Na+/H+ exchanger regulatory factor isoform-1 (NHERF1) is suggested to mediate the interaction between CFTR and ENaC indirectly, and even facilitate the reciprocal regulation of these channels (CFTR downregulates ENaC, whereas ENaC activates CFTR). Besides this mechanism, other authors showed that CFTR regulates ENaC through Cl^- secretion and water balance across the epithelia (König et al., 2001), while some indicate that lack of CFTR leads to ENaC hyperactivity by a direct

(Suaud et al., 2007) or indirect (Guggino & Stanton, 2006) interaction between the two channels.

In addition to ENaC, CFTR has also been correlated to the regulation of other Cl^- channels such as the outwardly rectifying Cl^- channels (ORCCs, defective in CF), renal outer medullary potassium K^+ channels (ROMK, important for K^+ homeostasis in kidney) and CaCCs (suggested to be upregulated in CF) (McNicholas et al. 1996; Schwiebert et al. 1998; Tarran et al. 2002). Finally, CFTR was also found to regulate the Solute carrier 26 family (SLC26), shown to be important in HCO_3^- transport (Ko et al., 2002).

3 Cystic Fibrosis Therapies

Up to very recently, conventional therapy for CF has focus on ameliorating the symptoms of the disease. But over the last years, research has been dedicated not only to understanding the effects of the various mutations reported but also to correcting the basic defect itself.

3.1 The "Bypass" Approach

As outlined above, as CFTR functions not only as Cl^- channel, but also as a regulator of other membrane proteins, it is necessary to devise strategies to modulate these other membrane transporters. It seems that the presence of CFTR at the cellular membrane (even the mutant protein) is enough for modulation of other proteins (Suaud et al., 2007).

Pharmacological approaches aiming to correct several dysfunctions caused by the absence of CFTR have been described (Amaral & Kunzelmann, 2007), examples include dysfunctions such as:

Firstly, absence of CFTR at the apical membrane leads to enhanced Na^+ conductance in surface airway epithelial cells leading to excessive absortion of electrolytes (Donaldson & Boucher, 2003). The responsible Na^+ channel ENaC can be blocked by specific inhibitors such as amiloride, benzamil or phenamil and probably by activation of PKC. Also, activation of purinergic receptors by ATP or UTP or denufosol inhibits ENaC (figure I.3.1A).

Secondly, stimulation of an alternative Cl^- channel, CaCCs, in CF airway epithelial cells by stimulation of luminal $P2Y_2$ purinergic receptors with ATP or UTP, has been demonstrated to restore Cl^- secretion (figure I.3.1B; Knowles et al. 1991).

Thirdly, increasing of electrical driving of luminal Cl^- secretion by stimulation of the basolateral Ca^{2+}-activated K^+ channel SK4 by the benzimidazol compound 1-EBIO (Roth et al., 2011) or activation of cAMP-regulated K^+ channels (KvLQT1) by agonists of the cAMP pathway, such as β-adrenergic compounds or blockers of phosphodiesterase (PDE) like amrinone or milrinone (figure I.3.1C; Kunzelmann & Mall 2001).

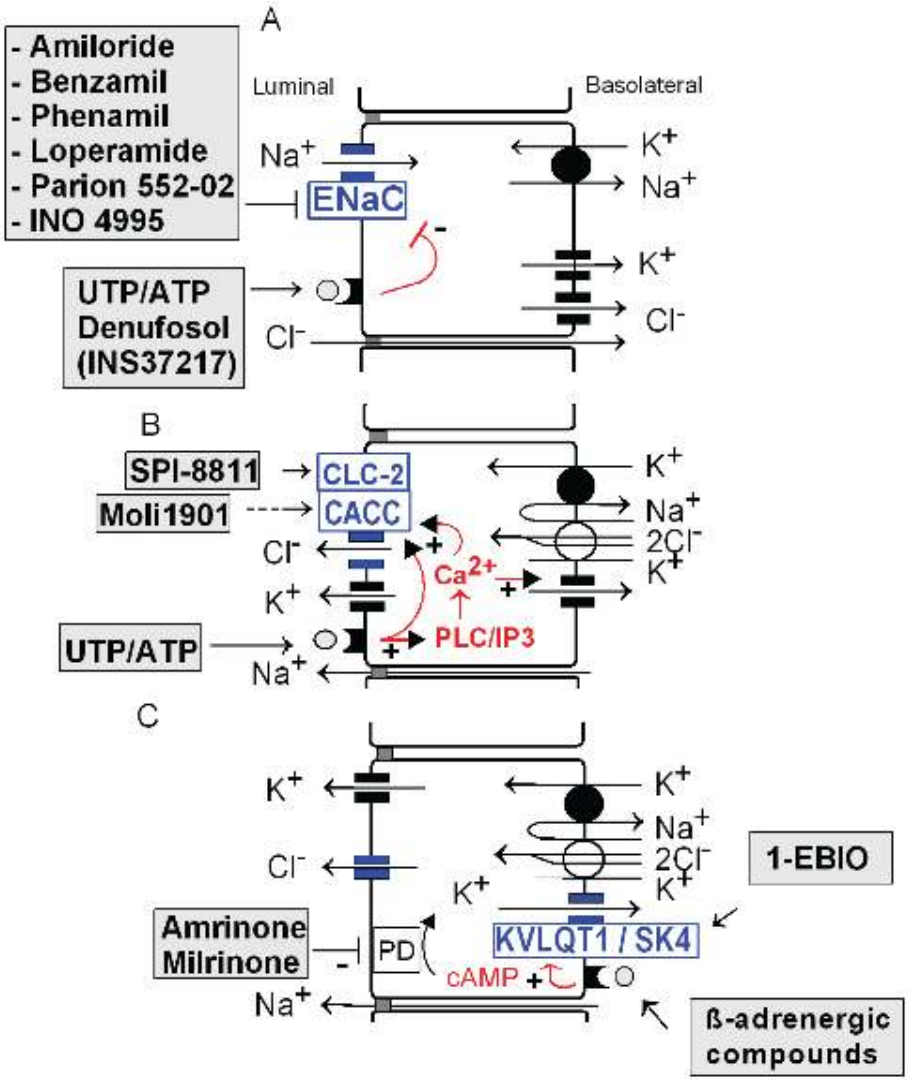


Figure III.3.1 – Pharmacological compounds used for the therapeutic strategies aimed at circumventing the ion channel defect in CF. Reproduced from Amaral and Kunzelmann 2007 (for details see text above).

3.2 Mutation-Specific Approaches

Chemical and pharmacological approaches to rescue CFTR dysfunction can be divided into two groups. Firstly, molecules that help mutant-CFTR to fold by generally favouring the cellular milieu, possibly through modulation of interacting proteins, such as chaperones; and secondly, specific molecules that rescue folding of CFTR mutants, putatively allowing them to function correctly at the plasma membrane. Due to their non-specificity, the former are called chemical chaperones, while the latter are termed pharmacological chaperones (or pharmaco-chaperones). Ideally such a pharmacological

chaperone would be able to act as a CFTR corrector (i.e. promoting mislocalised protein to the plasma membrane), and also act as a CFTR potentiator (i.e. increasing CFTR Cl^- channel function upon stimulation). Pharmacological rescue relies mostly on mutation-specific therapies (Brennan & Geddes, 2004) and can be divided according to the class of CFTR mutation targeted (Lim & Zeitlin, 2001).

Correction of class I mutations

These mutations produced mRNA transcripts with premature termination codons (PTCs) that are degraded by the nonsense mediated decay (NMD) system and fail to produce CFTR protein. It was demonstrated that aminoglycosides (G418 or gentamicin) are able to suppress those PTCs and to restore the ribosomal read-through activity for full-length CFTR (Howard et al. 1996).

PTC124 (3-[5-(2-fluorophenyl)-[1,2,4] oxadiazol-3-yl]- benzoic acid), an orally bioavailable non-aminoglycoside compound that was specifically developed to induce ribosomes to read through PTCs, but not normal stop codons (Kerem et al. 2008), is now in phase III of clinical trials. Results obtained in phase II of clinical trials show Cl^- transport increase in CF patients under treatment with PTC124, assessed by NPD measurements (Kerem et al. 2008).

Correction of class II mutations

Mutations in this class have a folding defect that results in their rapid targeting for degradation by the ERQC. The most common CFTR mutation, F508del, belongs to this class. Compounds that promote folding and allow mutant CFTR to escape from the ERQC and reach the cell surface will be good candidates to repair these mutations.

This group includes chemical, molecular or pharmacological chaperones (Pedemonte et al., 2005c). Chemical chaperones like glycerol or trimethylamine-N-oxide (TMAO) have been known to stabilize F508del-CFTR (Brown et al. 1996). Some authors also described that phenylbutyrate influences F508del-CFTR folding or trafficking in addition to also enhancing transcription (Zeitlin et al., 2002). Substituted benzo[c]quinolizinium derivates have also been reported to both rescue F508del-CFTR from the ER and activate its Cl^- conductance (Dormer et al., 2001). Other drugs such as

deoxyspergualin can partially restore function to F508del-CFTR through the modulation of interaction with molecular chaperones (Jiang et al., 1998). However, most of these compounds have not yet been used in clinical trials, either due to their inherent toxicity or because the mechanism by which they act is poorly characterized at the cellular level.

High throughput screens (HTS) have identified other agents, called correctors, as being able to rescue and correct the F508del trafficking defect. Currently, VX-809/Lumacaftor and VX-661 (Clancy et al., 2011; Van Goor et al., 2011), together with the potentiator VX-770 (Kalydeco/Ivacaftor, see above under *Correction of class III mutations;* (Accurso et al., 2010; Van Goor et al., 2009)), are under phase II of clinical trials. Interim results from the trial with VX-809 showed improvement in lung function in patients homozygous for the F508del mutation taking VX-809 plus VX-770.

Correction of class III mutations

CFTR mutants in this class have reduced responses to regulatory molecules. The principal representative mutation in this class is G551D. Also included is F508del that not only has a trafficking defect but also presents a gating impairment (Wang et al. 2000). CFTR potentiators such as genistein and other flavonoids can activate Cl^- conductance and overcome mutations in this class. The potentiator VX-770 (Kalydeco/Ivacaftor, Van Goor et al., 2009) has been shown to increase CFTR activity in CF patients carrying the G551D mutation and it is the first CFTR-modulator drug, aimed at correcting the basic defect of a CFTR-mutant, to be released to patients (Accurso et al., 2010). Moreover, VX-770 has also been shown to correct other gating defect CFTR mutants located at the cell surface (Yu et al., 2012), thereby opening new avenues for the treatment of the CF disease.

Correction of class IV mutations

Class IV mutants are associated with reduced Cl^- conductance or abnormal channel gating. Stimulation of CFTR conductance can be achieved by either increasing the overall cell surface content of these mutants, thus compensating for the reduced conductance or augmenting the existing activity of channels with potentiators. In these cases, enhancing CFTR channel activity can probably also be achieved with CFTR-potentiators, such as VX-770 referred to above (Yu et al., 2012).

Correction of class V mutations

This class includes mainly splicing mutations that generate both aberrant and correctly spliced mRNAs in variable amounts among patients. The major aim for therapy in these cases is to increase levels of correctly spliced transcripts. However, modulating the splicing process is very complex and in this case would probably need to be tissue specific. Nevertheless, one could also imagine a combined therapy using both CFTR correctors, such as VX-809, and potentiators, such as VX-770, to further increase trafficking of CFTR to the plasma membrane and channel activity.

3.2.1 Preclinical functional readouts to assess the activity of CFTR-modulators

Taking the example of the successful achievement with the VX-770 potentiator, one can conclude that a human bronchial epithelial (HBE) cell culture system derived from native tissue constitutes a good preclinical model to assess compound efficacy (Van Goor et al., 2009).

Having such a preclinical model is thus critical since final selection of molecules for clinical development rests, in part, on the potency and activity of the drug candidates in the chosen models (Ashlock & Olson, 2011).

The criteria for model selection can rely on whether the model faithfully reproduces relevant aspects of the disease and whether the pharmacological activity that is being measured is the same in the model and in a clinical trial (Ashlock & Olson, 2011).

Along the same line, one can also think of native tissue samples, such as rectal biopsies, as an *ex vivo* preclinical model. Both HBE and rectal biopsies can possible be obtained from from multiple individuals, and allow assessment of ion transport abnormalities in Ussing chambers, determination of CFTR maturation and ultimately avoids issues associated with studying CFTR in nonhuman species (Ostedgaard et al., 2007). This subject will be further discussed in *Chapter 3,* **III. Results & Discussion** section.

Overall, results from VX-770 and also VX- 809 in the HBE system (Van Goor et al., 2009, 2011), combined with clinical data on these two investigational drugs (Accurso et al., 2010; Clancy et al., 2011), strongly support the use of the HBE system as a valuable pharmacology model for CFTR modulator drug discovery.

3.2.2 Clinical trials: CFTR biomarkers and outcome measures

Spirometry, in particular FEV_1, has been used for decades to assess changes in pulmonary function in CF patients in clinical care and research. This is an outcome measure that reflects clinically meaningful benefits and that has been accepted in past and recent clinical trials for CF therapies as a primary outcome measure (Mayer-Hamblett et al. 2007). Notwithstanding, as the health of the CF population improves and the rate of FEV_1 decline decreases, it is becoming more difficult to use FEV_1 to detect potentially important changes and assess treatment effects that may alter the slope of FEV_1 decline and other aspects of lung disease (Corey, 2007). Also other issues, such as the number of hospitalizations, the time between pulmonary exacerbations, and antibiotic use, are included in CF therapeutic trials. These have been commonly used and complemented by the use of CF-specific patient-reported outcome instruments (Goss & Quittner, 2007).

In such clinical trials, it also of major importance to include outcome measures reflecting CFTR ion-channel function, such as NPD and sweat test (Accurso et al., 2010; Clancy et al., 2011), and probably also *ex vivo* measurements of ion transport in rectal biopsies.

The impact of CFTR modulators on abnormalities associated with CFTR dysfunction (e.g., bicarbonate and mucus secretion, propensity for infection, and inflammation, beyond Cl^- secretion) in various genotypes and multiple affected tissues may be particularly important for the development and validation of new outcome measures of CFTR function.

4 Objectives of the present work

Since measurements of CFTR function in rectal biopsies *ex vivo* have been used to give an insight into diagnosis and prognosis of CF disease and can eventually constitute an outcome measure in (pre-)clinical trials for CFTR modulators, the objectives of the present work were the following:

i) To further establish measurements of CFTR-mediated Cl^- secretion in human rectal biopsies *ex vivo* as a sensitive and robust biomarker for CF diagnosis and prognosis;

ii) To assess what is the best discriminator measurement, from the laboratory and clinical tools available, to distinguish non-CF individuals from CF patients with Classic (severe) and non-Classic (milder) CF disease;

iii) To evaluate rectal specimens viability for for *ex vivo* bioelectrical (Ussing chamber) measurements and biochemical (Western blot and immunofluorescence) laboratory analyses;

iv) To determinate the overall assessment (comfort, invasiveness, pain, sedation requirement, etc) of the rectal biopsy procedure from the patients' perspective,

v) To further evaluate how studies for the mechanism of action or efficacy of CFTR modulators in heterologous expression systems may be applied to the physiological relevant tissues (bronchial primary cells and native tissues) and ultimately, to *in vivo* situation.

II. Materials & Methods

1 Clinical Parameters

1.1 Subjects

Access to human tissues used in this study received approval from the Research Ethics Committee of the Faculty of Medical Sciences, State University of Campinas (UniCamp, ref. 503/2007). Signed informed consent was obtained from all patients (or parents/tutors, for those <18 yrs).

Regarding data on *Chapter 1* (section **III. Results & Discussion**), 524 freshly excised rectal biopsies were analysed from 118 Brazilian individuals, including CF patients with confirmed diagnosis (n=51), individuals with CF clinical suspicion (n=49) and age-matched non-CF controls (n=18) undergoing biopsing with no CF-related disorders and agreeing to participate in the study. Conclusive results were obtained for 113/118 subjects, i.e., 96% of the cases.

As for the data included on *Chapter II* (section **III. Results & Discussion**), we extended the previous studies to extra 14 individuals. Altogether, total of 580 freshly excised rectal biopsies were analysed from 132 Brazilian individuals, including CF patients (n=67) and age-matched non-CF individuals (n=65) agreeing to participate in the study. The number of biopsies found suitable for quantitative bioelectrical measurements was 404 (i.e., ~70%).

1.2 Clinical Assessment

Patient data collected included: age at diagnosis, sweat Cl^- values ($\geq$ 2), body mass index (BMI), Shwachman-Kulczycki scores, pancreatic status and pulmonary function. Additional clinical features included presence of MI, nasal polyposis, glucose intolerance (GI), diabetes, osteopenia, osteoporosis, hepatic involvement and lung pathogens.

1.3 Shwachman-Kulczycki scores

Shwachman-Kulczycki (SK) scores were perfomed as before (Shwachman & Kulczycki, 1958) tin order to evaluate the severity of CF disease, and to provide a perception of the overall clinical status of the patient. The SK score is divided into four domains namely, general activity; physical examination; nutrition; and radiological findings; each having five possible subscores, according to the degree of impairment. The scores of the four domains are

summed to obtain the final score, from which the condition of the patient is categorized as: excellent (86-100), good (71-85), average (56- 70), poor (41-55) or severe (<40) (Shwachman & Kulczycki, 1958). The SK score was calculated by two pediatric clinicians, pulmonologist and gastroenterologist, with expertise in CF, and included here with permission from the responsible clinician Dr. Antônio F. Ribeiro (Pediatrics Department – State University of Campinas, University Hospital of Campinas).

1.4 Pulmonary Function

For pulmonary function, different time point measurements (2-4 per individual) of forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV1) were evaluated from individuals >5-6 years of age, and expressed as a percentage of predicted normal values for sex, age, and height. Pulmonary function tests were performed by Maria A. Ribeiro (CIPED - Research Center in Pediatrics - State University of Campinas, Campinas, Brazil) and include here with permission.

1.5 Questionnaire used for patients' assessment of the rectal biopsy procedure

Individuals were approached by telephone to evaluate the overall procedure from bowel preparation to collection of the biopsy (full questionnaire in Figure II.1.1). Data were obtained for 75, divided into four age groups: divided into 4 age groups, namely (yrs): 0-9 (n=21); 10-9 (n=33); 20-29 (n=7); ≥30 (n=14). Most patients were children (median age=13 yrs). Individuals were asked to rate the discomfort in comparison to other procedures such as NPD, nasal brushing, spirometry, sweat test, bronchoscopy and blood collection. They were also asked to comment on how many times they would accept repeating the procedure.

Although several attempts have been made to define patient satisfaction an acceptable definition is that it represents a patient's cognitive or emotional evaluation of a health−care provider's performance and is based on relevant aspects of a patient's experiences and perceptions (Maciejewski et al. 1997). Thus, different domains of assessment with gastrointestinal endoscopy procedures were described (Yacavone et al. 2001) and may include (i) the quality of care (endoscopy staff and environment); (ii) the comfort and tolerability of the procedure; (iii) the provision of an adequate explanation of

the procedure; (iv) communication with the physicians before and after the procedure; and (v) waiting time or delays.

Here, we focus on the comfort and tolerability of rectal biopsy procedure using a questionnaire covering: i) the choice of being or not sedated; ii) the evaluation of the discomfort of the overall procedure, including the monitoring, bowel preparation, rectoscopy, biopsing and sedation procedure; iii) comparison of the rectal biopsy procedure with other clinical and/or research exams such as nasal potential difference, nasal brushing, spirometry, sweat test, bronchoscopy and blood collection; iv) classification of the pain deriving from the procedure; v) the concerns with this type of exam; and vi) the will to repeat the procedure if used as a research procedure (Figure II.1.1). Survey data was collected by Maria A. Ribeiro (CIPED - Research Center in Pediatrics - State University of Campinas, Campinas, Brazil) and Adriana M. Vinagre (Faculty of Medical Sciences – State University of Campinas, Campinas, Brazil) and include here with permission.

Rectal Biopsy Procedure Patient Assessment Questionnaire

Subject Name_____ **Procedure Date:**_____
Gender: _____ **Birth Date:** _____

1. Was it required to use sedation? **Yes / No**

2. Please classify the overall rectal biopsy procedure as:
A) Very uncomfortable B) Somewhat uncomfortable C) Not uncomfortable

3. Regarding the overall procedure, that includes:
i) the monitoring ii) the bowel preparation iii) the sigmoidoscopy iv) the biopsy v) the sedation (if used)

which one(s) did you consider to be:
A) Not uncomfortable B) The least uncomfortable C) The most uncomfortable

4. Regarding other clinical and/or research procedures that you have experienced, please classify the rectal biopsy procedure as being:
A) Less unpleasant or **B) More unpleasant**

than:
i) Nasal potential difference measurement
ii) Nasal brushing
iii) Spirometry
iv) Sweat test
v) Bronchoscopy
vi) Blood collection

5. Have you felt some pain during the biopsing procedure? **Yes / No**

In case of a positive response, please classify your pain as:
A) Very painful B) Acceptable Pain C) Almost unpainful

6. What bothered you more in this procedure: the *a priori* presumption that this would be an uncomfortable and painful procedure or the preconception and taboo of a rectal exam? _____

7. If the rectal biopsy was to be used as a future outcome measure in clinical trials, how many times do you think you would be willing to have it performed?
____ None
____ One time
____ Two times
____ Three times
____ Four times

8. Please express any additional comment you would like to provide us relative to this procedure.
_____

Figure II.1.1: Rectal Biopsy Procedure Patient Assessment Questionnaire

2 Laboratorial Diagnosis Techniques

2.1 CFTR Genotyping

To detect CFTR mutations that were not identified by screening the 6 most common CFTR mutations (F508del, G551D, G542X, R1162X, N1303K, R553X) in the region of Campinas (Brazil) (Correia, 2005), an extended CFTR mutation search was done consisting in two-step automatic DNA sequencing of all 27 exons and the respective flanking intronic regions of the CFTR gene. The first step in genotyping included detection of mutations for 15 exons (exons 3, 6a, 7, 9, 10, 11, 12, 13, 16, 17b, 18, 19, 20, 21 and 24), which show a detection frequency of 95.14% (Bonadia, 2011; Felício, 2011). Such test method with a mutation detection rate of 95%, gives us a 90% probability of finding two abnormal alleles, 10% probability of finding one abnormal allele and 0% probability of finding no abnormal alleles (Moskowitz et al. 2001; Strom et al. 2003). To detect CFTR mutations that were not identified by the above described screening method, we performed DNA sequencing of the remaining 12 exons of the CFTR gene for CF patients. The mutations were classified according to the European Consensus (Castellani et al. 2008) as: a) mutations that cause CF disease; b) mutations that result in a CFTR-related disorder; c) mutations with no known clinical consequence; and d) mutations of unproven or uncertain clinical relevance.

CFTR genotyping was performed by Luciana C. Bonadia (Faculty of Medical Sciences – State University of Campinas, Campinas, Brazil), and by Anabela S. Ramalho, Verónica Felício and Inna Ulyakina (BioFIG, Faculty of Sciences – University of Lisboa and Genetics Department – National Institute of Health Dr. Ricardo Jorge, Lisboa Portugal) and included here with permission.

2.2 Sweat-test

For sweat collection, we used the quantitative pilocarpine iontophoresis method, which remains the gold standard for this approach, and sweat was obtained from the flexor surface of either forearm of the individual. Pilocarpine nitrate (0.2–0.5%) was used at the anode device following which a current of 0.5 μA is applied initially, increasing to 4 μA; current is maintained for

between 3–5 minutes (Taylor et al. 2009). After resting for 4 minutes, sweat was collected on to pre-weighed chloride-free filter paper for between 20 and 30 minutes. A minimum sweat rate of 1 g/m^2 body surface area/min is required; thus a sweat volume of 50–100 ml was considered adequate. Sweat collected onto filter paper was eluted for a minimum of 40 min (Taylor et al. 2009). Thereafter, colorimetric technique was used to determine the concentration (in mmol/L) of both Na^+ and Cl^- in sweat (Taylor et al. 2009). Sweat [Cl^-] above 60mmol/L is classified as abnormal and values of [Cl^-] between 40 and 60mmol/L are considered borderline.

This technique was performed by Miss Jaqueline at the Pediatrics Department of the University Hospital of Campinas, Campinas, Brazil, and included here with permission from the responsible clinician Dr. Antônio F. Ribeiro.

2.3 Fecal Elastase Test

Stool samples were analyzed for Fecal Elastase E1 (FEE) with the use of a human monoclonal ELISA test (Pancreatic Stool Elastase Kit, ScheBo Biotech AG, Giessen, Germany). Pancreatic sufficiency (PS) was established by FEE values ($\geq$ 2) of above 200 µg/g stool, while FEE concentrations ranging between 100 and 200 µg/g were considered moderate PI. A history of malabsorption together with FEE values <100 µg/g determined PI (Borowitz et al., 2004). This technique was performed by Adriana M. Vinagre (Faculty of Medical Sciences – State University of Campinas, Campinas, Brazil) and included here with permission.

2.4 Rectal Biopsies Procedure

When using jumbo forceps (Endoflex® 3.4mm; Voerde, Germany) a colonoscope with a working channel of 3.8mm Ø and an external diameter of 12.8mm (Fujifilm® EC-590ZWL; Tokyo, Japan) was used. For the standard forceps (Olympus® 2.5mm; Shinjuku, Tokyo, Japan) we used either a Olympus® (CV-VL model; Shinjuku, Tokyo, Japan) or a Pentax Ricoh® (FG-27X model, Tokyo, Japan) scope with a working channel of 2.8mm Ø and an external diameter of 9mm.

Individuals were examined in the left lateral decubitus position. The distal 10 cm of the scope was lubricated with a water-soluble jelly and it was

then inserted into the rectum by exerting gentle pressure by the scope tip on the anal sphincter until it relaxed. Insertion of the scope was done as quickly as possible, thereby limiting patient discomfort. Once the scope is the rectum (10 to 20 cm inserted), fluid that may be present was suctioned and the lumen was located and inspected by moving the tip of the scope. The normal rectal mucosa demonstrates a non-friable vascular system. Little or no air insufflation was used to avoid abdominal pain and bowel distension and because it is easier to obtain superficial rectal biopsies. These were collected laterally at 15 to 20 cm from the anal verge in a low vascularized mucosa.

Superficial 4-6 rectal mucosa specimens (2-4 mm in diameter) were obtained by either jumbo or standard forceps with visual examination, avoiding the risk of bleeding, perforation or of collecting damaged tissue, and immediately stored in ice-cold RPMI1640 with 5% (v/v) Fetal Bovine Serum (FBS). The procedure was performed by two experienced paediatric gastro-endoscopists, Drs. Maria F. Servidoni and Silvia R. Cardososo (Endoscopy Unit - University Hospital of Campinas; and Gastrocentro – State University of Campinas, Campinas, Brazil) in 3-15 minutes.

2.4.1 Sedation

Intravenous sedation with dormonid or midazolam was performed for all individuals undergoing colonoscopy. Rectoscopy procedure was performed with or without sedation (depending on individuals' will, collaboration or anxiety) by sevoflurane inhalation (54%). In other cases intravenous sedation with propofol, rapfen or midazolam was applied (46%). In general, individuals were monitored for blood pressure, pulse, oxygen saturation and ECG tracing by an anaesthologist.

2.4.2 Bowel Preparation

Bowel preparation was done on-site by applying an enema of a) saline solution (0.9% NaCl, 0.25 to 1L) or b) glycerinated solution (12% glycerol, 0.25 to 1L) for individuals undergoing rectoscopy; or c) oral mannitol solution (20%, 0.5L) for individuals undergoing colonoscopy. Satisfactory bowel cleaning was achieved in 99.24% (131/132) of cases.

2.4.2 Macroscopic Evaluation and Preparation

Prior to mounting in Ussing chamber, tissues were macroscopically evaluated regarding bleeding, mucus, biopsy thickness (presence of submucosa or not), and also for friability (tissue breakdown from manipulation), in a scale from 0 to 3 (from absence to clear presence of the descriptor). Regarding tissue integrity resulting from the biopsing procedure, the scale was inverted to facilitate the technicians' evaluation, i.e., 0 means full integrity of the specimen while 3 means that the tissue is fully disrupted (i.e. integrity is not present). Data on specimens' macroscopic evaluation were obtained for 107 individuals and was averaged for 2-5 biopsies/ individual to obtain a single value per individual.

To minimize edge damage, we mount tissues under a stereomicroscope allowing for optimal orientation of the small tissue specimen over insert opening (circular aperture of 0.95 mm^2), appropriate for Ussing chamber measurements, and preventing tissue damage during manipulation with instruments, as before (Mall et al. 2004a; Hirtz et al. 2004; Sousa et al. 2012).

2.4.3 Histology Preparations

One out of the 4-6 rectal specimens collected per individual was fixed in 4% formaldehyde, embedded in paraffin and cut in thin sections (2-3 μM) for histological observation and data were obtained for 78 individuals. Sections were then deparaffinized dehydrated in xylene (twice, 10 min), re-hydrated with absolute ethanol (twice, 5 min), 95% ethanol (2 min), 70% ethanol (2) min and briefly rinsed with distilled water and stained with hematoxylin-eosin (HE) and Tricome's Masson as previously (Mall et al. 2004a). Slides were mounted with xylene-based mounting medium. Pathologists, Drs. Luciana Meirelles and Rita Carvalho (Pathological Anatomy Department – University Hospital of Campinas, Campinas, Brazil), were blinded for CF diagnosis, bowel preparation and biopsy forceps used, regarding both *Chapters I and II* (section **III. Results & Discussion**). Results from this stainings were included here with their permission.

Regarding *Chapter I* (section **III. Results & Discussion**), one biopsy per individual was histologically analysed to exclude inflammation, haemorrhage, infection or other tissue damage (Figure II.2.1).

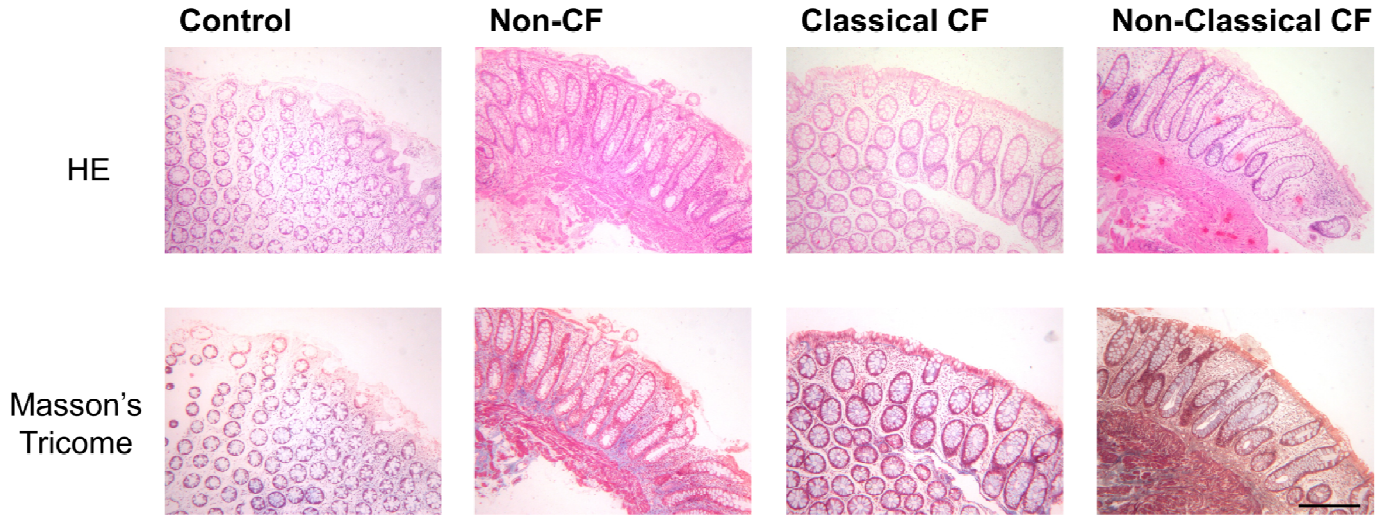


Figure II.2.1 – Histological evaluation of rectal biopsies by Hematoxilin-Eosin (HE) and Masson's Tricome stainings in control (transversal cut), Non-CF (longitudinal cut), Classical CF (transversal cut) and Non-Classical CF (longitudinal cut) showing a healthy epithelia, namely no fibrotic processes were observed and some biopsies presented inflammatory processes, independent of being CF or not. In HE stained sections we observe nuclei in blue and cytoplasm in pink to red. For Tricome's Masson we observe collagen in blue, nuclei black, and muscle and cytoplasm in red. Black scale bar represents 250 μM.

3 Cell Culture Techniques

3.1 Culture Conditions for BHK cells

We used Baby Hamster Kidney (BHK) cells stably expressing either wt- or F508del-CFTR proteins (Farinha et al. 2002). BHK cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 nutrient medium supplemented with 5% FBS and 200 µg/ml methotrexate (AAH Pharmaceuticals Ltd., Coventry, UK) at 37°C in a humidified atmosphere of 5% CO_2. In some experiments BHK cells expressing F508del-CFTR were cultured at 26°C to overcome its processing defect and promoting its delivery to cell membrane (see above under *4.1 Iodide Efflux*) (Denning et al., 1992).

3.2 Organotypic Cultures of CFBE41o- Cells Stably Expressing F508del- and wt-CFTR

Cystic Fibrosis Bronchial Epithelial (CFBE41o-) cells stably expressing wt-CFTR or F508del-CFTR (Bebok et al., 2005) were a generous gift from Dr. J.P. Clancy (University of Alabama at Birmingham, Birmingham, Alabama). CFBE cells were cultured in Modified Eagle Medium (MEM) with Earle's salts supplemented with 10% FBS and at 37°C in a humidified atmosphere of 5% CO_2. After reaching 80-100% confluence, cells were trypsinized to collagen IV coated porous membranes (Snapwell Inserts Ø12mm, from Corning-Costar®, New York, NY, USA) and grown for 6 to 9 days prior to the functional or biochemical assays.

_3.3 Isolation, Production and (organotypic) Culture of Primary Human Bronchial Epithelial Cells

The access to explanted lungs was established through a collaborative project between the University of Lisboa/Faculty of Sciences and the Cardio-Thoracic Surgery Department of the Hospital de Santa Marta (Lisboa), which received approval from the Ethics Committee of this Hospital, and primary HBE cells were isolated as described below (Fulcher et al. 2009; Fulcher et al. 2005).

The lungs were transported to the laboratory on cold phosphate buffer saline (PBS, on ice) and require significant dissection. The material that is used to dissect is previously sterilized and assembled on a clean bench. The first step consists in dissecting the airways by removing all excess of connective tissue and in cutting them into 5-10 cm segments. Then, the tissue segments are further cleaned, by removal of any additional connective tissue and lymph nodes and rinsing in PBS solution. Segments are slit longitudinally and cut into 1 x 2 cm portions with a scalpel. Finally, they are transferred to specimen cups containing chilled PBS solution. Because human tissue samples are likely to contain yeasts, bacteria, or fungi exposure to antibiotics should begin as soon as possible using a cocktail of common antibiotics – such as amphotericin, ceftazidime, tobramycin, and vancomycin (ACTV cocktail). "Wash Medium" is thus prepared by adding these antibiotics to Joklik's minimal essential medium (J-MEM) and dissected tissues are washed three times. Tissues from chronically infected patients containing abundant secretions were further treated to remove thick mucus and other debris with a soak solution containing supplemental antibiotics, dithiothreitol (DTT) and DNase, and washed again in the "Wash Medium" (Figure II.3.1[1]).

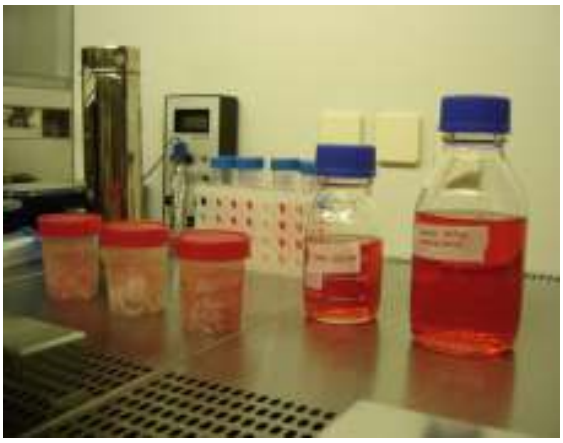


Figure II.3.1a - Solutions required for soaking and washing the tissue

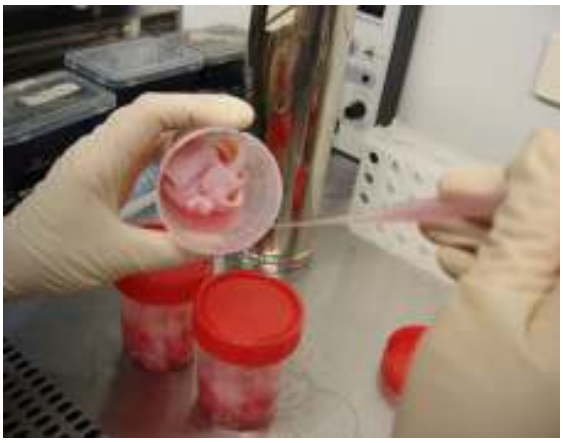


Figure II.3.1b - Rinsing tissue in "Wash Medium" to remove DTT/DNase

Previously washed tissue segments are transferred into 50-mL conical tubes containing 30 mL "Wash Medium" plus 4 mL Protease/DNase solution. The tubes are placed on a rocking platform (at 60 cycles/min), in the cold room (4°C), for 18-24h.

The day after this procedure, we harvest the cells following the standard sterile tissue-culture techniques in a laminar flow hood. To inhibit tissue

[1] Pictures by Marta Palma and included here with permission.

dissociation we transfer the contents of 50-mL tubes into a 150-mm tissue-culture dish, add FBS to a final concentration of 10% (v/v) to neutralize protease and scrapped epithelial surface with a convex surgical scalpel. Tissue surfaces are rinsed and cells collected from dish with PBS (Figure II.3.2[2]).

Solutions containing dissociated cells are pooled into 50-mL conical tubes. Finally, we centrifuge the latter at 500g for 5 min at 4°C, wash cells once in media, resuspended in a volume calculated to be approx 5 x 10^6 cells/mL, and counted the cells using a hemocytometer.

Figure II.3.2a - Epithelial cell removal after protease dissociation. The concave, luminal airway surface is gently scraped with a convex scalpel blade to remove adherent cells.

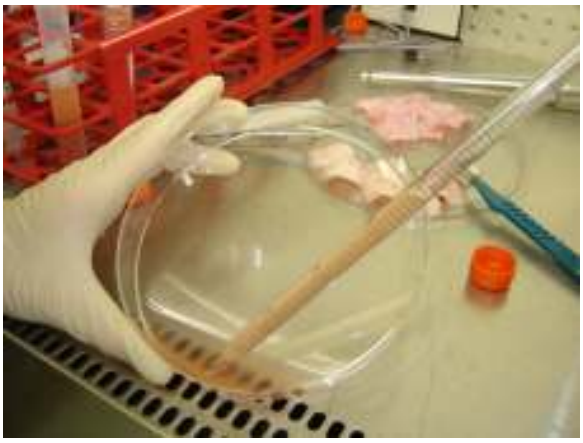


Figure II.3.2b - Rinsing and collecting the scraped cells.

These primary cells were then plated in bronchial epithelium growth medium (BEGM) on collagen-coated plastic dishes at a density of 2-6 x 10^6 per 100-mm dish. Primary cell media (BEGM) was supplemented with additional antibiotics (ACTV) for the first 3 days after plating, and changed every 2-3 days to prevent acidification. After reaching 70-90% confluence, cells were trypsinized (passage 1) to collagen IV coated porous membranes (Snapwell Inserts Ø12mm, from Corning-Costar®, New York, NY, USA) and grown in Air Liquid Interface (ALI) for four to five weeks prior to the functional or biochemical assays.

All chemicals (highest available purity) were from Sigma-Aldrich® (St Louis, MI, USA) and culture media was from GIBCO®/Invitrogen (Carlsbad, CA, USA). The isolation and production of primary HBE cells was done in collaboration with Marta Palma (Faculty of Sciences, Univ. of Lisboa, Portugal).

[2] Pictures by Marta Palma and included here with permission.

4 Functional Methods

4.1 Iodide Efflux

BHK cells expressing F508del-CFTR were seeded in plastic dishes (60mm) and incubated for 48h at 37 ºC or 26 ºC with the following compounds: DMSO 0.01% (last 24h), VRT-325 6.7 μM (last 24h) or C4a 10 μM (last 12 h, because of toxicity effects), before the assay. Due to binding affinity of small molecules to serum proteins, cells were culture with 0.5% FBS during incubation times with those drugs. CFTR-mediated iodide efflux was measured at room temperature (RT) as described (Lansdell et al. 1998) using the cAMP agonist forskolin (Fsk, 10 μM) and the CFTR potentiator genistein (50 μM). Prior to commencing experiments, cells were incubated for 1 h in loading buffer containing 13 mM NaI, 3 mM KNO_3, 2mM $Ca(NO_3)_2$, 20 mM Hepes, and 11 mM D-glucose, and vehicle (DMSO) or compounds referred above, pH 7.4, and then washed thoroughly with efflux buffer (136 mM $NaNO_3$ replacing NaI in the loading buffer). The amount of iodide in each sample of efflux buffer was determined using an iodide selective electrode (MP225, Thermo Electron Corp., Waltham, MA). Loading step and experiments were carried out at RT (23 °C).

4.2 Micro-Ussing chamber Experiments

The transepithelial resistance of the CFBE and primary HBE monolayers was measured using a Chopstick Electrode (STX2 from WPI®) and only monolayers with resistance values above 600 $\Omega.cm^2$ were mounted in modified micro-Ussing chambers and used for protein extracts.

To control for sample-to sample variability of *ex vivo* tissue specimens, we generally perform diagnostic bioelectric measurements on 2-5 biopsies per individual and data were averaged to obtain a single value for each individual.

Briefly, the luminal and basolateral surfaces of the rectal epithelium and primary HBE monolayers were continuously perfused (5 ml/min) with Ringer solution of the following composition (mmol/l): NaCl 145, KH_2PO_4 0.4, K_2HPO_4 1.6, D-glucose 5, $MgCl_2$ 1, Ca-gluconate 1.3. For the CFBE41o- monolayers, the apical bath solution was replaced by a low Cl- Ringer solution containing (mmol/l): NaCl 32; KH_2PO_4 0.4; K_2HPO_4 1.6; D-glucose 5; $MgCl_2$ 1; Ca-gluconate 5.7 and Na-gluconate 112. All bath solutions pH was adjusted to 7.4

and all experiments were carried out at 37 °C under open circuit conditions. HCO_3^- free buffer solutions were used to exclude a possible contribution of CFTR-independent electrogenic HCO_3^- secretion, which would be indistinguishable from electrogenic Cl^- secretion and thus may mimic residual Cl^- channel function in CF colonic epithelia (Mall et al. 2004b). Tissues or monolayers were equilibrated in the micro-Ussing chambers for 30 min in perfused Ringer solution before the above experimental protocol.

Values for the transepithelial voltage (V_{te}) were recorded continuously and were referred to the serosal surface of the epithelium. Transepithelial resistance (R_{te}) was determined by applying intermittent (1s) current pulses (0.5 μA). The equivalent short-circuit current (I_{sc}) was calculated according to Ohm's law ($I_{sc} = V_{te} / R_{te}$), after appropriate correction for fluid resistance (Mall et al. 1998, 2000; Mall et al. 2004a).

4.2.1 Protocol for the Assessment of cAMP-mediated CFTR Function in Human Rectal Biopsies

Regarding *Chapter I* (section **III. Results & Discussion**), values for basal R_{te} were similar for all groups of patients: $R_{te\ (Control)}$ = 18.63 ± 0.85 Ω.cm² (n = 18); $R_{te\ (non\text{-}CF)}$ = 19.12 ± 0.12 Ω.cm² (n = 28); $R_{te\ (Classic\ CF)}$ = 19.60 ± 1.16 Ω.cm² (n = 55); and $R_{te\ (Non\text{-}Classic\ CF)}$ = 21.68 ± 2.80 Ω.cm² (n=12). Amiloride (Amil, 20 μM, luminal) was added to block electrogenic Na^+ absorption through ENaC and Indomethacin (Indo, 10 μM, basolateral) was applied for 40-60 min to inhibit endogenous cAMP formation through prostaglandins (Mall et al. 1998a, 2000; Mall et al. 2004a; Hirtz et al. 2004; Mall et al. 2004b).

As before (Mall et al. 1999; Mall et al. 2004a; Hirtz et al. 2004), Na^+ absorption was significantly augmented in CF rectal tissues in comparison to control (p=0.041) or non-CF (p=0.014) rectal tissues: $I_{sc\text{-}Amil\ (Classic\ CF)}$ = 86.75 ± 17.03 μA/cm²; $I_{sc\text{-}Amil\ (Non\text{-}Classic\ CF)}$ = 64 . 39 ± 17.79 μA/cm²; $I_{sc\text{-}Amil\ (Control)}$ = 38.56 ± 5.90 μA/cm²; and $I_{sc\text{-}Amil\ (Non\text{-}CF)}$ = 37.63 ± 6.34 μA/cm².

As reported (Mall et al. 1998a; Mall et al. 2000), cAMP-dependent and cholinergic Cl^- secretion in human rectal tissues relies on functional CFTR. Thus, we used 3-isobutyl-1-methylxantine (IBMX, 100 μM, basolateral) and Fsk (2 μM, basolateral) to activate cAMP-dependent Cl^- secretion and carbachol (CCH, 100 μM, basolateral) for cholinergic co-activation (Mall et al. 1998a; Mall et al. 2000; Hirtz et al. 2004). Thus, percentage of CFTR function was calculated for maximal CFTR activation ($\Delta I_{sc\text{-}IBMX/Fsk} + \Delta I_{sc\text{-}CCH(IBMX/Fsk)}$) and

normalized to the correspondent mean value (-217.45 μA/cm^2) for the reference non-CF control group.

To test the effect of the correctors on fresh rectal biopsies, tissues were then incubated with the correctors indicated in *Chapter III* (section **III. Results &** Discussion) or with DMSO (vehicle) for 16-18h at 37ºC with oxygen perfusion (95% O_2 + 5% CO_2) in RPMI1640 media (supplemented with 100U/ml Penicilin, 100ug/ml Gentamicin, 2.5ug/ml Amphotericin, 25mM $NaHCO_3$ and 20-30mM HEPES) and studied in the Ussing chamber again by the same protocol described above.

4.2.2 Assessment of CFTR Cl^- Secretion in Organotypic Cultures Following Small Molecules Treatment

Cells were treated with at 37ºC with the following compounds added to the apical and basolateral cell culture media: DMSO 0.01% (last 48h), VRT-325 6.7 μM (24h), C4a 10 μM (16 h, because of toxicity effects) or VX-809 3 μM (48h).

Experiments were carried out in the presence of 20 μM Amiloride (luminal) to block electrogenic Na^+ absorption. To activate cAMP-dependent Cl^- secretion we used 2 μM forskolin (luminal) and, in order to achieve maximal CFTR activation, we applied next the potentiators genistein (25 μM, luminal) or VX-770 (5 μM, luminal). CFTR-mediated Cl^- secretion was then blocked with luminal application of 25 μM CFTR-inh172.

VRT-325, C4a and CFTR-inh172 were from Cystic Fibrosis Foundation Therapeutics consortium (Bethesda, MA, USA). VX-809 and VX-770 were from Selleck Chem (Houston, TX, USA). All the other chemicals and compounds (highest available purity) were from Sigma-Aldrich (St Louis, MI, USA) or Merck (Darmstadt, Germany).

5 Biochemical and Cell Biology Techniques

5.1 Western blot

For Western blot (WB), protein extracts from both CFBE41o- and primary HBE cells were prepared using cell lysis with Laemmli sample buffer (1.5 % (w/v) SDS; 10 % (v/v) glycerol; 0.001 % (w/v) bromophenol blue; 0.5 mM dithiotreitol; 31.25 mM Tris (pH 6.8)). DNA was sheared by treatment with with 1 µl benzonase® nuclease (5U) (Sigma-Aldrich, St Louis, MI, USA). This endonuclease is a genetically engineered from *Serratia marcescens* and degrades all forms of DNA and RNA.

Rectal biopsy specimens from normal individuals and patients with established CF genotypes (kept at -80ºC after bioelectrical measurements) were homogenized in ice-cold 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1mmol/L EDTA containing a protease inhibitor cocktail (Roche®, Basel, Switzerland). This material was then incubated 40 minutes with 1% (w/v) Nonidet P-40, 1% (w/v) sodium deoxycholate and 0.1% (w/v) sodium dodecyl sulfate. Insoluble material was removed by centrifugation and Laemmli buffer was added to supernatants.

All protein extracts were quantified by modified micro-Lowry method and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (7% acrylamide) separation (Farinha et al. 2004).

Western blots were probed with a cocktail (1:2000 dilution) of the monoclonal antibodies M3A7 and MM13-4 (Chemicon®, Millipore, Billerica, MA, USA) or anti-CFTR antibodies 570 and 596 (Cystic Fibrosis Foundation, Bethesda, MA, USA) which recognize separate CFTR epitopes to increase sensitivity to detect native CFTR protein (Mendes et al. 2004a; Mall et al. 2004b)(70 µg of total protein extract was applied in the gel). Chemiluminescent detection was performed using Super Signal® West Pico Chemiluminescent Substrate (Pierce Rockford, IL, USA) or ChemiDoc™ XRS+ System with Image Lab™ Software system (Bio-rad, Hercules, CA, USA).

5.2 Immunohistofluorescence

Thin sections (3-4 μm) of frozen rectal tissues were mounted on glass slides and stored at -80°C until immunofluorescence analyses. Briefly, sections were hydrated in phosphate-buffered saline for 5 minutes and fixed in methanol at -20ºC for 10 minutes5. After a blocking step (30 minutes in 1% (w/v) bovine serum albumin), sections were incubated with monoclonal anti-CFTR antibody 570 (Cystic Fibrosis Foundation, Bethesda, MA, USA) diluted 1:150 for 2h at room temperature (Mendes et al. 2004b) and after with Alexa 488-fluorescein labelled (Invitrogen, Carlsbad, CA, USA) diluted 1:300 for 1h, and mounted in Vectashield containing 4,6-diamidino-2-phenylindole (DAPI) to label nuclei (Vector Laboratories, Burlingame, CA, USA).

Immunofluorescence staining was observed and recorded on a confocal microscope Leica TCS SPE (Leica, Jehna, Germany).

6 Statistical analyses

Statistical analyses were performed with SPSS software (v. 19; SPSS Inc, Chicago, IL, USA) and a p value < 0.05 was accepted to indicate statistical difference. Unless otherwise stated, data are shown as mean + SEM (n = number of individuals studied).

For *Chapter 1* (section **III. Results & Discussion**), Pearson coefficients (r) were used to find correlations and partial correlations between clinical outcomes and CFTR function. As previously described (Cleveland, Zurakowski, Slattery, & Colin, 2009; Schaedel et al., 2002), a mixed model regression analysis was chosen to determine the rate of decline in FEV1 vs. Age among the established groups; and Kruskal-Wallis test for independent samples was used to find differences between the distribution of FEV1 vs. Age across those groups. For Crosstabs, Pearson Chi-Square Tests were used to determinate independence between the variables analysed. Monte Carlo estimates of the exact p-value are provided whenever the data are too sparse or unbalanced for the asymptotic results to be reliable.

A stepwise Discriminant Analysis with Wilks' Λ method was used to identify which variable or variables in study are able to discriminate with highest accuracy the established groups in this study (Tables VI.1.1 and 1.2). The assumptions of normality and homogeneity of variance-covariance matrices of each group were tested with Shapiro-Wilk (since one of the groups was small) and Box M tests, respectively. A Classification Analysis was also performed to obtain Fisher's linear classification functions that could predict in which group new cases would be classified.

For *Chapter 2* (section **III. Results & Discussion**), Pearson coefficients (r) were used to find correlations and partial correlations between descriptors for macroscopic evaluation and R_{te} of biopsies. ANOVA, with a Bonferroni post hoc correction when appropriated, was used to find differences between groups' means with a 90% confidence interval. For Crosstabs regarding patients questionnaires, Pearson's Chi-Square tests or Monte Carlo estimates of the exact p-value were used as above.

For *Chapter 3* (section **III. Results & Discussion**), two-tailed student t-test was used to find statistical significant differences between means of the different sets of experiments ($p < 0.05$).

Table VI.1.1 – Discriminant Functions: Eigenvalues and Wilk's Lambda statistics.

Eigenvalues

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	7,238[a]	90,4	90,4	,937
2	,765[a]	9,6	100,0	,658

a. First 2 canonical discriminant functions were used in the analysis.

Wilks' Lambda

Test of Function(s)	Wilks' Lambda	Chi-square	df	Sig.
1 through 2	,069	157,928	6	,000
2	,567	33,513	2	,000

NOTE: Eigenvalues show that the biggest proportion (90.4%) of variances (in terms of differences between groups) can be explained by first discriminant function (in this case CCH-induced I_{sc} (following IBMX/Fsk application). Wilks' Lambda (Λ) test of functions shows that both discriminant functions are significant in determining these differences between groups.

Table VI.1.2 – Canonical Discriminant Functions used in the Analysis.

Structure Matrix

	Function 1	Function 2
CCH-induced Isc (µA/cm2) (following IBMX/Fsk application)	,777*	,290
IBMX/Fsk-induced Isc (µA/cm2)[a]	,545*	,076
Fecal Elastase E1 (µg/g)	-,658	,705*
Sweat Chloride (mmol/L)	,414	,531*
Schwachman & Kulczycki Score[a]	,055	-,290*
FEV1 (% predicted)[a]	-,196	-,265*
Body Mass Index[a]	,072	-,144*

Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions
Variables ordered by absolute size of correlation within function.

*. Largest absolute correlation between each variable and any discriminant function
a. This variable not used in the analysis.

Standardized Canonical Discriminant Function Coefficients

	Function 1	Function 2
Sweat Chloride (mmol/L)	,322	,585
Fecal Elastase E1 (µg/g)	-,520	,821
CCH-induced Isc (µA/cm2) (following IBMX/Fsk application)	,675	,383

Functions at Group Centroids

CF Clinical Diagnosis - Consensus Guidelines	Function 1	Function 2
Classic CF	1,943	-,284
Non-Classic CF	-,861	1,945
Non-CF	-4,347	-,578

Unstandardized canonical discriminant functions evaluated at group means

NOTE: Structural coefficients matrix, showed on the left, explains that CCH-induced I_{sc} (following IBMX/Fsk application) is related to the first discriminant function, while Fecal Elastase E1 and Sweat Chloride values are correlated with the second discriminant function. Standardized coefficients, in the middle panel, also show the same: CCH-induced I_{sc} (following IBMX/Fsk application) is saturated for the first function, whereas both Fecal Elastase E1 and Sweat Chloride concentrations are saturated in the second one. Panel on the right shows discriminant functions at group centroids.

III. Results & Discussion

Chapter 1 **Measurements of CFTR-mediated Cl- Secretion in Human Rectal Biopsies Constitute a Robust Biomarker for Cystic Fibrosis Diagnosis and Prognosis**

(Manuscript submitted to *PLoS one*,
with minor alterations).

1 Summary

Cystic Fibrosis (CF) is caused by ~1,900 mutations in the *CF transmembrane conductance regulator* (*CFTR*) gene encoding for a cAMP-regulated chloride (Cl^-) channel expressed in several epithelia. Clinical features are dominated by respiratory symptoms, but there is variable organ involvement thus causing diagnostic dilemmas, especially for non-classic cases.

To further establish measurement of CFTR function as a sensitive and robust biomarker for diagnosis and prognosis of CF, we assessed cholinergic and cAMP-CFTR-mediated Cl^- secretion in 524 freshly excised rectal biopsies from 118 individuals, including patients with confirmed CF clinical diagnosis (n=51), individuals with clinical CF suspicion (n=49) and age-matched non-CF controls (n=18). Conclusive measurements were obtained for 96% of cases.

Patients with "Classic CF", presenting earlier onset of symptoms, pancreatic insufficiency, severe lung disease and low Shwachman-Kulczycki scores were found to lack CFTR-mediated Cl^- secretion (≤5%). Individuals with milder CF disease presented residual CFTR-mediated Cl^- secretion (10-57%) and non-CF controls show CFTR-mediated Cl^- secretion ≥30-35% and data evidenced good correlations with various clinical parameters. Finally, comparison of these values with those in "CF suspicion" individuals allowed to confirm CF in 16/49 individuals (33%) and exclude it in 28/49 (57%). Statistical discriminant analyses showed that colonic measurements of CFTR-mediated Cl^- secretion are the best discriminator among Classic/Non-Classic CF and non-CF groups.

Determination of CFTR-mediated Cl^- secretion in rectal biopsies is demonstrated here to be a sensitive, reproducible and robust predictive

biomarker for the diagnosis and prognosis of CF. The method also has very high potential for (pre-)clinical trials of CFTR-modulator therapies.

Keywords: CFTR function; CF biomarkers; outcome measures innovative therapeutics; diagnosis-algorithm prediction.

2 Introduction

Cystic Fibrosis (CF), the most common severe autosomal recessive disease in Caucasians, is caused by mutations in the *CF transmembrane conductance regulator* (*CFTR*) gene (Rommens et al. 1989; Kerem et al. 1989; Riordan et al. 1989) which encodes a cAMP-regulated chloride (Cl^-) channel expressed at the apical membrane of epithelial cells to control salt and water transport (Rich et al., 1990). Clinically, CF is characterized by multiple manifestations in different organs, but is dominated by the respiratory disease, the main cause of morbidity and mortality. Airway obstruction by thick mucus and chronic infections, especially by *Pseudomonas aeruginosa* (Pa), eventually lead to impairment of respiratory function (Rowe et al. 2005). Other CF symptoms include pancreatic insufficiency, intestinal obstruction, elevated sweat electrolytes and male infertility (Collins, 1992). About 1,900 CFTR mutations were reported, but one (F508del) found in association with a severe CF, accounts for ~70% of CF chromosomes worldwide (Collins, 1992). Despite impressive advances in our understanding of the molecular basis of CF, life expectancy and quality of life for CF patients are still limited (Rowe et al. 2005).

For the vast majority of patients, the diagnosis of classic forms of CF is established early in life and suggested by one or more characteristic clinical features, a history of CF in a sibling or, more recently, by a positive newborn screening result (Rosenstein & Cutting 1998; Farrell et al. 2008). Such diagnosis is usually supported by evidence of CFTR dysfunction through identification of two CF-disease causing mutations, two abnormal sweat-Cl^- tests (≥60mEq/L), and/or distinctive transepithelial nasal potential difference (NPD) measurements (Rosenstein &Cutting 1998; Farrell et al. 2008).

However, depending on the ethnic background of the populations tested (Bobadilla et al., 2002) there is a fraction of patients escaping such diagnosis criteria by presenting "non-classic" symptoms, i.e., milder disease and often inconclusive evidence of CFTR dysfunction from the available diagnostic tools

(De Boeck et al. 2006; Boyle 2003). For such individuals with clinical phenotypes not fully meeting the CF diagnostic criteria it is also difficult to exclude CF. These have described as having "CFTR-opathies" (Noone & Knowles 2001) or CFTR-related disorders (CFTR-RD) (Bombieri et al., 2011; Paranjape & Zeitlin, 2008). Moreover, the recently implemented extensive newborn screening programs identify increasing numbers of asymptomatic CF patients merely identified by elevated serum concentrations of immunoreactive trypsinogen (IRT), posing new challenges to the CF diagnosis paradigm (Farrell et al. 2008; Parad & Comeau 2005; Taylor et al. 2009), especially when associated with borderline sweat [Cl^-] and/or inconclusive *CFTR* genotypes (Boyle 2003; Parad & Comeau 2005; Taylor et al. 2009; De Boeck et al. 2006). To confirm/exclude a CF diagnosis in such increasing numbers of individuals, besides close clinical follow-up, further laboratory support is required (Taylor et al. 2009), in particular, there is a need for robust methods relying on the functional assessment of CFTR.

Assessment of CFTR (dys)function in native colonic epithelia *ex vivo*, as we previously reported, constitutes a good approach to this end (Mall et al. 2004a; Hirtz et al. 2004). However, since those data were reported, other groups have investigated the abnormalities in electrogenic Cl^- secretion in the intestinal epithelium of CF patients using Ussing chamber measurements by different protocols (van Barneveld et al. 2006; Derichs et al. 2010; van Barneveld et al. 2010). Unfortunately, the final composite parameter used by some results from a combination of experimental readouts not all relying on direct measurement of CFTR-mediated Cl^- secretion, thus leading to conflicting results and precluding good correlations with clinical symptoms (van Barneveld et al. 2006; Derichs et al. 2010; van Barneveld et al. 2010).

Since our previous results have established that quantification of rectal CFTR-mediated Cl^- secretion is a sensitive test for the diagnosis and prognosis of CF disease (Hirtz et al., 2004), hereunder we applied it to the largest cohort of CF patients and highest number of rectal biopsies ever assessed in a single study analyzing CFTR-mediated Cl^- secretion in native tissue *ex vivo* to evaluate its robustness as a diagnosis/prognosis biomarker. Our current data demonstrate significant correlations with CF clinical symptoms, evidencing the value of this method as a superior laboratory tool to aid the clinical practice. Furthermore, here we also applied this technique to individuals with clinical suspicion of CF to confirm/exclude a CF diagnosis. Through comparison of the

values for CFTR-mediated Cl^- secretion in these individuals to those both of the CF reference group and of a non-CF control group, we could confirm CF in 16/49 individuals (33%) and exclude it in 28/49 (57%), remaining 5 inconclusive. Finally, using the Ussing chamber data in statistical discriminant analyses, together with the clinical outcomes and other laboratory measurements, has shown that colonic measurements of CFTR-mediated Cl^- secretion, alone or in combination with sweat-Cl^- and fecal elastase E1 (FEE), provides the best discriminator factor among patients with Classic CF, Non-Classic CF and non-CF individuals.

3 Results

3.1 Subjects under study and overview of clinical data

The clinical diagnosis of CF was established based on consensus clinical criteria (Rosenstein and Cutting 1998; Farrell et al. 2008), namely: 1) presence of one or more characteristic phenotypic features (chronic sinopulmonary disease; gastrointestinal/ nutritional abnormalities; obstructive azoospermia or salt-loss syndrome) and 2) evidence of a CFTR abnormality (increased sweat [Cl^-] (>60 mEq/L) and/or detection of two CF-disease causing mutations). Using these criteria and the current CF classification terminology (De Boeck et al. 2006), two different sub-groups of CF patients (n = 51) were established (detailed clinical data in section **VI. Appendix**): (a) Classic CF patients (n = 46) presenting a severe phenotype and classic disease manifestations (high sweat-Cl^-; PI; nutrition deficiencies; chronic sinopulmonary disease); and (b) Non-Classic CF patients (n = 5) with an atypical phenotype or milder disease (pancreatic sufficiency-PS; adulthood diagnosis; less serious lung involvement; and at least one organ with CF phenotype).

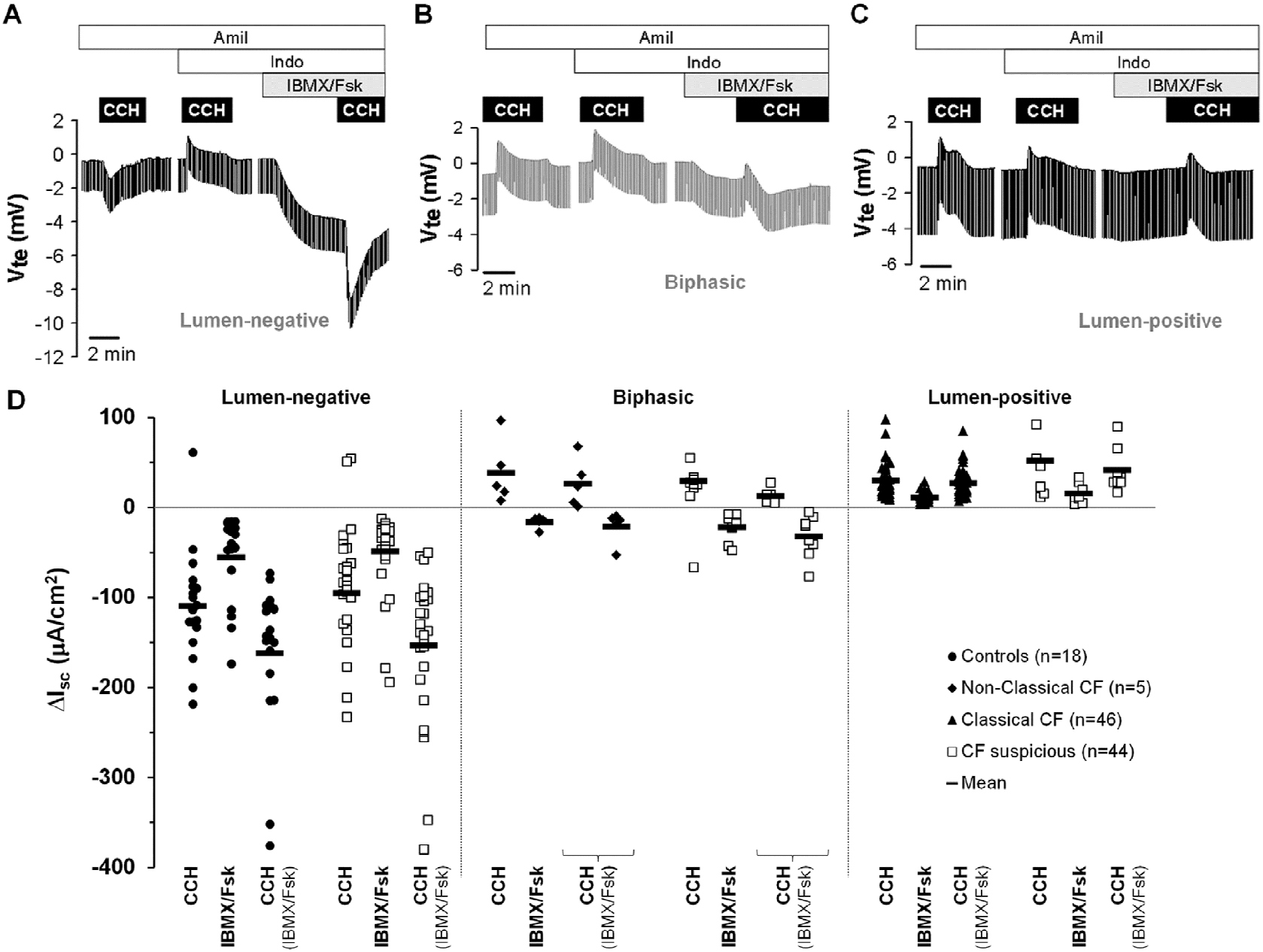
A third group included individuals with a clinical suspicion of CF (n = 49) of which 27% had only one abnormal sweat-Cl^- value; others presented borderline (22%) or normal (20%) sweat-Cl^- values; and 31% had not been tested for sweat-Cl^- at the time. Most of these individuals (69%) had inconclusive genetic testing (20% had only one CF-disease causing mutation identified) and the remainder had not been CFTR-genotyped at the time (**VI. Appendix**). Most of them (68%) showed mono-symptomatic features, including: respiratory symptoms in 29% (nasal polyps, chronic

cough/bronchitis, pneumonia, bronchiectasis or pansinusopathy); 35% had abnormal gastrointestinal signs (nutrients malabsorption; failure to thrive; hepatobiliary disease; chronic diarrhoea; recurrent pancreatitis; diabetes and GI); others presented osteopenia/osteoporosis, liver disease or male infertility. Fourteen percent of these "CF suspicion" individuals presented relatively mild lung disease and nutrition abnormalities, one presenting GI and another azoospermia. A group of 9 patients with severe phenotype (both respiratory and gastrointestinal) were initially included in this CF suspicion group (18%) due to their recent identification and while waiting for confirmation of a CF diagnosis (**VI. Appendix**).

3.2 Assessment of CFTR-mediated Cl^- secretion in rectal biopsies

CFTR-mediated Cl^- secretion was assessed in rectal biopsies from the above two sub-groups of CF patients (Classic and Non-Classic CF) as CF reference. As non-CF control group, we also analysed CFTR function in age-matched individuals undergoing routine colonoscopy for non-CF related reasons. Finally, measurements were carried out in the CF suspicion individuals to establish/exclude a CF diagnosis, following comparison with values from the CF patients and control groups.

As shown previously (Mall et al. 2004a; Hirtz et al. 2004; Mall et al. 1998b; Mall et al. 2000), application of carbachol (CCH) under basal conditions elicited lumen-positive responses in CF and lumen-negative in non-CF tissues (Figure III.1.1-A, B and C). Nevertheless, due to variable levels of endogenous prostaglandins, lumen-positive responses can also be observed in non-CF control tissues (Mall et al. 2004a; Mall et al. 2000). Thus, when CCH was applied for a second time, now under indomethacin to completely inhibit endogenous cAMP (and thus CFTR-mediated Cl^- secretion), all tissues presented lumen-positive responses correspondent to potassium (K^+) exiting the cell (Figure III.1.1-A, B and C) (Mall et al. 2004a; Mall et al. 2000). Next, when we used 3-isobutyl-1-methylxantine (IBMX) and forskolin (Fsk) to activate cAMP-dependent CFTR-mediated Cl^- secretion (Mall et al. 2004a; Hirtz et al. 2004) ($I_{sc\text{-}IBMX/Fsk}$), we observed lumen-negative responses in tissues from individuals in both the "non-CF control" group (Figure III.1.1-A) and "Non-Classic CF" sub-group (Figure III.1.1-B) but lumen-positive responses for those in the "Classic CF" sub-group (Figure III.1.1-C).


A
Amil
Indo
IBMX/Fsk
CCH
CCH
CCH
Vte (mV)
2
0
-2
-4
-6
-8
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-2
-4
-6
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Lumen-positive
D
Lumen-negative
Biphasic
Lumen-positive
ΔIsc (μA/cm²)
100
0
-100
-200
-300
-400
CCH
IBMX/Fsk
CCH (IBMX/Fsk)
Controls (n=18)
Non-Classical CF (n=5)
Classical CF (n=46)
CF suspicious (n=44)
Mean

Figure 1. Results from Ussing chamber measurements in rectal biopsies from 113 individuals. Original recordings of the effects of cholinergic (CCH, 100μM) and cAMP-dependent (IBMX, 100μM and Fsk, 2μM, basolateral) activation on transepithelial voltage in rectal tissues from **(A)** a healthy control presenting CCH lumen-negative responses; **(B)** a Non-Classic CF patient showing biphasic responses, thus presenting residual CFTR function; and **(C)** a Classic CF patient with no detectable Cl- secretion, presenting only lumen-positive responses. All the experiments were performed in the presence of Amiloride (Amil, 20μM, luminal) and Indomethacin (Indo, 10μM, basolateral). **(D)** Summary of activated short-circuit currents (ΔI_{sc}) for basal CCH ($I_{sc\text{-}CCH}$), IBMX/Fsk ($I_{sc\text{-}IBMX/Fsk}$) and CCH following IBMX/Fsk application ($I_{sc\text{-}CCH(IBMX/Fsk)}$) in rectal biopsies from controls (lumen-negative, filled circles, n=18), Non-Classic CF (biphasic, filled diamonds, n=5), Classic CF (lumen positive, filled triangles, n=46) and CF suspicious patients (open squares, n=44) showing lumen-negative, biphasic and lumen-positive responses. Data represent the mean of the mean measurements on 2-5 rectal biopsies per individual. Black solid line shows mean ΔI_{sc} values for each group represented in $\mu A/cm^2$: Controls ($I_{sc\text{-}CCH}$ = -109.28±14.47; $I_{sc\text{-}IBMX/Fsk}$ = -55.38±11.12; $I_{sc\text{-}CCH(IBMX/Fsk)}$ = -162.07±19.64); Non-Classic CF ($I_{sc\text{-}CCH}$ = 47.20±17.86; $I_{sc\text{-}IBMX/Fsk}$ = -16.27±2.83; $I_{sc\text{-}CCH(IBMX/Fsk)}$ = -21.64±8.01); Classic CF ($I_{sc\text{-}CCH}$ = 30.13±2.66; $I_{sc\text{-}IBMX/Fsk}$ = 11.30±0.78; $I_{sc\text{-}CCH(IBMX/Fsk)}$ = 27.17±2.22); and CF suspicious with similar ΔI_{sc} distribution.

Finally, and as previously described (Mall et al. 2004a; Hirtz et al. 2004; Mall et al. 1998b; Mall et al. 2000; Mall et al. 1998a), following stimulation with CCH in the presence of IBMX/Fsk ($I_{sc\text{-}CCH(IBMX/Fsk)}$), three different response patterns were observed: (i) monophasic lumen-negative (Cl^--secretory) in tissues from "non-CF controls", and we quantified such negative peak (Figure III.1.1-A); (ii) monophasic lumen-positive (K^+-secretory) in tissues from the "Classic CF" subgroup, and we determined both peak and plateau (Figure III.1.1-C); and finally (iii) biphasic responses, in the "Non-Classic CF" sub-group, and we determined both positive and negative peaks (Figure III.1.1-B).

3.3 Confirmation / Exclusion of a CF diagnosis and correlation with genotypes

The equivalent evoked-short-circuit currents (ΔI_{sc}) calculated for individuals in the "CF suspicion" group (Figure III.1.1-D, open squares, ΔI_{sc} values in **VI. Appendix**) were compared with the reference values for the CF ("Classic CF" and "non-Classic CF" sub-groups, Figure III.1.1-D black triangles and diamonds, respectively) and control groups (Figure III.1.1-D, black circles). Accordingly, each individual with a clinical suspicion of CF could be associated with one of these two groups (n = 44): for those with a lumen-negative response (Figure III.1.1-A and D) a CF diagnosis was excluded and

those presenting only lumen-positive or biphasic responses (Figure III.1.1-B, C and D) were considered as CF. For the remainder 5 data were inconclusive due to inviability of biopsies. Extended genotyping was performed for all CF-suspicious individuals and two CF-disease causing mutations were eventually detected in individuals with absence or residual CFTR-mediated Cl^- secretion.

One "CF suspicion" patient with severe CF symptoms and the F508del/S549R genotype, evidenced very low levels of CFTR function (~5% normalized to controls) thus setting the threshold for severe disease at this value. In contrast, among the individuals in the "CF suspicion" group who showed lumen-negative responses, i.e., CFTR function (Non-CF($I_{sc\text{-}CCH(IBMX/Fsk)}$) = -153.38±15.33 μA/cm^2) in the same range as the non-CF control group (Controls($I_{sc\text{-}CCH(IBMX/Fsk)}$) = -162.07±19.64 μA/cm^2) we could only detect one CF-disease causing mutation in one individual and 2 other mutations (4428insGA and D1152H) in two other individuals heterozygous for W1282X and F508del, respectively (see Table III.1.1 and **VI. Appendix**). These individuals were later classified as CFTR-RD and 4428insGA and D1152H as CFTR-RD mutations.

Overall, based on the combined data, i.e., CFTR-mediated Cl^- secretion in the colon and *CFTR* genotypes, we could classify the individuals in the "CF suspicion" group (n = 49) as: Classic CF (n = 9), Non-Classic CF (n = 7), CFTR-RD (n = 2) and Non-CF (n = 26). As to the 5 individuals showing inconclusive Ussing chamber measurements, one individual had one CF-disease causing mutation (G542X) and two individuals had RD-related mutations (V562I and G576A). As we could not confirm/exclude CF in these 5 individuals, they were not included in the hereunder correlations.

Functional classification of rarer mutations also results from these analyses, namely (Table III.1.1 and **VI. Appendix**): 3120+1G>A as class I (2 siblings with 3120+1G>A/R1066C, absence of CFTR-function and severe phenotypes); 1716+18672A>G as class V (2 other siblings with F508del/1716+18672A>G, residual CFTR function -28-34%- and mild CF); I618T as class IV (in a patient with G542X/I618T, 37% CFTR function and mild disease); and L206W as class IV or CFTR-RD mutation (in a patient with F508del/L206W and the highest CFTR function -57%- and very mild disease).

Table S2. CFTR mutations found in individuals under study. Gene and protein localization. mutation classification and frequency from the present study are designated. Traditional and HGVS standard nomenclature[a] for CFTR mutations are also indicated.

Traditional Nomenclature	HGVS Nomenclature[a]		Localization (CFTR gene)[d]	Consequence	Protein localiz.	Freq. (%)	Mutation Classific.	Predicted Functional Class
	cDNA name	Protein name						
F508del	c.1521_1523delCTT	p.Phe508del	Exon 10	Point deletion	NBD1	63.89	A	II
G542X	c.1624G>T	p.Gly542X	Exon 11	Nonsense	NBD1	7.64	A	I
R1162X	c.3484C>T	p.Arg1162X	Exon 19	Nonsense	ICL4	2.08	A	I
R334W	c.1000C>T	p.Arg334Trp	Exon 7	Missense	TM6	2.08	A	IV
3120+1G>A	c.2988+1G>A	-	IVS16	Splicing	-	2.08	A	I
W1282X	c.3846G>A	p.Trp1282X	Exon 20	Nonsense	NBD2	1.39	A	I
P205S	c.613C>T	p.Pro205Ser	Exon 6a	Missense	TM3	1.39	A	IV
1716+18672A>	c.1584+18672A>G[b]	-	IVS 10	Splicing[c]	-	1.39	A	V
1717-1G>A	c.1585-1G>A	-	IVS11	Splicing	-	1.39	A	I
1812-1G>A	c.1680-1G>A	-	IVS12	Splicing	-	1.39	A	I
2183AA>G	c.2051_2052delAA insG	p.Lys684Ser fsX38	Exon 13	Frameshift	RD	1.39	A	I
A561E	c.1682C>A	p.Ala561Glu	Exon 12	Missense	NBD1	1.39	A	II
3272-26A>G	c.3140-26A>G	-	IVS17b	Splicing	-	0.69	A	V
G85E	c.254G>A	p.Gly85Glu	Exon 3	Missense	TM1	0.69	A	II
I618T	c.1853T>C	p.Ile618Thr	Exon 13	Missense	NBD1/ RD	0.69	A	IV
N1303K	c.3909C>G	p.Asn1303Lys	Exon 21	Missense	NBD2	0.69	A	II
R1066C	c.3196C>T	p.Arg1066Cys	Exon 17b	Missense	ICL4	2.08	A	II
R553X	c.1657C>T	p.Arg553X	Exon 11	Nonsense	NBD1	0.69	A	I
S549R(T>G)	c.1647T>G	p.Ser549Arg	Exon 11	Missense	NBD1	0.69	A	III

V562I	c.1684G>A	p.Val562Ile	Exon 12	Missense	NBD1	0.69	B	
L206W	c. 617T>G	p.Leu206Trp	Exon 6a	Missense	TM3	0.69	A / B	IV
4428insGA	c.4296_4297insGA	p.Ser1435GlyfsX	Exon 24	Frameshift	C-terminus	0.69	B	VI
D1152H	c.3454G>C	p.Asp1152His	Exon 18	Missense	TM12/NBD2	0.69	B / A	IV
G576A	c.1727G>C	p.Gly576Ala	Exon 12	Missense/Splic	NBD1	0.69	B / A	V
IVS8-5T	-	-	Exon 9	Exon 9 skiping	-	2.78	B / C	

NOTE: A - CF-causing mutation; B - CFTR-RD mutation; C - Mutation with no clinical consequence.

[a] Reference CFTR sequence accession number: NM_000492.3, nucleotide number 1 corresponds to the A of the ATG translation initiation codon, in the reference sequence is numbered as 133.
[b] According to the HVGS guidelines this mutation should be named: 1585-9412bp A>G.
[c] Inclusion of 104bp criptic exon between exon 10 and exon 11 in the CFTR transcripts.
[d] Traditional Nomenclature.

3.4 Correlation between CFTR-mediated Cl^- secretion and clinical outcomes

In order to assess the value of CFTR-mediated Cl^- secretion in rectal biopsies as a predictive tool for CF, we attempted to statistically correlate these values with the accepted CF-characteristic parameters (Table III.1.2), namely: sweat [Cl^-] (Figures III.1.2-A and 1.3-A); FEE (Figures III.1.2-B and 1.3-B); BMI (Figure III.1.3-C); and age at diagnosis (Figures III.1.2-D and I.3-D). For the correlations involving SK scores (Figures III.1.2-C and 1.3-E) and FEV_1 (Figures III.1.2-E and 1.3-F), we subdivided patients into 4 age-groups (in years): 0-9; 10-19; 20-29; and ≥30, since these parameters have been shown to decline with age (Schaedel et al., 2002).

Table III.1.2 - Correlations between clinical outcomes and CFTR-mediated I_{sc} in rectal biopsies.

	Assessment of CFTR Function in Rectal Biopsies			
	$I_{sc\text{-}IBMX/Fsk}$		**$I_{sc\text{-}CCH\ (IBMX/Fsk)}$**	
	Pearson (*r*)	p-value	Pearson (*r*)	p-value
Sweat Chloride	**+0.495**	**3.41×10^{-7}**	**+0.677**	**4.73×10^{-14}**
Fecal Elastase E1	**-0.721**	**6.83×10^{-14}**	**-0.770**	**1.10×10^{-16}**
Age at Diagnosis	**-0.728**	**1.34×10^{-12}**	**-0.713**	**6.18×10^{-12}**
BMI	-0.169	0.101	**-0.226**	**0.028**
BMI *Age at study*	-0.104	0.319	-0.121	0.244
SK Score	**-0.274**	**0.009**	**-0.362**	**4.59×10^{-4}**
SK Score *Age at study*	**-0.302**	**0.004**	**-0.411**	**6.44×10^{-5}**
FEV_1	-0.205	0.071	**-0.240**	**0.035**
FEV_1 *Age at study*	**-0.251**	**0.028**	**-0.301**	**0.008**

NOTE: Pearson coefficients (r) and p-values showing statistical correlations ($p < 0.05$) are highlighted (n=95).

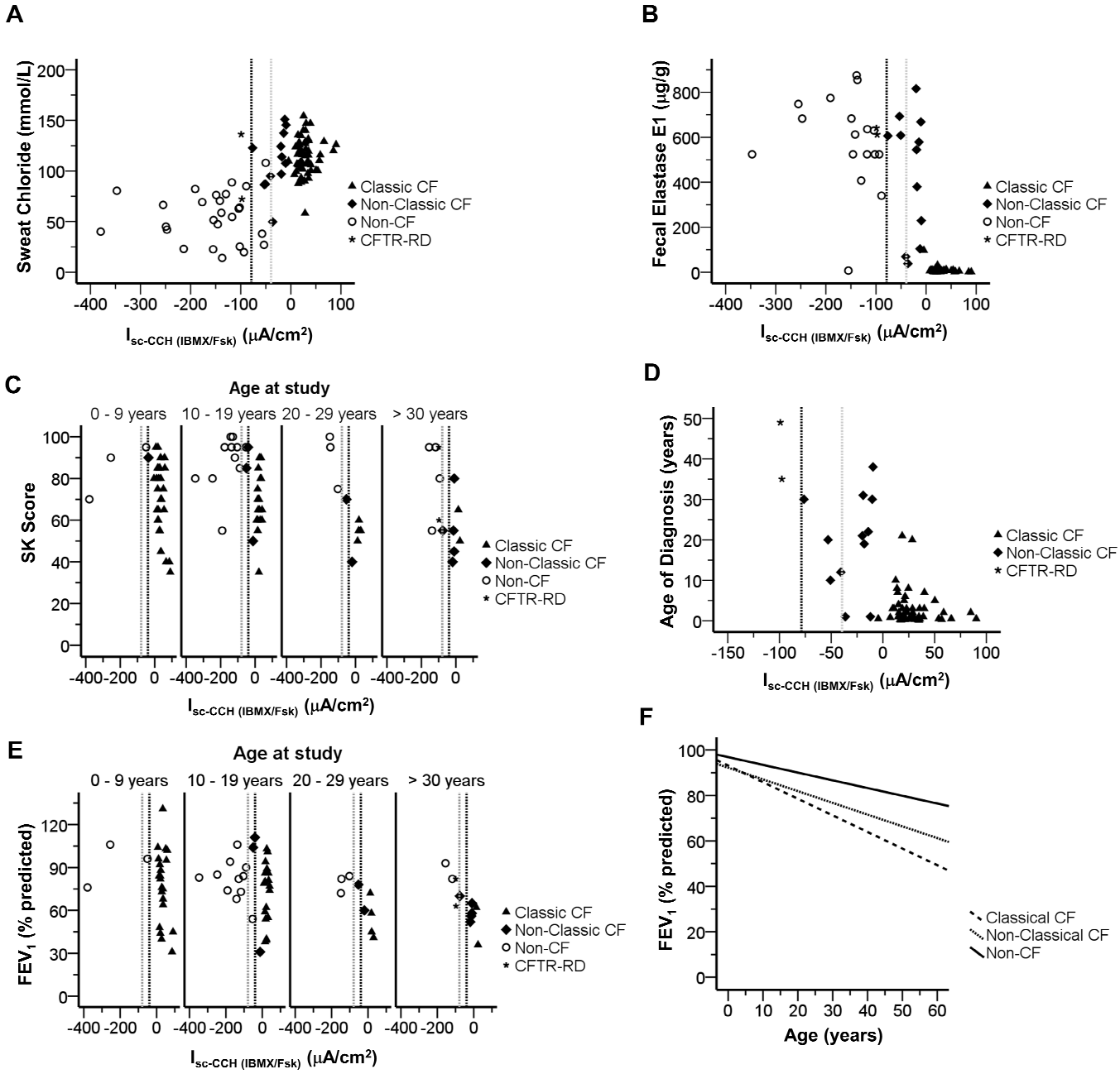


Figure III.1.2: Correlations between CF clinical features and CCH-induced short circuit currents following IBMX/Fsk application ($I_{sc\text{-}CCH(IBMX/Fsk)}$). Scatter-plot summarizing the distribution of $I_{sc\text{-}CCH(IBMX/Fsk)}$ against **(A)** sweat [Cl^-] (mmol/l); **(B)** FEE concentrations (µg/g of stools); **(C)** SK clinical scores distributed by groups of ages; **(D)** age at diagnosis (in years); and **(E)** FEV_1 distributed by groups of ages, for the individuals included in the study showing conclusive results (n=113) and classified according to CF Clinical Diagnosis Consensus Guidelines as: Classic CF (filled triangles, n=55); Non-Classic CF (filled diamonds, n=12); CFTR-RD (star, n=2); and non-CF (open circles, n=26). Vertical dashed black line represents subtraction of one STD of the mean value calculated $I_{sc\text{-}CCH\ (IBMX/Fsk)}$ (ΔI_{sc}=-78.77 µA/cm²) in non-CF controls ("grey zone"). Vertical dotted grey line represents addition of one STD of the mean value calculated for $I_{sc\text{-}CCH(IBMX/Fsk)}$ (ΔI_{sc}=-39.55 µA/cm²) in reference sub-group of Non-Classic CF patients. **(F)** Mixed regression model for decline rates in *FEV_1 vs. Age* (n=232 measurements) for Classic CF (y=93.15–0.73x), Non-Classic CF (y=92.23–0.52x), and Non-CF (y=96.86–0.34x) groups.

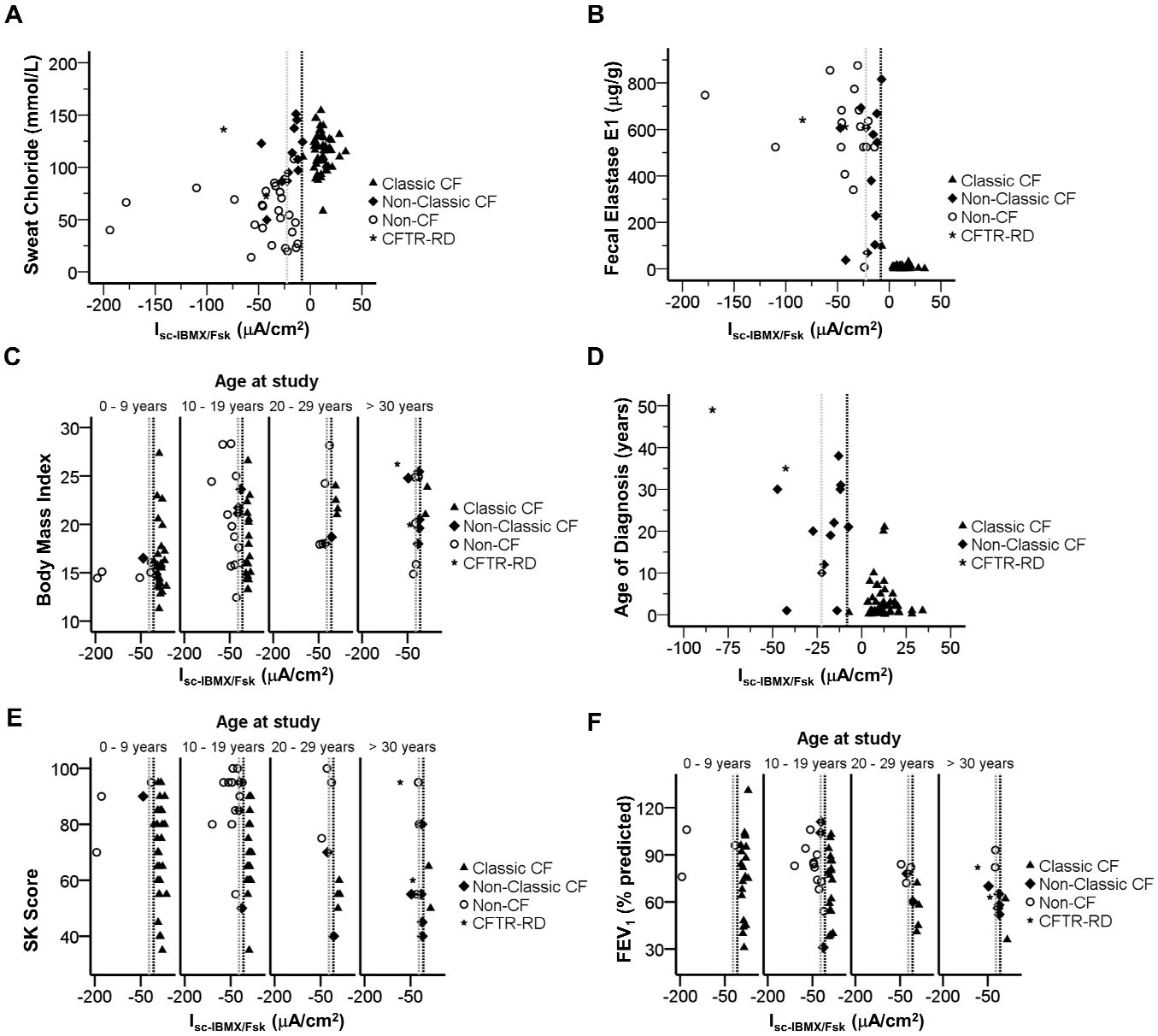


Figure III.1.3: Correlations between CF clinical features and IBMX/Fsk-induced short circuit currents ($I_{sc\text{-}IBMX/Fsk}$). Scatter-plot summarizing the distribution $I_{sc\text{-}IBMX/Fsk}$ against **(A)** sweat [Cl^-] (mmol/l); **(B)** age at diagnosis (in years); **(C)** BMI distributed by groups of ages; **(D)** FEE concentrations (µg/g of stools); **(E)** SK clinical scores distributed by groups of ages; and **(F)** FEV_1 distributed by groups of ages, for the individuals included in the study showing conclusive results (n=113) and classified according to CF Clinical Diagnosis Consensus Guidelines as: Classic CF (filled triangles, n=55); Non-Classic CF (filled diamonds, n=12); CFTR-RD (star, n=2); and non-CF (open circles, n=26). Vertical dashed black line represents subtraction of one standard deviation (STD) of the mean value calculated for $I_{sc\text{-}IBMX/Fsk}$ in non-CF controls (ΔI_{sc} = -8.18 µA/cm²). Vertical dashed grey line represents addition of one STD of the mean value calculated for $I_{sc\text{-}IBMX/Fsk}$ in reference sub-group of Non-Classic CF patients (ΔI_{sc} = -22.60 µA/cm²).

Our data (Figures III.1.1 and 1.2 and Tables III.1.2) show high correlations between CFTR-mediated Cl^- secretion values and these parameters describing CF phenotypes. Indeed, patients with absence/very low CFTR-mediated Cl^- secretion (≤5%) had higher sweat-Cl^- values (114.23±2.33 mmol/l), lower FEE concentrations (9.08±2.04 µg/g), earlier age at diagnosis (2.7±0.6 yrs), lower SK scores ($mean_1$ = 71±3, $mean_2$ = 69±3; $mean_3$ = 56±2; $mean_4$ = 58±8), and lower FEV_1 ($mean_1$ = 77±6; $mean_2$ = 72±5; $mean_3$ = 55±5; $mean_4$ = 49±13) than individuals with normal values of CFTR-mediated Cl^- secretion (Table III.1.3). Moreover, CF patients with residual values of CFTR-mediated Cl^- secretion had intermediate values in those parameters, namely: sweat-Cl^- values (109.80±8.34 mmol/l); FEE concentrations (444.57±78.05 µg/g), age at diagnosis (19.6±3.4 yrs), SK scores ($mean_1$ = 90; $mean_2$ = 77±14; $mean_3$ = 63±12; $mean_4$ = 55±7); and FEV_1 ($mean_1$ = n.a.; $mean_2$ = 82±26; $mean_3$ = 61±9; $mean_4$ = 60±3) (Table III1.3). Moreover, patients with classic CF and ≤5% CFTR-mediated Cl^- secretion consistently presented faster decline rates of pulmonary function (Cleveland et al., 2009; Schaedel et al., 2002) (Fig.III.1.1-E, dashed line), than Non-Classic CF patients retaining residual (Fig.III.1.1-E, dotted line) or normal CFTR functions (Fig.III.1.1-E, solid line, p=0.001 by Kruskal-Wallis test). Regarding BMI, only a modest trend was observed for lower BMI values in CF patients with absence of Cl^- secretion, not related to age differences (Figure III.1.2-C and Table III.1.2).

Next, we analysed the distribution of other clinical features (MI, nasal polyposis, diabetes/GI, osteopenia/osteoporosis) and also presence of lung pathogens in the three groups under study. Significant differences (Table III.1.4) were found between the groups under study for the distribution of lung pathogens (p = 1.75 x 10^{-6}, 88% of CF patients with pathogens), with predominance of Pa, and MI (p = 0.0015, 24% of CF patients with MI). Altogether, these data indicate that our approach to measure the level of CFTR (dys)function in rectal biopsies provides data evidencing good correlation with the CF severity.

Table III.1.3 - Mean and standard error of the mean values for clinical parameters among the 3 groups of individuals analyzed in this study: Classic CF; Non-Classic CF and Non-CF.

	CF Clinical Diagnosis											
	Classic CF ($I_{sc\text{-}CCH(IBMX/Fsk)}$ = 28.68±2.22 µA/cm²; n=55)				**Non-Classic CF** ($I_{sc\text{-}CCH(IBMX/Fsk)}$ = -30.06±6.15 µA/cm²; n=12)				**Non-CF** ($I_{sc\text{-}CCH(IBMX/Fsk)}$ = -153.38±15.33 µA/cm²; n=28)			
Sweat Cl^- (mmol/l)	114.23 ± 2.33				109.80 ± 8.34				58.84 ± 5.38			
FE E1 (µg/g)	9.08 ± 2.04				444.57 ± 78.05				585.44 ± 44.99			
BMI	17.37 ± 0.52				21.15 ± 0.92				19.76 ± 0.92			
Age at diagnosis (yrs)	2.7 ± 0.6				19.6 ± 3.4				-			
Aged-grouped	*0-9 yrs*	*10-19 yrs*	*20-29 yrs*	*≥ 30 yrs*	*0-9 yrs*	*10-19 yrs*	*20-29 yrs*	*≥ 30 yrs*	*0-9 yrs*	*10-19 yrs*	*20-29 yrs*	*≥ 30 yrs*
FEV1 (% predicted)	77 ± 6	72 ± 5	55 ± 5	49 ± 13	-	82 ± 26	61 ± 9	60 ± 3	93 ± 9	81 ± 4	79 ± 4	80 ± 6
FVC (% predicted)	85 ± 6	79 ± 5	71 ± 8	69 ± 22	-	83 ± 17	69 ± 15	74 ± 4	108 ± 11	88 ± 4	83 ± 7	84 ± 8
SK score	71 ± 3	69 ± 3	56 ± 2	58 ± 8	90	77 ± 14	63 ± 12	55 ± 7	85 ± 8	88 ± 4	90 ± 8	80 ± 8

Table III.1.4 - Distribution of clinical features among the 3 groups of individuals analysed here: Classic CF; Non-Classic CF and Non-CF.

		CF Clinical Diagnosis			
		Classic CF ($I_{sc\text{-}CCH(IBMX/Fsk)}$ = 28.68±2.22 μA/cm²; n=55)	**Non Classic CF** ($I_{sc\text{-}CCH(IBMX/Fsk)}$ = -30.06±6.15 μA/cm²; n=12)	**Non-CF** ($I_{sc\text{-}CCH(IBMX/Fsk)}$ = -153.38±15.33 μA/cm²; n=28)	Total
Lung Pathogens (***p* = 1.75 x 10⁻⁶**)	Pa	7	3	1	**11**
	Pa + other pathogens	21	5	0	**26**
	Other pathogens	20	3	2	**25**
	Negative	2	0	7	**9**
	n.a.	5	1	18	**24**
Meconium Ileus (***p* = 0.0015**)	Positive	16	0	0	**16**
	Negative	39	12	26	**77**
	n.a.	0	0	2	**2**
Nasal Polyposis (*p* = 0.591)	Positive	10	4	6	**20**
	Negative	44	8	20	**72**
	n.a.	1	0	2	**3**
GI Diabetes (*p* = 0.303)	GI	5	1	0	**6**
	Diabetes	5	2	2	**9**
	GI/Diabetes	5	0	0	**5**
	Negative	39	9	23	**71**
	n.a.	1	0	3	**4**
Osteopenia Osteoporosis (*p* = 0.511)	Osteopenia	2	0	2	**4**
	Osteoporosis	6	3	2	**11**
	Negative	46	9	22	**77**
	n.a.	1	0	2	**3**

NOTE: n.a., Not analysed. p-values showing statistical differences among groups under study ($p < 0.05$) are highlighted (n = 95).

3.5 Evaluation of the best tool for discriminating CF patients from Non-CF individuals

Following the above findings, we attempted to establish a CF diagnosis tool which could also serve for disease prognosis. We thus used a stepwise discriminant analysis to evaluate which one(s) among the clinical and laboratory measurements available (sweat-Cl^-; FEE; BMI; SK score; FEV_1; $I_{sc\text{-}IBMX/Fsk}$; and $I_{sc\text{-}CCH(IBMX/Fsk)}$), constitutes the best discriminator factor between patients with Classic and Non-Classic CF and also between these groups and non-CF individuals (see **II. Materials & Methods** section).

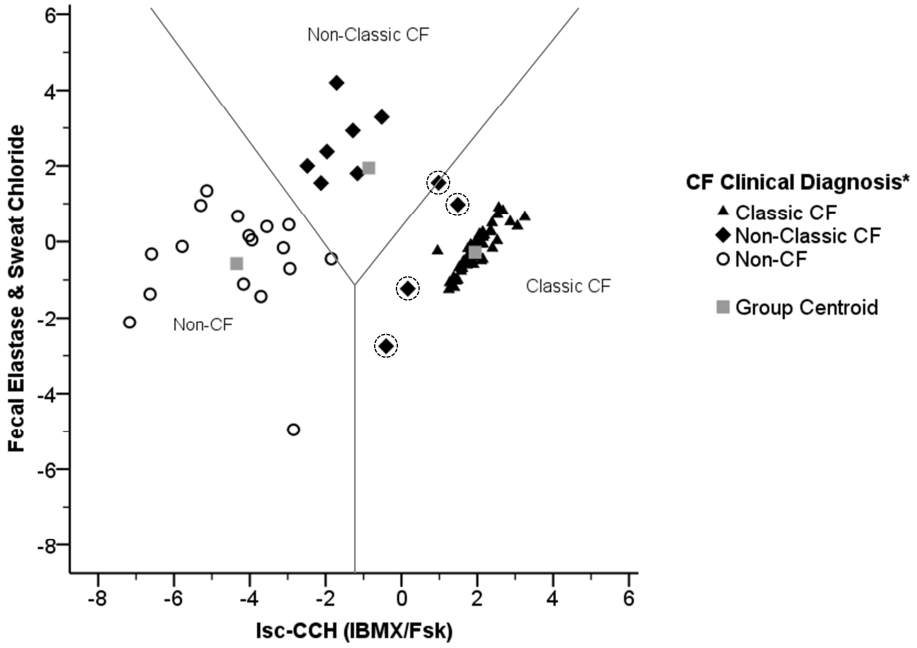


Figure 4. Distribution showing the relative position of each individual in study according to the scores obtained by discriminant analysis (territorial map)**.** Analysis performed for individuals having complete information about $I_{sc\text{-}CCH(IBMX/Fsk)}$, FEE concentration in stools and sweat-Cl^- (n=75): Classic CF (filled triangles, n=47); Non-Classic CF (filled diamonds, n=11); and non-CF (open circles, n=17). Misclassified cases are marked with dotted circles. Grey squares represent the group centroids and grey lines the barriers between each group.

As shown in Figure III.1.4, discriminant function 1 (x-axis) corresponding to CFTR-mediated Cl^- secretion measurements in rectal biopsies under CCH ($I_{sc\text{-}CCH(IBMX/Fsk)}$) is the best option to explain the differences between the three groups in 90.4% of cases (Tables III.1.5 and 1.6). Additionally, FEE concentration and sweat-Cl^- correspond to discriminant function 2 (y-axis) enabling separation of an additional ~6% of the remainder individuals (see **II. Materials & Methods** section, Tables VI.1.1 and 1.2). Notwithstanding, four

Non-Classic CF patients were still misclassified as Classic CF (Figure III.1.4, black diamonds with dotted circles). Furthermore, to translate this discriminant analyses into a diagnosis algorithm, we have obtained its respective Fisher's linear classification functions as (see **II. Materials & Methods** section, Tables VI.1.2):

Classic CF = 0.317(Sweat Chloride) + 0.005(Fecal Elastase) + 0.012($I_{sc\text{-}CCH(IBMX/Fsk)}$) –18.909

Non-Classic CF = 0.338(Sweat Chloride) + 0.028(Fecal Elastase) - 0.015($I_{sc\text{-}CCH(IBMX/Fsk)}$) –28.485

Non-CF = 0.202(Sweat Chloride) + 0.026(Fecal Elastase) - 0.102($I_{sc\text{-}CCH(IBMX/Fsk)}$) –23.414

These allow classification of a new CF suspicion case by replacing in these 3 equations the values from laboratory measurements obtained for a given individual. The function giving the highest value will correspond to the to the CF classification group best describing the individual.

4 Discussion

The wide spectrum of CF phenotypes, high variability of CF lung disease, the uncertain (dys)function of many rare CFTR mutations together with increasing numbers of asymptomatic patients identified in recent newborn CF screens, have posed major challenges to clinicians for the establishment of CF diagnoses and prognosis (Farrell et al. 2008; Boyle 2003; De Boeck et al. 2006). Such hurdles make it difficult for caregivers to provide adequate genetic counseling and medical care, risking worsening of symptoms and organ damage.

Good correlations between CFTR-mediated Cl^- secretion and CF parameters

To evaluate the robustness of colonic CFTR-mediated Cl^- secretion as a diagnosis/prognosis biomarker and thus help overcoming such difficulties, we assessed CFTR (dys)function *ex vivo* in 524 rectal biopsies from 118 individuals, including the largest cohort of CF patients ever analysed by this approach (n=51), a non-CF (control) group (n=18) and individuals with clinical

CF suspicion to confirm/ exclude a CF diagnosis (n=49). The functional data, demonstrating good correlations with most CF-defining parameters, have also provided key information to adjust the clinical judgment of a CF diagnosis and prognosis.

Our approach consists in direct measurements of colonic CFTR function assessed through both cAMP-dependent and cholinergic Cl^- secretion which, as previously shown (Mall et al. 1998a; Mall et al. 1998b; Mall et al. 2000), are strictly dependent on the presence of functional CFTR. Our data here show that CFTR-mediated Cl^- secretion is absent or present at almost undetectable levels (<5%) in 244 biopsies from patients with classic forms of CF (Figure III.1.1 and **VI. Appendix**), as defined by consensus criteria (Rosenstein & Cutting 1998; Farrell et al. 2008; De Boeck et al. 2006) including: early age at diagnosis (2.7±0.6 yrs), very high sweat-Cl^- (114.23±2.33 mmol/l) and PI (9.08±2.04 μg/g) (Figures III.1.2 and 1.3 and Table III.1.3). This group of Classic CF patients also presented other severe CF symptoms, like MI (29%), associated diabetes and/or GI (27%), lung pathogens (87%) and also hepatic involvement (29%) (Table III.1.4 and **VI. Appendix**). In contrast, a group of patients with milder symptoms, classified as Non-Classic CF by established guidelines (Rosenstein and Cutting 1998; Farrell et al. 2008; De Boeck et al. 2006), evidenced residual CFTR-mediated Cl^- secretion (10 to 57%), consistently with our previous data (Hirtz et al., 2004). Indeed, most of patients in this group were diagnosed at an older age (19.6±3.4 yrs) and were PS (444.57±78.05 μg/g) (Figures III.1.2 and 1.3 and Table III.1.3). Additionally, by stratifying patients into four different age groups, our functional data also showed good correlations with lung function (FEV_1) and SK scores, where patients with highest values evidenced residual colonic CFTR-mediated Cl^- secretion (Table III.1.2, r =-0.301 and -0.411, respectively).

Comparison with other bioelectric methods

By applying a different Ussing chamber protocol to bioelectric measurements in rectal biopsies, other authors used an overall parameter resulting from a combination of different readouts, some relying on indirect activation of CFTR and thereof proposed a cut-off value (Derichs et al., 2010). Yet, application of such protocol in another study reported a classic CF patient evidencing readout values higher than the cut-off (van Barneveld et al., 2010). So, it is our conviction that such cut-off value cannot be clearly established for

usage among different laboratories (Derichs et al., 2010; van Barneveld et al., 2010). Moreover, several technical aspects differed between the current and such protocol. Firstly, here we use bicarbonate (HCO_3^-)-free buffer solutions to exclude a possible contribution of electrogenic HCO_3^- secretion to lumen-negative V_{te}/I_{sc} (Hirtz et al. 2004; Pratha et al. 2000; Mall et al. 2004b). Secondly, our measurements evidence stable baselines in contrast to the drifting baselines reported in those studies where agonist responses have to be determined from estimated baselines (van Barneveld et al., 2010). Thirdly, our readout is based on direct CFTR-mediated Cl^- secretion under complete inhibition of endogenous cAMP (Mall et al. 2004a; Hirtz et al. 2004; Mall et al. 1998b; Mall et al. 2000; Mall et al. 1998a; Mall et al. 2004b), instead of complex bioelectric responses involving two different Ca^{2+}-dependent agonists and an anion-transporter inhibitor following incomplete prostaglandin inhibition (Derichs et al. 2010; van Barneveld et al. 2006; van Barneveld et al. 2010). Our protocol has the major advantage of using continuous perfusion which allows for pairwise examination of agonists. Altogether, these characteristics, together with the demonstration of its extreme sensitiveness (accurate calculation of CFTR activity down to <5%, see **VI. Appendix**), and high reproducibility (very stable baseline currents during the >2h course of the experiments and similar recordings in different biopsies from the same individual, Figure III.1.5) make the current approach a superior contribution for CF diagnosis and prognosis (Figure III.1.4).

Another technique to support a CF diagnosis is NPD measurements. The outcome of this approach, however, relies on a composite score including both ENaC- and CFTR-mediated responses (Knowles et al. 1981; Ho et al. 1997; Walker et al. 1997; Wallace et al. 2003; Standaert et al. 2004; Sermet-Gaudelus et al. 2010), where two abnormal NPD recordings on separate days evidence CFTR dysfunction (Rosenstein and Cutting 1998; Farrell et al. 2008). The technique, however, has variable applicability for adults and children (Sermet-Gaudelus et al. 2010). Moreover, this *in vivo* procedure requires patient immobilization and sedation (for children <6yrs) for at least ½h (Sermet-Gaudelus et al. 2010), while the rectal biopsing only takes ~10 min allowing for multiple *ex vivo* measurements of different samples from the same individual and lead to conclusive results (96% here) for a highest percentage of individuals than NPD (91% (Sermet-Gaudelus et al. 2010)). Moreover, NPD values considerably overlap among from CF patients, carriers and non-CF

controls (Sermet-Gaudelus et al. 2010; Ho et al. 1997; Walker et al. 1997; Wallace et al. 2003). This may be due to the fact that NPD is not a truly quantitative measurement but relies on the pure measurement of voltages.

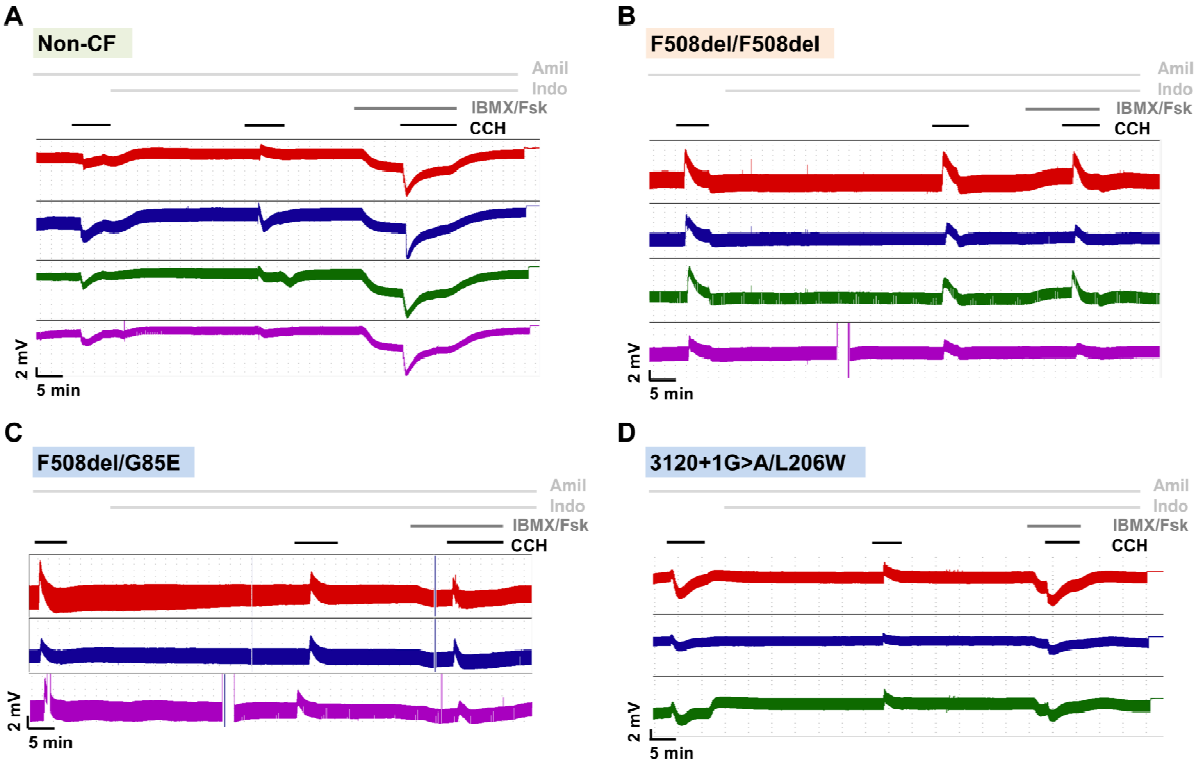


Figure III.1.5 – Original recordings of transepithelial voltage (V_{te}) measurements in Ussing chambers obtained in 3-4 rectal biopsies from the same individual evidencing the high reproducibility of the method. Rectal biopsies from **(A)** Non-CF individual showing large cholinergic (carbachol, CCH, 100 µM, basolateral) and cAMP-dependent (3-isobutyl-1-methylxantine, IBMX, 100 µM, and forskolin, Fsk, 2µM, basolateral) Chloride (Cl^-) secretion (lumen-negative responses); **(B)** CF patient homozygous for F508del-CFTR mutation with absence of Cl^- secretion (only lumen-positive responses, reflecting potassium (K^+) secretion, were observed); **(C)** CF patient (genotype: F508del/G85E-CFTR) showing very little (~12%) cAMP-dependent Cl^- secretion (biphasic responses observed upon co-cholinergic stimulation with CCH); and **(D)** CF patient (genotype: 3120+1G>A/L206W-CFTR) presenting larger CFTR residual function (~57%) and milder phenotype than in **(C)**. All the experiments were performed in the presence of Amiloride (Amil, 20 µM, luminal) and Indomethacin (Indo, 10 µM, basolateral). Transepithelial resistance (R_{te}) was determined from V_{te} deflections obtained by pulse current injection (0.5 µA).

Validation as a biomarker for CF diagnosis / prognosis

Importantly, we show here how assessment of colonic CFTR function was a key tool to exclude CF in 28 individuals (2 of classified as CFTR-RD patients). In fact, despite that the clinical features of these individuals suggested CF, both genotyping and sweat test were inconclusive (**VI.**

Appendix). This leads us to conclude of the importance of this approach for patients in the "grey" zone (Figure III.1.2 and 1.3) for the establishment/ exclusion of a final diagnosis of CF or CFTR-RD.

Interestingly, our data are also highly informative to correlate CFTR function with *CFTR* genotypes (Figure III.1.6). For instance, we were able to detect very low function (~5% CFTR function vs non-CF controls) in a patient bearing S549R, recently described as Class III (Yu et al., 2012), whereas significant CFTR-mediated Cl^- secretion was found in two patients bearing 4428insGA and D1152H (84 and 64%, respectively). Thus, we classify the former here as CF-DC and the two latter as CF-RD mutations.

In the present study, we have also used a statistical discriminant analysis to identify which clinical and functional parameter(s) better reflects the differences between Classic CF, Non-Classic CF and Non-CF groups for prognosis. Results show that $I_{sc\text{-}CCH(IBMX/Fsk)}$ measurements are more discriminative than $I_{sc\text{-}IBMX/Fsk}$, evidencing the highest discriminant power (90.4%) among the three groups studied. The second and third parameters with higher discriminant power are FEE and sweat-Cl^- concentration, respectively. Using such analysis (Figure III.1.4), one can for example predict that individuals in the "Non-Classic CF" group with values lying closer to the "Non-CF" cluster, have better prognosis than those with values closer to the "Classic CF" cluster. In fact, the only four cases with "Non-Classic CF" which were misclassified as "Classic CF" by this analysis, correspond to individuals with moderate PI and/or low levels of CFTR function. It may, thus be expected that these four patients develop severe CF earlier than most Non-Classic ones. Interestingly, and corroborating such prediction among these four, there is a 19-year old patient with the F508del/G85E genotype, moderate PI (FEE=103.89 μg/g) and ~12% of CFTR function (Figure III.1.5-C and **VI. Appendix**) who recently (last 1½ yr) started to progress from a relatively mild to moderate-severe lung disease, with five pulmonary exacerbations plus surgery to remove nasal polyps associated with a strong sinusitis.

Recently, we demonstrated that the current approach may be used in pre-clinical assessment of therapeutic compounds efficacy directly on native tissues (Roth et al., 2011) and similarly it may be used to identify CF patients (and CFTR mutations) who will respond to innovative therapeutic strategies, namely those aimed at increasing the residual CFTR activity and already

approved for clinical use for other mutations towards a predictive personalized-medicine approach (Accurso et al., 2010; Hirtz et al., 2004; Ramsey et al., 2011; Roth et al., 2011; Shah, 2011; Van Goor et al., 2009). For example, the patient with S549R could be tested *ex vivo* for the correction with the FDA-approved potentiator Ivacaftor, as suggested by the *in vitro* data (Yu et al., 2012). Moreover, our data also indicate that this approach, together with sweat-Cl^- is probably the best biomarker in clinical trials aimed at modulating CFTR (Accurso et al., 2010; Clancy et al., 2011).

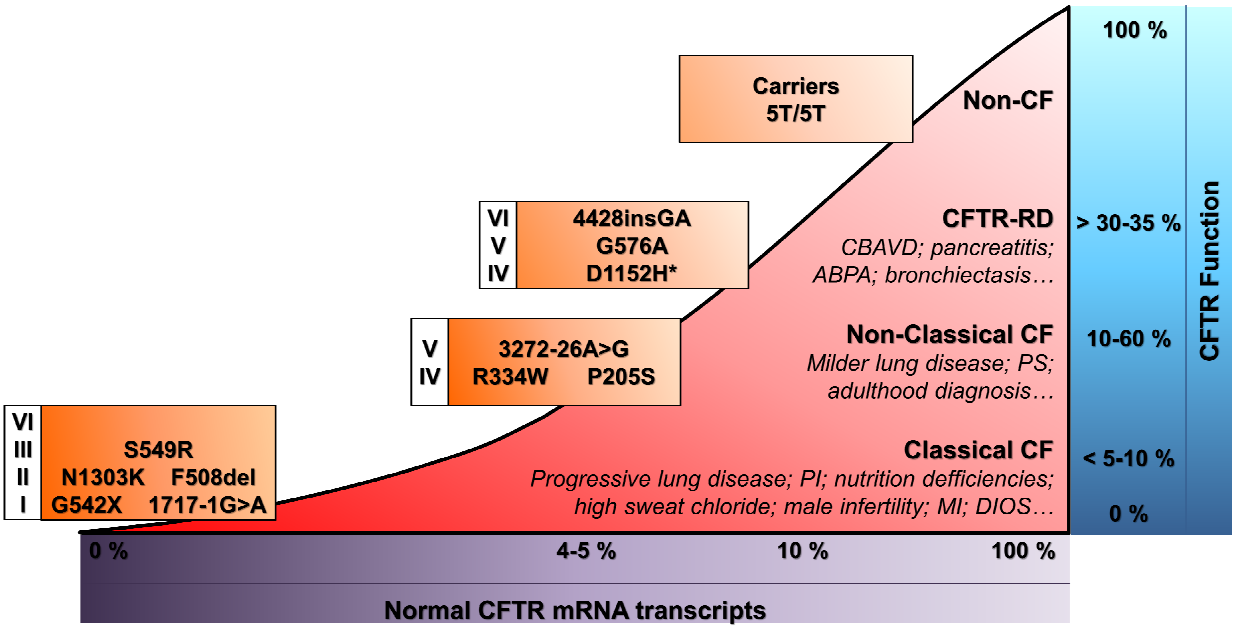


Figure III.1.6 - Association of CF clinical phenotypes with the levels of normal CFTR mRNA transcripts, CFTR function (as percentage of maximal CFTR activation normalized to control group) **and CFTR mutations** (grouped by functional classes of mutations) with examples found in this study. Indicated values are speculative. *D1152H may belong either to CFTR-RD or CFTR-disease causing mutations. CBAVD, Congenital Bilateral Absence of the Vas Deferens; ABPA, Allergic Bronchopulmonary Aspergillosis; PS, Pancreatic Sufficiency; PI, Pancreatic Insufficiency; MI, Meconium Ileus; DIOS, Distal Intestinal Obstruction Syndrome.

What is the functional CFTR threshold to avoid CF?

Finally, in an attempt to further answer the old question "how much functional CFTR would be enough to avoid CF?" (Amaral, 2005) we have put together the current and previous findings including those concerning mRNA levels to identify the threshold for CF (Amaral et al., 2001; Beck et al., 1999; Hirtz et al., 2004; Ramalho et al., 2002) (Figure III1.6). Indeed, we previously showed that ~5% of normal *CFTR transcripts* (relative to non-CF individuals) is

sufficient to attenuate CF severity (Amaral et al., 2001; Beck et al., 1999; Ramalho et al., 2002). From our current and previous data, we propose that CFTR values above ~10% of normal CFTR *function* are required for a better CF prognosis. Indeed, CF patients with CFTR function below 10% evidence more severe CF (see **VI. Appendix**). Moreover, non-CF controls and individuals for whom a CF diagnosis was discarded show CFTR-mediated Cl^- secretion ≥30-35%, as before (Hirtz et al., 2004).

Altogether, our current approach to measure CFTR-mediated Cl^- secretion in rectal biopsies is demonstrated here to be a sensitive, reproducible and robust predictive biomarker for the diagnosis/prognosis of CF. Moreover this method has very high potential for (pre-)clinical trials of innovative therapeutic approaches involving CFTR-modulators.

Chapter 2 **Rectal Biopsies Procedure in Cystic Fibrosis: Technical Aspects and Patients Perspective for Clinical Trials Feasibility**

(Manuscript submitted to *J Gastroenterology*,
with minor alterations).

1 Summary

Measurements of CFTR function in rectal biopsies *ex vivo* have been used for diagnosis and prognosis of Cystic Fibrosis (CF) disease. Here, we aimed to evaluate the technical aspects of this procedure regarding: (i) viability of the rectal specimens for ex vivo bioelectrical and biochemical laboratory analyses; and (ii) overall assessment (comfort, invasiveness, pain, sedation requirement, etc) of the rectal biopsy procedure from the patients perspective to assess its feasibility as an outcome measure in (pre-)clinical trials.

Here, we compared three bowel preparation solutions (NaCl 0.9%, glycerol 12%, oral mannitol), and two biopsy forceps (standard and jumbo) in 580 rectal specimens from 132 individuals (CF and non-CF). Assessment of the overall rectal biopsy procedure by patients, was carried out by telephone surveys to 75 individuals who underwent the procedure.

Our data shows that integrity and friability of the tissue specimens correlate with their transepithelial resistance (r=-0.438 and -0.305, respectively) and are influenced by the bowel preparation solution and biopsy forceps used, being NaCl and jumbo forceps the most compatible methods with the electrophysiological analysis. The great majority of the individuals (76%) did not report major discomfort due to the short procedure time (max 15 min) and considered it relatively painless (79%). Importantly, most (53%) accept repeating it four more times.

Obtaining rectal biopsies with a flexible endoscope and jumbo biopsy forceps after bowel preparation with NaCl isotonic solution is the recommended procedure to obtain viable specimens for CFTR bioelectrical/biochemical analyses. The procedure is well tolerated by patients, demonstrating its feasibility as an outcome measure in (pre-)clinical trials.

Keywords: quality control; patient comfort; forceps; outcome measures clinical trial.

2 Introduction

Cystic Fibrosis (CF), the most common severe autosomal recessive disease of Caucasian populations, is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene encoding a chloride (Cl^-) channel expressed at the apical membrane of epithelial cells, a major regulator of salt and water transport in epithelia (Rich et al., 1990). CF is dominated by respiratory disease but other organs are also affected including the pancreas, intestine and sweat gland as well as male reproductive tract (Collins, 1992).

Although the clinical diagnosis of classic (severe) forms of CF is straightforward, for other patients there is wide variability in the clinical presentation and organ involvement, thus making the CF diagnosis more challenging (Rosenstein & Cutting 1998; Farrell et al. 2008; Wallis 2003; Paranjape & Zeitlin 2008; Boyle 2003). Moreover, increasing numbers of asymptomatic patients are currently identified through extended programs of CF newborn screening (Farrell et al. 2008; De Boeck et al. 2006; Taylor et al. 2009; Com et al. 2010; Sermet-Gaudelus et al. 2010).

One of the most useful laboratory parameters used for the diagnosis and prognosis of CF, is *ex vivo* assessment of CFTR-mediated Cl- secretion channel in freshly collected rectal biopsies (De Boeck et al. 2006; Taylor et al. 2009; Hirtz et al. 2004; Mall et al. 2004a; Sousa et al. 2012; de Jonge et al. 2004; Derichs et al. 2010), which we have shown to constitute a sensitive, reproducible and robust predictive biomarker for the diagnosis and prognosis of CF disease (Hirtz et al., 2004; Sousa et al., 2012).

Moreover, ongoing clinical trials of novel therapeutic CFTR-modulators require improved and robust biomarkers to adequately assess their *in vivo* efficacy on CFTR. Indeed, there is also great potential to exploit this method to pre-clinically assess compound efficacy directly on human tissues *ex vivo*, as we previously showed (Roth et al., 2011) or as a biomarker in clinical trials of novel CFTR-modulators (Accurso et al., 2010; Clancy et al., 2011; Shah, 2011). Moreover, it may even be used to evaluate patient/CFTR genotype responsiveness to a drug through a personalized-medicine approach.

For such disseminated usage of this method, standardized operational procedures (SOP) for bowel preparation and biopsing are essential to ensure

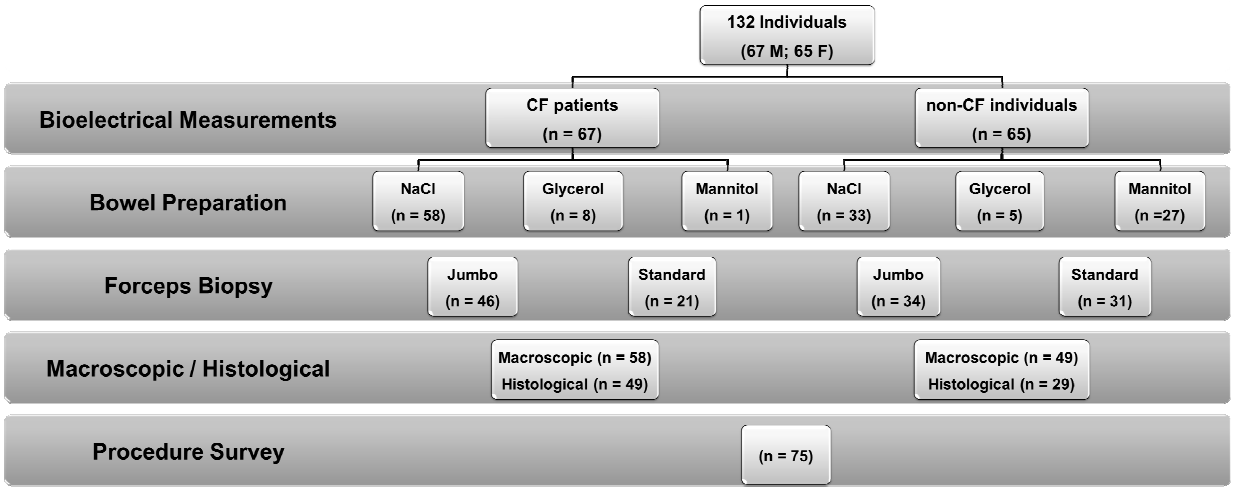
good tissue viability for the quantitative assessment of the bioelectric parameters (Mall et al. 2004a). Moreover, since the procedure involves biopsing, a somewhat invasive procedure possibly triggering psychological rejection, there should be clear information on how it is perceived from the patients' perspective (comfort, invasiveness, pain, sedation requirement, etc) to obtain an overall assessment of the method.

Our aim here was to evaluate (a) the technical procedure regarding the quality for bioelectrical/ biochemical laboratory analyses of 580 rectal specimens from 132 individuals (CF and non-CF), namely by comparing different bowel preparations and different biopsy forceps; and (b) to determine individuals' assessment regarding the rectal biopsy procedure feasibility to be possibly used as an outcome measure in (pre-)clinical trials, as described in other studies (Anton et al., 2011; McGowan et al., 2007).

Our results demonstrate that best tissue viability for Ussing chamber measurements results after bowel preparation with isotonic solution (NaCl 0.9%) and usage of jumbo (*vs* standard size) biopsy forceps allowing collection of larger specimens without tissue integrity. Data collected on patient's comfort show that the great majority of individuals (76%) did not report major discomfort due to the short procedure time (max 15 min) and considered it relatively painless (79%), regardless of sedation. Moreover, most individuals accept repeating the procedure another four times (53%), supporting the feasibility of the current approach as an outcome measure in (pre-)clinical trials.

3 Results

A flow-chart summarizing the technical biopsing aspects assessed in



the present study is shown in Figure III.2.1.

Figure III.2.1: Flow-chart of the technical biopsing aspects assessed in the present study. Bioelectrical measurements were performed for rectal biopsies (n = 580) from all the individuals enrolled in the study (n = 132) to assess tissue viability(Sousa et al., 2012). Bowel preparation included enemas of either NaCl 0.9%, glycerol 12% (v/v) or oral mannitol 20% (w/v) solutions. Two different biopsy forceps were tested, namely jumbo (3.4mm Ø) and standard (2.5mm Ø), independently of bowel preparation. Macroscopic and histologic evaluation of rectal biopsies was achieved for 107 and 78 individuals, respectively. Patient assessment surveys were carried out for 75 individuals undergoing sigmoidoscopy with rectal biopsy collection, divided into 4 age groups, namely (yrs): 0-9; 10-9; 20-29; ≥30.

3.1 Bowel preparation and biopsy forceps

The bowel cleaning procedures, consisting in administration of an oral laxative (for colonoscopy) or by enema (for sigmoidoscopy) allowed equally good visualization of the rectal mucosa and forceps during the sigmoidoscopy/ colonscopy procedure. Expectedly, jumbo biopsy forceps (3.4mm Ø) generated larger specimens than standard forceps (2.5mm Ø), thus facilitating the mounting of the tissue in Ussing chamber inserts.

Data referring to bowel preparation (Tables III.2.1 and 2.2) show that there are statistically significant differences (p=0.054, 90% confidence interval) for tissue integrity between the preparations using NaCl 0.9% (1.19±0.12) and

glycerol 12% (1.82±0.21). Oral mannitol was not statistically different from isotonic saline, independently of the biopsy forceps used (Table III.2.1).

Table III.2.1 – Summary of macroscopic evaluation data and bioelectrical measurements (R_{te}) of rectal biopsies *vs.* bowel preparation and biopsy forceps.

Bowel Preparation		**NaCl 0.9%**		**Glycerol 12%**		**Mannitol 20%**	
Biopsy Forceps		**Jumbo**	**Standard**	**Jumbo**	**Standard**	**Jumbo**	**Standard**
Tissue integrity		1.00±0.11	2.00±0.35	1.48±0.31	2.21±0.23	1.02±0.02	1.97±0.30
Friability		1.23±0.12	1.36±0.24	1.50±0.31	1.81±0.22	1.13±0.33	1.33±0.25
Bleeding		1.01±0.08	1.21±0.18	1.27±0.21	1.16±0.29	1.05±0.22	0.83±0.32
Mucus		1.12±0.11	1.18±0.17	0.91±0.24	0.88±0.13	0.74±0.13	1.06±0.13
Sub-mucosa (n)	Yes	16	3	3	1	2	1
	No	42	9	4	4	12	10
R_{te} ($\Omega.cm^2$)		21.82±1.03	14.37±1.21	18.75±2.66	13.57±2.70	18.69±0.98	13.32±1.11

NOTE: Results are mean ± SEM; n = 107.

Table III.2.2 – Summary of macroscopic evaluation data and bioelectrical measurements (R_{te}) of rectal biopsies *vs.* bowel preparation.

	NaCl 0.9%	**Glycerol 12%**	**Mannitol 20%**
Tissue integrity	1.19 ± 0.12	1.82 ±0.21	1.55 ±0.21
Friability	1.26 ± 0.10	1.65 ± 0.19	1.24 ± 0.19
Bleeding	1.04 ± 0.07	1.22 ± 0.17	0.99 ± 0.18
Mucus	1.13 ± 0.09	0.89 ± 0.14	0.88 ± 0.10
R_{te} ($\Omega.cm^2$)	19.12 ± 0.87	16.36 ± 1.96	16.20 ± 0.89

NOTE: Results are mean ± SEM; n = 107. ANOVA analyses indicates statistical differences between distribution of among the three groups for bowel preparation (p=0.039). Bonferroni post hoc test shows that difference is between NaCl 0.9% and Glycerol 12% (p=0.054; 90% confidence interval).

Regarding biopsy forceps, mean values for tissue integrity (Jumbo=1.07±0.09 *vs* Standard=2.04±0.18) and also for R_{te} (Jumbo=20.97±0.81 $\Omega.cm^2$ *vs* Standard=14.01±0.86 $\Omega.cm^2$) are also statistically different for the isotonic saline and glycerol procedures ($p=5.51x10^{-7}$ and $p=8.42x10^{-8}$, respectively, Tables III.2.1 and 2.3). Friability (i.e. tissue susceptibility to breakdown with manipulation) is the only

parameter significantly affected by the presence of sub-mucosa (Table III.2.4, p=0.008), but this may result from more tissue manipulation being required to remove the sub-mucosa. Regarding bleeding and mucus, their presence/abundance are not influenced by neither bowel preparation nor biopsy forceps (Table III.2.1).

Table III.2.3 – Summary of macroscopic evaluation data and bioelectrical measurements (R_{te}) of rectal biopsies *vs.* biopsy forceps.

	Jumbo	**Standard**
Tissue integrity	1.49 ± 0.16	1.32 ±0.12
Friability	1.65 ± 0.18	1.18 ± 0.09
Bleeding	1.15 ± 0.14	1.01 ± 0.07
Mucus	1.20 ± 0.16	0.97 ± 0.06
R_{te} ($\Omega.cm^2$)	20.11 ± 1.45	18.95 ± 0.91

NOTE: Results are mean ± SEM; n=107. There are significant differences in tissue integrity ($p=5.51x10^{-7}$) and also between means of R_{te} ($p=8.42x10^{-8}$) for biopsy forceps.

Table III.2.4 – Summary of macroscopic evaluation data and bioelectrical measurements (R_{te}) of rectal biopsies *vs.* presence of sub-mucosa.

	Sub-mucosa presence	
	Yes	**No**
Tissue integrity	1.49 ± 0.16	1.32 ±0.12
Friability	1.65 ± 0.18	1.18 ± 0.09
Bleeding	1.15 ± 0.14	1.01 ± 0.07
Mucus	1.20 ± 0.16	0.97 ± 0.06
R_{te} ($\Omega.cm^2$)	20.11 ± 1.45	18.95 ± 0.91

NOTE: Results are mean ± SEM; n = 107. Statistically significant differences were found between mean of friability regarding the presence of sub-mucosa (p=0.008).

In addition, we also found statistically significant correlations (Figure III.2.2 and Table III.2.5) between R_{te} and tissue integrity (r=-0.438, $p=8.51x10^{-5}$) and between R_{te} and friability (r=-0.305, $p=6.65x10^{-3}$), which is supported by partial correlations with bowel preparation and biopsy forceps. Data concerning presence/abundance of blood and mucus do not correlate with tissue viability-R_{te} (Figure III.2.2 and Table III.2.5).

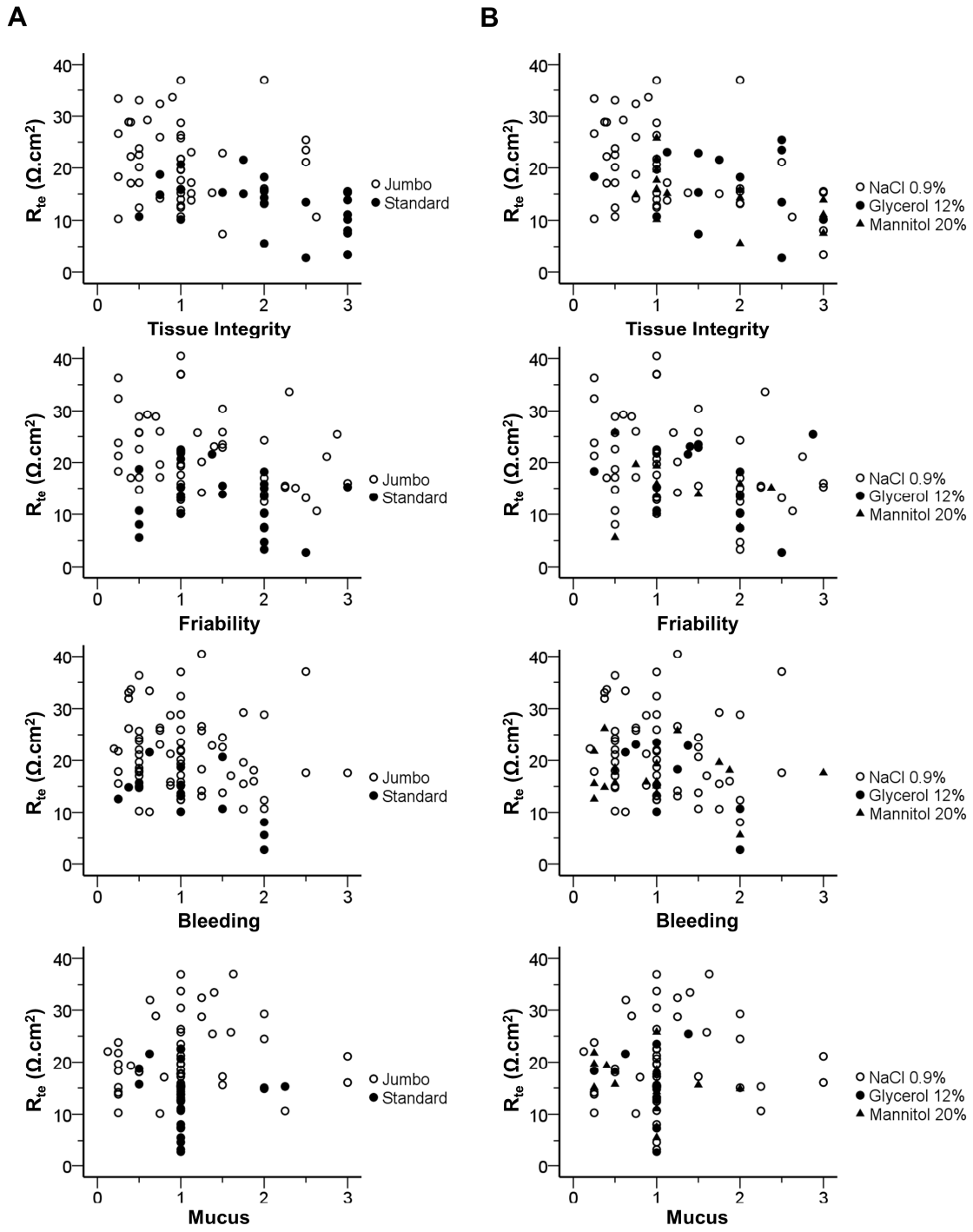


Figure III.2.2: Correlations between tissue transepithelial resistance (R_{te}) and macroscopic descriptors (tissue integrity, friability, bleeding and mucus) according to **(A)** biopsy forceps and **(B)** bowel preparation (n = 107 individuals).

Table III.2.5 – Summary of the correlations and partial correlations (by bowel preparation and biopsy forceps) between tissue transepithelial resistance (R_{te}) and macroscopic evaluation of biopsies.

	Transepithelial resistance (R_{te})	
	Pearson (*r*)	**p-value**
Tissue integrity	**-0.438**	**8.51×10^{-5}**
Bowel preparation	**-0.403**	**3.74×10^{-4}**
Biopsy forceps	**-0.244**	**0.036**
Friability	**-0.305**	**6.65×10^{-3}**
Bowel preparation	**-0.301**	**7.88×10^{-3}**
Biopsy forceps	**-0.277**	**0.015**
Bleeding	-0.176	0.110
Bowel preparation	-0.185	0.093
Biopsy forceps	-0.182	0.100
Mucus	+0.078	0.507
Bowel preparation	+0.032	0.787
Biopsy forceps	+0.115	0.330

NOTE: Pearson (*r*) and p-values indicating significant correlations are highlighted (n = 107).

3.2 Histological and macroscopic evaluation of rectal tissues and biochemical analysis

Histological examination of rectal specimens revealed no obvious abnormalities and only some inflammatory cells were found, independently of both tissue origin (CF or non-CF) or bowel preparation (Figure III.2.3-A).

Tissues were evaluated for integrity, friability, bleeding, mucus, and thickness of biopsy (presence of sub-mucosa or not) under a dissecting microscope by two different technicians in blinded way (Table III.2.1). As shown above, the scores obtained from this evaluation were correlated with bowel preparation, biopsy forceps and measurements of transepithelial resistance (R_{te}) to assess tissue viability (Table III.2.5).

We have also used these native colonic tissues to further look at maturation pattern and localization of wt- and mutant-CFTR protein, which can serve as a valuable tool to evaluate the effects of CFTR-modulators together with Ussing chamber measurements of CFTR-mediated Cl^- secretion. As shown in Figure III.2.3-B, we were able to detect CFTR protein at the

plasma membrane for both wt-CFTR and F508del/P205S-CFTR (P205S is a class IV mutation), but F508del/F508del-CFTR was retained into cytoplasm, close to the basolateral membrane. These findings were in accordance with the maturation pattern of CFTR protein (Figure III.2.3-C), which was found to be fully-glycosylated (presence of band C) for wt-CFTR and another class IV CFTR-mutant (R334W), but failed to mature in the biopsy from a F508del/F508del patient.

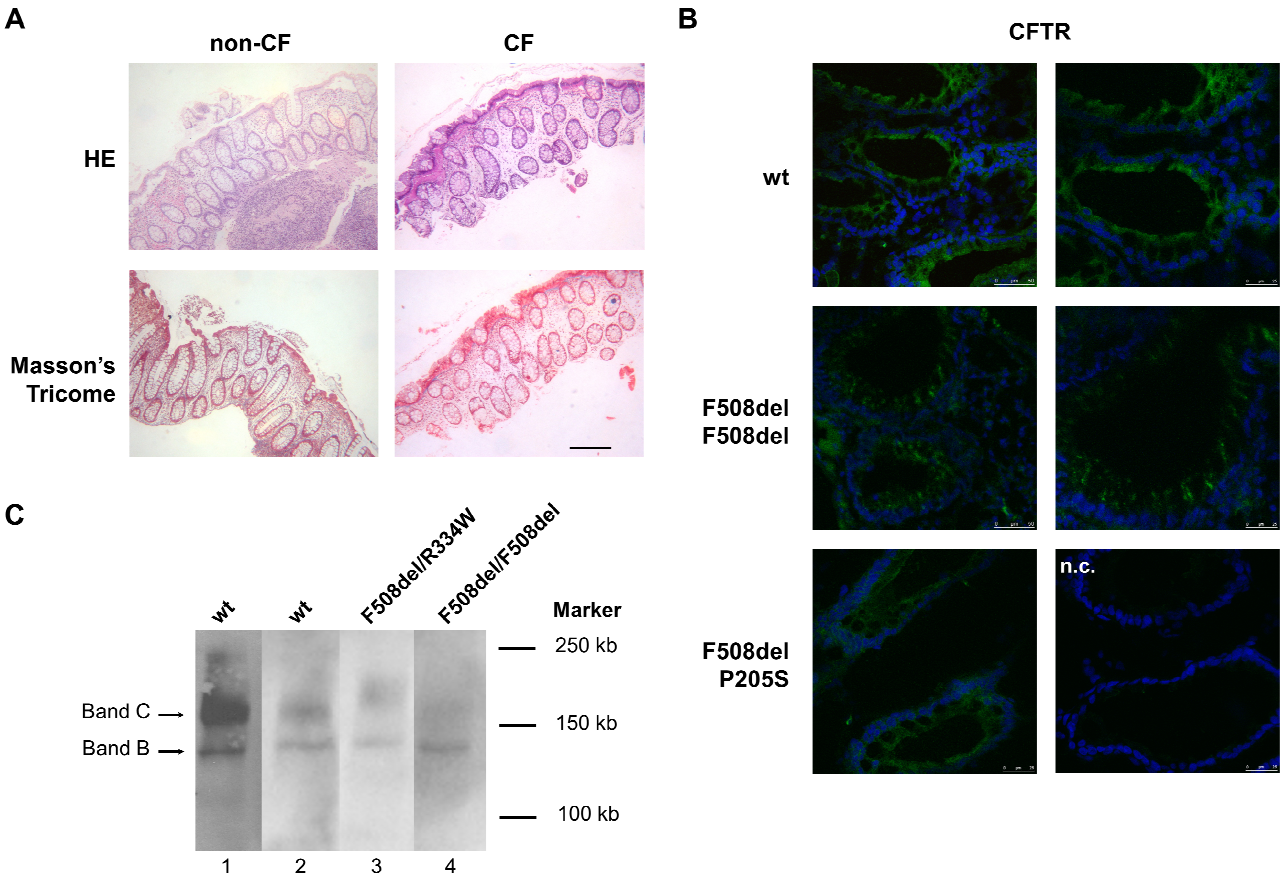


Figure III.2.3: (A) Rectal biopsies (longitudinal cuts) were histologically evaluated by Hematoxilin-Eosin (HE) and Masson's Tricome stainings in non-CF and CF tissues. Images show healthy epithelia, with no fibrotic processes and only some inflammatory cells were detected. Images shown are representative of the total and correspond to biopsies from a non-CF individual (left) and a CF patient (right) performed with standard (3.4mm Ø) forceps after bowel preparation with glycerol (non-CF) or isotonic saline (CF). In HE-stained sections (top), nuclei are stained in blue and cytoplasm in red. In Tricome's Masson-stained sections (bottom) collagen (fibrotic biomarker) is stained in blue, nuclei in black, and muscle and cytoplasm in red. Black scale bar represents 250 µM. **(B)** Immunohistofluorescence of rectal biopsies showing nuclei in blue (DAPI staining) and CFTR in green. Images evidence CFTR at the membrane in a non-CF tissue (top panels) and also, albeit weaker, in a biopsy from a CF patient with the F508del/P205S-CFTR genotype (middle panels). In contrast, a biopsy from a F508del-homozygous CF patient evidences intracellular CFTR staining (bottom panels). A negative control (no primary antibody n.c.) was also performed. Scale bar represents 25 µm. **(C)** Western blot of a single rectal biopsy from non-CF individuals (wt-CFTR, lanes 1-2) and from a CF patient with F508del/R334W-CFTR genotype (lane 3)

evidence the presence of both immature and mature forms of CFTR (bands B and C, respectively; and from a CF patient with the F508del/F508del-CFTR genotype (lane 4) evidencing only immature form (band B) which is characteristic of the endoplasmic reticulum (ER) and thus corroborating the traffic defect associated with this mutation.

3.3 Patient safety and comfort with overall procedure

No major complications (perforation, haemorrhage) were reported following NaCl 0.9% bowel preparation or jumbo biopsy forceps (allowing larger and more viable rectal specimens), thus making this a safe procedure. There was only one CF patient complaining about abdominal pain who was observed for 4h after the procedure, but had no other complications and recovered by then from such pain. This was the only patient with inappropriate bowel cleaning, where more insufflation had to be used in order to perform the procedure. We believe this was the reason for the abdominal pain. This patient also had a clinical history of surgical interventions for meconium ileus and colonic adhesions. We found no influence by usage of sedation in the procedure regarding tissue viability (data not shown).

Patients were asked by telephone to assess the rectal biopsy procedure by posing several questions targeting several aspects of patient assessment (**II. Materials & Methods** – *Figure II.1.1*). The questions were divided into 3 broad categories (see **II. Materials & Methods** section): *i)* procedural pain/discomfort and sedation requirement (questions 1, 2, 3 and 5); *ii)* comparison with other clinical/diagnosis procedures (question 4); and *iii)* acceptance towards the possible introduction of this method as an outcome measure in clinical trials (question 7). In addition, there was also a question regarding preconceptual concerns or discomfort and pain associated *a priori* with this procedure (question 6).

Data collected on patients' (dis)comfort show that 57/75 (76%) of the interviewed individuals did not report high levels of discomfort, independently of sedation or age, but shows statistically significant differences ($p = 0.032$) regarding gender (Table III.2.6): as there were more female patients saying that the overall rectal biopsy procedure is "somewhat uncomfortable". Nevertheless, the majority of both female (32/41) and male (25/34) reported low levels of discomfort. Also, the great majority of the individuals inquired (78.7%) reported that this is a painless procedure, regardless of sedation, age or gender (Table III.2.6). Only 2 individuals assessed the procedure as "Very painful" (data not shown). Moreover most of the individuals (53%) accept

repeating this procedure for four more times, while only a minority (12%) do not wish to repeat it (Table III.2.6), also independently of sedation, age and gender.

Table III.2.6 – Evaluation of comfort, pain and future repetition of the rectal biopsy procedure assessed by patients by gender, age group and sedation (n=75).

		Comfort			Pain		Future Procedure				
		Very Comfortable (18)	Somewhat Uncomfortable (27)	Not Uncomfortable (30)	No (59)	Yes (16)	0 (9)	1 (14)	2 (9)	3 (3)	4 (40)
Gender	Female	9	20	12	34	7	3	8	6	0	24
	Male	9	7	18	25	9	6	6	3	3	16
Age Groups (years)	0–9	7	3	11	15	6	3	2	2	2	12
	10–19	6	16	11	27	6	5	7	5	1	15
	20–29	2	3	2	6	1	0	2	1	0	4
	≥ 30	3	5	6	11	3	1	3	1	0	9
Sedation	No	10	15	12	28	9	6	7	3	1	20
	Yes	8	12	18	31	7	3	7	6	2	20

NOTE: Results are n = number of individuals. Statistically significant differences were found between classification of procedure overall comfort regarding gender (p = 0.032).

When asked to indicate which steps of the procedure they considered as the least/ most uncomfortable (Table III.2.7), data shows that "the monitoring" was considered by the highest percentage (89.3%) as "Not uncomfortable" (76%) or "Least uncomfortable" (13.3%), followed by "the biopsing" (70.7%) and "the bowel preparation" (70.7%), and finally "the sigmoidoscopy" (66.7%). For the individuals being sedated, "the sedation" step was also well-tolerated, as much as "the monitoring step" (Table III.2.7). Furthermore, as somewhat expected, sedation significantly enhances the rate of "Not uncomfortable" responses regarding "the sigmoidoscopy" (p = 0.016) and "the biopsing" (p = 0.003) procedures. No differences were found regarding gender or age (Table III.2.7).

Table III.2.7 – Evaluation of the overall rectal biopsy procedure (n=75).

	Not uncomfortable	Least Uncomfortable	Most uncomfortable	n.a.
Monitoring	57	10	6	2
Bowel preparation	27	26	20	2
Sigmoidoscopy	36	14	23	2
Biopsy	42	11	20	2
Sedation	28	4	5	38

NOTE: Results are n = number of individuals. Statistically significant differences were found between classification of comfort for sigmoidoscopy (p = 0.016) and biopsy (p = 0.003) regarding sedation. n.a. means not applicable.

Concerning the comparative assesment of the rectal biopsy procedure with other clinical / diagnosis examinations (Table III.2.8), most of the individuals classified the overall rectal biopsy procedure as being "more unpleasant" than sweat test (76%), followed by spirometry (64%) and blood collection (53.3%), regardless of gender, age or sedation. No conclusions could be drawn concerning NPD, nasal brushing or bronchoscopy, because more than 65% of the individuals had not experienced those procedures or were unable to establish a comparison.

Table III.2.8 – Comparison of the rectal biopsy procedure with other clinical / diagnosis procedures (n=75).

	Less unpleasant	More unpleasant	n.a.
Nasal Potential Difference	9	11	55
Nasal Brushing	12	14	49
Spirometry	12	48	15
Sweat test	10	57	8
Bronchoscopy	3	7	65
Blood collection	25	40	10

NOTE: Results are n = number of individuals. n.a. means not applicable.

Finally, when patients where asked about their preconceptual concerns regarding the procedure (question 6), the reported answers were similar: 46.7% for preconception /taboo and 41.3% for discomfort /pain. The remaining 12% did not answer (data not shown).

4 Discussion

Measurements of CFTR function in rectal biopsy specimens have proven its value in the fine-diagnosis of patients with milder or "non-classical" forms of CF, in particular when sweat test results are equivocal or borderline and/or if CFTR-disease causing mutations are not readily identified by DNA mutation analysis (Hirtz et al. 2004; Sousa et al. 2012; Mall et al. 2004a). This approach also serves as a sensitive test to predict the prognosis when rare CFTR mutations are not identified by standard screening tests (Hirtz et al., 2004; Sousa et al., 2012). Compared to the airways, the rectal epithelium is easily accessible at any age, expresses higher levels of CFTR, thus increasing robustness of the measurements and, as shown here (Figure III.2.3-A), does not undergo major secondary tissue destruction/remodelling as those occurring in CF airways (Mall et al. 2004a).

Because functional and biochemical data are not available for most rare mutations, these specimens can also be used to establish functional and biochemical correlations with rare CFTR genotypes (Figure III.2.3-B and 2.3-C). Furthermore, this approach may be used as well to validate efficacy of novel CFTR-modulator compound/drugs directly on human native tissues for such rare mutations (Accurso et al., 2010; Clancy et al., 2011; Shah, 2011). Indeed, rectal biopsies are already in use as outcome measures in clinical trials for other diseases (Anton et al., 2011; McGowan et al., 2007) and for some a high number of biopsies (n = 28) per patient has been reported without complications. The same can thus be translated into the CF field.

Since good tissue viability is critical for quantitative assessment of bioelectric responses our recommendation is that specimens are maintained in appropriate medium on ice until used in functional measurements, which should be performed immediately (Mall et al. 2004a). To minimize edge damage (and consequent liquid/electric leakage), tissues should be mounted under a dissecting microscope for optimal orientation of biopsy on the insert opening and to prevent tissue damage with excessive instrument manipulation.

The present study, aimed to determine whether and how bowel preparation for sigmoidoscopy and choice of biopsy forceps influence tissue viability for subsequent laboratory analyses, in particular, bioelectric measurements. We compared two commonly used solutions for bowel preparation, namely glycerol and isotonic saline enema (for sigmoidoscopy) as well as as oral mannitol (for colonoscopy). Our data show that isotonic saline

solution, which we speculate is less harmful for the mucosa, is superior to glycerol-based preparation, but there are no significant differences between NaCl-enema and oral mannitol. Indeed, there is a major difference between mean values for the tissue integrity parameter between biopsies obtained after NaCl- and glycerol-based bowel preparations, the latter severely compromising tissue viability (Tables III.2.1 and 2.2).

Regarding the biopsing procedure, we choose to perform it with forceps instead of a suction device, as the forceps utilize endoscope which allows direct visualization of both the mucosa and the forceps during the procedure and identification of any abnormal vascular structures thus potentially increasing its safety profile. It also allows avoiding biopsing the same site twice. Excessive bleeding, although proven in this study as not being a problem, would be rapidly identified and could be immediately managed. Moreover, our goal was also to compare the forceps size (standard *vs* jumbo forceps) in terms of how sample size might affect its viability and also regarding safety of the procedure. Overall, mean values for tissue integrity (jumbo=1.07±0.09 *vs* standard=2.04±0.18) and also for R_{te} (jumbo=20.97±0.81 $\Omega.cm^2$ *vs* standard=14.01±0.86 $\Omega.cm^2$) were shown to be statistically different ($p=5.51x10^{-7}$ and $p=8.42x10^{-8}$, respectively, Tables III.2.1 and 2.3), with jumbo forceps providing the best results.

Importantly, data for both tissue integrity and friability show good correlations (positive and negative, respectively) with tissue viability (assessed by R_{te} measurements) and are influenced by bowel preparation and biopsy forceps (Figure III.2.2 and Table III.2.5). Our data also show that collection of superficial rectal biopsies with (jumbo/standard) forceps constitutes a safe procedure, as we observed no complications, similarly to what others previously reported for studies on Hirschsprung (Hirsch et al. 2011) or inflammatory bowel disease (Elmunzer et al. 2008).

Data concerning presence/abundance of bleeding and mucus do not correlate with tissue viability (R_{te}), but interestingly there is a trend positive correlation between mucus and R_{te} (r=+0.078), indicating that presence of mucus (i.e. probably resulting from a less "aggressive" bowel cleaning) could somehow serve to preserve tissue viability (Figure III.2.2 and Table III.2.5).

Therefore, performing bowel preparation with isotonic saline and obtaining the rectal biopsies with jumbo forceps are demonstrated to constitute the best combination for the procedure (Table III.2.1) with the highest mean values for

R_{te} (21.82±1.03 Ω.cm^2) and the best for tissue integrity (1.00±0.11). On the other hand, usage of glycerol-based enema and smaller (standard) forceps produce the less viable tissues, namely (Table III.2.1): worst tissue integrity (2.21±0.23) and R_{te} (13.57±2.70 Ω.cm^2), and highest friability (1.81±0.22), thus rendering quantitative determination of CFTR-mediated Cl^- secretion less reliable. Alternative for bowel preparation in children could be sodium citrate + sodium lauryl sulfoacetate, together with glycerol + sorbitol ("Microlax®") or dioctyl sulfosuccinic acid sodium salt + sorbitol ("Clyss-go®"), which he had previously experienced to be less harmful for the mucosa if done the day before sigmoidoscopy (data not shown), probably allowing rectal mucosa to recover from this enema procedure. However, as these procedures were not rigorously assessed, they cannot be recommended.

The easy access to the patients rectum and the low innervation of this area minimizing pain, makes this approach to be expectedly well tolerated (Hirtz et al., 2004; Sousa et al., 2012). Moreover, modern gastroenterology techniques and instruments currently applied in outcome measures for clinical trials have made this approach increasingly simpler and easier (Anton et al., 2011). Indeed, in the present study, sedation was used primarily to reduce anxiety and ensure cooperation. In cooperative, non-anxious patients, our experience recommends that the procedure is performed with no anaesthesia. The present study also shows that this procedure is safe to be applied from young children to adults (age range was 6 mo to 52 yrs). Although we have no experience, it also expected that it can be safely applied to newborns, namely those identified in increasing numbers as asymptomatic CF patients by the recently implemented extensive newborn screening programs, merely based on elevated serum concentrations of immunoreactive trypsinogen (IRT). Indeed, these patients, posing new challenges to the CF diagnosis and prognosis are likely candidates to undergo this procedure to find evidence of CFTR (dys)function.

Patient enquiries demonstrate that for the majority of the individuals (76%) the rectal biopsy procedure is not associated with high levels of discomfort due to the short procedure time (max 15 min), regardless of sedation (Table III.2.6). Moreover, this shows to be also a relatively painless procedure, as 79% of the individuals did not report pain, Table III.2.6). Nevertheless, "the sigmoidoscopy step" was associated with the highest level of discomfort (Table III.2.7). Also, the individuals interviewed classified the rectal

biopsy procedure as more unpleasant than sweat test, spirometry or blood collection (Table III.2.8). But if these individuals are required to repeat the biopsy procedure, despite some preconception concerns comprising prejudice and discomfort, they accept doing it up to four more times (53%).

In conclusion, results from the present study recommend that rectal biopsies for bioelectric measurements for CF studies should be obtained with a flexible endoscope and jumbo biopsy forceps after bowel preparation with NaCl isotonic solution to obtain viable specimens. The procedure is well tolerated from the patients' perspective, demonstrating its feasibility as an outcome measure in (pre-)clinical trials.

Chapter 3 **Correction of the Ion Transport Defect in Cystic Fibrosis by Small Molecules**

(Manuscript in preparation)

1 Summary

Cystic Fibrosis (CF) is caused by loss of function of the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a chloride (Cl^-) channel that regulates salt and water transport in epithelia. The most common CF causing mutation, F508del, disrupts traffic and function of CFTR, probably due to protein misfolding. High-throughput screens identified several novel small molecules with potential to treat the basic defect in CF and some are starting hit the clinical setting. These include correctors like VX-661 and VX-809-Lumacaftor (both in Phase IIb clinical trial) that partially rescue the trafficking defect of F508del-CFTR and potentiators likeVX-770-Ivacaftor (FDA-approved drug) that corrects the gating defect of G551D-CFTR. However, to accelerate the entry into the clinic of novel compounds, the respective mechanism of action (MoA) should be established what usually requires studies in heterologous expression cellular systems. Nonetheless, at pre-clinical stage determining whether compounds may proceed into clinical trials efficacy in CF airway primary cutures/native human tissues needs to be determined. Comparative efficacy assessment between heterologous expression systems and airway primary cutures/native human tissues is thus of the ultimate importance before CFTR modulators reach the clinical setting.

Here, we aim to: i) evaluate of impact of different small molecules correctors and potentiators on CFTR-protein maturation and Cl^- channel activity in heterologous cellular systems (BHK and CFBE41o- cells), so as to better understand their mechanism of action; and ii) to comparatively determine their efficacy in modulating CFTR activity in those heterologous cellular systems and directly on primary cultures of human bronchial epithelial (HBE) cells/ native human tissues *ex vivo.*

Functional data for F508del-CFTR rescue by iodide efflux in heterologous systems (BHK cells) shows that VRT-325, C4a, VRT-640 and VRT-532 rescue CFTR function at varying levels, namely: 26, 33, 19 and 35%,

respectively of wt-CFTR function. . On polarized F508del-CFTR transduced CFBE cells, C4a treatment resulted in a 1.5-fold increase in CFTR function relative to control F508del-CFTR cells. In contrast, preliminary data from functional analysis conducted in primary HBE cells indicate that the efficiency of the correctors VRT-325 and C4a is lower than in BHK cells. Remarkably, VX-809 treatment resulted in a ~5.7-fold increase in CFTR-mediated Cl^- secretion, which represents a recovery of ~16% of the CFTR function observed in primary non-CF HBE cells.

Preliminary results in biopsies from F508del/F508del patients did not evidence a clear effect for VRT-325 nor C4a, but showed a modest effect for a novel corrector compound (TS-01-02-D8) which we recently identified.

Altogether, these results suggest that the effect of a given compound in rescuing F508del-CFTR activity in heterologous systems is poorly predictive of its rescuing capacity in native tissues and indicates that pre-clinical validation in native tissues *ex vivo* is highly recommended.

Keywords: CFTR modulators; CFTR maturation; CFTR function; cellular systems.

2 Introduction

Cystic Fibrosis (CF) is the most common severe autosomal recessive disease of Caucasian populations, caused by mutations in the CF transmembrane conductance regulator (CFTR) gene (Riordan et al. 1989; Rommens et al. 1989; Kerem et al. 1989), that encodes for a chloride (Cl^-) channel expressed at the apical membrane of epithelial cells, regulating salt and water transport in epithelia (Rich et al., 1990). Most CF mutations cause disease by disrupting either its surface expression or function as a Cl^- channel.

The biosynthesis and maturation of CFTR is a complex and inefficient process, even in for the wild-type (wt) protein. Correctly folded CFTR traffics from the ER through the Golgi (where it undergoes fully-glycosylation) to the plasma membrane. In contrast, when the protein misfolds, it is retained in the ER, remaining thus core-glycosylated, and is rapidly targeted for ER-associated degradation (ERAD) via the ubiquitin-proteasomal pathway (UPP) (Chang et al. 1999; Farinha et al. 2002; De Carvalho et al. 2002).

One single mutation, named F508del (a 3bp deletion that causes the loss of the phenylalanine residue at position 508 of CFTR) accounts for about

70% of CF chromosomes worldwide (Collins, 1992). This mutation is associated with a severe clinical phenotype (Collins 1992; Rowe et al. 2005). F508del disrupts the function of CFTR in three ways: firstly, F508del causes defects in protein folding that prevent the traffic of CFTR to its correct cellular location, the apical membrane of epithelial cells (Cheng et al., 1990). Secondly, F508del causes defects in channel regulation that impair greatly channel opening (Wang et al. 2000; Schultz et al. 1999). Thirdly, when rescued to cell surface F508del-CFTR presents a highly decreased plasma membrane (PM) half-life due to both accelerated endocytosis and fast ubiquitin-dependent turnover (Sharma et al., 2004; Swiatecka-Urban et al., 2005). Knowledge of how F508del and other CF mutations cause a loss of Cl^- channel function is leading to new, more rational, approaches to therapy for CF patients aiming to correct such basic defects.

Experiments carried out by Denning and collaborators (Denning et al., 1992) show that F508del-CFTR could be rescued to the cell surface by prolonged (24-48h) incubation of cells at low temperature (26-28°C), thus demonstrating that the folding defect associated with the F508del mutation is temperature-sensitive, but also, and very importantly, susceptible of rescue. Soon after, Sato et al. (1996) showed that similarly, 10% (v/v) glycerol rescued the cell surface expression of F508del in recombinant cells. However, at these concentrations glycerol is highly toxic precluding its use in CF patients. These findings, however, triggered a quest for drugs that correct the folding defect of F508del-CFTR, termed chemical or pharmacological chaperones, depending on their specificity in promoting protein folding (Amaral, 2004).

Ever since, high-throughput screening (HTS) efforts have produced a number of candidate compounds, so as to achieve two goals, namely: a) "correctors" (Van Goor et al. 2006) to overcome the folding defect of F508del-CFTR; and b) "potentiators" (Pedemonte, et al. 2005a) to restore Cl^- channel gating. These compounds have become increasingly potent, i.e., effective at lower concentrations, more specific and less toxic. These include "laboratory reagent correctors" like VRT-325, VRT-640, compound 4a (C4a) or pre-clinical correctors like VX-661 or VX-809 and also "laboratory reagent potentiators" like VRT-532 or FDA-approved potentiator VX-770 (Ivacaftor) (Van Goor et al. 2006; Pedemonte et al. 2005b; Van Goor et al. 2011; Van Goor et al. 2009; Shah 2011).

Some of these correctors, such as C4a, potentiator VRT-532 (also with corrector capacity) and VX-809, have been described to promote maturation of F508del-CFTR in a specific way (Wang et al. 2006; Van Goor et al. 2011), while others are uspecific. VRT-325 for instance promotes maturation of both P-glycoprotein- and CFTR-processing mutants (Wang et al. 2007a; Van Goor et al. 2006). Moreover, Wang and collaborators (Wang et al. 2007b) reported that correctors could have an additive effect on maturation of CFTR-processing mutants, thus opening avenues for combination therapies.

However, the mechanism of action of these compounds/drugs remains largely undetermined. Moreover, recent studies have highlighted that not only cell background can influence the pharmacological rescue of mutant CFTR (Pedemonte et al. 2010), but also the polarization status in the case of epithelial cells (Rowe et al. 2010). Furthermore, for "laboratory reagent modulators" there is no knowledge on their comparative efficacy in heterologous expression systems and native human tissues, which may be critical to validate MoA. Importantly, even for compounds already in clinical trials/ practice (like VX-770, VX-661 or VX-809) the pre-clinical tests have only been performed in cultured human (primary) cells but not directly in human native tissues. Indeed, poor tissue penetrance may be the cause for the reported limited success of VX-809 in clinical trials

Several studies have investigated the potential abnormalities in electrogenic Cl^- secretion in the intestinal epithelium of CF patients by Ussing chamber measurements using cell cultures, jejunal or rectal tissue biopsies (Veeze et al. 1994; Mall et al. 1998a; Mall et al. 1999; Hirtz et al. 2004). Results from these studies established that quantification of rectal CFTR-mediated Cl^- secretion is a sensitive test for the diagnosis and prognosis of CF disease and also to identify CF patients who can benefit from therapeutic strategies aimed at increasing the residual CFTR activity (Hirtz et al., 2004; Sousa et al., 2012). We have also recently demonstrated how human native tissues can be used for pre-clinical validation of CFTR activation by potentially therapeutic compounds (Roth et al., 2011).Here, our goal is two-fold, namely: 1) to evaluate of impact of different small molecules correctors and potentiators on CFTR-protein maturation and Cl- channel activity in heterologous cellular systems (BHK and CFBE41o- cells), so as to better understand their mechanism of action (MoA); and 2) to comparatively determine the efficacy of these compounds in modulating CFTR activity in

such heterologous cellular systems and directly on native human tissues *ex vivo* and primary cultures of human bronchial epithelial (HBE) cells. Regarding the first goal we have investigated the compounds MoA by determining the additivity of these small molecules F508del-CFTR rescuing with that achieved by low temperature (LT) incubation, which we recently proposed to rescue F508del-CFTR through bypassing the ERQC (Farinha et al. 2012).

3 Results

As referred above, several novel small molecules identified in HTS show the ability to restore the trafficking defect and function of F508del-CFTR, some of these compounds are just "laboratory reagents", others are (pre-)drugs. In the search for the MoA used by these compounds to rescue the cell surface expression of F508del-CFTR and assessment of its native tissue efficacy, we tested the following ones:

1) VRT-325, a "laboratory reagent" quinazoline derivative known to rescue F508del-CFTR and also P-glycoprotein mutants (corrector) (Loo, Bartlett, Wang, & Clarke, 2006).

2) VRT-532, a "laboratory reagent" known to specifically activate rescue F508del-CFTR (potentiator), but also shown to rescue its trafficking defect at higher doses (Wang et al. 2006).

3) VRT-640, a "laboratory reagent" corrector of F508del-CFTR. This small molecule was chemically engineered based on the structure of another corrector compound (VRT-325).

4) C4a, a "laboratory reagent" corrector previously described to rescue F508del-CFTR in human bronchial cells (Pedemonte et al. 2005c).

6) VX-809, a CFTR pre-drug corrector showing the ability to rescue F508del-CFTR to the plasma membrane in primary HBE cells *in vitro* (Van Goor et al., 2011), and currently on clinical trials as combined therapy with the potentiator VX-770.

3.1 Channel Activity Rescue of Heterologous F508del-CFTR by VRT-325, VRT-532, VRT-640, C4a and Low Temperature in BHK cells

Iodide efflux experiments were carried out in order to comparatively assess activity levels of F508del-CFTR rescued by small molecules and that following low-temperature (LT) rescuing. We were able to detect F508del-CFTR function after 12-24h-incubation with all small molecules tested here (Figure III.3.1), thus confirming their rescuing effect as CFTR correctors. Moreover, some brought about a shift in the iodide efflux peak from 3 to 2 min: VRT-325, C4a, Comparison of the iodide efflux peak for F508del-CFTR cells rescued by VRT-325 or LT alone to that obtained for VRT-325/ LT together, shows that rescuing of F508del-CFTR activity by VRT-325 is additive to LT by an additional 39%. Recently we proposed that LT allows F508del-CFTR to bypass the ERQC (Farinha et al. 2012) still with some degree of misfolding. Interestingly, C4a evidenced an even more pronounced additive effect with LT rescue by an additional 47%, achieving ~80% activity of wt-CFTR. In contrast, rescuing of F508del-CFTR by VRT-640 or VRT-532 did not show an additive effect to LT.

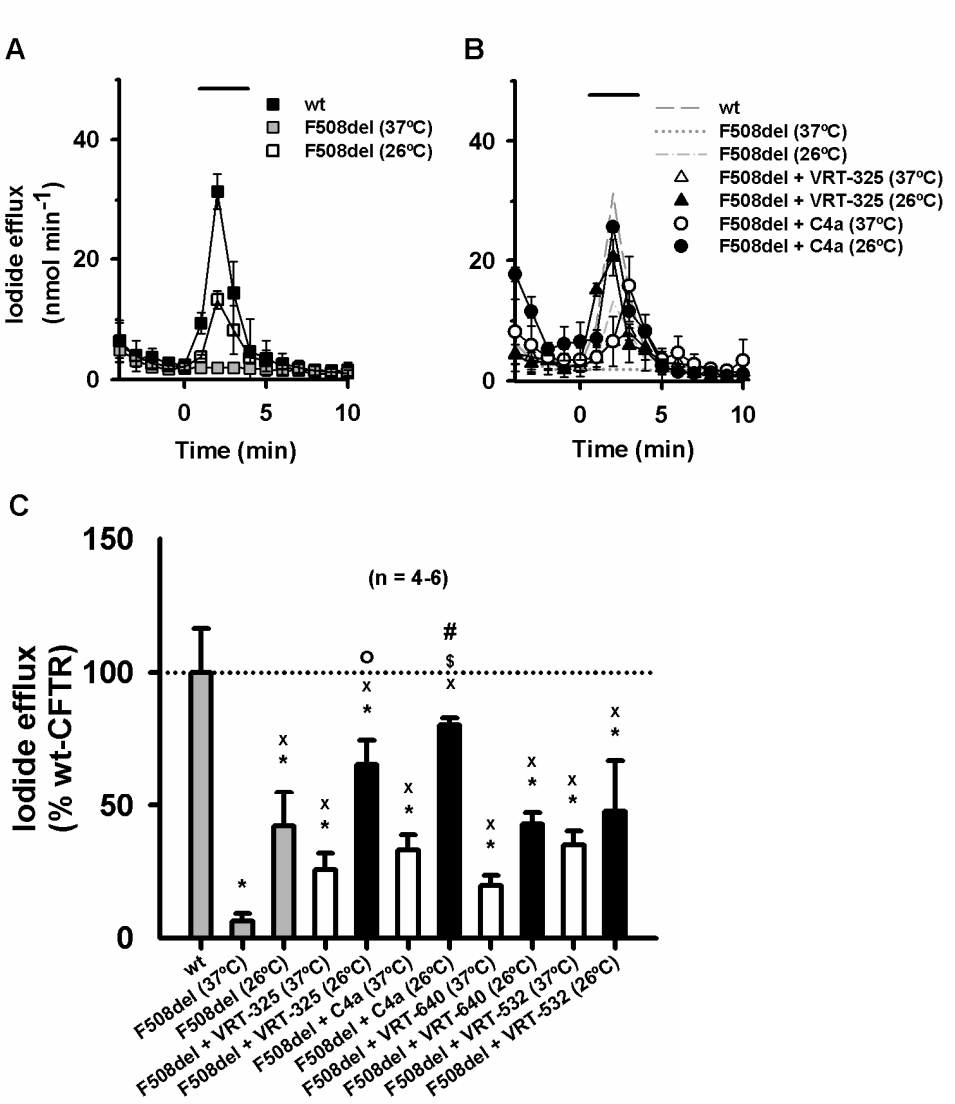


Figure III.3.1: (A, B) Iodide efflux from BHK cells stably expressing F508del-CFTR pre-treated with VRT-325, 6.7 μM (24 h) or C4a, 10 μM (12 h) and rescued by low temperature (26 ºC, 48 h). Cells were stimulated with Forskolin (10 μM) and Genistein (50 μM) in the period indicated by the black solid line above graph of time-course iodide efflux measurements. **(C) Graph summarizing data of I^- efflux peak magnitude generated by the different treatments of BHK F508del-CFTR cells expressed as a percentage of wt-CFTR activity**, showing that: i) VRT-325 restores F508del-CFTR function to ~26% of wt-CFTR. VRT-325 in combination with low temperature rescue F508del-CFTR function to ~65% of wt-CFTR activity; ii) C4a restores F508del-CFTR function to ~33% of wt-CFTR at 37 ºC and in combination with low temperature it is able to enhance F508del- function up to ~80% of wt-CFTR activity; iii) VRT-640 (6 μM, 24h) restores F508del-CFTR function to ~19% of wt-CFTR. VRT-640 in combination with low temperature rescue F508del-CFTR function to ~42% of wt-CFTR activity; and iv) VRT-532 (20 μM, 48h) restores F508del-CFTR function to ~35% of wt-CFTR. VRT-532 in combination with low temperature rescue F508del-CFTR function to ~47% of wt-CFTR activity. Data are means ± SEM at each point (n = 4-6). Where error bars are not visible, the symbol has obscured them. * indicates statistically significant differences ($p < 0.05$) relative to wt; *x* relative to F508del (37°C); *o* relative to F508del-VRT-325 (37°C); *$* relative to F508del (26°C); and # relative F508del-C4a (37°C) treated cells.

3.2 Channel Activity Rescue of Heterologous F508del-CFTR by VRT-325, C4a and Low Temperature in Polarized CFBE cells

We then investigated how VRT-325 and C4a correctors and LT affected the restoration of channel activity in polarized CFBE41o- cells stably expressing F508del-CFTR following letiviral transduction (Bebok et al., 2005). Unfortunately, the toxicity of these correctors (C4a, in particular) which had been previously reported (Chiaw et al. 2010; Jurkuvenaite et al. 2010), severely compromised the cellular monolayer integrity. Indeed, such increased monolayer susceptibility, confirmed by Ussing chamber measurements of transepithelial resistance (R_{te}) (Figure III.3.2-A), led to impairment of an accurate estimation of cAMP-induced Cl^- transport in corrector-treated cells, particularly in C4a.

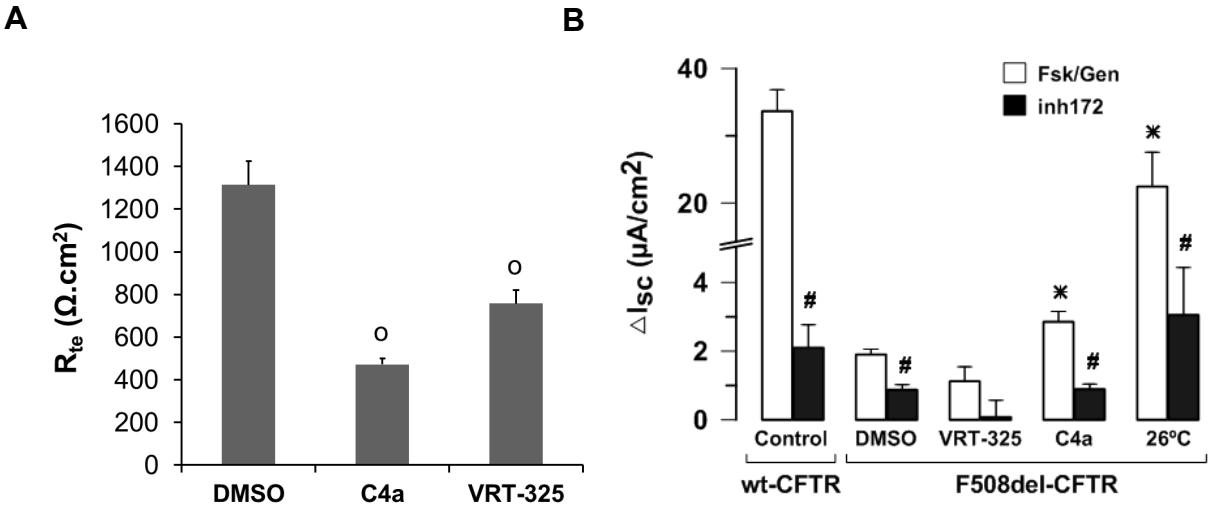


Figure III.3.2 – Micro-Ussing chamber experiments carried out on polarized CFBE41o- at 37°C and treated with vehicle (DMSO, control) or correctors VRT-325 (25 μM), C4a (15 μM) and low temperature (26ºC), as indicated. (A) Summary of the transepithelial resistance (R_{te}) for the F508del-CFTR CFBE monolayers treated with DMSO or CFTR-correctors. **(B)** Summary of the induced short-circuit currents (ΔI_{sc}) upon luminal stimulation with forskolin (2 μM) and genistein (50 μM) (Fsk/Gen) and blocked with CFTR inh172 (30 μM) from CFBE41o- polarized monolayers. Data are means ± SEM. *o* and * indicates statistically significant differences ($p < 0.05$) relative to control (DMSO) cells and # significant inhibition ($p < 0.05$) relative to CFTR inh-172 untreated cells.

Nevertheless, equivalent short-circuit currents (I_{sc}) derived from 15 μM C4a-treated cells (24h) showed an enhancement of corrector-induced, CFTR-mediated Cl^- transport compatible with the effects observed in BHK cells, whereas cells exposed to VRT-325 (25 μM) revealed a much smaller response,

consistent with reported inhibitory effect of this compound on cAMP-induced channel activity (Chiaw et al. 2010) at higher concentrations (Figure III.3.2-B). On the other hand, LT-incubation of F508del-CFTR CFBE cells (26°C, 24h) restored CFTR function to higher values (~65% of wt-CFTR) than those observed in (non-polarized) BHK cells (~42% of wt-CFTR, see above). This is in accordance with other studies reporting high levels of LT-rescue for F508del-CFTR in CFBE cells (Rowe et al. 2010).

3.3 Channel Activity Rescue of Endogenous F508del-CFTR by VRT-325, C4a and VX-809 in Human Primary Bronchial Epithelial Cells

To test the effect of these CFTR modulators in rescuing endogenous F508del-CFTR on a system closer to *in vivo* conditions, we treated primary cultures of human bronchial epithelial (HBE) cells with the compounds. Firstly, we assessed the effects of the treatment with increasing concentrations (from 0.1 to 100 μM, 48h) of corrector VX-809 on non-CF and CF HBE polarized cells (Figure III.3.3), so to obtain a dose-curve response with this compound. The effects were analysed both at the level of function (CFTR-mediated Cl- secretion through Ussing chamber measurements) and protein processing (Western blot to detect for the presence of mature form of CFTR).

Data shows that VX-809 is able to rescue both F508del-CFTR function in response to the cAMP agonist forskolin or the CFTR potentiator genistein (Figure III.3.3-B) and also to restore its processing (Figure III.3.3-C). Also in non-CF primary HBE cells, increasing concentrations of VX-809 treatment revealed concomitant increase in forskolin or genistein-induced CFTR-mediated I_{sc}., being however, significantly higher under genistein. High concentrations of VX-809 (100 μM) had cytotoxic effects, making it impossible to carry out bioelectrical measurements (data not shown).

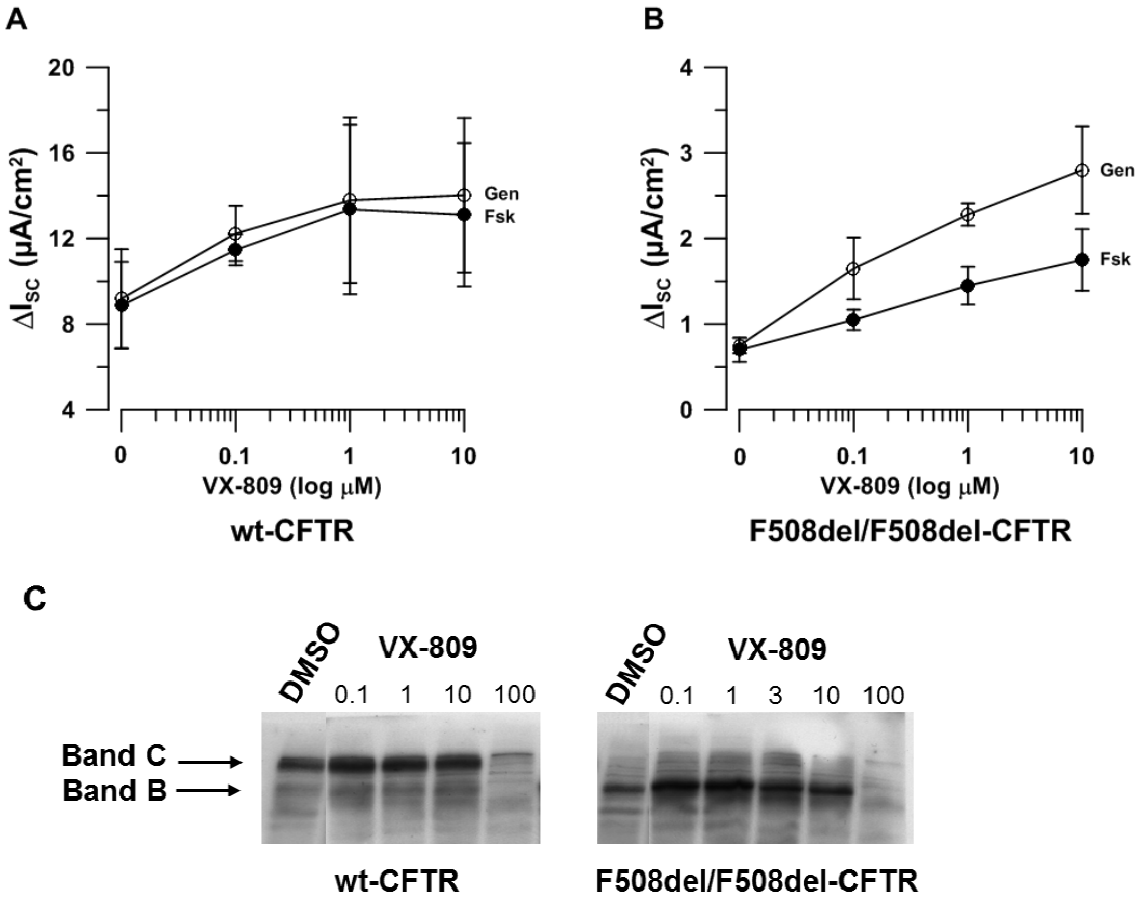


Figure III.3.3 – Dose-curve responses obtained for the treatment of primary human bronchial epithelial (HBE) monolayers with VX-809 (48h). Monolayers from a non-CF donor **(A)** or a F508del/F508del-CFTR patient **(B)** were treated for 48h with increasing concentrations (from 0.1 to 100 μM) of the corrector VX-809 or with vehicle (DMSO, 0 μM) and studied in micro-Ussing chambers by stimulation with CFTR agonists, namely Forskolin (Fsk, 2 μM) and Genistein (Gen, 25 μM) (duplicates for each donor). **(C)** Representative Western blots analysis from the monolayers following the treatments indicated above blots are shown.

In comparison to control primary HBE cells (DMSO), VX-809 treatment (3 μM, 48h) resulted in a ~5.7-fold increase in Cl^- transport in response to forskolin and genistein, whereas VRT-325 (6.7 μM, 24h) or C4a (10 μM, 16h) resulted in a fold-increase of only ~1.5 and ~1.2, respectively (Figure III.3.4). This ~6-fold increase in channel activity represented a recovery of activity equivalent to ~16% of normal CFTR function, i.e., that observed in primary HBE cells from a non-CF individual. A potential interference by the epithelial sodium (Na^+) channel (ENaC) on V_{te} recordings was excluded by luminal exposure of all primary cells to the ENaC specific inhibitor Amiloride. Moreover, these effects were specifically blocked by CFTR inh-172 (Figure III.3.4-A).

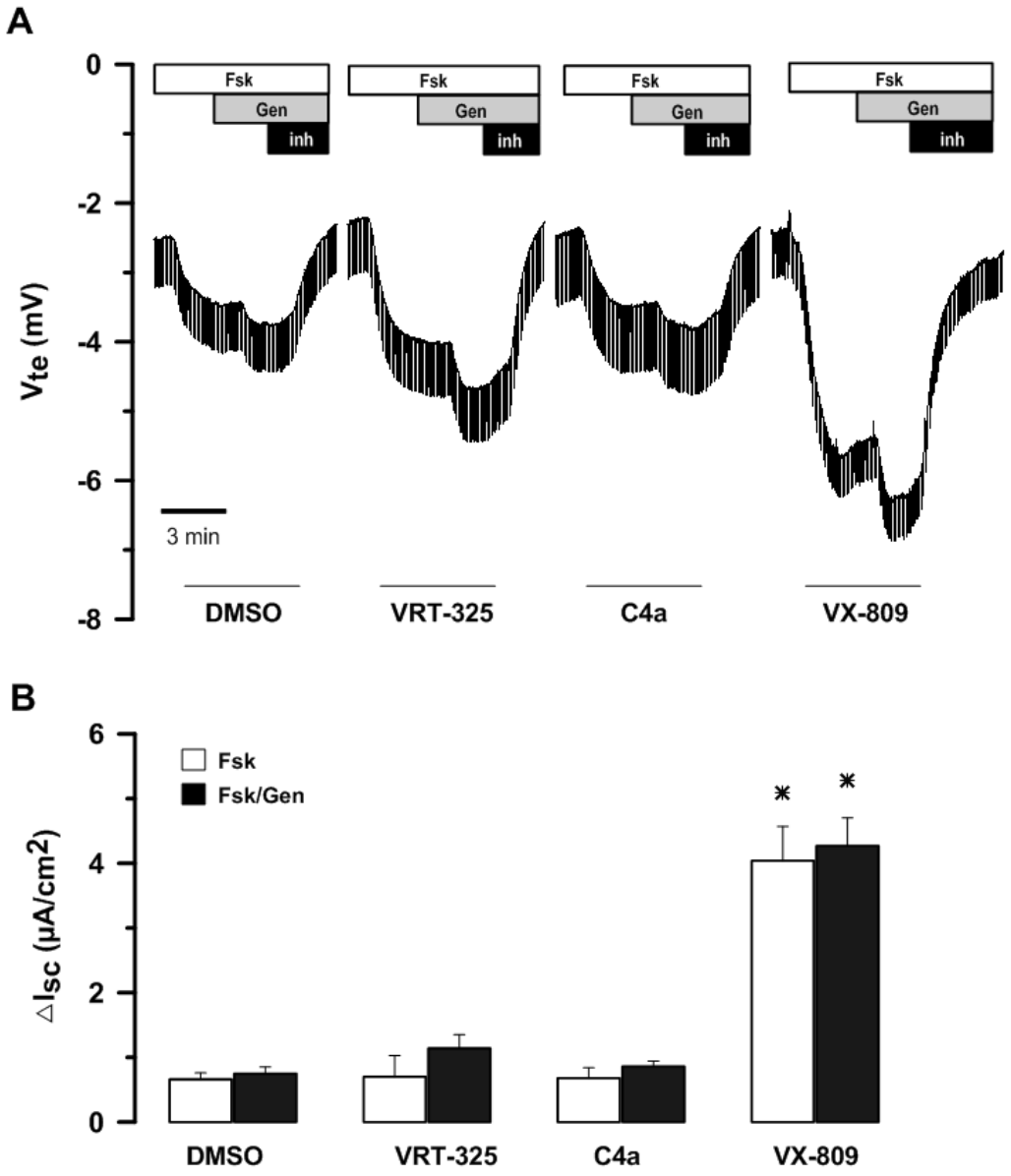


Figure III.3.4 - Ussing chamber results obtained for the analysis of CF (F508del/F508del) primary HBE polarized monolayers with small molecule correctors treatments. A) Original Ussing chamber recordings obtained for the treatments with DMSO (0.01%, 48h), VRT-325 (6.7 μM, 24h), C4a (10 μM, 16h) or VX-809 (3 μM, 48h). Note that, in primary F508del/F508del monolayers, VX-809 enhances the negative transepithelial voltage (V_{te}) deflection following the application of luminal forskolin (Fsk, 2 μM), which is potentiated by genistein (Gen, 25 μM), and completely inhibited by CFTRinh-172 (inh, 30 μM). **B)** Summary of sensitive-I_{sc} induced upon correctors and CFTR agonists exposure as referred in **(A)**. Data are means ± SEM (triplicates from the same donor). * indicates statistically significant differences ($p < 0.05$) relative to control (DMSO) cells.

Although preliminary, (because they were conducted in primary HBE cells from only one single non-CF individual and one F508del-homozygous CF

patient) these results are in accordance with recently published findings by Van Goor et cols (Neuberger et al. 2011; Van Goor et al., 2011).

3.4 Channel Activity Rescue of Endogenous F508del-CFTR by VRT-325, C4a and VRT-532 on Human Rectal Biopsies *Ex vivo*

In order to assess the effect of these CFTR modulators in rescuing endogenous F508del-CFTR-mediated Cl^- secretion in the most physiologically relevant systems, i.e., native CF epithelia, we used rectal biopsies from CF patients with two CF-disease causing mutations identified in the *CFTR* gene, namely with the F508del/F508del and F508del/G542X CFTR genotypes. In parallel, the compound effects were also tested on colonic tissue from non-CF individuals (biopsied or undergoing surgery for reasons unrelated to CF). The rectal biopsies (3-4 mm) were obtained by forceps and investigated as described in **II. Materials & Methods** section.

More recently, we screened a 250 small-molecule library in a collaboration with Sygnature Discovery, (UK) in the scope of the EU-TargetScreen2 consortium. Data from this screen, using both quantitative Western blot in CFBE41o- cells in our lab and differential scanning fluorimetry (DSC) to assess F508del-NBD1 folding (in collaboration with C Gomes' lab, ITQB) has identified four novel F508del-CFTR primary lead correctors, among which is TS-01-02-D8 (Palma et al., 2010).

To test the effect of potentiator VRT-532, correctors VRT-325 and C4a and also of TS-01-02-D8 on fresh rectal biopsies, we mounted these tissues into a perfused micro Ussing chamber as above (for further details see **II. Materials & Methods** section).

Correctors VRT-325 and C4a in Non-CF native tissue

After addition of CFTR agonists, 100 μM IBMX (3-isobutyl-1-methylxanthine) and 2 μM forskolin (Fsk) to biopsies from non-CF individuals, we observed a large lumen-negative deflection which is further enhanced as lumen-negative after the addition of carbachol (CCH, 100 μM, is stimulating K^+ channels and exit of K^+ from the epithelium that further increases the driving force for the exit of Cl^-). These biopsies where then incubated with VRT-325 6.7 μM, C4a 10 μM or with DMSO 0.01% (vehicle) for 16h-18h (as described in **II. Materials & Methods** section) as shown in Figure III.3.5.

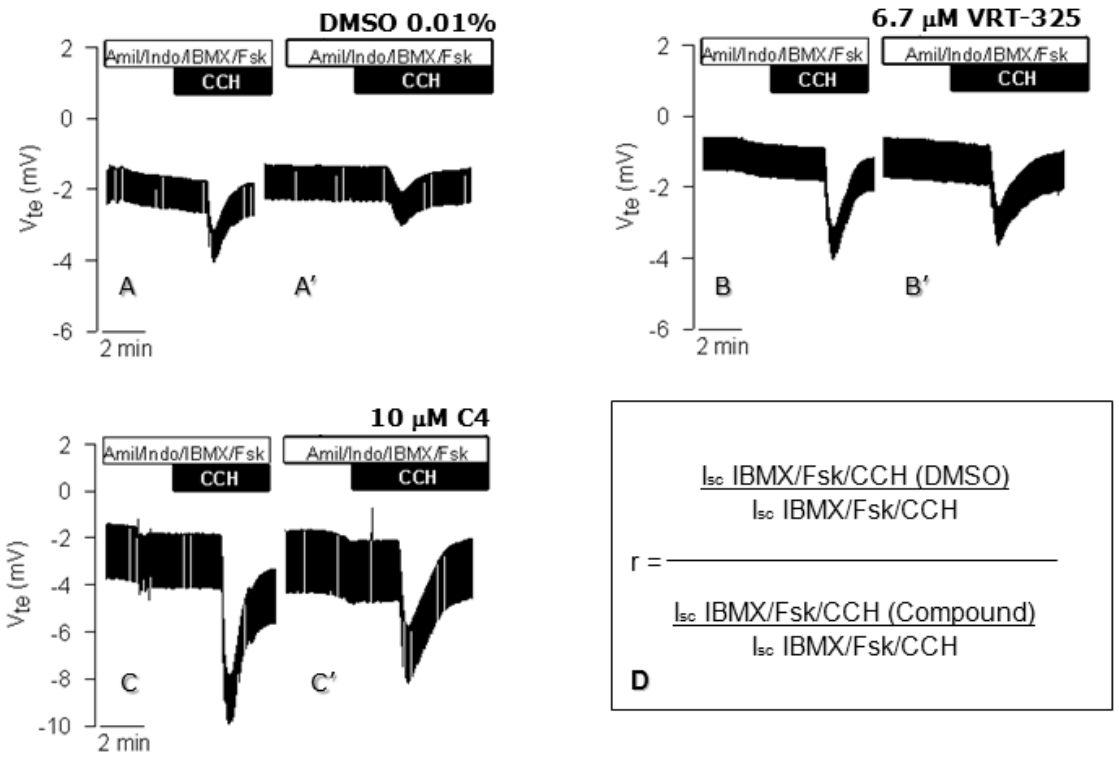


Figure III.3.5 - Effect of CFTR modulators on Non-CF human native tissues *ex vivo*. Original Ussing chamber recordings obtained for measurements on rectal biopsies from the same non-CF individual analyzed (with protocol indicated above traces): immediately after biopsy excision (A, B and C) or after incubation with modulators (indicated above traces) or the vehicle, DMSO, for 16-18h at 37°C (A', B' and C'). Analysis of data (D) shows that VRT-325 and C4a increase CFTR-mediated Cl^- currents by ~25% and ~33% (n=3), respectively, in comparison to incubation with the vehicle alone.

In order to assess the effect of correctors in enhancing the CFTR-mediated Cl^- currents, we estimated first the ratio of the peak currents before and after incubation with corrector or vehicle (control) as indicated in Figure III.3.5-D. Then, the corrector rescuing efficiency (i.e., the increase in the IBMX/Fsk/CCH-induced I_{sc} upon treatment with corrector) was obtained by the ratio (*r)* of these two ratios (Figure III.3.5-D).

Correctors VRT-325, C4a and TS-01-02-D8 in CF native tissue

After stimulation of CF rectal biopsies with IBMX/ Fsk), we observe a small lumen-positive deflection, then, upon CCH addition, a lumen-positive peak deflection is observed, reflecting the exit of K^+ from luminal potassium channels. The biopsies where then incubated with VRT-325 6.7 µM, C4a 10 µM, TS-01-02-D8 10 µM or with DMSO 0.01% (vehicle) for 16h-18h (Figure III.3.6).

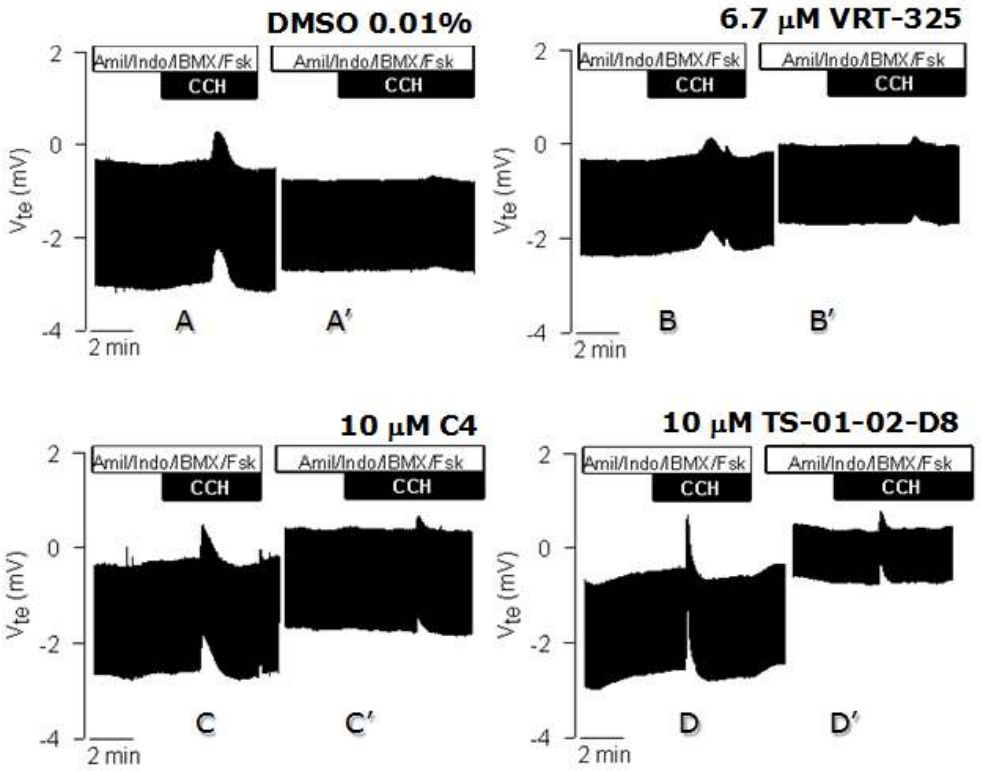


Figure III.3.6 - Effect of CFTR modulators on CF human native tissues *ex vivo*. Original Ussing chamber recordings obtained for measurements on rectal biopsies from the same CF patient analyzed in A, B and C (p.F508del/G542X) and for other CF patient in D (p.F508del/p.F508del) with protocol indicated above traces: immediately after biopsy excision (A, B, C and D) or after incubation with modulators (indicated above traces) or the vehicle, DMSO, for 16-18h at 37ºC (A', B', C' and D').

These preliminary results showed no clear effect in the activation of CFTR-mediated Cl- secretion after 16-18h incubation with VRT-325 or C4a, because V_{te} deflection still reflects K^+ secretion (n=3). On the other hand, we were able to identify a modest effect of TS-01-02-D8 (16-18h incubation) in activating CFTR-mediated Cl- currents by ~5% (n=2) in comparison to normal values for CFTR Cl^- secretion. Analysis of data also shows that part (18-64%) of the IBMX/Fsk/CCH-sensitive K^+ secretion was reduced after incubation with the all the compounds above or vehicle. These data, although preliminary (n = 3) already evidence that VRT-325 and C4a have a positive effect on CFTR-mediated Cl^- currents in non-CF tissue. On the other hand, preliminary results (n = 2) in biopsies from CF patients did not evidence a clear effect for VRT-325 nor for C4a, but showed a modest effect for TS-01-02-D8. Nevertheless, these experiments demonstrate the potential of human native tissues and this technique to assess the effect of compounds *ex vivo*.

Potentiator VRT-532 in non-CF native tissues

The effect of potentiator VRT-532 was tested acutely (i.e., without incubation) as indicated in Figure III.3.7, showing a further deflection towards the lumen-negative side and indicating exit of more Cl^- from the epithelium.

These data, although preliminary (n = 6) indicate a clear trend for VRT-532 (of ~ 26%) to positively stimulate CFTR-mediated Cl^- currents. It is believed that this effect can be intensified in samples from CF patients where there is more scope to enhance CFTR function, due to the deficient gating of the mutant channel.

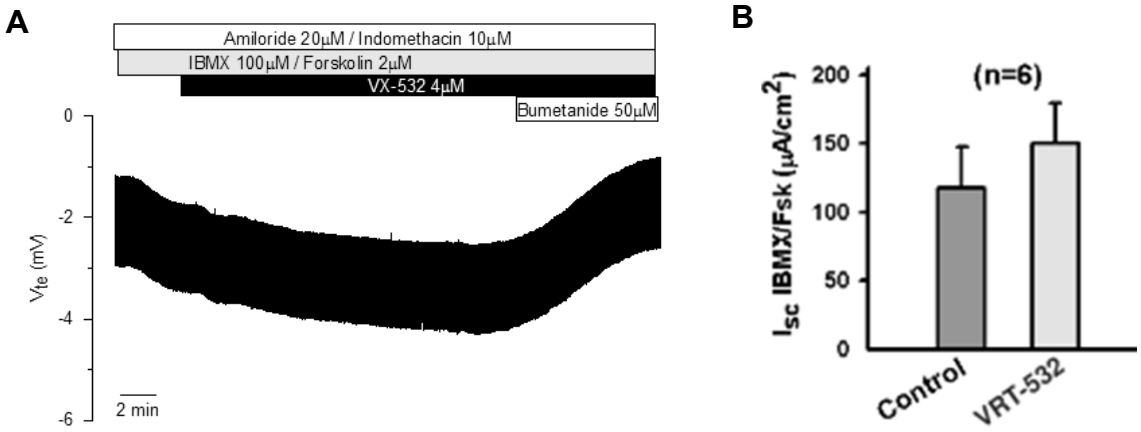


Figure III.3.7 - Effect of the potentiator VRT-532 on human native tissue. (A) Original Ussing chamber tracing obtained for the analysis (with compounds as indicated above traces) of a rectal biopsy from a non-CF individual analysed immediately after excision. In (B) summary of the data obtained for the same time of experiment with 4 μM VRT-532 or with the vehicle (control) is shown. Data are means ± SEM.

4 Discussion

Several high-throughput screening efforts identified several novel small molecules with potential to treat the basic defect in CF. These include correctors (like VRT-325, VRT-640, C4a and VX-809) that partially rescue the trafficking defect of F508del-CFTR (Loo et al. 2006; Pedemonte et al. 2005c; Van Goor et al. 2011) and potentiators (like VRT-532 and VX-770) that correct the gating defects associated with the functional classes III and IV of CFTR mutants (Wang et al. 2006; Van Goor et al. 2009).

In the great majority of these studies, FRT (Fisher Rat Thyroid) cells have been used to identify a number of small molecules that rescue F508del-CFTR activity, and the positive findings require confirmation in cells

potentially more plysiologically relevant and representative of the CF airway. CFTR correctors, compared with potentiators, are more difficult to identify and usually bring about a relatively modest efficacy, particularly in primary airway epithelial cells from CF patients (Van Goor et al. 2011; Pedemonte et al. 2010). Their potency and efficacy is still limited, as shown in recent outcomes from clinical trials involving the CFTR corrector VX-809 (Clancy et al., 2011). Moreover, their specific MoA is still unknown and the efficacy of these compounds in native human tissues affected by the CF disease still needs to be addressed, so as to give better prediction of their *in vivo* efficacy.

Insight into small molecule correctors' mechanism of action

To start addressing the MoA and cell specificity of correctors, we have shown here that VRT-325, C4a, VRT-640 and VRT-532 are all able to rescue heterologous F508del-CFTR function to variable levels, in BHK celle namely: 26, 33, 19 and 35%, respectively of wt-CFTR function, as assessed by iodide efflux measurements. Moreover, both VRT-325 and C4a show additive effects to the rescue achieved by low temperature (LT), reaching values up to ~65-80% of wt-CFTR function, respectively.

VRT-325 rescuing of F508del-CFTR activity is also additive to that observed by the genetic revertant 4RK, but not with that of the genetic revertant G550E, indicating as well that this compound is probably acting at the same level as G550E, i.e. folding (Roxo-Rosa et al., 2006; Schmidt, 2008). C4a is not additive either to G550E or to 4RK (Schmidt, 2008), but shows additivity with LT and also VRT-325 (Farinha et al. 2012), indicating that it is probably involved in a different step of protein folding.

Regarding VRT-640 and VRT-532, no significant additive effects with LT were observed here, neither with genetic revertants G550E or 4RK, (Schmidt, 2008), suggesting that they probably act at different steps/binding pockets of CFTR folding or at distinct cellular checkpoints. VRT-640, a chemical derivative of VRT-325, was shown to stabilize the immature form (band B) of F508del-CFTR (Schmidt 2008) and one could speculate that it is probably this accumulation in the ER that leads some protein to "bypass" the ERQC and reach the plasma membrane. Indeed, it has been known for long that strategies leading to accumulation of immature F508del-CFTR like protein overexpression lead to its cell surface expression (Cheng et al., 1995).

VRT-532, first described as a potentiator, was also shown to act as a corrector of F508del-CFTR at higher concentrations (20 μM) (Wang et al., 2006). In contrast to VRT-325, VRT-532 was shown not not to rescue p-glycoprotein (Wang et al. 2006), suggesting that this small molecule acts specifically on F508del-CFTR.

Small molecule effects on airway cells

We then assessed the effects of the compounds with higher percentage of rescue, namely VRT-325 and C4a, at higher doses in a more relevant heterologous system, CFBE41o- cells which are human epithelial respiratory cells which are able to polarize forming epithelial monolayers. Indeed, recent studies indicate that CFTR processing and activity are strongly influenced by the cell model system of study, with enhanced maturation and surface stability demonstrated in models that polarize compared to non-polarizing systems (Rowe et al. 2010). We, thus used F508del-transduced CFBE41o- polarized cells (Bebok et al., 2005) and measured CFTR-mediated I_{sc} changes in Ussing chambers upon stimulation with CFTR agonists and treatment with CFTR modulators. Although at the higher compound concentrations these were shown to be toxic (data nor shown), we were neverthelss able to demosntrate that C4a treatment resulted in a 1.5-fold increase in $I_{sc\text{-}Fsk/Gen}$ relative to control (DMSO) in F508del-CFTR CFBE cells. On the other hand, high concentrations of VRT-325 showed to be inhibitory on cAMP-induced channel activity, as reported by others (Chiaw et al. 2010). Although, we used different functional assays (namely iodide efflux and Ussing chamber measurements) to assess CFTR function in BHK cells and CFBE monolayers, both VRT-325 and C4a showed less efficiency in restoring F508del-CFTR activity in CFBE monolayers (3.32 and 8.5% of wt-CFTR, respectively) than in BHK cells (26 and 33% of wt-CFTR, respectively).

Preliminary data resulting from the studies conducted in primary HBE cells indicates that the efficiency of this correctors (VRT-325 and C4a) are even more reduced (4.18 and 3.16% of wt-CFTR, respectively) than in heterologous systems (BHK and CFBE41o-), which is in accordance with recent published data (Rowe et al. 2010). Remarkably, VX-809 treatment resulted in a ~5.7-fold increase in $I_{sc\text{-}Fsk/Gen}$, representing a recovery of ~16% of the CFTR function observed in primary non-CF HBE cells. Notwithstanding, these results might not fully translate into native tissues or the *in vivo* situation. In fact I_{SC} data in

primary HBE cell cultures for the CFTR-inh-172 inhibitor indicate its strong potency in inhibiting CFTR-mediated Cl^- transport, which, however cannot be recapitulated in human native tissue (rectal biopsies) experiments (data not shown).

Small molecule effects on human native tissues

Rectal biopsies used to confirm/exclude a CF diagnosis, were also used here to test the effects of 6.7µM VRT-325, 10µM C4 and 10µM TS-01-02-D8 after 16-18h incubation in order to assess the effects of CFTR-modulators on native tissues *ex vivo*. Preliminary results in biopsies from F508del/F508del and F508del/G542X patients did not evidence a clear effect for VRT-325 and C4 correctors, but showed a modest effect for TS-01-02-D8.

On other hand, the incubation of non-CF tissues with these small molecules showed that VRT-325 and C4a increased CFTR-mediated Cl^- currents by ~25% and ~33%, respectively, in comparison to incubation with the vehicle alone. Data regarding the potentiator VRT-532 indicate a clear trend (~26%) to positively stimulate CFTR-mediated Cl^- currents.

Despite their limited impact, these experiments demonstrate nevertheless the potential of using human native tissues and this technique to assess the effect of correctors *ex vivo*, as we had already shown for regulators of ion transport (Roth et al., 2011).Future work will involve extending these studies to test efficacy of novel compounds on native tissues from more CF patients as well as in primary HBE cultures cells (CF and non-CF).

Altogether these results suggest that restoration of F508del-CFTR activity in primary HBE cells and on native tissues requires a greater efficacy from the above CFTR-modulators compared with cell lines, like BHK or even CFBE cells. Thus, differences in cellular processing efficiency, in addition to cell-specific effects at the plasma membrane, might contribute to the differences regarding VRT-325 and C4a rescuing efficiencies observed between polarized (3.32 and 8.5% of wt-CFTR function in CFBE cells, respectively) and non-polarized cells (26 and 33% of wt-CFTR function in BHK cells, respectively). This can also be valid for the differences found between efficacy of those CFTR-modulators in heterologous systems (BHK and CFBE cells) *vs* native cells (primary HBE).

Despite the fact that we have used different potentiators to stimulate F508del-CFTR activity in cellular systems (genistein) and in native tissues (IBMX), our data also reveals differences in the ability of small molecules, VRT-325 and C4a, in restore CFTR activity in native tissues (rectal biopsies) and in primary HBE cells. Indeed VRT-325 and C4a compounds are able to restore CFTR activity to 4.18 and 3.16% of wt-CFTR in CF primary HBE cell cultures (Fig.III.3.4), while in human native CF rectal biopsies no correction of CFTR activity by these compounds was observed (Fig.III.3.6).

This strongly evidences the existence of tissue-specific barriers (e.g, the extracellular matrix) in native tissues which are absent in cell culture, but which also have to be overcome by compounds *in vivo*. Thus, our results also highlight that the effects of a compound in one cell line expressing F508del-CFTR are poorly predictive of its activity in native tissue. Moreover, it also emphasizes the importance of pre-clinical compound testing directly on human native tissues.

It has also been a major challenge to have good clinical trials outcomes for the assessment of CFTR modulators efficacy. For instance, cAMP-dependent stimuli used in current nasal potential difference (NPD) protocols may be insufficient to fully activate F508del-CFTR, as reported by the reduced measurable rescue of CFTR activity reported in recent clinical trials (Clancy et al., 2011). Probably, the introduction of a potentiator (like genistein) in those protocols would significantly facilitate activation of mutant-CFTR and better assess the levels of protein rescued to the plasma membrane. An alternative could be the assessment of CFTR activity in rectal biopsies *ex vivo* which allows more flexible protocols. For instance, we could also compare the results obtained here for the effects of small molecules in CF rectal biopsies, where CFTR-mediated Cl^- secretion was induced with potentiator IBMX, with potentiators like genistein or even VX-770/Ivacaftor for fully activation of F508del-CFTR.

In conclusion, this kind of comparisons are highly relevant to drug discovery, namely pre-clinical validation of potential drugs particularly because results from pre-clinical studies serve as the evidence for selecting and prioritizing those potentially therapeutic CFTR-restoring agents that will move forward to clinical trials.

IV. Conclusions & Perspectives

Despite the enormous advances in CF research since the identification of the CFTR gene in 1989, a cure cannot yet be offered to patients suffering from CF. The most severe CF symptoms occur in the lungs where CFTR absence/ reduced activity at the surface of airway impairs ionic homeostasis and airway hydration, leading to increased mucus viscosity and compromising mucociliary clearance, promoting chronic infection / inflammation, and ultimately lung failure and thus reduced life expectancy. Other classical symptoms of the CF disease include meconium ileus, pancreatic insufficiency and male infertility.

For the vast majority of patients, the diagnosis of classic forms of CF is established early in life and suggested by one or more characteristic clinical features, a history of CF in a sibling or by a positive newborn screening result. Such diagnosis should usually be supported by evidence of CFTR dysfunction through identification of two CF-disease causing mutations, two abnormal sweat-Cl^- tests, and/or distinctive NPD measurements. However, there is a portion of patients being diagnosed from infancy to adulthood that present "non-classical" or milder forms of the disease, representing 2% to 10% of all CF diagnoses and depending on the ethnic background of the populations tested. To confirm/exclude a CF diagnosis in individuals with questionable diagnosis criteria, further laboratory support is required. In particular, there is a critical need for robust methods relying on the functional assessment of CFTR.

The **first objective** of the present doctoral work was to establish a sensitive and robust biomarker for CF diagnosis and prognosis. With that purpose we assessed cholinergic and cAMP-CFTR-mediated Cl^- secretion (i.e. CFTR function) by Ussing chamber measurements in 524 freshly excised rectal biopsies from 118 individuals, including patients with confirmed CF clinical diagnosis (n=51), individuals with clinical CF suspicion (n=49) and age-matched non-CF controls (n=18). Our data show that patients with "Classic CF" present earlier onset of symptoms, pancreatic insufficiency, severe lung disease and lack CFTR-mediated Cl^- secretion ($\leq$5% of normalized wt-CFTR function). Individuals with "Non-Classic" forms of CF presented residual CFTR-mediated Cl^- secretion (10-57%) and non-CF controls showed higher values for CFTR-mediated Cl^- secretion ($\geq$30-35%). Comparison of these values with those in "CF suspicion" individuals allowed to confirm CF in 16/49 individuals (33%) and exclude it in 28/49 (57%). Moreover, these data

evidenced good correlations with various clinical parameters, namely age at diagnosis, sweat Cl^- concentrations, pancreatic function (by fecal elastase E1 measurements), Shwachman-Kulczycki scores and pulmonary function (FEV1). In conclusion, determination of CFTR-mediated Cl^- secretion in rectal biopsies was demonstrated here to be a sensitive, reproducible and robust predictive biomarker for the diagnosis and prognosis of CF. Moreover, our current approach to measure CFTR-mediated Cl^- secretion in rectal biopsies is demonstrated here to be the best discriminator among Classic CF, Non-Classic CF and non-CF groups.

Still using these data of CFTR-mediated Cl^- secretion in rectal biopsies, together with previous findings concerning mRNA levels we have made an attempt to further answer the old question of "how much functional CFTR would be enough to avoid CF?" so as to define the funtional threshold necessary to avoid CF. Our previous published data showed that ~5% of normal CFTR transcripts (relative to non-CF individuals) are sufficient to attenuate CF severity (Ramalho et al., 2002). From our current data, we can now propose that CFTR levels above ~10% of normal CFTR function are required for a better CF prognosis. Indeed, CF patients with CFTR function below 10% evidence more severe CF. Moreover, non-CF controls and individuals for whom a CF diagnosis was discarded show levels of CFTR-mediated Cl^- secretion $\geq$30-35%, as also shown in our previous studies (Hirtz et al., 2004).

The **second objective** of the current work was to assess the feasibility the rectal biopsing procedure as an outcome measure in (pre-)clinical trials for CFTR modulators. To this end, we evaluated its technical aspects regarding: (i) viability of the rectal specimens for *ex vivo* bioelectrical (Ussing chamber) measurements and biochemical (Western blot and immunofluorescence) laboratory analyses; and (ii) overall assessment (comfort, invasiveness, pain, sedation requirement, etc) of the rectal biopsy procedure from the patients' perspective. We thus compared the viability of biopsies (assessed by transepithelial resistance measurements) when three different solutions for bowel preparation (NaCl 0.9%, glycerol 12% and oral mannitol), and two biopsy forceps (standard and jumbo) were used. Similar correlations were performed regarding macroscopic descriptors of the biopsies, such as levels of tissue integrity, friability, mucus and bleeding. Our results indicate that higher tissue integrity and lower friability of the specimens obtained correlate

with higher transepithelial resistance (i.e, viability) and are influenced by the solution used for bowel preparation and biopsy forceps, being the isotonic saline the most compatible with analysis and jumbo the best forceps.

Regarding the assessment of the individuals' for the overall rectal biopsy procedure (comfort, invasiveness, pain, sedation requirement, etc), the great majority of the individuals (76%) did not report high levels of discomfort due to the short procedure time (max 15 min) and relatively painless procedure (79%). Importantly, most individuals (53%) accept repeating it up to four times. The major conclusion of this survey is that rectal biopsing is a well-tolerated procedure by patients (with or without sedation), demonstrating its feasibility as an outcome measure for (pre-)clinical trials.

Despite the major advances in understanding the molecular basis of CF disease, further studies on the biosynthesis, localization and trafficking of CFTR are necessary for a more comprehensive understanding of its cellular determinants. Namely, studies from native cells and tissues which are still scarce and often contradictory, partly due to technical difficulties. Only recently those small molecules capable of restoring F508del-CFTR folding and trafficking defects have started to hit the clinical setting. For instance, correctors VX-661 and VX-809-Lumacaftor (both in Phase IIb clinical trial) that partially rescue the trafficking defect of F508del-CFTR; and potentiators like VX-770-Ivacaftor (FDA-approved drug) that corrects the gating defect of G551D-CFTR. Another drug presently under trial together with the correctors is potentiator VX-770 and is capable of stimulating CFTR function at the cell membrane.

However, the mechanism of action of these compounds should be established, which usually requires studies in heterologous expression cellular systems. Nonetheless, at pre-clinical stage determining whether compounds may proceed into clinical trials, efficacy in CF airway primary cultures/native human tissues needs to be determined. Comparative efficacy assessment between heterologous expression systems and airway primary cultures is thus of the ultimate importance before CFTR modulators reach the clinical setting. In addition, one can also envisage native tissue samples, such as rectal biopsies, as a suitable ex vivo preclinical model. Both airway primary cultures and rectal biopsies can possible be obtained from multiple individuals, and allow assessment of ion transport abnormalities, determination of CFTR

maturation and ultimately avoids issues associated with studying CFTR in non-human species.

The **third objective** of the current work was to evaluate how studies for the mechanism of action (MoA) or efficacy of CFTR modulators in heterologous expression systems may be applied to the physiological relevant tissues and ultimately, to *in vivo* situation. To this end, we performed studies with CFTR modulator compounds (correctors and potentiators) in distinct cellular systems to: i) evaluate of impact of these different small molecules on CFTR-protein maturation and Cl^- channel activity in heterologous cellular systems (BHK and CFBE41o- cells), so as to better understand their MoA; and ii) to comparatively determine their efficacy in modulating CFTR activity in those heterologous cellular systems and directly on primary cultures of human bronchial epithelial (HBE) cells/ native human tissues *ex vivo*.

Functional data obtained by iodide efflux for F508del-CFTR stably expressed in BHK cells shows that VRT-325, C4a, VRT-640 and VRT-532 rescue CFTR function at varying levels, namely: 26, 33, 19 and 35%, respectively of wt-CFTR function. In combination with low temperature (26ºC) treatment those values go up to 65, 80, 42 and 47% of wt-CFTR activity for VRT-325, C4a, VRT-640 and VRT-532, respectively.

On polarized F508del-CFTR transduced CFBE cells, C4a treatment resulted in a 1.5-fold increase in CFTR function relative to control F508del-CFTR cells. In contrast, preliminary data from Ussing chamber functional analysis conducted in primary HBE cells indicate that the efficiency of the correctors VRT-325 and C4a is lower than in BHK and in CFBE cells (4.18 and 3.16% of wt-CFTR function, respectively). Remarkably, VX-809 treatment resulted in a ~6-fold increase in CFTR-mediated Cl^- secretion, which represents a recovery of ~16% of the CFTR function observed in primary non-CF HBE cells. Furthermore, preliminary results in biopsies from F508del/F508del and F508drl/G542X patients did not evidence a clear effect for VRT-325 nor C4a, but showed a modest effect for a novel corrector compound (TS-01-02-D8) which we recently identified. On the other hand, compounds VRT-532, showed here, and 1-EBIO, in another recent collaborative study from our lab (Roth et al., 2011), demonstrate being able to potentiate cAMP-induced Cl^- secretion in excised rectal biopsies from non-CF patients, and further indicates the potential of this approach for pre-clinical validation of CFTR-modulators.

Altogether, these results suggest that the effect of a given compound in rescuing F508del-CFTR activity in heterologous systems is poorly predictive of its rescuing capacity in primary bronchial epithelial cells and more strikingly in native tissues, indicating that pre-clinical validation in native tissues *ex vivo* is highly recommended.

Concluding remarks and perspectives for future work

It has been suggested that different small molecule correctors could act at different steps along the folding pathway and thus multi-drug approach to CF disease might be appropriate to target this and other misfolding disorders. Moreover, the combination of a molecule that promotes folding of mutant-CFTR (corrector) with another that corrects its gating defect (potentiator), or a single molecule that comprises this dual-activity, can uncover even more promising results and reveal structural "hot-spots" for therapeutic purposes. In this regard, the outcoming results from both clinical trials (phase 2b) of the combination of correctors VX-661 or VX-809/Lumacaftor with potentiator VX-770/Ivacaftor would shed some light into the efficacy of correctors and combiantion of therapies. It is likely that a third compound may still be needed with a different function to render rescuing more efficient, i.e.: 1) correcting, 2) stimulating and, 3) stabilizing F508del-CFTR at the plasma membrane. This could be achieved by using compounds or factors that either prevent its increased rate of internalization or enhance its anchoring and retention at the cell surface.

From the results presented along this doctoral work, we envisage the need to achieve a corrective therapy able to restore at least 10% of wt-CFTR function in order to prevent the severe symptoms of CF disease. Notwithstanding, we propose that a cure can only be probably achieved when 30-35% levels of wt-CFTR function are reached. Future work should focus on the search for combined therapies aiming to achieve these levels of CFTR-activity restoration.

Ongoing clinical drug trials have demanded a search for more reliable CF diagnostic tools and more robust biomarkers to assess CF corrective therapies. For instance, the nasal potential difference (NPD) protocols currently used for both diagnosis and therapy assessment rely on cAMP-dependent *stimuli* which may be insufficient to fully activate F508del-CFTR, as

reported by the reduced measurable rescue of CFTR activity in recent clinical trials concerning VX-809/Lumacaftor. Probably, the introduction of a potentiator (like genistein or VX-770) in those protocols would significantly facilitate activation of mutant-CFTR and better assess the levels of protein rescued to the plasma membrane. In previous and present studies, we have demonstrated that assessment of CFTR-mediated Cl^- secretion in rectal biopsies is a feasible, highly informative and reproductible approach to this end. A recent study from another group (Gonska et al., 2009), introduced a novel technique to assess CFTR function in vivo by measuring the transductal voltage of sweat glands at the skin surface (Sweat Potential Difference - SPD), showing promising results as a new diagnostic tool for CF. In this regard, both measurements of SPD and rectal CFTR-mediated Cl^- secretion appear to be comparably good outcomes for monitoring changing levels of CFTR function in therapeutic clinical trials. Further studies need to focus on monitoring the efficay of CFTR-modulators in physiolologically relevant tissues, e.g., primary cultures of human bronchial epithelial cells and rectal biopsies so as to establish a pre-clinical model which is highly predictive of the *in vivo* MoA and response to those modulators. Future studies like those performed here in rectal biopsies *ex vivo*, should proceed with validation of the disruptive effects caused by different mutations on CFTR expression and function. The same studies can simultaneously pre-clinically assess the efficacy of the novel CFTR modulators in rescuing those defects. Furthermore, the same rectal biopsy measurements approach may then be used as a feasible outcome measure in clinical trials for the same patients being treated by these CFTR modulators.

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VI. Apendix

Table VI.1-A - Overview of data for all CF patients in the reference group:

ID	Gender	Group Age (years)	*CFTR* Genotype		Age at Diagnosis (years)	Sweat Sodium Mean (mmol/L)	Sweat Chloride Mean (mmol/L)	Fecal Elastase (μg/g)	BMI	SK score	Last FEV1 (% predicted)	Last FVC (% predicted)
106/2010	F	0 - 9	F508del	G542X	2	101.6	116.1	10	22.62	80	88	86
84/2010	F	0 - 9	F508del	R1066C*	3	76	99.45	7.41	15.64	80	96	105
127/2007[a]	F	0 - 9	F508del	F508del	8	108.8	116.5	2.17	15	65	84	93
126/2007[a]	F	0 - 9	F508del	2183AA>G*	3	111.7	119.5	8.57	14.59	70	68	75
54/2010	F	10 - 19	F508del	F508del	4	79	105.9	n.a.	16.06	55	38	52
107/2010	F	10 - 19	F508del[1]	-	6	89	109.3	10	20.54	70	62	75
35/2010	M	20 - 29	F508del	F508del	21	89.75	119.56	2.44	21.58	55	58	89
19-101/2010	M	10 - 19	F508del	F508del	0.5	117	140	6.81	14.65	60	101	78
73/2010	F	0 - 9	F508del	G542X	0.5	77.3	89	1	14.55	85	84	95
45/2010	F	0 - 9	F508del	F508del	0.4	110	130.03	7.83	15.59	70	n.a.	n.a.
109/2007	M	0 - 9	F508del	R1162X	2	109.5	120	1	12.81	40	31	27
25/2010	F	0 - 9	F508del	F508del	0.5	61.9	90.1	15.6	11.29	55	40	54
49/2010	M	10 - 19	F508del	F508del	1	71.6	106.4	2.67	26.56	70	86	97
92/2010	F	0 - 9	F508del	F508del	0.5	-	108.5	16.97	20.57	95	n.a.	n.a.
88/2010	M	0 - 9	F508del	F508del	1	109	131.6	2.83	17.22	80	75	72
51/2010	M	0 - 9	F508del	F508del	0.2	96	103	3.85	16.9	60	92	99
103/2010	F	0 - 9	F508del	F508del	5	70.6	100.8	6.65	17.71	75	103	104
53/2010	F	0 - 9	F508del	F508del	1	76.82	121.6	5.38	14.61	85	n.a.	n.a.
46/2010	M	0 - 9	F508del	F508del	0.8	82	96.5	5.3	15.92	95	104	97
91/2010	M	0 - 9	F508del	1717-1G>A*	2	77.7	116	1.98	15.8	85	102	97
105/2010	F	0 - 9	F508del	1812-1G>A*	0.5	83.2	100.2	10	19.9	65	95	92
44/2010	M	10 - 19	F508del	F508del	2	92.6	106.8	1	17.94	60	80	88
21/2010	F	20 - 29	F508del	R553X	1.5	74	135	6.71	21	60	45	61
30/2010	F	10 - 19	F508del	F508del	3	98.2	118.5	30.76	15.04	70	40	35
31/2010	F	10 - 19	F508del	G542X	3	81	121	16.56	20.21	85	54	59
06/2010	M	0 - 9	F508del	F508del	0.5	68.3	92.7	n.a.	13.59	45	64	92
48/2010	M	10 - 19	F508del	F508del	7	99.5	106	12.19	21.16	65	77	74
139/2007	F	10 - 19	F508del	F508del	0.3	89.2	107.5	1	13.27	35	39	46
52/2010	M	0 - 9	F508del	F508del	5	82.5	93.2	7.19	27.32	65	82	92
78/2010	F	0 - 9	F508del	F508del	3	94.73	127.22	10	15.5	85	76	117
85/2010	F	10 - 19	F508del	G542X	8	118	140	n.a.	14.27	65	103	108
40/2010[b]	M	0 - 9	F508del	F508del	0.3	89.07	108.4	10	13.44	60	44	48
41/2010[b]	M	0 - 9	F508del	F508del	0.3	87.6	128.28	5.28	13.86	85	48	59
83/2010	F	10 - 19	F508del	F508del	0.3	92.8	125.6	10	14.31	65	94	116
108/2007	M	0 - 9	F508del	G542X	0.3	69.8	110.3	1	13.99	90	n.a.	n.a.
87/2010	M	10 - 19	F508del	F508del	0.5	86.2	97.5	n.a.	16.64	90	86	83
65/2010	M	20 - 29	F508del	N1303K	0.3	136	147	5.17	23.98	55	41	48
37-81/2010	M	> 30	F508del	F508del	7	104.9	135.6	4.43	21.02	65	62	90
34/2010	F	10 - 19	F508del	F508del	0.4	84.6	106.7	7.19	13.29	80	55	65
71/2010	F	10 - 19	F508del	G542X	1	54.3	87.7	7.83	22.33	75	59	87
133/2007	M	10 - 19	F508del	F508del	0.5	114.3	154.4	6.87	15.91	90	89	97
82/2010	M	20 - 29	F508del	F508del	10	68.9	106.9	9.83	22.49	50	72	87
55/2010	F	10 - 19	F508del	F508del	3	92.2	100.4	10	22.99	60	74	77
110/2007	M	> 30	G542X	2183AA>G*	2	91.7	101.86	n.a.	23.84	50	36	47
75/2010	F	10 - 19	F508del	1812-1G>A*	0.1	114.6	115.8	n.a.	18.77	80	81	89
50/2010	M	10 - 19	F508del	F508del	3	107.6	123.8	10	14.94	60	79	89
63/2010[c]	F	> 30	F508del	R334W	38	94	145.4	229.16	25.44	80	65	79
64/2010[c]	M	> 30	F508del	R334W	22	111.8	137.4	578.82	17.99	55	56	65
96/2010	F	10 - 19	F508del	G85E*	1	83.8	151	103.89	23.62	50	31	49
66/2010	F	> 30	F508del	3272-26A>G*[1]	31	89.9	97	544.53	20.49	40	52	85
111/2007	F	20 - 29	G542X	I618T*	20	83.5	86.5	693.11	18	70	78	95

n.a. - Not analysed

* - Data not known at the time of the study

$ - Sa, *Staphylococcus aureus;* Pa, *Pseudomonas aeruginosa;* Sm, *Stenotrophomonas maltophilia;* Bc, *Burkholderia cepacia;* Ax, *Achromobacter xylosoxidans;* Af, *Aspergillus fumigatus;* Mt, *Mycobacterium tuberculosis*

- Maximum lumen-positive or lumen-negative peak (and lumen-negative plateau) CCH-induced I_{sc} in the presence of IBMX/Fsk are shown

§ - Percentage (%) of CFTR function was calculated for maximal CFTR activation ($\Delta I_{sc\text{-}IBMX/Fsk} + \Delta I_{sc\text{-}CCH(IBMX/Fsk)}$) and normalized to the correspondent mean value for the reference non-CF control group

a - First cousins

b - Monozygotic twins

c - Siblings

1 - TG10T9/TG11T7

genotypes, clinical phenotypes and Ussing chamber measurements, leading to confirmation / exclusion of a CF Diagnosis

Pathogens detected (Lungs)$	Nasal Polyposis	Meconium Ileus	Glucose Intolerance (GI) / Diabetes	Osteopenia / Osteoporosis	Other Clinical Features	basal Isc-CCH ($\mu A/cm^2$)	Isc-IBMX/Fsk ($\mu A/cm^2$)	Isc-CCH (IBMX/Fsk) ($\mu A/cm^2$)#	% CFTR Function§	Clinical Diagnosis
Sa, Pa, Af	Negative	Negative	Diabetes	Negative	Hepatic Steatosis; Verminosis	29.42	20.37	22.77 (-5.29)	2	Classic CF
Sa, Pa	Negative	Negative	Negative	Negative	Hepatic Steatosis	12.65	3.59	10.73 (-2.48)	1	Classic CF
Sa	Negative	Negative	Diabetes / GI	Negative	-	18.93	4.55	13.61 (0)	0	Classic CF
Sa	Negative	Negative	Diabetes / GI	Negative	Hepatic Steatosis	25.4	7.95	35.42 (0)	0	Classic CF
Sa	Positive	Negative	GI	Osteoporosis	-	17.39	6.02	15.1 (0)	0	Classic CF
Pa, Sa	Positive	Negative	GI	Osteoporosis	-	45.43	12.91	21.42 (-5.25)	2	Classic CF
Sa, Pa	Negative	Negative	GI	Osteopenia	-	15.96	12.82	18.56 (-3.94)	2	Classic CF
Pa, Sa	Negative	Negative	Negative	Negative	Chronic liver disease	43.69	9.61	29.22 (-9.19)	4	Classic CF
Sa	Positive	Positive	Negative	Negative	-	20.62	5.03	20.3 (-3.55)	2	Classic CF
Sa, Pa	Negative	Negative	Negative	Negative	-	43.41	6.75	35.59 (0)	0	Classic CF
n.a.	Negative	Positive	Negative	Negative	Chronic liver disease	35.1	14.57	84.89 (0)	0	Classic CF
Sa, Bc, Pa	Negative	Positive	Negative	Negative	Digital clubbing; lung hyperinflation;	34.77	10.35	27.37 (-2.54)	1	Classic CF
Pa	Negative	Negative	Negative	Osteoporosis	Chronic liver disease	20	10.21	14.84 (-1.12)	1	Classic CF
Normal Flora	Negative	Negative	Negative	Negative	-	19.15	7.2	18.66 (-9.32)	4	Classic CF
Normal Flora	Negative	Negative	Negative	n.a.	-	55.52	28.22	34.79 (-4.83)	2	Classic CF
Sa	Negative	Positive	Negative	Negative	-	15.32	5.94	17.74 (0)	0	Classic CF
Sa	Negative	Negative	Negative	Negative	Digital clubbing; steatorrea	45.07	17.43	50.2 (0)	0	Classic CF
Pa	Negative	Positive	Negative	Negative	Biliary lithiasis	25.18	5.7	29.76 (0)	0	Classic CF
Pa	Negative	Negative	Negative	Negative	Failure to thrive; malabsorption	19	16.15	7.07 (-2.39)	1	Classic CF
Pa, Sa	Negative	Positive	Negative	Negative	-	97.63	18.41	58.68 (-2.66)	1	Classic CF
Sa	Negative	Negative	Diabetes	Negative	-	32.12	21.16	53.9 (-4.96)	2	Classic CF
Sa, Pa	Negative	Negative	Negative	Negative	-	21.36	12.5	15.39 (-1.5)	1	Classic CF
Pa	Positive	Negative	Diabetes / GI	Osteoporosis	Digital clubbing; colilithiasis	9.4	10.55	19.91 (0)	0	Classic CF
Sa, Bc	Negative	Negative	Negative	Negative	Renal lithiasis; liver disease	24.88	18.58	22.78 (0)	0	Classic CF
Sa, Ax, Af	Negative	Negative	Negative	Negative	Esophagitis	10.72	12.26	18.78 (-0.31)	0	Classic CF
Pa	Negative	Positive	Negative	Negative	Digital clubbing; gastrostomy	81.67	6.66	35.47 (-2.34)	1	Classic CF
Sa	Positive	Negative	Negative	Negative	Failure to thrive; hepatic steatosis	41.34	8.27	40.1 (-1.34)	1	Classic CF
n.a.	Negative	Negative	Negative	Osteoporosis	Oxygen dependent; lobectomy; liver	21.54	10.33	21.84 (0)	0	Classic CF
Sa	Positive	Positive	n.a.	Negative	Bilateral nasal polypectomy	18.69	10.52	20.43 (0)	0	Classic CF
Pa, Sa, Sm	Negative	Positive	Diabetes	Negative	Hepatic steatosis	28.27	18.48	28.52 (0)	0	Classic CF
Sa	Negative	Negative	Negative	Negative	-	34.09	12.55	24.63 (0)	0	Classic CF
Sa	Negative	Positive	Negative	Negative	-	24.01	12.34	20.88 (0)	0	Classic CF
Sa	Negative	Positive	Negative	Negative	Hepatic steatosis	25.85	13	16.52 (0)	0	Classic CF
Sa, Pa	Positive	Negative	Negative	Negative	Hepatic steatosis; IgA deficiency	31.7	4.72	17.36 (0)	0	Classic CF
n.a.	Negative	Negative	Negative	Negative	Failure to thrive; metabolic alkalosis	50	12.2	56.87 (0)	0	Classic CF
Pa	Positive	Negative	Diabetes	Negative	Failure to thrive	50.55	15.6	33.91 (-4.42)	2	Classic CF
Pa, Sa, Bc	Negative	Negative	Diabetes	Osteopenia	Two sisters with CF	31.83	5.8	30.37 (0)	0	Classic CF
Sa, Pa	Negative	Negative	Diabetes / GI	Negative	Hepatic steatosis; Barrett's esophagus	49.22	9.02	14.22 (0)	0	Classic CF
Pa, Sa	Negative	Negative	Negative	Negative	-	57.6	9.74	31.18 (-5.34)	2	Classic CF
Pa	Positive	Negative	GI	Negative	-	10.43	7.35	14.84 (-2.80)	1	Classic CF
Pa, Sa	Negative	Negative	Negative	Negative	-	14.46	10.32	25.36 (0)	0	Classic CF
Bc, Sa, Af	Negative	Negative	GI	Osteoporosis	Digital clubbing; azoospermia	18.12	6.74	12.27 (0)	0	Classic CF
Sa, Pa, Af	Negative	Positive	Negative	Negative	-	24.17	16.08	39.93 (0)	0	Classic CF
Sm, Af	Negative	Negative	Negative	Negative	-	21.65	15.17	23.47 (0)	0	Classic CF
Sa, Pa	Positive	Negative	Negative	Negative	-	30	12.95	34.87 (0)	0	Classic CF
Sa, Pa, Af	Negative	Negative	Negative	Negative	-	25.85	3.32	9.4 (-3.39)	2	Classic CF
Pa	Negative	Negative	Negative	Negative	-	7.49	-12.94	5.31 (-9.59)	10	Non-Classic CF
Sa, Pa	Negative	Negative	Negative	Negative	-	17.33	-15.54	8.7 (-14.24)	14	Non-Classic CF
Pa, Bc	Positive	Negative	Diabetes	Negative	-	96.36	-13.8	67.47 (-12.14)	12	Non-Classic CF
Pa, Sa	Negative	Negative	Negative	Negative	-	23.87	-11.74	23.12 (-19.13)	14	Non-Classic CF
Sa, Af	Negative	Negative	Negative	Negative	Allergic Bronchopulmonary	46.89	-27.31	36.17 (-53.07)	37	Non-Classic CF

Table VI.1-B - Overview of data for all individuals in the CF-suspicious group:

ID	Gender	Group Age (years)	*CFTR* Genotype		Age at Diagnosis (years)	Sweat Sodium Mean (mmol/L)	Sweat Chloride Mean (mmol/L)	Fecal Elastase E1 (µg/g)	BMI	SK score	Last FEV1 (% predicted)	Last FVC (% predicted)
95/2010	F	0 - 9	F508del*	F508del*	0.5	-	126*	1	13	35*	45	61
38/2010	F	20 - 29	A561E*	A561E*	20	54.4*	58.3*	11.02	17.83	60	57	70
119/2007	F	0 - 9	R1162X*	R1162X*	1	70.34¥	115¥	1	13.61	55	n.a.	n.a.
09/2010	M	0 - 9	F508del*	F508del*	0.2	-	120*	1.37*	22.93	75*	n.a.	n.a.
43/2010[a]	M	0 - 9	F508del*	F508del*	1	104¥	147¥	8.1	15.77	70	n.a.	n.a.
118/2007	F	0 - 9	F508del	1717-1G>A*	0.2	90¥	110¥	n.a.	16.26	90	131	142
33/2010[b]	M	0 - 9	3120+1G>A*	R1066C*[1]	2	95.9¥	129¥	10	13.99	55	73	75
32/2010[b]	M	0 - 9	3120+1G>A*	R1066C*[1]	0.5	115.6¥	129.2¥	4.18	14.34	40	n.a.	n.a.
86/2010	F	0 - 9	F508del	S549R(T>G)*[2]	0.5	71.76¥	109.8¥	97.51	16.19	80	n.a.	n.a.
27/2010[c]	F	0 - 9	W1282X[3]	-	1	-	49.7*	37.74	16.5	90*	n.a.	n.a.
60-98/2010	F	> 30	3120+1G>A*	L206W*[4]	30	86.3¥	122.8¥	606.13*	24.77	55	70	76
136/2007[d]	M	10 - 19	F508del	1716+18672 A>G*	12	65.9¥	95¥	68.89*	21.72	95	111	102
135/2007[d]	F	10 - 19	F508del	1716+18672 A>G*	10	83.6*	87*	608.36*	21.12	85	104	99
123/2007	F	> 30	F508del	P205S*	30	78¥	107.6¥	668.19*	19.61	45*	58	67
137/2007	M	20 - 29	G542X	P205S*	21	97.6	124.3	816.07*	18.69	40*	60	43
124/2007	F	20 - 29	G542X*	R334W*	20	99.8	113.9	379.96*	25.89	80	46	69
15/2010	F	0 - 9	Negative for: F508del and R1162X		-	64.75	66.39	747.57*	15.1	90	106*	128*
131/2007	F	> 30	n.i.*[5]		-	54.2	58.65	875.4*	14.87	55	n.a.	n.a.
90/2010	F	0 - 9	Negative for: F508del[1]		-	-	45*	n.a.	14.48	n.a.	n.a.	n.a.
72/2010	F	10 - 19	Negative for: F508del, G551D, G542X,		-	55.3	54.5	635.8*	17.62	90*	73	92
128/2007	F	10 - 19	Negative for: F508del and N1303K		-	18.8	26.9	n.a.	16.01	95*	54	72
121/2007	M	> 30	n.i.*[2]		-	26.3*	22.6*	6.66	20.11	95	93	94
13/2010	M	10 - 19	Negative for: F508del*		-	65.3¥	62.8¥	629.43*	28.34	95*	84	90
89/2010	M	0 - 9	Negative for: F508del*[6]		-	51	40	n.a.	14.44	70*	76*	104*
102/2010	M	> 30	Negative for: F508del*[1]		-	-	23	n.a.	24.91	n.a.	n.a.	n.a.
68/2010	M	20 - 29	n.i.*[7]		-	45.3*	47.3*	524.25	28.15	95*	82	92
130/2007	M	20 - 29	n.i.*[8]		-	53.5	63.9	524.25	17.92	75	84	70
132/2007	F	10 - 19	n.i.*[7]		-	77.4	82.2	774.4*	15.82	55	74	71
114/2007	F	20 - 29	Negative for: F508del*		-	-	25.3	n.a.	17.99	n.a.	n.a.	n.a.
122/2007	M	20 - 29	n.i.*[7]		-	71.7	76.3	683.15*	24.22	100*	72	88
140/2007	M	10 - 19	n.i.*		-	66.6	80.4	524.25	24.42	80*	83*	101*
18/2010	F	10 - 19	Negative for: F508del*		-	75.1	77.2	407.4*	19.81	100*	82*	78*
08/2010	M	10 - 19	Negative for: F508del		-	73.3	69.2	n.a.	28.26	95*	94	96
117/2007	M	10 - 19	Negative for: F508del, G542X, R1162X and		-	18.7¥	14¥	855.07*	21	95*	106*	94*
115/2007	M	0 - 9	Negative for: F508del*		-	-	38	n.a.	15.03	n.a.	n.a.	n.a.
116/2007	M	> 30	n.i.*[1]		-	-	19.7	524.25	15.85	80	n.a.	n.a.
42/2010[a]	M	0 - 9	F508del*	-	-	93¥	108¥	n.a.	16.06	95	96	91
74/2010	M	10 - 19	Negative for: F508del, R1162X and N1303K		-	84	85	340*	18.72	85	90	94
61/2010	M	> 30	n.i.*[1]		-	84¥	88.7¥	524.25	24.86	95	82	92
70/2010[e]	F	10 - 19	Negative for: F508del and R1162X		-	65.8	70.5	612.3*	12.43	100*	68	74
100/2010	F	10 - 19	Negative for: F508del*[7]		-	-	42	682.83*	15.67	80	85	106
138/2007	M	10 - 19	Negative for: F508del*		-	41	51.5	n.a.	25	n.a.	n.a.	n.a.
99/2010	F	> 30	F508del	D1152H*	35	71.1*	72.25*	611.55*	19.96	60*	63	60
76/2010[c]	M	> 30	W1282X	4428insGA*	49	105.5*	136.2*	640.57*	26.22	95*	82*	91*
134/2007	F	0 - 9	G576A*	-		31.1	31.8	773.57*	15.41	95*		
07/2010	M	20 - 29	V562I*	n.i.*[8]		53.3	52.6	592.72*	21.91	75	40	21
125/2007	M	0 - 9	G542X	Negative for: F508del,	-	14.6	20	n.a.	16.84	n.a.	n.a.	n.a.
26/2010	M	0 - 9	Negative for: F508del and R1162X		-	49.6	50.5	n.a.	14.87	90*	n.a.	n.a.
69/2010[e]	M	10 - 19	Negative for: F508del		-	84	85	n.a.	16.55	100*	97*	98*

n.a. - Not analysed

n.i. - All 27 exons and intronic flanking regions of CFTR gene were sequenced and no other mutations were identified

*** - Data not known at the time of the study**

¥ - Only one sweat test done at the time of the study

$ - Sa, *Staphylococcus aureus;* Pa, *Pseudomonas aeruginosa;* Sm, *Stenotrophomonas maltophilia;* Bc, *Burkholderia cepacia;* Ax, *Achromobacter xylosoxidans;* Af, *Aspergillus fumigatus;* Mt, *Mycobacterium tuberculosis*

 # - Maximum lumen-positive or lumen-negative peak (and lumen-negative plateau) CCH-induced I_{sc} in the presence of IBMX/Fsk are shown

§ - Percentage (%) of CFTR function was calculated for maximal CFTR activation ($\Delta I_{sc\text{-}IBMX/Fsk} + \Delta I_{sc\text{-}CCH(IBMX/Fsk)}$) and normalized to the correspondent mean value for the reference non-CF control group

genotypes, clinical phenotypes and Ussing chamber measurements, leading to confirmation / exclusion of a CF Diagnosis

Pathogens detected (Lungs)$	Nasal Polyposis	Meconium Ileus	Glucose Intolerance (GI) / Diabetes	Osteopenia / Osteoporosis	Other Clinical Features	basal Isc-CCH ($\mu A/cm^2$)	Isc-IBMX/Fsk ($\mu A/cm^2$)	Isc-CCH (IBMX/Fsk) ($\mu A/cm^2$)#	% CFTR Function§	Clinical Diagnosis
n.a.	n.a.	Positive*	Negative*	Negative*	-	47.3	20.4	90.19 (0)	0	Classic CF*
Sa, Pa, Sm	Negative*	Negative*	Diabetes / GI	Negative	Digital clubbing	23.1	12.23	28.31 (0)	0	Classic CF*
Sa	Negative*	Negative*	Negative*	Negative*	Failure to thrive	22.42	34.22	28.66 (0)	0	Classic CF*
n.a.	Negative*	Positive*	Negative*	Negative*	-	92.5	3.95	17.08 (-8.51)	4	Classic CF*
Sa, Pa, Bc	Negative*	Negative	Negative	Negative	-	15.9	5.04	38.99 (0)	0	Classic CF*
Sa	Negative*	Positive	Negative	Negative	Chronic liver disease; steatorrea;	11.46	28.26	33.89 (0)	0	Classic CF*
Sa, Pa	Negative*	Negative*	Negative	Negative*	-	46.2	10.08	28.85 (0)	0	Classic CF*
Candida	Negative*	Positive	Negative	Negative*	-	55.09	11.74	66.05 (0)	0	Classic CF*
Sa	Negative*	Negative	Negative	Negative*	Metabolic alkalosis	13.17	-7.08	14.72 (-4.51)	5	Classic CF*
n.a.	Negative*	Negative*	Negative*	Negative*	Congenital cardiopathy	55.5	-42.1	6.73 (-36.36)	36	Non-Classic CF*
Pa, Sa, Af	Negative*	Negative*	Negative*	Osteoporosis	Bronchiectasis; lung exacerbations	30.84	-47.31	5.47 (-76.34)	57	Non-Classic CF*
Sa	Positive	Negative	Negative	Osteoporosis	-	22.57	-21.12	8.47 (-40.7)	28	Non-Classic CF*
Sa	Positive	Negative	GI	Negative	-	34.16	-22.36	27.81 (-50.71)	34	Non-Classic CF*
Pa	Negative*	Negative*	Negative*	Negative	Hypothyroidism	28.13	-12.03	7.68 (-10.37)	10	Non-Classic CF*
Pa	Negative	Negative	Diabetes	Osteoporosis	Azoospermia; hepatic steatosis;	25.67	-7.42	14.72 (-19.91)	13	Non-Classic CF*
Sa, Pa	Positive	Negative	Negative	Negative	-	26.3	-17.5	14.52 (-18.1)	16	Non-Classic CF*
Sa, Mt*	Negative*	Negative*	Negative*	Negative*	-	-149.68	-178.11	-254.89	199	Non-CF*
n.a.	Positive	Negative*	Negative*	Osteoporosis	-	-86.6	-30.58	-138.61	78	Non-CF*
n.a.	Negative	Negative*	Negative	Negative*	Pneumonia; diarrhea	-211.11	-53.66	-249.34	139	Non-CF*
n.a.	Negative*	Negative*	Negative*	Negative*	-	-124.27	-20.29	-117.3	63	Non-CF*
n.a.	n.a.	n.a.	n.a.	Negative	-	-96.25	-12.22	-53.59	30	Non-CF*
Normal Flora	Negative	Negative	Diabetes	Negative	Severe steatorrhea	-83.01	-24.14	-155.09	82	Non-CF*
n.a.	Negative	Negative	n.a.	Negative	Obesity; dyspnea; thalassemia trait	-176.86	-45.9	-104.02	69	Non-CF*
n.a.	Negative	Negative	Negative	Negative	-	-100.02	-193.88	-379.46	263	Non-CF*
Normal Flora	Positive	Negative	Negative	n.a.	Normal spermogram; repetitive	-232.43	-13.68	-213.78	104	Non-CF*
n.a.	Negative*	Negative	Negative*	Negative	-	-66.11	-14.33	-145.89	73	Non-CF*
Normal Flora	Negative*	Negative	Negative*	Negative	Oligozoospermia	-39.67	-46.56	-102.25	68	Non-CF*
Normal Flora	Negative*	Negative*	Negative*	Osteopenia	Digital clubbing; liver disease;	-30.34	-33.71	-190.95	103	Non-CF*
n.a.	Positive	Negative	Negative	Negative	Bronchiectasis; pansinusopathy;	-61.24	-37.2	-101.98	64	Non-CF*
n.a.	Negative*	Negative*	Negative*	Negative*	Good ponderal status	-80.79	-29.24	-149.01	82	Non-CF*
n.a.	Negative	Negative*	Negative*	Osteopenia	Chronic diarrhea; dermatomyositis;	-44.96	-110.05	-347.21	210	Non-CF*
n.a.	Negative	Negative*	Negative*	Negative*	Repeated pancreatitis	-136.1	-42.95	-129.51	79	Non-CF*
n.a.	Positive	Negative*	Negative*	Negative*	Normal spermogram; nasal	-45.67	-73.39	-176.82	115	Non-CF*
n.a.	Negative	Negative	Negative	Negative	Failure to thrive; steatorrhea; asthma;	-65.35	-57.23	-137.09	89	Non-CF*
Normal Flora	Negative	Negative	Negative	Negative	Duodenal ulcer; pancreatic calculus;	-91.11	-17.49	-57.36	34	Non-CF*
Pa	Negative	Negative	Negative	Negative	Normal spermogram; Bronchiectasis	-61.72	-22.04	-93.59	53	Non-CF*
Normal Flora	Negative*	Negative	Negative	Negative	-	-24.07	-15.78	-50.16	30	Non-CF*
Sa	Negative	Negative	Diabetes	Negative	IgA deficiency	-70	-34.72	-89	57	Non-CF*
n.a.	Negative	Negative	Negative	Negative	Repetitive pneumonias; obesity	-67.09	-24.91	-117.72	65	Non-CF*
n.a.	Positive	Negative	Negative	Negative	-	-128.84	-27.83	-141.64	78	Non-CF*
Normal Flora	Negative	Negative	Negative	Negative	Pansinusopathy	-406.51	-46.11	-247.23	135	Non-CF*
n.a.	n.a.	n.a.	n.a.	n.a.	CT scan: bronchial wall thickening	-93.91	-28.87	-154.23	84	Non-CF*
n.a.	Negative	Negative	Negative	Osteoporosis	-	51.47	-42.69	-97.69	64	CFTR-RD*
n.a.	Positive	Negative	Negative	Negative*	Obstructive Azoospermia (CBAVD)	54.77	-83.85	-99.19	84	CFTR-RD*
n.a.	Negative	Negative	Negative	Negative	Neuroblastoma*	-	-	-	-	Inconclusive
Sa, Pa	Positive	Negative	Negative	Osteoporosis	Bronchiectasis and obstructing	-	-	-	-	Inconclusive
n.a.	Negative	Negative	Negative	Negative	-	-	-	-	-	Inconclusive
n.a.	Negative*	Negative*	Negative*	Negative*	-	-	-	-	-	Inconclusive
n.a.	Positive	Negative	Negative	Negative	Repetitive pneumonias	-	-	-	-	Inconclusive

a - Siblings
b - Siblings
c - Patient 76/2010 is father (by in vitro fertilization) of patient 27/2010
d - Siblings
e - Siblings

1 - TG11T7/TG11T7
2 - TG10T9/TG11T7
3 - TG10T7/TG12T5
4 - TG9T9/TG10T7
5 - TG12T7/TG12T7
6 - TG10T7/TG12T7
7 - TG10T7/TG10T7
8 - TG10T7/TG11T5
9 - TG11T5/TG11T7