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SPECTRUM OF CFTR MUTATIONS IN CYSTIC FIBROSIS AND IN CYSTIC FIBROSIS RELATED DISORDERS

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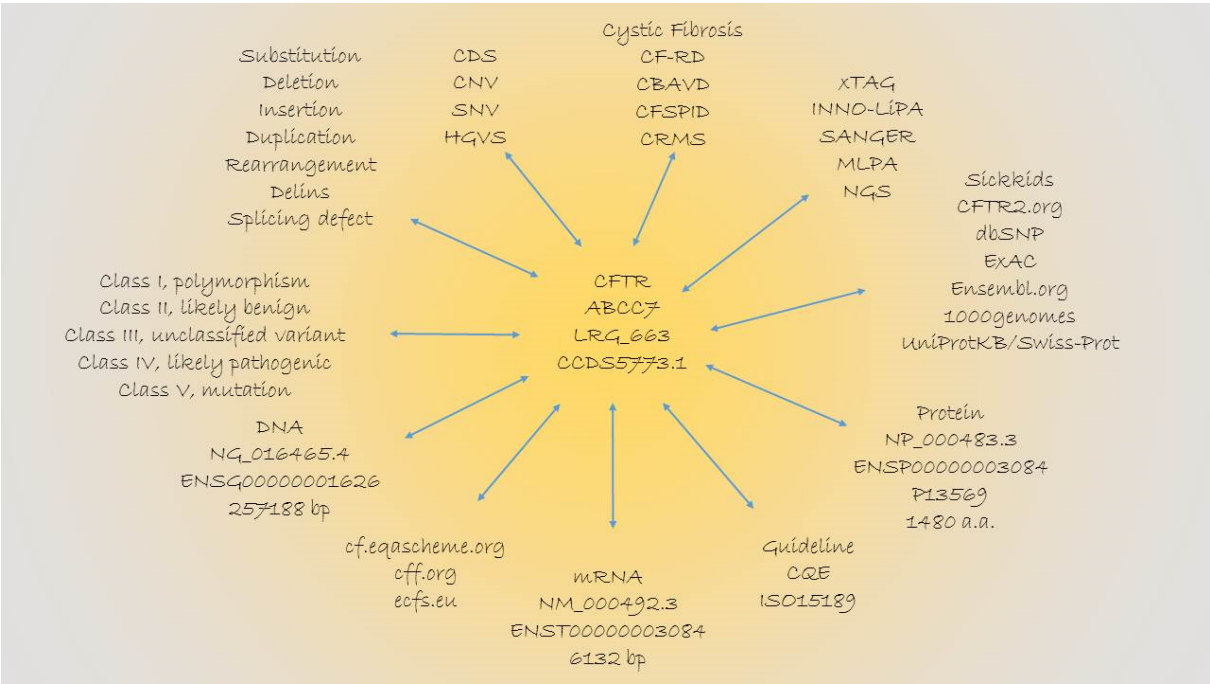

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Ce doctorat a permis de joindre deux choses qui me tiennent à cœur : aider la connaissance du diagnostic moléculaire de la mucoviscidose et le développement technologique en Argentine.

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List of abbreviations

1000 genomes	1000 Genomes Project
ABCC7	Adenosine triphosphate binding cassette sub-family C member 7
ADIPOR2	adiponectin receptor 2
ABLM	Association Belge de Lutte contre la Mucoviscidose
ACMG	American College of Medical Genetics
ACOG	American College of Obstetricians and Gynecologists
AMP	Adenosine monophosphate
cAMP	Cyclic adenosine monophosphate
CBAVD	Congenital atrophy or absence of the *vas deferens*
CDS	Coding exons
CF	Cystic fibrosis
CFRD	Cystic Fibrosis related diabetes
CFTR	Cystic Fibrosis transmembrane conductance regulator gene
CFTR	Cystic Fibrosis transmembrane conductance regulator protein
CFGAC	Cystic Fibrosis Genetic Analysis Consortium
CFSPID	CF screening positive inconclusive diagnosis
CNV	Copy number variation
CRMS	CFTR-related metabolic syndrome
DIOS	Distal obstruction syndrome
DNA	Deoxyribose nucleic acid
ECFS	European Cystic Fibrosis Society
EDNRA	Endothelin receptor gene A
ENaC	Epithelin receptor A
EurocareCF	European Coordination Action for Research in Cystic Fibrosis
ExAC	Exome Aggregation Consortium
ICP	Idiopathic chronic pancreatitis
IgE	Immunoglobulin E
IRT	Immunoreactive trypsinogen

INNO-LiPA	Method by Fujirebio Europe N.V. company
ISO15189	International Organisation for Standardization for Medical laboratories
kb	Kilobase
MBL2	Mannose binding lectin 2
MIF	Migration inhibitory factor
MLPA	Multiplex Ligation-dependent Probe Amplification
mRNA	Messenger RNA
MSD	Membrane-spanning domain
NBD	Nucleotide-binding domain
NCBI	National Center for Biotechnology Information
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
PI	Pancreatic insufficient
PK	Protein kinase
PRSS1	protease, serine 1
PS	Pancreatic sufficient
QPIT	Quantitative pilocarpine iontophoresis sweat test
R domain	Regulatory domain
SLC4A4	solute carrier family 4 member 4
SPINK1	serine peptidase inhibitor, Kazal type 1
SNV	single nucleotide variation
Src	Rous Sarcoma virus oncogene Cellular homolog
TGF-β	Transforming growth Factor beta
TLR4	Toll-Like Receptor 4
TNFα	Tumor Necrosis Factor α
ULB	Université Libre de Bruxelles
UCL	Université catholique de Louvain
ULg	Université de Liège
UCSC	University of California, Santa Cruz
USA	United States of America
xTAG	Method by Luminex company

Abstract

A large spectrum of more than 2,000 *CFTR* mutations have been reported, associated with a very diverse clinical phenotype of Cystic Fibrosis (CF). In this work we analyzed the spectrum of *CFTR* mutations in CF and in CF-related disorders. The project specifically aims at investigating the following points:

1) Validation of a new diagnostic screening method xTAG (Luminex; panel of 71 mutations), for routine analysis compared to the INNO-LiPA® (Innogenetics; panel of 36 mutations), used as a reference. Reproducible and concording results were obtained on the Luminex platform from DNA Innogenetics positive samples, using DNA extracted from different biological matrices, including blood samples, blood spots from Guthrie cards, chorionic villi and amniotic fluid. The new panel significantly increases the detection rate for patients of southern European origin.

2) Study of frequencies of *CFTR* mutations in central Argentina in the Santa Fe province, which has never been characterized. A cohort of 83 patients out of an initial local selection of 121 was analysed. The results were combined with those of a previous study of the neighboring Cordoba province, leading to the proposal of a unique panel of 21 *CFTR* mutations for a first line molecular diagnosis in central Argentina.

3) Analysis of the effects of mutations in the *PRSS1*, *CFTR* or *SPINK1* genes on the severity of sporadic idiopathic pancreatitis. A retrospective cohort of 68 patients carrying mutations in the genes was compared to a paired cohort of age- and sex-matched patients with idiopathic pancreatitis and negative genetic testing. Clinical and morphological characteristics of patients were taken into account in the analysis. Clinical parameters were similar in the two cohorts, except for the age of pancreatic disease onset. A significantly higher occurrence of pancreas cancer was observed in the case group, particularly in patients carrying mutations in

the *CFTR* gene. We therefore suggest that *CFTR* variants present a risk factor for pancreatic cancer.

4) Updating the molecular analysis of the *CFTR* gene in a cohort of patients with allergic bronchopulmonary aspergillosis syndrome (ABPA). Samples from 18 patients previously analysed using a panel of 13 mutations and reported in a paper published in 2001 were re-analysed in 2010 using complete exon sequencing. Compared to the first analysis, 8 cases were found carrying one *CFTR* mutation and 4 with two mutations. The study considerably extends previous findings by demonstrating a strong link between ABPA in adults and *CFTR* mutations.

Altogether, these studies contribute to shed new light on the molecular diagnosis of the *CFTR* gene in CF and in CF-related disorders.

Résumé

Un large spectre de plus de 2000 mutations *CFTR* ont été associées à un phénotype clinique très variable de la mucoviscidose. Dans ce travail, nous avons analysé le spectre des mutations du gène *CFTR* chez des patients atteints de mucoviscidose et dans les troubles associés à la mucoviscidose. Le projet vise spécifiquement à étudier les points suivants:

1) Validation d'une nouvelle méthode de dépistage xTAG (Luminex, panel de 71 mutations), pour l'analyse de routine par rapport à l'INNO-LiPA® (Innogenetics, panel de 36 mutations), utilisé comme référence. Des résultats reproductibles et concordants ont été obtenus sur la plate-forme Luminex à partir d'échantillons ADN Innogenetics positifs utilisant de l'ADN extrait de différentes matrices biologiques, y compris des échantillons de sang, des taches de sang de cartes Guthrie, des villosités chorioniques et du liquide amniotique. Le nouveau panel augmente significativement le taux de détection pour les patients d'origine sud-européenne.

2) Etude des fréquences des mutations du *CFTR* dans le centre de l'Argentine, dans la province de Santa Fe, qui n'a jamais été caractérisée. Une cohorte de 83 patients sur une sélection locale initiale de 121 a été analysée. Les résultats ont été combinés avec ceux d'une étude précédente de la province voisine de Cordoba, aboutissant à la proposition d'un panel unique de 21 mutations du *CFTR* pour un diagnostic moléculaire de première ligne au centre de l'Argentine.

3) Analyse des effets des mutations dans les gènes *PRSS1*, *CFTR* ou *SPINK1* sur la gravité de la pancréatite idiopathique sporadique. Une cohorte rétrospective de 68 patients porteurs de mutations dans ces gènes a été comparée à une cohorte, pairée pour l'âge et le sexe, de patients atteints de pancréatite idiopathique avec des tests génétiques négatifs. Les caractéristiques cliniques et morphologiques des patients ont été prises en compte dans l'analyse. Les paramètres cliniques étaient similaires dans les deux cohortes, à l'exception de

l'âge d'apparition de la maladie pancréatique. Une augmentation significative du cancer du pancréas a été observée dans le groupe des patients porteurs de mutations, en particulier dans le gène *CFTR*. Nous suggérons donc que les variants de *CFTR* présentent un facteur de risque pour le cancer du pancréas.

4) Mise à jour de l'analyse moléculaire du gène *CFTR* dans une cohorte de patients porteurs d'un syndrome d'aspergillose bronchopulmonaire allergique (ABPA). Les échantillons de 18 patients précédemment analysés à l'aide d'un panel de 13 mutations et rapportés dans un article publié en 2001 ont été ré-analysés en 2010 en utilisant le séquençage complet des exons. Comparativement à la première analyse, 8 cas ont été trouvés porteurs d'une mutation *CFTR* et 4 de deux mutations. L'étude a considérablement étendu les résultats précédents en démontrant une forte relation entre ABPA chez les adultes et mutations du *CFTR*.

Au total, ces études contribuent à jeter un éclairage nouveau sur le diagnostic moléculaire du gène *CFTR* dans les cas de patients atteints de mucoviscidose et de syndromes associés à la mucoviscidose.

INTRODUCTION

Introduction

1. Historical overview

In the 18th century, the adage that said "Woe to that child which kissed on the forehead tastes salty. He is bewitched and will soon die." was an early reference describing the fatal disease nowadays known as Cystic Fibrosis (CF).

During the last century, broad knowledge was progressively acquired to better characterize the disease and to understand its pathophysiology. The medical literature of the disease started in 1936 when Guido Fanconi, a Swiss pediatrician, reported in his PhD thesis, written in German, the case of a child suspected of carrying a celiac disease, and described it as “fibrocystic disease of the pancreas and bronchiectasis” (1). Two years later, Dorothy Andersen, from the Pathological Laboratory, Babies Hospital and the Department of Pathology, Royal College of Physicians and Surgeons, Columbia University in New York, made, based on the review of one thousand autopsies, a clear description of the “fibrocystic disease of the pancreas”, pointing out damage of the pancreatic tissue and associating meconium ileus to the disease. For the first time, CF was recognized as a separate entity, different from celiac disease (2). Prior to this description, the association made by Karl Landsteiner (in 1905) between meconium ileus and pancreas fibrosis was erroneously believed to result from an enzyme deficiency (3). Two years after Andersen’s description, Blackfan and May reported on thirty-five children with atrophy and fibrosis of the pancreas due to thickening of secretions and dilatation of ducts and acini (4). The current terminology of Cystic Fibrosis was introduced in 1943 by Sydney Farber (5).

A recessive transmission of a life-threatening familial condition combining steatorrhea and bronchopneumonia, probably corresponding to CF, was suspected by Sir Archibald Garrod in 1912 (6). An autosomal recessive inheritance was suggested in 1945 by Anderson and Hodges following the observation of about a hundred families with CF (7).

Before the discovery of the gene responsible for the disease in 1989 by the Lap-Chee group, the pathogenesis of CF was not understood, even though primary investigations revealed that the salt loss occurred *via* the sweat glands (8,9). This major advance allowed to establish an accurate diagnostic test of CF consisting in measuring an elevated sweat chloride concentration following pilocarpine stimulation by iontophoresis, as originally reported by Gibson and Cooke in 1959 (10). In the 80's, more knowledge was brought up with Paul Quinton's work about chloride impermeability in the sweat gland (11).

The modern history of CF is dominated by the identification of the CF transmembrane conductance regulator (*CFTR*) gene and of the most clinically relevant F508del (c.1521_1523delCTT or p.Phe508del) mutation (12-14). Consequently, the basic defect in CF has been better understood as a loss of function of the cyclic AMP-dependent CFTR chloride channel. In the 90's, a number of studies to characterize molecular defects of the gene, culminating with the development of an open CFTR1 mutation database (http://www.genet.sickkids.on.ca/app), have been addressed. The main goal of the database is to assemble information on *CFTR* mutations, more than 2,000 of them having been reported up to now. More recently, the CFTR2 database (http://www.cftr2.org/), collecting clinical and molecular data from more than 88,000 patients from 41 different countries has been created with the aim of studying genotype-phenotype correlations.

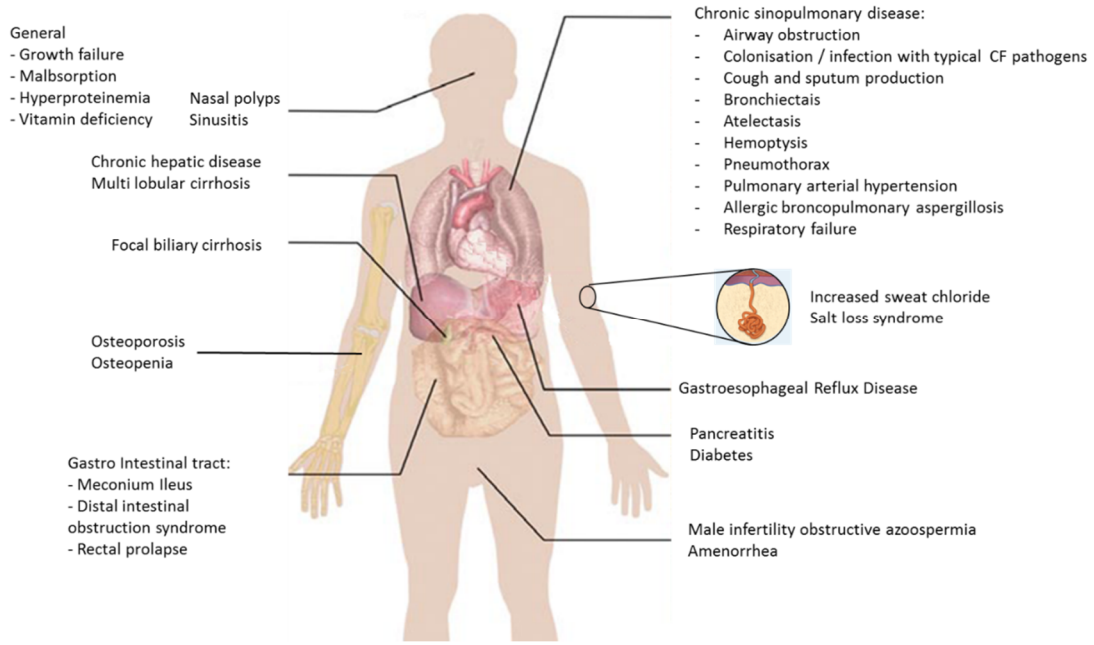
Due to early referral to specialized, multi-disciplinary reference centers for CF (1955 US National CF Research Foundation; 1959 Canada foundation of CF; 1965 in Paris the CF International Association) (15) and to more comprehensive care, survival has improved over time. While in 1938, 70% of CF babies died within the first year of life (2), the median life expectancy of patients in the US reached 40.7 years in 2013 (16). Together with a rational nutritional care, multi-disciplinary symptomatic management of patients with CF, including chest physiotherapy, inhaled bronchodilators, mucolytic and anti-inflammatory agents to

improve mucus clearance and to limit chronic infections, and aggressive antibiotic therapy, has contributed to improve survival and quality of life. More recently, numerous efforts have been made to find a cure for CF by developing strategies addressing the basic defects of the disease. As a proof of evidence that the basic defect is drug targetable, ivacaftor (Kalydeco®) has been approved as the first drug to treat the underlying cause of the disease. The drug is a small molecule acting as a CFTR potentiator, meaning that it increases the gating function of CFTR proteins of class III CFTR mutations, such as G551D, normally expressed at the plasma membrane of epithelial cells (17); more details on class mutations are given in section 4. The number of mutations that can be targeted by ivacaftor has expanded (currently 10 mutations are targeted: G551D, G1244E, G1349D, G178R, G551S, S1251N, S1255P, S549N, S549R and R117H). Used as a monotherapy, the drug targets about 5% of the CF community. In July 2015, Orkambi®, the combination of ivacaftor and lumacaftor, was approved for patients displaying at least one F508del allele. Lumacaftor is a small molecule acting as a corrector of the misfolded F508del mutated protein, with good preclinical correcting effects *in vitro* and *ex vivo* (18) but with only modest effects in patients (19, 20). Translarna™ (ataluren, PTC124) is another small molecule currently in phase III clinical development for read-through of *CFTR* nonsense mutations. The drug is believed to interact with ribosomes to enable read-through premature nonsense stop signals on mRNA, thus allowing cells to produce a full length protein; the drug is used to target class I mutations, such as G542X (21). Ataluren has recently received conditional approval for Duchenne muscular dystrophy, another disease associated with premature stop codons. Despite these developments, we still lack answers to many questions on the pathogenesis of the disease, and there is still no cure for CF.

2. Clinical description of CF

CF is the commonest lethal recessive autosomal disorder in Caucasian populations, with an average incidence of 1 in 3,000 live births in European countries (22). It is an exocrine gland multisystemic disorder, in which chronic respiratory disease and pancreatic insufficiency dominate the clinical picture. Beside sino-pulmonary disease and exocrine pancreatic insufficiency, male infertility and high concentrations of sweat electrolytes are among the most typical manifestations. The full spectra of phenotypic characteristics in CF are shown on Figure 1. CF is one of the most frequent types of chronic lung disease in children and young adults, and pulmonary involvement is the major cause of morbidity and mortality. Meconium ileus occurs at birth in 15 to 20% of newborns with CF (23). In 85 to 90% of children with CF, the pancreatic parenchyma is gradually destroyed, leading to exocrine pancreatic insufficiency with protein and fat malabsorption (22). As patients with CF now live longer than before, glucose intolerance and cystic fibrosis-related diabetes (CFRD) are becoming common complications. Extensive exocrine pancreatic damage is associated with progressive loss of endocrine pancreatic tissue, represented by a reduction of the number of Langerhans islets, that may contribute to the development of CFRD, the etiology of which is still not completely understood (24). More than 95% of males with CF are infertile as a result of obstructive azoospermia caused by atrophic, fibrotic or even absent Wolffian duct structures; their complete absence is known as CBAVD (for congenital bilateral absence of vas deferens).

Figure 1. Spectrum of clinical manifestations in Cystic Fibrosis



The phenotypic spectrum spans from mono- to polysymptomatic disease, and from atypical to classical CF (25). The severity of involvement within a specific organ, as well as the number of affected organs, varies widely from patient to patient, even among those displaying the same genotype. The factors related to the large and complex variability are not fully understood. Moreover, oligo- or mono-symptomatic patients are usually diagnosed later in life, presenting diagnostic, prognostic and therapeutic challenges, in particular considering that rare and low prevalent mutations are usually found in such cases. It has been recognized that beside genetic factors directly related to *CFTR* mutations, the presence of residual chloride secretion, either by CFTR channels or by alternative chloride channels and of environmental factors (e.g., recurrent pulmonary infections, tobacco (26), pollution (27), socioeconomic status (28), or compliance to therapy), can influence the CF phenotype. Moreover, genetic factors extrinsic to CFTR known as modifier genes have been recently studied. Several genes involved in the modulation of the inflammatory response have been pointed out. Convincing candidates have been highlighted: the presence of alleles linked to a reduced expression of the MBL2 (mannose-binding lectin 2, located at the chromosome 10q),

or to an exaggerated expression of TGF-β (transforming growth factor beta, located at chromosome 19q) and of EDNRA (endothelin receptor gene A, located at chromosome 4q), (29). Additionally, a microsatellite CATT localized in the macrophage migration inhibitory factor (MIF) promoter has been reported to be a possible candidate capable of influencing the disease severity in patients homozygous for the F508del mutation (30).

Clinically, typical CF is characterized by the accumulation of viscous mucus in the airways, gastrointestinal tract and numerous other organs. In the respiratory tract, this leads to chronic obstruction, fibrosis, and ultimately destruction of the lung tissue architecture. The most prominent symptoms in the airways are cough, tachypnea and wheezing due to recurrent and chronic bronchopulmonary infections. The intestinal malabsorption and pancreatic insufficiency lead to steatorrhea and failure to thrive. Exploring sweat gland abnormalities, characterized by production of sweat with elevated chloride and sodium concentrations, provides the rationale for the development of the sweat test, the gold standard diagnostic method.

2.1 Respiratory tract

At birth, the macroscopic and microscopic appearance of the airways has been described as normal, suggesting that airway disease develops postnatally (31). Over the first months of life, pathology starts in small airways, bronchioles become plugged with mucus and bronchiolar mucosae become inflamed (32,33). However, studies have shown that inflammation is present very early in life in CF infants, even in the absence of any detectable infection in response to current or previous infection (34-37).

Progressive accumulation of highly viscous mucus in the airways impairs mucociliary clearance and causes bronchiolitis and bronchitis, and eventually bronchiolectasis and

bronchiectasis. At end-stages, disseminated bronchiectasis is recognized as irreversible dilatation of thick-walled bronchi with airflow obstruction, inflammation and collapse. The airway submucosal glands and goblet cells, both involved in mucus production, also seem normal at birth, but progressively become obstructed with mucus resulting in dilatations and hypertrophy. Mucus hyperproduction and plugging that typifies CF is also a consequence of mucous cell metaplasia and up-regulation of mucin gene expression by inflammation. Altered airway cilia structure and function, also found in patients with CF, may contribute to impairing mucociliary clearance (38,39). At the end-stage of the lung disease, final disruption of lung architecture leads to sustained decline in lung function to such an extent that lung transplantation becomes the only option for improving quality of life and survival.

Most CF patients have chronic rhinitis with inflammation and irritation of the nasopharyngeal mucosa that can contribute to the development of nasal polyps. As a matter of fact, beginning in preschool age, during their lives, up to 50% of patients with CF experience obstructing nasal polyposis. Enlargement of terminal phalanges of fingers and toes, known as digital clubbing, is fairly common in patients with CF and is generally considered indicative of extensive pulmonary disease.

The progression of lung disease is associated with repeated chronic lung infections by opportunistic pathogens. Typically, *Staphylococcus aureus* and *Haemophilus influenza* are the first detected microbial agents (40), and infection with *Pseudomonas aeruginosa*, a predominant biofilm-forming pyogenic bacterial pathogen in CF, overcomes at later stages. Colonization with *P. aeruginosa* is associated with an faster decline of pulmonary function. In end-stage CF lung disease, *P. aeruginosa* can co-exist with other microorganisms competing for the same pool of resources. Additionally, *Burkholderia cepacia* complex organisms are

important respiratory pathogens in patients with CF. Infection with *B. cepacia* complex organisms can result in increased mortality and morbidity (41).

Although the exact mechanism of lung infection is not yet fully understood, it seems clear that accumulation of thick mucus with impaired mucociliary clearance creates a favourable environment for development of pathogens.

2.2 Gastrointestinal tract

Pathological changes of the mucosa of the gastrointestinal tract are minimal and consist in dilatation of ducts and acinar lumens of the Brunner glands in the duodenum due to mucus accumulation. As in airways, goblet cell hyperplasia is a common finding, especially in the appendix, together with increased amounts of mucous material within crypts and lumen of the gastrointestinal tract.

Thick meconium plugs in the lumen of the gastrointestinal tract is the first clinical manifestation in CF observed in prenatal stages as early as 17 weeks of gestation (42-44), both from autopsy material and from *in utero* echography examinations. These gastrointestinal abnormalities can occur irrespective of changes in any other organ (45). The accumulation of meconium plugs can develop into total obstruction of the distal ileum, failure to pass meconium, abdominal distension and emesis. A meconium plug syndrome occurs in 10 to 15% of newborns with CF, usually presenting within 48 h after birth, or even earlier when complications such as volvulus, intestinal wall perforation or meconium peritonitis arise (46,47). Babies with meconium ileus should always be evaluated for CF, as very few other conditions can cause it and up to 90% of infants with the clinical picture have CF (48).

The pathogenesis of meconium ileus has been attributed to pancreatic insufficiency and to consequent indigestion of the intestinal intraluminal contents (49). Abnormal transmucosal

transport and dehydration of extramucosal liquid layers leading to increased viscosity of the intestinal mucus contribute to the underlying pathophysiological mechanism (50,51). Meconium ileus is clearly associated with homozygosity for the F508del mutation, commonly causing pancreatic insufficiency, whereas mutations that do not impair pancreatic function are only rarely found in association with meconium ileus (52). Moreover, non-*CFTR* genes, known as modifier genes, also influence the risk of developing meconium ileus. Genome-wide analyses have suggested ADIPOR2 and SLC4A4 as candidate modifier genes, although the mechanisms underlying the relationship with meconium ileus are still unknown (53).

After the neonatal period, distal intestinal obstruction syndrome (DIOS, formerly designated "meconium ileus equivalent"), and constipation with complete or incomplete intestinal obstruction of viscid fecal accumulation in the terminal ileum and proximal colon occur in about 20% of patients with CF.

Bicarbonate secretion in the duodenum, a protective mechanism neutralizing gastric acidic secretions, has been shown to be defective in CF. Indeed, it has been shown that functional CFTR is required for cAMP-stimulated electroneutral bicarbonate secretion involving the Cl-/HCO3- exchanger (54). This impairment might contribute to the gastrointestinal complaints of patients with CF.

2.3 Pancreas

Exocrine pancreatic insufficiency is present in the vast majority (up to 95 %) of patients with CF (55), with pathological pancreatic changes, consisting of dilated ducts and acini owing to thick and inspissated pancreatic secretions, already detectable during intrauterine life (56). Detection of high levels of immunoreactive trypsin-like activity (IRT) by newborn screening programs demonstrates the capacity of the neonatal parenchyma pancreas to secrete enzymes,

but excretion is impaired due to ductal obstruction (57). The major role of CFTR in pancreatic ducts is to dilute and to alkalinize the protein-rich acinar secretions. Progressive loss of acinar cells and areas of destruction are replaced by fibrous tissue and fat. Later in the progression of the disease, islets of Langerhans, the functional units of the endocrine pancreas, become affected with deformation by fibrous tissue and destruction of insulin producing β-cells. These abnormalities contribute to the development of glucose intolerance and CFRD.

The severity of the pancreatic enzyme and bicarbonate deficiency is variable. It increases with age (58-60) and seems to be genetically determined (61). Fat loss in stools (steatorrhea), due to insufficient secretion of lipolytic enzymes to digest and absorb fat associated with deficiency in fat-soluble vitamins, correlates with massive (more than 90%) destruction of pancreatic exocrine tissue. Patients displaying steatorrhea are classified as pancreatic insufficient (PI), while those (10-15%) showing less severe pancreatic changes with some remaining functional pancreatic tissue are classified as pancreatic sufficient (PS). The PI status is associated with more severe mutations like F508del while the PS status seems to better correlate with milder *CFTR* mutations. Pancreatic insufficiency is defined by faecal fat loss above 7 g/day, fat absorption below 93% during a 3-day faecal fat balance or faecal elastase below 200 μg/g. While fat loss is increased in stools, faecal elastase-1 activities are increased in PI patients. (62). Symptomatic treatment of the pancreatic insufficiency status includes pancreatic enzyme supplements (63) and structured follow up of nutritional status.

2.4 Hepatobiliary tract

CFTR is normally expressed in bile ducts. It has long been recognized in post-mortem analyses that liver abnormalities, found in up to 50% of autopsies of patients with CF, integrate the spectra of phenotypic characteristics of the disease (64,65). Liver morphological

changes include inspissated secretions, biliary duct proliferation, periportal inflammation and fibrosis. Fibrotic areas surround patches of normal liver parenchyma with a lobular aspect. Additionally, gallbladder abnormalities include hypoplasia of the gallbladder, stones containing calcium and protein, and thick, white, mucous contents (66).

Symptomatic liver disease is found in approximately 5-10% of patients. Multilobular cirrhosis occurs early in the first decades of life, with signs of portal hypertension and liver failure developing later in childhood (67). Clinical manifestations may include hyperbilirubinemia, ascites, peripheral edema, or hematemesis due to eosophageal varices (68). In contrast to the pancreatic involvement in CF disease, there is no clear genotype-phenotype association in CF liver disease (69).

2.5 Genital tract

Male patients with CF develop normal secondary sexual characteristics and sexual maturation, yet almost all (~ 97%) of them are infertile due to obstructive azoospermia attributed to congenital absence of the vas deferens (CBAVD). Genital male abnormalities encountered in CF include bilateral atrophic or absent vas deferens and body and tail of the epididymis, and seminal vesicles are dilated, fibrotic, or also absent (70). As a consequence, no spermatozoa are present in the ejaculate due to obstruction of the vas deferens, whereas testicular biopsies show that spermatogenesis is preserved. With increasing survival of patients with CF, a need of micro-assisted reproduction techniques, such as testicular sperm extraction and intracytoplasmic sperm injection, have emerged as beneficial methods to enable these patients to father children of their own (71).

Reduced female fertility might exist through reduced water content of the cervical mucus, which hinders sperm passage (72). Other disturbing factors are chronic pulmonary sepsis initiating menstrual irregularities, and possible cysts in the ovaries (73).

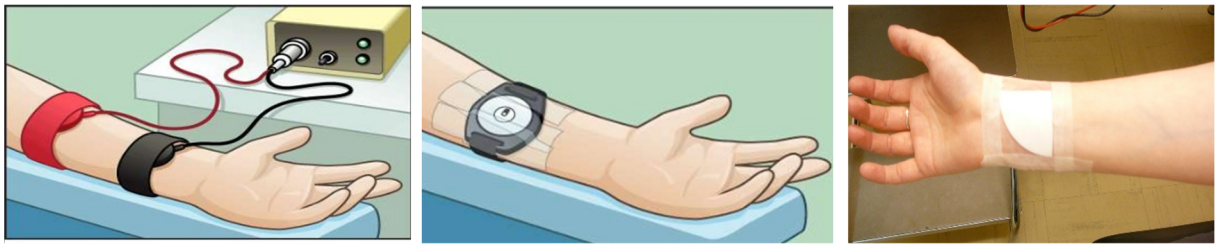
2.6 Sweat glands

During the summer of 1949 in New York City, an unusually intense heat wave struck, bringing to Dr Paul di Sant 'Agnese, a clinician, the suspicion that the accentuated dehydration presented by children with CF was due to pure salt loss. Gibson and Cooke meticulously demonstrated that sweat glands were the site of the ion loss in these children (G&C). This was the basis of the introduction of the "Quantitative Pilocarpine Iontophoresis Sweat Test" (QPIT) still used nowadays as the gold standard diagnostic test in CF.

In practice, sweat gland secretion is stimulated by iontophoresis of pilocarpine, a cholinergic agent, and sweat is collected for 30 minutes using either filter paper or the Macroduct sweat collection system (Wescor Inc, Logan, Utah) (Figure 2). Sweat chloride concentration is measured by quantitative analysis techniques such as colorimetry, coulometry, etc. Currently, according to the European Coordination Action for Research in Cystic Fibrosis (EurocareCF) (http://www.eurocarecf.eu) and the European Cystic Fibrosis Society (ECFS) Diagnostic Network Working Group (http://www.ecfsoc.org), established cutoff sweat chloride concentrations are: normal: <30 mmol/L; intermediate: 30-60 mmol/L; and abnormal :> 60 mmol/L. However, it has been well established that a fraction of patients with a diagnosis of CF have a sweat chloride value < 60 mmol/L. As a matter of fact, some mutations, *i.e.* A455E, 3849+10kbC>T, R117H, L206W, … are described with borderline (30-60 mmmol/L) or normal (<30mmmol/L) chloride concentrations (74,75).

Figure 2. Collection methods of sweat test. A iontophoresis electrodes on the forearm. B Wescor collection method. C Filter paper collection.

A B C



3. CFTR-related disorders

A CFTR-related disorder has been described as « a clinical entity associated with CFTR dysfunction that does not fulfil diagnostic criteria for CF » (24,76). The picture is comprised of three main clinical entities, all associated with CFTR dysfunction: CBAVD (congenital bilateral absence of the vas deferens), acute recurrent or chronic pancreatitis and disseminated bronchiectasis (24). Additionally, ABPA has been considered as a CF-related disorder (77). More recently, CFSPID (CF screening positive inconclusive diagnosis) has been suggested as a CF-related disorder. The terminology has been adopted to describe the sometimes challenging case of a child with a positive newborn screening test, later changed into an inconclusive diagnosis of CF (78). In clinical practice, it is advisable that the child graduates to a proper CF care unit, assuming the possibility, even limited, of conversion to CF. The follow-up should include repeating the sweat test (at 6 and 12 months of age, then annually) and the fecal elastase test later. To assist in the diagnosis, other diagnostic tests such as the nasal potential difference test and intestinal current measurements in tissues obtained from rectal biopsies are helpful. In the USA, the CFSPID has been termed "CFTR-related metabolic syndrome" (CRMS) (79).

3.1 CBAVD

CBAVD is the clearest example of a CFTR-related disorder. As such, isolated CBAVD occurs in men with mild pulmonary or gastrointestinal manifestations of CF or even without any detectable CFTR dysfunction. The expression "CFTR-related disorder" for isolated CBAVD associated with at least one CFTR-causing mutation has been considered more appropriate than other common terminologies such as mild, atypical or non-classic CF. The absence of vas deferens is part of the clinical picture of classical CF. In all cases of CBAVD, affected men have azoospermia (absence of sperm in the semen) and are infertile. Isolated CBAVD accounts for approximately 3% of cases of male infertility (incidence around 1:1000 males). In contrast with classical forms of CF, the prevalence of isolated CBAVD does not appear to be linked to ethnic factors and does not vary among populations.

In subjects with CBAVD, the most common CF-causing mutation is p.Phe508del varying from 21-33% (in Canada, USA, Northern Europe) to 12-18% (in India and Southern Europe). These frequencies are much higher than those found (4%) in control European populations. The frequency of the 5T allele in the general population is 5%, but it is higher in the CBAVD populations (Indians 25%, Japanese 30%, Egyptians 44%, Taiwanese 44%). In about 70-90% of cases with CBAVD, a compound heterozygous status is identified with combination, in *trans* position, of a « severe » (class I to III) and a « mild » CFTR mutation (class IV to V). The two most common compound heterozygous genotypes found in European subjects with CBAVD are p.Phe508del with a 5T allele (28%) and p.Phe508del with p.Arg117His (6%). The 5T allele is considered as a CBAVD mutation with incomplete penetrance. It has been well established that the combination of the T(5-9) repeats with that of TG dinucleotide, usually varying from 10-13 repeats and lying immediately upstream of the former, influences

the efficiency of exon 9 splicing (80). Longer TGm repeats in *cis* with shorter Tn repeats are associated with increased exon 9 skipping, and lead to production of misfolded and nonfunctional CFTR protein and correlate with a more severe CBAVD or CFTR-related phenotype. (81,82).

The diagnosis of CFTR-related CBAVD is established in males with azoospermia with low volume (<2 mL; normal: 3-5 mL) of ejaculated semen and absence of vas deferens, possibly associated with abnormalities of seminal vesicles. Rarely, a thin fibrous cord representing a rudimentary vas deferens may be present. Additionally, a specific chemical profile including acidic sperm (average pH <6.8; normal pH >8), elevated citric acid concentration (>2000 mg/100 mL; normal: 400-1500 mg/100 mL), elevated acid phosphatase concentration (760-1140 mμ/mL; normal: 140-290 mμ/mL) and low fructose concentration (30-80 mg/100 mL; normal: 250-720 mg/100 mL) can be found.

3.2 Idiopathic Chronic Pancreatitis

Chronic pancreatitis is defined as a long-standing inflammatory destruction of the pancreatic parenchyma, finally leading to tissue fibrosis. The disease is characterized by variable abdominal pain, calcifications, necrosis, fatty replacement, fibrosis and scarring, and other complications. Chronic pancreatitis rarely causes diabetes. Diabetes due to chronic pancreatitis is characterized by a low incidence of ketosis and a high incidence of insulin-induced hypoglycaemia. Obstructive pancreatitis is due to an obstacle (tumours, scars) in the pancreatic duct. Calcifications, also signing the chronic inflammation, are like stones embedded in the tissue itself or in the pancreatic duct.

The estimated incidence of chronic pancreatitis in adults is 3.5-10 cases per 100,000. In developed countries, 60–70% of patients with chronic pancreatitis have a history of excessive

alcohol intake, while in 30% of cases, the disease is considered idiopathic. Variability in susceptibility to idiopathic chronic pancreatitis seems to be related to genetic differences among patients. Plugging of the smaller pancreatic ducts is a frequent early finding in idiopathic chronic pancreatitis (ICP) in which progressive obstruction finally leads to pancreatic injury.

CFTR protein is expressed at the external membranes of epithelial cells lining the normal ducts of the human exocrine pancreas, where it regulates bicarbonate secretion through a cAMP-dependent pathway. The major role of CFTR in pancreatic ducts is to dilute and to neutralize acinar solutions that are rich in protein. Through this mechanism, CFTR protects the tissue against formation of protein aggregates and tissue damage. The fact that a loss-of-function of CFTR protein of > 98% is needed to produce pancreatic insufficiency (i.e. homozygous p.Phe508del) illustrates the large functional reserve of the pancreas.

About 30% of patients with ICP display *CFTR* mutations. About two-thirds of them carry common *CFTR* mutations and a small fraction (~ 6%) is compound heterozygous combining a CF-causing mutation and a milder *CFTR* allele. Although there is no clear genotype-phenotype relationship regarding the development of ICP, rare class IV or class V mutations are generally associated with pancreatitis (24). The pancreatitis risk increases 40-fold in the presence of two CFTR mutations (83).

An association between some genetic non-*CFTR* mutations and chronic pancreatitis (CP) has been recognized for a long time. ICP can also result from mutations in the cationic trypsinogen gene (*PRSS1*), and the serine protease inhibitor Kazal 1 (*SPINK1*) genes. In 1996, the first mutation associated with hereditary pancreatitis, namely the R122H mutation in the *PRSS1*, was identified (84). Several other mutations (A16V, K23R, N29I, N29T, R122C) and triplication as well as duplication of the PRSS1 locus have been subsequently described (85).

The effect of mutations in the *SPINK1* gene, considered as a susceptibility gene, on the onset of pancreatitis was reported in 2000 (86,87). The most frequent *SPINK1* mutation is N34S. The pancreatitis risk increases 20-fold in the presence of N34S mutation in the *SPINK1* gene and 900-fold in the presence of both *CFTR* and *SPINK1* mutations (83).

3.3 Allergic Bronchopulmonary Aspergillosis

Allergic bronchopulmonary aspergillosis (ABPA) is a pulmonary hypersensitivity mediated by an allergic response to *Aspergillus fumigatus*. There are approximately 250 different species of *Arpergillus*, but only a few of them are known human pathogens. Depending on the host/pathogen equation, the respiratory diseases caused by *Aspergillus* are classified as allergic (allergic Aspergillus sinusitis, hypersensitivity pneumonia and ABPA), saprophytic (aspergilloma) and invasive (airway invasive aspergillosis, chronic necrotizing aspergillosis, and invasive aspergillosis).

Multiple immunological features combining immediate type 1 hypersensitivity, antigen-antibody type III reactions, and eosinophil-rich inflammatory type IVb responses have been described in ABPA (88). However, a predominant T-helper 2 lymphocyte response to *Aspergillus fumigatus* infection without tissue invasion seems to be well characterised, leading to mucus production, airway hyperactivity and, finally, bronchiectasis.

Clinically, ABPA manifests itself as chronic poorly controlled asthma, recurrent pulmonary infiltrates and bronchiectasis. In asthma or CF, most patients with ABPA present with wheezing, bronchial hyperreactivity, hemoptysis, productive cough, low-grade fever, malaise, weight loss, and/or worsening symptoms; however asymptomatic forms are recognised. The diagnosis of ABPA is currently based on the most recent criteria reported in 2012 in the Journal of Allergy and Clinical Immunology (89). According to the paper, the minimal

criteria to establish the diagnosis include deterioration of lung function in patients with asthma or CF, positive test for immediate skin reactivity to Aspergillus species (prick skin test), elevated total serum IgE (> 1000ng/mL), increased Aspergillus species-specific IgE and IgG antibodies and the presence of chest radiographic infiltrates (90,91). Despite these criteria (89), the diagnosis of ABPA in patients with CF remains challenging. It is believed that ABPA most probably remains underdiagnosed.

In the general population, the prevalence of ABPA is unknown but it is believed to be as high as 2.5% (92). Although ABPA is most commonly diagnosed in adults, its prevalence in children is increasing and the condition is very unusually found in children outside the context of CF. In patients with CF, the prevalence of ABPA has been reported as varying from 2 to 15% (93). Its increased frequency in patients with asthma or CF is consistent with a genetic susceptibility to ABPA.

Patients with disseminated bronchiectasis carry at least 1 CFTR mutation in 10-50% of the cases, and 5-20% carry 2 mutations. In a letter published in Thorax (94) extending an earlier DNA analysis, it has been shown that the CFTR mutation carrier frequency can be as high as 67% (12/18), which is close to the estimate for CBAVD.

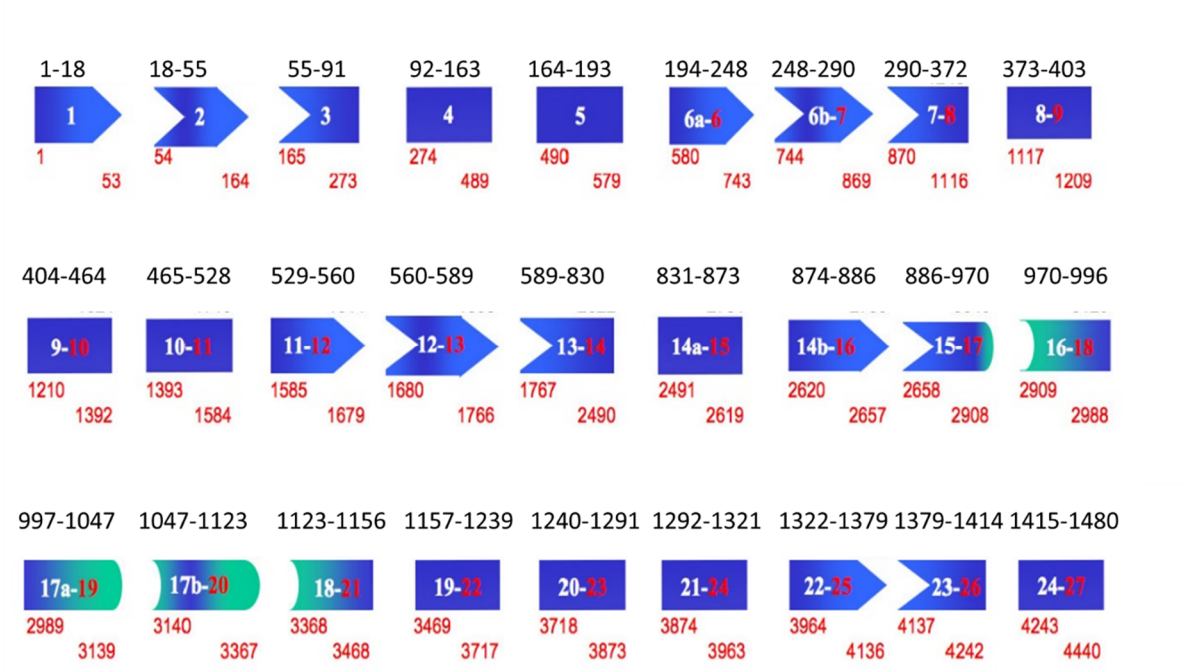
4. The *CFTR* gene

The large spectrum of phenotypes (detailed in paragraph 3) associated with variable degrees of severity of typical and atypical CF disease is caused by mutations in the *CFTR* gene (NG_016465.4). The gene is large, spanning approximately 230kb on the chromosome 7q31.2 and contains 27 exons, numbered as illustrated in Figure 3.

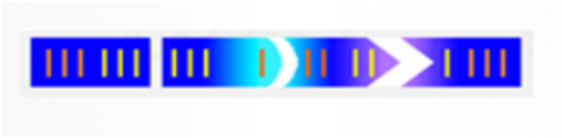
Figure 3. Structure of the CFTR gene.

A)

B)



C)



A) Exons numbering. Rescaled exon scheme (introns are not drawn to scale). Numbers above (1 to 24) correspond to the old nomenclature (legacy name). Numbers below (1 to 27) correspond to the new nomenclature (HGVS).

B) Corresponding numbering and reading frame for exons and nucleotides. Inside each box, white numbers refer to exons labelling corresponding to the old nomenclature (legacy name) and red numbers to the new nomenclature (HGVS). Numbers in black correspond to codon numbers. Red numbers under boxes correspond to nucleotide numbers in the CDS (NM_000492.3).

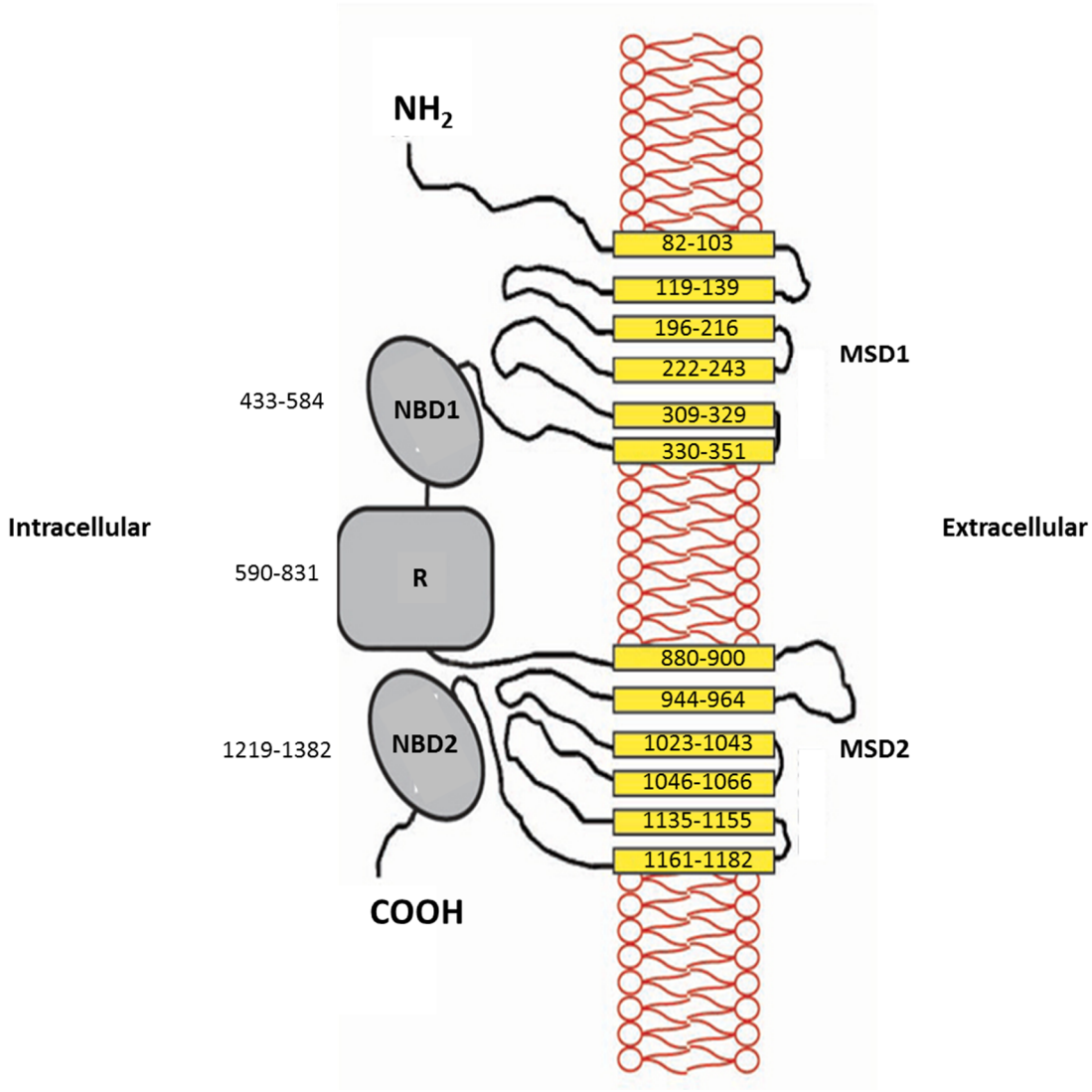
C) Meaning of boxes' edges: round-shaped edges indicate exon ending at the first nucleotide; arrow-shaped edges indicate exon ending at the second nucleotide; flat edges indicate exons ending at the third nucleotide.

5. The CFTR protein

The encoded mRNA (NM_000492.3) is about 6.5 kb in length and is translated into a protein product of 1480 amino acids (NP_000483.3). A putative structure of the CFTR protein has been drawn on the basis of the amino acid sequence. It is a member of the superfamily of ATP-binding cassette (ABC) transporters, in particular it is categorised as a sub-family C member 7 (ABCC7). The predicted protein structure is composed of two repeated units, each consisting in a membrane spanning domain (MSD) comprised of six hydrophobic transmembrane helices, followed by a nucleotide-binding domain (NBD) that interacts with ATP. Ten of the 12 transmembrane helices contain one or more charged amino acids, and two potential glycosylation sites are found between helices 7 and 8. The 6-helical domains of MSD1 span from residues 82 to 103, 119 to 139, 196 to 216, 222 to 243, 309 to 329 and 330 to 351 interlinked by three short extracellular loops and two longer intracellular loops (Figure 4). The 6-helical domains of MSD2, also interlinked by two intracellular and three extracellular loops, span from residues 880 to 900, 944 to 964, 1023 to 1043, 1046 to 1066, 1135 to 1155, and 1161 to 1182. NBD1 and NBD2 correspond to the amino acid sequences 433-584 and 1219-1382 respectively (Figure 4). The two repeated units are linked by a single regulatory (R) domain that spans from amino acid residues 590 to 831 and contains 9 of the 10 consensus sites for phosphorylation by protein kinase A (PKA) and 7 of the phosphorylation sites for protein kinase C (PKC). The R domain is unique to CFTR as it is not present in any other member of the ABC superfamily. The transmembrane helices assemble to line the pore of the anion-selective channel (95) through which chloride ions can flow across the plasma membrane (96). Anion flow through the channel is believed to be gated by cAMP-dependent PKA phosphorylation of the R domain (97,98) and by binding of ATP to NBD sites that induces conformational changes in the protein, finally controlling its open probability

(96,99-102).

Figure 4. Protein CFTR structure



Numbers correspond to amino acid positions in the polypeptide chain.

6. Mutations in the *CFTR* gene

Under physiological conditions, the *CFTR* gene undergoes transcription and is translated into a CFTR protein that trafficks to the cell membrane where it mainly functions as a chloride channel (Figure 4). In CF, the majority of CFTR mutations involve changes in three or fewer nucleotides and result in amino acid substitutions, frame shifts, splice site, or nonsense

mutations. The most common and also the first identified mutation, p.Phe508del, corresponds to a three base pair deletion that codes for a phenylalanine at position 508 of the protein. However, the relative frequency of the p.Phe508del mutation in families carrying the CF gene varies among ethnic groups. An increasing South East–North West gradient has been noticed for its relative frequency across European countries: the highest frequency of 82% is reached in Denmark but the mutation is much less frequent in Mediterranean regions, where less than 50% of chromosomes with the *CFTR* gene have the mutation (103). The overall frequency of non-p.Phe508del mutations is low, except for some rare alleles that segregate with a specific ethnic group. For instance, the W1282X, a stop codon mutation, accounts for 48% of CF chromosomes in Ashkenazi Jews (104), and 23% of French Canadian CF chromosomes carry the 621+1G>T variant (105,106). It is the presence of the p.Phe508del mutation that increases the frequency of CF in Caucasian population relative to other races.

CFTR mutations have been described in six different classes (Table 1). Class I mutations result in an absence of functional CFTR protein (caused by unstable truncated RNA); they correspond to nonsense, frameshift canonical splice. This class contains for example p.Gly542*, p.Trp1282*, p.Arg553*, c.489+1G>T mutations. Class II result in CFTR trafficking defects (full length CFTR RNA but protease destruction of misfolder CFTR); they correspond to missense or amino acid deletion. Examples of class II mutations include p.Phe508del, p.Asn1303Lys, p.Ile507del, p.Arg560Thr mutation. Class III produce a CFTR protein at the cytoplasmic membrane but with defective channel regulation; they correspond to missense or amino acid change. This class contains for example the p.Gly551Asp, p.Gly178Arg, p.Gly551Ser, p.Ser549Asn mutations. Class IV produce a CFTR protein at the cytoplasmic membrane but with a defective CFTR channel with decreased channel conductance; they correspond to missense or amino acid change. This class contains for example the p.Arg117His, p.Arg347Pro, p.Arg117Cys, p.Arg334Trp mutations. Class V

induce a reduced synthesis of CFTR caused by splice defect or missense. This class contains for example the 3849+10kbC>T, 2789+5G>A, 3120+1G>A and 5T mutations. Class VI lead to decreased CFTR membrane stability; they correspond to missense or amino acid change. This class contains for example the c.4196_4197delTC, p.Gln1412* and c.4147_4148insA mutations.

Table 1. Classes of CF mutations

Class	Resulting CFTR defect	Mutation Type	Examples
I	No protein	Premature stop codon, larges deletions, out-of-frame deletion, insertion	p.Gly542* p.Trp1282* p.Arg553* c.489+1G>T
II	Processing defect	Missense, amino acid deletion	p.Phe508del p.Asn1303Lys p.Ile507del p.Arg560Thr
III	Regulation defect	Missense	p.Gly551Asp p.Gly178Arg p.Gly551Ser p.Ser549Asn
IV	Decreased conductance	Missense	p.Arg117His p.Arg347Pro p.Arg117Cys p.Arg334Trp
V	Reduce synthesis	Missense, change in splicing efficiency	3849+10kbC>T 2789+5G>A 3120+1G>A 5T
VI	Altered channel stability	Nonsense, frameshift	c.4196_4197delTC p.Gln1412* c.4147_4148insA

7. Molecular diagnosis of *CFTR*

The consensus strategy for molecular diagnosis is divided in two groups. The first line strategy aims at detecting known mutations using commercial kits. The second line aims at detecting unknown mutations, using a gold standard DNA sequencing method.

In Europe, due to heterogeneity of distribution of mutations, a local detection rate higher than 95% is difficult to achieve (with mutation frequencies of more than 1%). Panels of mutations commonly used recognise from 20 to 86 CF-causing mutations.

In the USA, the situation is unambiguous. The American College of Medical Genetics (ACMG)/American College of Obstetricians and Gynecologists (ACOG) have recommended a core panel of 23 mutations to be included in the first line strategy.

When a CF phenotype is suspected and no or only one known mutation in the panel has been detected, a search is made for a rare CF-causing mutation. This second line sequences the coding exons (CDS) and bordering intronic regions for punctual mutations (SNV), and the Multiplex Ligation-dependent Probe Amplification (MLPA) for large rearrangements (CNV). Nowadays, the complete intronic nucleotide sequencing is not routinely applied in molecular diagnostic laboratories.

8. CFTR1 database

In 1990 the international Cystic Fibrosis Genetic Analysis Consortium (CFGAC) created the 'cftr1' mutation database (http://www.genet.sickkids.on.ca/PicturePage.html) in the CF Centre at the Hospital for Sick Children in Toronto. On August 25 2016, 2009 variants had been introduced in the database. Their distribution into categories is given on Table 2.

Table 2. Distribution of variants in the *CFTR* gene

Mutation Type	Count	Frequency %
Missense	795	39.57
Frameshift	313	15.58
Splicing	228	11.35
Nonsense	167	8.31
In frame in/del	41	2.04
Large in/del	52	2.59
Promoter	15	0.75
Sequence variation	269	13.39
Unknown	129	6.42

The usefulness of the database is limited by its composition: a list of individual patients with a CF- or a CF-related phenotype and carrying a new suspected pathological variant in the *CFTR* gene.

9. CFTR2 database

More recently, a second CF mutation database "CFTR2" for "Clinical and Functional Translation of CFTR" (http://www.cftr2.org/) was created, in an attempt to correlate phenotype with genotype (including the allelic distribution in *cis* or in *trans*) in groups of patients carrying the same mutation. It was based on the collection of clinical data of almost 88,000 patients from 25 European and North-American datasets, namely CF registries and CF centers. Despite the very large size of the set of patients, only 273 mutations have been included (http://cftr2.org/progress.php). Clinical data taken into consideration are sweat chloride, lung function, pancreatic status and presence of *Pseudomonas.* The database classifies mutations into four categories labelled « disease-causing », « neutral », « mutation

of varying clinical consequences » and "mutations of unknown clinical significance", and provides clinical information about complex alleles containing more than one variant (107).

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OBJECTIVES

Objectives

The general aim of this work is to analyse the spectrum of CFTR mutations in CF and in CF-related disorders. The severity of classic forms of the disease and the number of affected organs varies substantially from patient to patient, even among those displaying the same genotype. The presence of CFTR mutations can also be associated with CF-related disorders, such as ABPA and pancreatitis. The factors related to the large and complex variability in CF are not well recognized and a large spectrum (more than 2,000) of CFTR mutations has been reported.

This project specifically aims at investigating the following points:

1) Validation of a new diagnostic screening method (xTAG Luminex) for routine analysis compared to the INNO-LiPA® method (Innogenetics) used as a reference.
2) Characterisation of frequencies of CFTR mutations in central Argentina with the final aim of developing a new first line panel of mutations with high sensitivity for routine use in this population.
3) Study of the clinical manifestations, including the occurrence of pancreas cancer, in patients with sporadic idiopathic pancreatitis who display mutations in the *CFTR*, *PRSS1* and *SPINK1* genes.
4) Better characterisation of the nature of CFTR mutations, by sequence analyses, in a group of adult patients with ABPA.

PERSONAL RESEARCH

Personal Research

New Molecular Screening Assay for Increased Detection Rate of CFTR Mutations in European Populations

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In 2009, all Belgian Genetics centers used the same panel of 36 mutations (the gold standard at the national level) for the first line molecular diagnosis of the *CFTR* gene. At that time, a US-based company contacted me with an alternative single panel of the 71 most frequent mutations for Europe as the whole. The rationale was that “it was better suited for patients of southern European descent”, who are characterised by a large diversity of mutations.

This work has been designed to address the first objective of the thesis project, namely, to investigate the validation of a new diagnostic screening method (xTAG Luminex) for routine analysis compared to the INNO-LiPA® method (Innogenetics) used as a reference. The paper below compares the two panels and presents mutation detection rates for countries from northern, central and southern Europe separately, with data from the national registries. This paper tried to answer the question : “what is the advantage of using a panel with twice the number of mutations in the first line molecular diagnosis?”. Since then, the UCL and ULg Genetics centers use the new panel in first line routine diagnosis.

New Molecular Screening Assay for Increased Detection Rate of *CFTR* Mutations in European Populations

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Abstract

The xTAG® Cystic Fibrosis 71 v2 kit (Luminex 200) is a new semi-quantitative, fully automated molecular genotyping test developed to simultaneously detect and identify an enlarged number of mutations and variants in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. During a validation process, the Luminex method was compared with INNO-LiPA® (Innogenetics), used as a reference method. A total of 120 DNA samples extracted from different biological matrices, including blood samples, blood spots from Guthrie cards, chorionic villi and amniotic fluid, were tested. Reproducible and concordant results were obtained on the Luminex platform from all DNA Innogenetics positive samples. However, differences were observed in the sensitivity of the methods based on distinct compositions of mutation panels. Indeed, 43 additional *CFTR* changes beyond those present in the Innogenetics panel are tested in the Luminex platform; of those, 40 are known mutations and three are recommended interference benign variants of F508del (F508C, I507V and I506V). Extension of the mutation panel to include Q552X, 711+5G>A, S1251N and nine other mutations is currently undergoing a validation process and will place Luminex xTAG as the most sensitive platform for routine *CFTR* molecular diagnosis in European countries.

Keywords

Cystic fibrosis, cystic fibrosis transmembrane conductance regulator (CFTR), screening, molecular diagnosis

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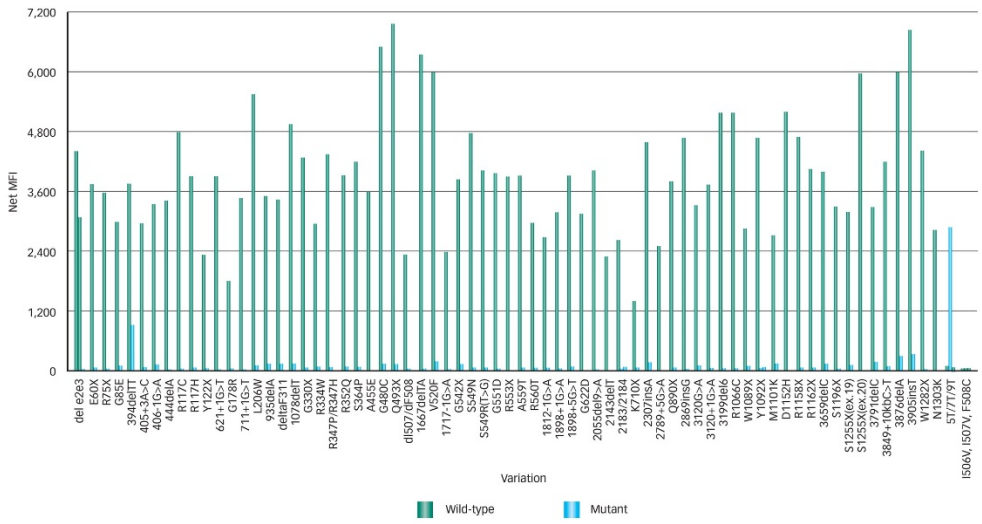
Cystic fibrosis (CF) is the most common and lethal hereditary recessive disease in Caucasian populations. The disease is caused by mutations of the CF transmembrane conductance regulator (*CFTR*) gene, located on the 7q31.2 locus, whose predicted translation product is a 1,480 amino acid protein. The protein, expressed in a variety of target tissues, organs and exocrine glands, functions as a cyclic adenosine monophosphate (cAMP)-dependent low-conductance chloride channel. The disease is less common in Hispanics and African-Americans[1] and is relatively rare in Asian Americans, with an incidence of one in 32,100. In central Europeans its frequency reaches about one in 2,500.

To date, over 1,600 different mutations have been identified (www.genet.sickkids.on.ca/). The most common mutation worldwide results in a deletion of a single phenylalanine residue at position 508 (F508del) and causes defective synthesis and folding of the mutant protein, which fails to escape the endoplasmic reticulum and reach the apical membrane of many epithelial cells.[2] Apart from the F508del mutation, which is responsible for approximately two-thirds of all CF chromosomes with a clear north-west–south-west gradient in its frequency across Europe,[3] there is a core of 38 less common mutations that occur with a relative frequency in European countries of 0.1% or greater.[4] The remainder, considered as rare mutations, have been identified in few individuals. Ethnic and racial backgrounds influence both the nature and the distribution of *CFTR* mutations. CF disease has a complex phenotype with variable disease severity and multiple clinical manifestations, including sino-pulmonary disease, exocrine pancreatic insufficiency, male infertility and high concentrations of sweat electrolytes. Patients displaying classic characteristics of CF from infancy usually have a relatively poor prognosis. There has been growing recognition of atypical, milder, pauci-organ disease cases of CF presenting in adolescence or adulthood and displaying normal or borderline sweat test and a better prognosis for survival.[5] Additionally, growing evidence of an association between *CFTR* mutations and other diseases, including isolated idiopathic pancreatitis, chronic rhinosinusitis, nasal polyposis, idiopathic bronchiectasis, allergic bronchoalveolar aspergillosis and congenital absence of vas deferens, has been added to this complex picture.[5] Atypical CF and CF-related disorders have made the diagnosis of CF less straightforward for clinicians and for geneticists, and have also required further extensive genetic screening.

Multiple *CFTR* molecular assays using diverse technologies are commercially available for routine analyses. Screening panels of *CFTR* mutations have been designed based on selected mutations having higher frequency among North Caucasian populations. Validation of a

Figure 1: Histogram of Wild-type and Mutant Net Signals of Variants Obtained from an Individual Wild-type *CFTR* DNA Sample



Expressed as median fluorescence intensity (MFI). The polythymidine tract of intron 8 shows a homozygous 7T/7T genotype.

CFTR molecular test should integrate distinct analytical steps encompassing sensitivity, specificity, accuracy, signal discrimination and no call signal or repeat rate. Practical aspects comprising integration of the required platform into laboratory workflow, data interpretation methods and required software, hands-on or start-to-finish time and cost analyses should also be taken into account. The mutation panel and its sensitivity to the target populations are pivotal decisional factors. Recommendations of the American College of Medical Genetics (ACMG) include a minimal list of 23 *CFTR* mutations as well as reflex testing for R117H of the Tn polymorphic alleles in intron 8 and interference testing for benign F508del variants such as F508C, I507V and I506V.[6]

Over 10 years of experience with *CFTR* molecular tests, our laboratory has used screening platforms allowing continuously upgrading mutation panels, from 13 in 1999 to 20 up to 2002 (ARMS, Elucigene CF), then to 29 up to 2004 and finally to 36 until 2008 (INNO-LiPACFTR17+TnUpdate and INNO-LiPACFTR19 kit; AUTO-LiPA instrument, Innogenetics, Ghent, Belgium). More recently, we have been involved in the pilot validation process of a new molecular screening method, the xTAG® Cystic Fibrosis 71 v2 kit (Luminex 200 Instrument; Luminex Co, Austin, Texas, US). During the validation process, the Innogenetics method was used as a reference method.

Luminex xTAG is a semi-quantitative, fully automated genotyping test developed to simultaneously detect and identify an enlarged number of mutations and variants in the *CFTR* gene. The Luminex platform is a fast, easy, robust and highly specific DNA hybridisation test incorporating multiplex polymerase chain reaction (PCR) and multiplex allele-specific primer extension with Luminex tagged primers, which are composed of an inventive array of fluorescently labelled microsphere beads. Signals generated for each specific mutation and variant are analysed on the proprietary platform and are finally expressed as net values, allelic ratios and thresholds, which are calculated by comparison with those signals generated for the corresponding wild-type alleles. Output files are then generated and a four-page report including a one-page colour histogram can be printed out for each individual mutation and assay run. A colour histogram obtained from an individual wild-type *CFTR* DNA sample is depicted in *Figure 1*. An example of a heterozygous F508del DNA sample is shown in *Figure 2*.

Internal Validation Process of the Luminex Method

During an internal validation process, a total of 120 DNA samples extracted from different biological matrices, including blood samples, blood spots obtained from Guthrie cards, chorionic villi and amniotic fluid, were tested by both the new and the reference method. Each sample was analysed in triplicate, in three different runs performed on different days by three different operators. Data analyses were carried out by applying working protocols according to the corresponding manufacturer's recommendations. Data interpretation of Luminex test products was performed using Luminex software. Costs as well as hands-on time were similar for both methods. Start-to-finish time for a run of 45 DNA samples on the Luminex platform averaged one day of a technician's time. No significant inter-operator variability was noted. No call rate was acceptable (<1%). Reproducible and concordant results were obtained on the Luminex platform from all DNA Innogenetics positive samples. A comparison between methods obtained for the length of the intron 8 polytymidine tract, tested in both panels, showed that they were identical. However, differences were observed in the sensitivity of the methods based on the distinct composition of mutation panels (see *Table 1*). Indeed, 43 additional *CFTR* changes, beyond those present in the Innogenetics panel, are tested in the Luminex platform; of these,

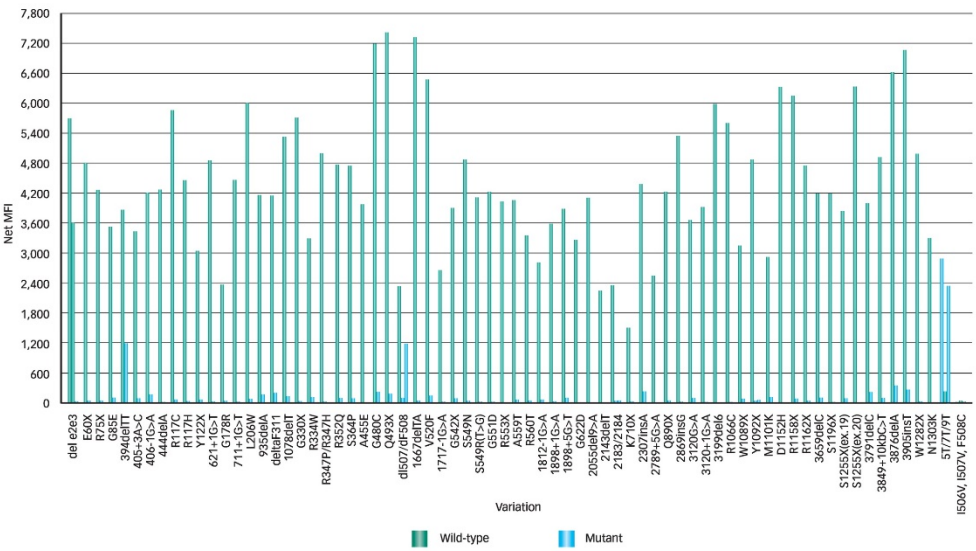
Table 1: Overview of Mutation Panels of Luminex xTAG Cystic Fibrosis 71 v2 and INNO-LiPA CFTR19 + CFTR17 +Tn Update, and Their Sensitivity in Different Selected Populations

				xTAG CF71v2 (Luminex)				
INNO-LiPA CFTR19 + CFTR17+Tn Update (Innogenetics)								
F508del	621+1G>T	R117H	3199del6	Y122X	M1101K	L206W	1812-1G>A	R1158X
1078delT	711+1G>T	R334W	CFTRdel2,3	R347H	S1255X	935delA	G622D	3791delC
1717-1G>A	A455E	R347P	3905insT	V520F	3876delA	F311del	2055del9>A	S1196X
1898+1G>A	I507del	R553X	394delTT	A559T	R75X	G330X	K710X	3120G>A
2184delA	G542X	R560T	5T/7T/9T	S549N	405+3A>C	R352Q	Q890X	F508C*
2789+5G>A	G551D	W1282X	Q552X**	S549R	406-1G>A	S364P	2869insG	I1507V*
3120+1G>A	G85E	E60X	711+5G>A**	1898+5G>T	444delA	G480C	R1066C	I506V*
3659delC	N1303K	2143delT	3272-26A>G**	2307insA	R117C	Q493X	W1089X	
3849+10kbC>T	R1162X	2813AA>G	S1251N**	Y1092X	G178R	1677delTA	D1152H	

Mutation Detection Rate %	Innogenetics	Luminex	Reference (number of alleles tested)
Denmark	93.51	94.10	[3] (n=678)
UK	86.64	87.74	[4] (n=11,402)
Spain	72.71	75.44	[3] (n=1,356)
Argentina	75.92	76.38	[9] (n=440)
Italy	69.27	69.44	[1] (n=3,524)
Belgium	83.59	82.26	[8] (n=2,114)

Immunogenetics mutation panel contains only those mutations in the shaded panel; Luminex mutation panel contains the mutations in both the shaded and unshaded panels, apart from those indicated by a double asterisk (**). Innogenetics mutation panel also includes I148T, recently recognised as a low-penetrance allele and not listed here.
**American College of Medical Genetics (ACMG)-recommended variant.[6]*

Figure 2: Histogram of Wild-type and Mutant Net Signals of a Variant Obtained from an Individual Heterozygous F508del *CFTR* DNA Sample



Expressed as median fluorescence intensity (MFI). The polythymidine tract of intron 8 shows a 5T/9T genotype.

40 are known mutations and three are recommended interference benign variants of F508del (F508C, I507V and I506V).[6] I148T is not included in the Luminex panel as it has been recognised as a low-penetrance allele.[7] In the study, when a new substitution was detected with the Luminex and not with the Innogenetics method, a *CFTR* exonic sequencing, processed by means of BDT3.1/313xl (Applied Biosystems, US) was performed and allowed confirmation of the change. Among discordant samples, three benign variants (F508C, I507V and I506V) identified by the Luminex method in a heterozygous state were confirmed by *CFTR* sequencing.

Post-validation Experience with the Luminex Platform

Following the successful validation procedures described above, about 400 screening tests using the Luminex platform have been performed in routine analyses in our laboratory.

The Luminex method advantageously favours an increased mutation detection rate in different countries across Europe. In Denmark, the European country with the highest relative frequency of F508del mutation,[3] a higher mutation detection rate is obtained. In the UK, a differential mutation detection rate of 1.1% is advantageously recorded (see *Table 1*).[4] A difference of 2.73% between mutation detection rates of the two panels is obtained in Spain.[3] A higher sensitivity is confirmed in a non-European Spanish-speaking population such as Argentina.[9] Among the mutations tested with the Luminex platform, four are common (relative frequency higher than 2.5%) in the following European or Mediterranean countries: R1066C, 3.1% in Portugal; S549R, 2.6% in Algeria; 1677delTA, 3.8% in South of Bulgaria and 2.8% in Turkey; and R347H, 2.8% in Turkey.[3] R352Q is found with a relative frequency of 1% in Lombardy, an Italian region near Milan.[3] A higher sensitivity of the Luminex test is also recorded in the general Italian population.[3] A further extension of the Luminex mutation panel containing 12 additional changes, notably three (S1251N, Q552X, 711+5G>A) of the four missing mutations that are exclusively tested in the Innogenetics panel, is currently being validated in our laboratory. Based on the 2007 annual data report available from May 2009 from the CF Belgian Registry,[8] inclusion of the three mutations will enable increasing the detection rate of 1.27% for the Luminex platform and quite similar sensitivities will then be reached for both methods in Belgium (see *Table 1*).

Finally, due to the large number of mutations found in the *CFTR* gene and their variable frequency among different ethnic and racial groups, it has been difficult to develop a screening assay that covers all mutations and is suitable for all populations. Our experience with the Luminex platform is that this fully automated, semi-quantitative, highly specific and robust platform including ACMG-recommended mutations and variants[6] has been tailored for populations, such as central Europeans, in which migration and miscegenation have increasingly arisen. The platform favours detection of twice as many *CFTR* mutations. Extension of the mutation panel to include Q552X, 711+5G>A, S1251N and nine other mutations, currently under validation in our laboratory, will place Luminex xTAG as the most sensitive platform for routine CFTR molecular diagnosis in European countries. ■

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Identification and frequencies of cystic fibrosis mutations in central Argentina

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In 2009 in South-America, a small number of publications identified the mutations of the *CFTR* gene present in the continent. Especially in Argentina, only two papers reported the distributions and frequencies of *CFTR* mutations, one for the Cordoba province and the other for Buenos Aires, the capital city. In contrast, all European countries and regions have been studied to determine the natures and frequencies of *CFTR* mutations (and large differences have been observed between regions). Argentina was largely populated by a European immigration at the end of the 19th century. The rationale of the paper was: "Can *CFTR* mutations of putative precolombian origin be found in a country mainly populated by Italians, Spanish, Irishmen, Germans, French, … Europeans?" And a second question arose: "Are frequencies similar to those found in Europe?"

To answer the questions and to address the second objective of my thesis project, namely to characterize the frequencies of CFTR mutations in central Argentina with the final aim of developing a new first line panel of mutations with high sensitivity for routine use in this population, I used my personal contacts in the country. A questionnaire was designed on purpose by Prof. Daniel Baran to select a cohort of more than 100 children with classical CF in the Santa Fe province of Argentina, and I performed a complete molecular CFTR screening of the cohort according to the best European practices. The results are described in the following paper.

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ABSTRACT

Background: The Argentinian population is mainly of Caucasian origin, with a small contingent of indigenous descent. The aim of this study is to test the hypothesis that a panel of mutations designed for European countries is not optimal as a first-line molecular diagnosis for routine use in this country of mixed European origin.

Methods: Phenotype analyses combined with a European screening panel of 71 mutations followed by Sanger sequencing and large rearrangement study, were used to characterize the identification and distribution of CFTR mutations in the Santa Fe province of Argentina.

Results: Clinical review of 121 subjects suspected of CF during childhood led to selection of 83 unrelated patients. Thirty four different mutations, including two new ones, c.2554dupT and p.Leu49Pro, were detected. The total sensitivity was 91% (n = 151/166 alleles).

Conclusions: Frequencies of *CFTR* mutations in Argentinian populations differ from those of their European ancestry. A new first line panel of 21 *CFTR* mutations with a sensitivity of 84% is proposed for routine use in central Argentina.

1. Introduction

Cystic fibrosis (CF, MIM#219700) is the most common lethal autosomal recessive disease in Caucasian populations. Incidence is between 1/2000 and 1/3000 for the European ancestry. Over 1900 different mutations have been reported in the CFTR1 database [1]. Due to the variability of the phenotype, the CFTR2 database [2] has been built to correlate genotype and phenotype. Most studies on frequencies of *CFTR* mutations have been performed in European countries but only a few in South America.

The present work studies the identification and frequency of *CFTR* mutations in a densely populated province of Argentina. This country has more than 40 million inhabitants. The Santa Fe province (area similar to that of Greece) contains 3.36 million inhabitants [3]. Located in this province, Rosario is the third largest Argentinian city (1.2 million inhabitants). Santa Fe City is the second largest city of the province (0.57 million inhabitants). The majority of the population of the province originated from Europe and descended from the large wave of immigration between the middle of the nineteenth to the first half of the twentieth centuries. Italians (mainly from Piemont), Spaniards, Swiss and Germans represent the largest ethnic groups of immigrants. Since 1970, internal migrants coming mainly from the Northern provinces have moved to Rosario. A minority of the population of Rosario (20%) consists of a mixed white European and Amerindian ancestry. A small fraction (2–4%) comes from full-blood Indians.

This study was designed to test the hypothesis that a panel of mutations designed for European countries is not optimal as a first-line molecular diagnosis for routine use in this country of mixed European origin. Using a European panel such as Luminex® xTAG containing 71 mutations, we showed a detection rate of 80.6%, as previously reported [4]. By sequencing analysis, we identified two new *CFTR* mutations. Based on a meta-analysis of our data with those of the province of Cordoba [5], we propose a new panel of a smaller number of 21 mutations with an increased detection rate of 84% for routine use in central Argentina.

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0009-9120/

2. Methods

2.1. Patients

Patients attending the pediatric ward of a hospital in Rosario or in Santa Fe City, located in the Santa Fe province, were enrolled in this study. All patients, regularly followed up since childhood, had suspected CF based on local medical evaluation. The Local Ethics Committees approved the study and written informed consent was obtained from patients or parents or legal guardians, as adequate.

2.2. Study design and molecular analyses

In this study, patients with suspected CF were selected from the largest children hospital of Santa Fe City (the Hospital de Niños "Orlando Alassia") and of Rosario (Hospital de Niños "Víctor J. Vilela"). To collect detailed clinical and laboratory data of selected patients, a comprehensive specially designed questionnaire was filled for each patient by local caretakers. Whole blood samples were taken at the time of filling out the questionnaire, and DNA was extracted by the salting-out method at the STEM® laboratory in Rosario. The extracts were sent to the Human Genetic Center in Brussels for genetic analyses.

A first-line molecular screening was conducted by applying a classical mutation panel designed for European populations including the 71 most frequent *CFTR* (reference sequence NG_016465.1, NM_000492.3, NP_000483.3) mutations in European populations (xTAG Cystic Fibrosis 71 Kit v2; Luminex Co®, Austin, Texas, US). In the case of detection of two different mutations, no additional molecular studies were undertaken. When one 5T allele was detected by the xTAG method, double-strand direct sequencing of the end of intron 8 was carried out by Dye Terminator Sequencing v3.1/ABI 3130xl (Applied Biosystems®, Foster City, CA, USA). The latter manipulation defined the combination of 5T and TG repeats. If a single mutation, at a homozygous or a heterozygous status, or if no mutation was identified, a Multiplex Ligation Polymerase Amplification (MLPA) method (MRC-Holland®, Amsterdam, The Netherlands) was applied to search for large deletion/duplication rearrangements of the *CFTR* gene. When a single or no mutation was detected, a second-line diagnostic procedure consisting of double-strand direct sequencing of all coding exons and flanking intronic regions, was carried out by Sanger sequencing.

2.3. Clinical data

The following data were collected: age, height, weight, symptoms at diagnosis, history of meconium ileus and family history, defined as the presence of affected siblings. Immunoreactive trypsin (IRT) values of newborn screening were interpreted locally. Sweat chloride concentration was measured according to Gibson and Cooke [6]. Pancreatic insufficiency was defined by fecal fat loss [7] during a 3-day fecal fat balance with reference values for children following recommendations [8] or fecal elastase below 200 μg/g. CF-related diabetes was defined by requirement of insulin therapy. Whenever possible, microbiology tests were performed in sputum samples. In patients who did not expectorate spontaneously, a deep pharyngeal aspirate was undergone after a session of physiotherapy [9].

Sputum microbiology cultures, regularly performed at every 3-monthly clinic visit, were conducted in a polyvalent non-selective medium for bacteria (Gram negative bacteria for *Pseudomonas aeruginosa*, *Mycobacteria*, *Burkholderia cepacia*, *Stenotrophomonas*) and for yeasts (atypical and fungi). Patients with positive cultures of *P. aeruginosa* in the bronchial tree persisting for at least 6 months, with at least three consecutive positive cultures at one-month intervals, were considered as chronically infected [10]. Respiratory involvement was assessed by lung function testing using MultiSPIRO™ spirometers (WinDX Revelation 1.0.64; Creative Biomedics, San Clemente, CA, USA) at the time of blood sampling [11].

2.4. Statistical analysis

Exclusion criteria, including Rosenstein's criteria [12], were applied to the initial population of 121 children with suspected CF from the two largest children hospitals of the province. Data were analyzed using SAS-JMP11 software (SAS Institute, Cary, NC, USA). Prior to statistical analysis, data were tested for normal distribution including the Shapiro–Wilk tests. Non-parametric variables are expressed as median (range), and between-group comparisons were evaluated using median rank scores. Between-group comparisons of normally distributed data were evaluated using one-way analysis of variance with posthoc comparisons being made using Student *t* test or Tukey–Kramer honestly significant difference test for two or more than two x-levels, respectively. Relative distributions of non-continuous variables are expressed as percentages. Differences between expected and observed frequencies in group categories were tested by the chi-square test. Null hypothesis was rejected at $p < 0.05$.

3. Results

DNA samples were obtained from 121 patients with suspected CF. Exclusion criteria included the presence of an affected sibling taking part in the study (n = 7), suspected CF in adulthood (n = 3) and any possible suspected CF diagnosis following clinical review by a CF clinician (DB) following Rosenstein criteria (n = 28) [12]. In the case of siblings, the most severely affected one was selected for the study. Among the 28 excluded subjects based on Rosenstein criteria, no mutation was found in 16 children; a single CF-causing mutation was found in 9 children, two of whom presented a TG11-5T or a TG12-5T allele and one child presented an unclassified CFTR variant, c.2620-15C>G. The c.4242+13A>G unclassified variant II was detected in two children without any mutation. Two CF-causing mutations (p.Phe508del/p.Arg334Trp) were detected in a child who was excluded from the series based on not evocative and/or unavailable clinical data. Finally, 83 patients were selected for further clinical and genetic data interpretation (Fig. 1).

3.1. Genetic analyses

Comprehensive analysis of the 166 alleles from the selected patients identified 34 different mutations while only 18 were detected by the first-line screening which reached a detection rate of 80.6%. After Sanger sequencing, it increased to 91% (n = 151/166 alleles). No large rearrangement was detected. No mutation was found in 15 alleles. Frequencies of individual mutations are shown in Table 1. p.Phe508del was the most frequently found mutation (n = 94/166 alleles; 56.6%). Among the 34 mutations, 11 were observed at least twice (n = 128/166 alleles). Two previously unpublished mutations, c.2554dupT (p.Tyr852Leufs*44) and c.146T>C (p.Leu49Pro), were submitted to the CFTR1 database in 2012 and 2013 respectively [13,14]. Their description followed the traditional and the Human Genome Variation Society (HGVS) nomenclature systems [15,16]. Phenotype/genotype correlations have not been characterized in CFTR2 database for 10 of the 34 mutations identified. In one patient, the variants c.1727G > C (p.Gly576Ala) and c.2002C > T (p.Arg668Cys) were observed. We assumed that the two variants were located in *cis*. No DNA analysis could be performed in family members of tested children.

Distribution analysis of genotypes is summarized in Table 2. The presence of p.Phe508del homozygous status was detected in 36.1% (30/83) and the mutation was recorded in at least one allele in 77.1% (64/83 patients). In three patients from the selected series of 83 carrying a p.Phe508del, no other pathological mutation was found. A compound heterozygous genotype without p.Phe508del was found in 11 patients. A single or no mutation was identified in 11 patients whose

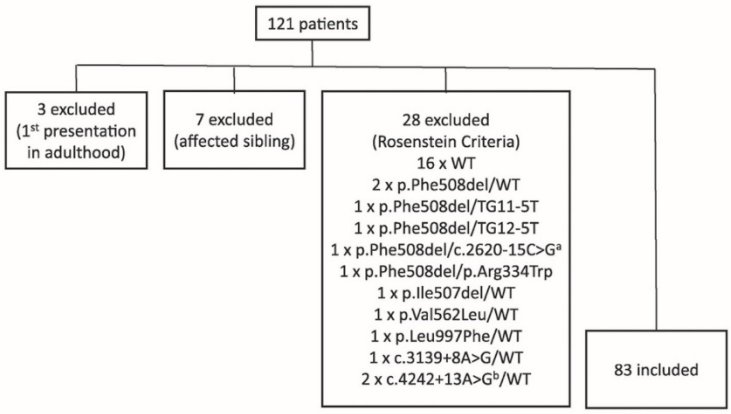

Fig. 1. Flow chart of the study a = unclassified variant class II. Corresponding c.2620-15C > G; legacy name: 2752-15C > G; HGVS name: dbSNP name: rs139379077. b = unclassified variant class II. Corresponding c.4242 + 13A > G; legacy name: 4374 + 13A > G; HGVS name: dbSNP name: rs76179227.

parents are native from the Santa Fe province with no additional data on ethnic origin from prior generations available. Genotypes were classified in five categories (Tables 2 and 3). Categories I to III contain patients with two identified mutations, p.Phe508del being detected in the first two categories at a homozygous (category I) or a heterozygous (category II) status. Categories IV and V contain patients with one or no mutation respectively.

Table 1
Identification and frequencies of *CFTR* mutations in a population with suspected CF from Santa Fe province, Argentina.

rs name	HGVS p. name	HGVS c. name	Legacy name	n (%)	Screening panel	CFTR1 database	CFTR2 database
rs199826652	p.Phe508del	c.1521_1523delCTT	F508del	94 (56.6)	Yes	Yes	CF-causing
rs113993959	p.Gly542*	c.1624G > T	G542X	7 (4.2)	Yes	Yes	CF-causing
No	p.Asn1303Lys	c.3909C > G	N1303K	5 (3)	Yes	Yes	CF-causing
rs74767530	p.Arg1162*	c.3484C > T	R1162X	4 (2.4)	Yes	Yes	CF-causing
rs75961395	p.Gly85Glu	c.254G > A	G85E	3 (1.8)	Yes	Yes	CF-causing
rs78756941	NA	c.489 + 1G > T	621 + 1G > T	3 (1.8)	Yes	Yes	CF-causing
rs76713772	NA	c.1585-1G > A	1717-1G > A	3 (1.8)	Yes	Yes	CF-causing
No	p.Lys684Serfs*38	c.2051_2052delAAinsG	2183AA > G	3 (1.8)	Yes	Yes	CF-causing
rs397508173	p.Ser4*	c.11C > A	S4X	2 (1.2)	No	Yes	No
rs121909011	p.Arg334Trp	c.1000C > T	R334W	2 (1.2)	Yes	Yes	CF-causing
rs77010898	p.Trp1282*	c.3846G > A	W1282X	2 (1.2)	Yes	Yes	CF-causing
rs397508141	p.Leu34_Gln39del	c.100_117delTTGTCAGACATATACCAA	232del18	1 (0.6)	No	Yes	No
No	p.Leu49Pro	c.146 T > C	L49P §	1 (0.6)	No	No	No
rs77834169	p.Arg117Cys	c.349C > T	R117C	1 (0.6)	Yes	Yes	CF-causing
No	p.Arg117Pro	c.350G > C	R117P	1 (0.6)	No	Yes	No
rs80282562	p.Gly178Arg	c.532G > A	G178R	1 (0.6)	Yes	Yes	CF-causing
rs121908803	p.Pro205Ser	c.613C > T	P205S	1 (0.6)	No	Yes	CF-causing
rs121908752	p.Leu206Trp	c.617 T > G	L206W	1 (0.6)	Yes	Yes	CF-causing
No	p.Arg347Pro	c.1040G > C	R347P	1 (0.6)	Yes	Yes	CF-causing
rs397508155	p.Tyr362*	c.1086 T > A	Y362X	1 (0.6)	No	Yes	No
rs74597325	p.Arg553*	c.1657C > T	R553X	1 (0.6)	Yes	Yes	CF-causing
rs1800098 + rs1800100	p.[Gly576Ala(;)Arg668Cys]	c.[1727G > C(;)2002C > T]	G576A-R668C	1 (0.6)	No	Yes in trans	non CF-causing in trans
No	p.Ser589Ile	c.1766G > T	S589I	1 (0.6)	No	Yes	No
No	NA	c.1766 + 1G > A	1898 + 1G > A	1 (0.6)	Yes	Yes	CF-causing
rs186089140	p.Ser737Phe	c.2210C > T	S737F	1 (0.6)	No	Yes	No
rs397508376	p.Leu812Phefs*11	c.2434_2435insT	2566insT	1 (0.6)	No	Yes	No
No	p.Ser821Argfs*4	c.2462_2463delGT	2594delGT	1 (0.6)	No	Yes	CF-causing
No	p.Tyr852Leufs*44	c.2554dupT	c.2554dupT §	1 (0.6)	No	No	No
rs80224560	NA	c.2657 + 5G > A	2789 + 5G > A	1 (0.6)	No	Yes	CF-causing
rs75096551	NA	c.2988 + 1G > A	3120 + 1G > A	1 (0.6)	Yes	Yes	CF-causing
rs76151804	NA	c.3140-26A > G	3272-26A > G	1 (0.6)	No	Yes	CF-causing
rs143570767	NA	c.3873 + 1G > A	4005 + 1G > A	1 (0.6)	No	Yes	CF-causing
rs397508631	p.Ser1297Phefs*5	c.3884_3885insT	4016insT	1 (0.6)	No	Yes	CF-causing
No	p.Leu1414Phe	c.4242_4242 + 1delGGinsTT	4374_4374 + 1GG > TT	1 (0.6)	No	Yes	No
No	NA	c.1210-12T [5]	TG11-5T	1 (0.6)	Yes	Yes	Varying clinical consequence
-	p.=	c.=	WT	14 (8.4)	NA	NA	NA

HGVS (Human Genoma Variation Society) used for protein nomenclature [15,16].
§ = new mutation identified in this study; NA = not applicable.

Table 2
Categorization of genotypes in a population with suspected CF from Santa Fe province, Argentina.

	Genotype	N	Frequency (%)	Total N	Total frequency (%)
Category I: p.Phe508del/p.Phe508del	p.Phe508del/p.Phe508del	30	36.1	30	36.1
Category II: p.Phe508del/Other	p.Phe508del/p.Gly542*	5	6		
	p.Phe508del/p.Asn1303Lys	3	3.6		
	p.Phe508del/p.Gly85Glu	2	2.4		
	p.Phe508del/c.1585-1G > A	2	2.4		
	p.Phe508del/c.2051_2052delAAinsG	2	2.4		
	p.Phe508del/p.Trp1282*	2	2.4		
	p.Phe508del/p.Arg117Pro	1	1.2		
	p.Phe508del/p.Pro205Ser	1	1.2		
	p.Phe508del/p.Leu206Trp	1	1.2		
	p.Phe508del/p.Arg553*	1	1.2		
	p.Phe508del/p.Ser589Ile	1	1.2		
	p.Phe508del/p.Ser737Phe	1	1.2		
	p.Phe508del/p.Arg1162*	1	1.2		
	p.Phe508del/c.1766 + 1G > A	1	1.2		
	p.Phe508del/p.Leu34_Gln39del	1	1.2		
	p.Phe508del/p.Leu812Phefs*11	1	1.2		
	p.Phe508del/c.3140-26A > G	1	1.2		
	p.Phe508del/c.3873 + 1G > A	1	1.2		
	p.Phe508del/p.Ser1297Phefs*5	1	1.2		
	p.Phe508del/c.4242_4242 + 1delGGinsTT	1	1.2		
	p.Phe508del/c.489 + 1G > T	1	1.2		
				31	37.5
Category III:	p.Gly542*/p.Asn1303Lys	1	1.2		
Other/other	p.Asn1303Lys/p.Gly85Glu	1	1.2		
	c.489 + 1G > T/p.Lys684Serfs*38	1	1.2		
	c.489 + 1G > T/p.Gly542*	1	1.2		
	p.Arg1162*/p.Ser4*	1	1.2		
	p.Arg1162*/p.Tyr362*	1	1.2		
	p.Arg334Trp/c.1585-1G > A	1	1.2		
	p.Arg334Trp/p.Ser821Argfs*4	1	1.2		
	p.Arg347Pro/p.Ser4*	1	1.2		
	c.2657 + 5G > A/p.Tyr852Leufs*44 #	1	1.2		
	p.Arg1162*/p.Leu49Pro #	1	1.2		
				11	13.2
Category IV:	p.Phe508del/WT	3	3.6		
A single mutation	c.2988 + 1G > A/WT	1	1.2		
	p.Arg117Cys/WT	1	1.2		
	p.Gly178Arg/WT	1	1.2		
	p.[Gly576Ala(;)Arg668Cys]/TG11-5T	1	1.2		
				7	8.4
Category V:					
Wild type				4	4.8

#: new mutation submitted to CFTR1 database [1]; other = other mutation than p.Phe508del.

3.2. Phenotype description

Table 3 summarizes the main clinical and laboratory characteristics of patients. Median age at diagnosis of the whole population was three months, and was significantly lower ($p = 0.044$, median test) in p.Phe508del homozygous patients (category I). Family history was detected in about 1/5 of patients, without significant difference between categories. Meconium ileus was only observed in association with at least one p.Phe508del allele (categories I and II; $p = 0.0393$, chi-square test). Almost all p.Phe508del homozygous patients were pancreatic insufficient and the prevalence of pancreatic insufficiency decreased from categories I to V. Sweat chloride, obtained in 76 patients, showed normal distribution with positive mean values in all categories without significant difference between categories ($p = 0.88$, ANOVA). *P. aeruginosa* colonization was almost exclusively found in association with the presence of at least one p.Phe508del mutation (14/16). Respiratory function, performed in about half of patients (42/83), was mildly preserved without significant difference between categories.

Data collection of symptoms during the twelve-month period prior to sampling revealed the presence of sinusitis (12/83, 11 with 2 identified mutations), nasal polyps (3/83), need of oxygenotherapy (3/83) and pneumothorax (1/83). Sinusitis was associated in 9/12 cases with p.Phe508del (5 homozygous and 4 compound heterozygous). Genotypes associated with nasal polyps were p.Phe508del/p.Phe508del, p.Phe508del/p.Gly85Glu and p.Gly542*/p.Asn1303Lys. Oxygenotherapy was needed continuously in two children in categories I and IV, and only during the night in another child in category I. A child with pneumothorax had a p.Phe508del/p.Arg553* genotype. Complications such as CF-related diabetes and hemoptysis were not observed.

3.3. Phenotype details of children presenting new mutations

We describe here the clinical presentation of the two children with new mutations, the c.2554dupT and the c.146T > C, which were identified and submitted to CFTR1 database [13,14].

The c.2554dupT was characterized by duplication of a thymidine at position 2554 of the nucleotide sequence thus leading to a frameshift at tyrosine 852 and predicting the addition of 43 aberrant amino acids before encountering a premature stop codon (p.Tyr852Leufs*44). The mutation was identified in a 2-year old Argentinian girl without family history with parents from Rosario. The child also carried a c.2657+5G>A mutation. Parental DNA analysis could not be performed to assign allele *trans* position. Diagnosis was raised in the first months of life due to a positive neonatal screening (IRT = 135 ng/ml; positive value > 70 ng/ml). At the time of diagnosis, pulmonary symptoms were present and sweat chloride concentration was 105 mmol/l. At

Table 3
Main clinical and laboratory characteristics in a population with suspected CF from Santa Fe province, Argentina.

	Whole population	Category I p.Phe508del/p.Phe508del	Category II p.Phe508del/Other	Category III Other/Other	Category IV a single mutation	Category V WT/WT
n (%)	83 (100)	30 (36)	31 (37)	12 (14)	6 (7.2)	4 (4.8)
Age at diagnosis, median in months [range]	3 [0;240]	1 [0;82]	3[0;240]	8 [0.5;148]	3.75 [2;6]	89.5 [59;166]
Age at sampling, median in months [range]	102 [3;329]	90 [3;294]	113 [8;329]	84 [6;288]	113 [21;192]	144 [78;222]
Male sex, n (%)	41 (49.4)	16 (53.3)	11 (35)	7 (58)	4 (67)	3 (75)
Family history, n (%)	17 (20.5)	5 (16.7)	9 (29)	3 (25)	0	0
Symptoms at diagnosis						
Respiratory, n (%)	56 (67.5)	15 (50)	24 (77)	10 (83)	5 (83)	2 (50)
Digestive, n (%)	53 (63.8)	22 (73.3)	20 (64.5)	4 (33)	5 (83)	2 (5)
Meconium ileus, n (%)	14 (16.9)	8 (26.7)	6 (19)	0	0	0
Respiratory + digestive, n (%)	36 (43.4)	14 (46.7)	15 (48.4)	3 (25)	4 (67)	0
Failure to thrive, n (%)	31 (37.3)	10 (33.3)	14 (45)	3 (25)	3 (50)	1 (25)
Neonatal screening (neonatal trypsin), n (%)	47 (56.6)	17 (56.7)	18 (58)	9 (75)	3 (50)	0
Local interpretation as positive trypsin, n (%)	34 (72.3)	15 (50)	12 (39)	5 (42)	2 (33)	0
Pancreatic insufficiency, n (%)	71 (85.5)	29 (96.7)	27 (87.1)	9 (75)	4 (66.7)	2 (50)
Sweat Cl, mmol/l mean [lower; upper 95% CI]	87 [82.2;92.7]	85.0 [76.9;93]	87.4 [79.5;95.3]	87.3 [68.8;105.8]	101.4 [73.6;129.2]	87 [64.9;109.1]
Microbiology						
P. aeruginosa, n (%)	16 (19.3)	7 (23.3)	7 (23)	1 (8.3)	1 (17)	0
B. cepacia, n (%)	7 (8.4)	4 (13.3)	3 (9.7)	0	0	0
S. aureus MRSA, n (%)	18 (21.7)	6 (20)	9 (29)	2 (17)	1 (17)	0
S. aureus MSSA, n (%)	43 (51.8)	15 (50)	17 (55)	5 (42)	3 (50)	2 (50)
H. influenza, n (%)	18 (21.7)	5 (16.7)	5 (16)	3 (25)	3 (50)	2 (50)
B. achromobacter, n (%)	7 (8.4)	4 (13.3)	2 (6.5)	0	1 (17)	0
No pathogens, n (%)	7 (8.4)	2 (6.7)	2 (6.5)	2 (17)	0	1 (25)
Respiratory function, n	42	15	17	5	0	3
% FEV1 mean [lower;upper 95% CI]	80 [71.1;89.4]	82.1 [67.3;97]	79.2 [64.6;93.7]	95.3 [88.8;101.8]	ND	93 [69.5;116.5]
% FVC mean [lower; upper 95% CI]	84 [77.6;91]	85.6 [76.5;94.7]	80 [67.5;92.4]	105.9 [96.8;115]	66.5 [65.5;67.5]	84 [61.3;106.7]

Frequency data presented as number with percentage in parentheses. Respiratory function data expressed as mean and lower; upper 95% confidence interval (CI) in brackets. FEV1: forced expiratory volume in 1 s and FVC: forced vital capacity expressed as percentage of predictive value for age, sex and height (%pred) [11]. Age expressed as median with range in brackets. other = other mutation than p.Phe508del; WT = wild-type; and ND = no data.

1 year of age, weight was between the 10th and 25th percentiles and height at the 50th percentile. Seven months later, a one-week hospitalization for antibiotherapy occurred following isolation of a methicillin-sensitive *Staphylococcus aureus*.

At that time, a flattening of the height curve, without change in the weight curve, was observed with height declining to the 3rd percentile. Pancreatic insufficiency was not detected.

The c.146 T > C was characterized by a substitution of a thymine by a cytosine at nucleotide position 146. The substitution leads to a missense mutation replacing a leucine, an amino acid highly phylogenetically conserved at position 49, by a proline (p.Leu49Pro). The mutation was identified in a 1-year old Argentinian boy without family history with parents from the North of the Santa Fe province. The child also displayed the p.Arg1162*. Parental DNA analysis could not be performed to assign allele *trans* position. He was born after a 42-week gestation. His weight was 4400 g, and he was asymptomatic. Diagnosis was raised due to a positive IRT. At the time of diagnosis, pulmonary symptoms were present without failure to thrive (97th percentile for height and weight). Chloride concentrations reached 76 and 66 mMol/l in two different sweat tests.

3.4. Phenotype details of subjects excluded from the analysis but presenting two mutations

Among the subjects excluded from clinical data interpretation (Fig. 1), all but one presented one or no mutation. The child presenting two classical mutations (p.Phe508del/p.Arg334Trp) was initially excluded because no evocative symptoms of CF were present up to nine years of age when DNA was sampled. The child, enrolled in newborn screening, presented a normal IRT. At the time of questionnaire filling, lung function was preserved (FEV1 90% pred) and *P. aeruginosa* colonization was absent. No failure to thrive was detected and weight and height were at the 75th percentile. Sweat chloride was not available. Fecal elastase was over 500 mg/g. Although a classical diagnosis of CF could not be established at the time of analysis, a discreet follow up was advised in the perspective of a possible delayed development of symptoms, around twenty years. Indeed, patients with p.Phe508del/p.Arg334Trp have, at least at younger ages (<20 years), a more moderate phenotype with a better preserved lung function than p.Phe508del homozygous patients [2].

4. Discussion

We studied here the identification and frequencies of *CFTR* mutations in Argentina and we showed that even the largest European panel was not optimal as a first-line central Argentinian molecular diagnosis for routine use. A smaller panel specially designed may provide higher detection rates.

In this study, comprehensive *CFTR* molecular analysis of the clinically selected patients identified two unpublished mutations [13,14] and reached a 91% detection rate. This figure is in line with 94.2% found for the neighboring province, Cordoba [5] and with 90% reported for the southern Brazilian population [17]. However, a lower 83.45% detection rate was found in 220 unrelated patients from the Argentinian province of Buenos Aires [18]. The differences in detection rates found between our study and that of Visich et al. [18] could be due to ethnic origin of the populations, criteria used for selecting patients, molecular methodology used in the analysis and degree of extension of the *CFTR* including exon-intron boundaries.

The three most common mutations were p.Phe508del, p.Gly542* and p.Asn1303Lys. The p.Phe508del frequency (56.6%) was similar to those reported in studies conducted for the adjacent area around the capital (58.6% and 57%) [18,19] and in the neighboring Cordoba province (60.2%) [5]. However, lower frequencies have been reported in Uruguay (40.4%) [20] and in southern Brazil (45.54%) [17]. In our work, the frequency was higher than the highest one noted in Chile although the two areas are located roughly at the same latitude (31–33° S) but separated by the Andes. Indeed, it has been recognized that in Chile, the frequency of p.Phe508del followed a south–north gradient with about 29% detection rate in the center and in the south and 45% in the north [21].

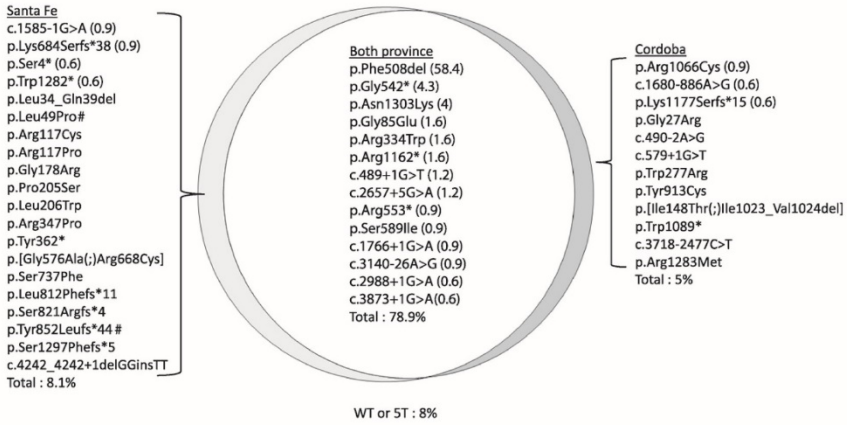

Fig. 2. Venn diagram comparing identification and frequencies of *CFTR* mutations between the Santa Fe (n = 83) and the neighboring Cordoba (n = 78) provinces. In bold, the 21 CFTR mutations detected at least twice in both studies. #: new mutations identified during this study. Data expressed as number with percentage in parenthesis. Percentage of mutations when not indicate corresponds to 0.3.

The 4.2% frequency of p.Gly542* is similar to that of the Cordoba province (4.49%) [5] and Buenos Aires (4.1% and 3.94%) [18,19]. It was lower than in Uruguay (5.7%) [20] and in southern Brazil (6.25%) [17]. The highest rates of p.Gly542* found in the south of Spain (14.4%) and in Tunisia (8.9%) allow postulating that caravels accosting at the Atlantic coast during the conquest could explain a p.Gly542* land-to-coast gradient.

The frequency we found for the p.Asn1303Lys mutation (3%) is of the same order of magnitude as that reported in studies conducted in Uruguay (2.9%) [20] or in Buenos Aires (2.73%) [18]. Yet, large discrepancies between frequencies of the mutation in the southern areas of the South American countries have been reported. Indeed, while a null frequency has been observed in Chile [21], it was present at different detection rates in other neighboring countries. In a study conducted in Buenos Aires, the mutation was detected at 1.75% [19] and higher frequencies (4.5–5.1%) were reported in the Argentinian province of Cordoba [5] and in the southern region of Brazil [17].

The two new c.2554dupT and p.Leu49Pro variants we identified are likely disease-causing mutations; they are not reported in human genome databases [22,23]. When analyzing "in silico" software for estimation of protein loss-of-function, homogeneous predictions can be observed: probably damaging (1.000 score by PolyPhen-2) [24], disease causing (p-value of 1 by Mutation Taster) [25] and deleterious (0.04 score by SIFT) [26].

We showed here that the distribution of *CFTR* mutation frequencies in Argentina differ from that of European ancestry. The population has some specificities justifying adaptation of mutation panels currently available in Europe. In the perspective of developing a new first-line screening panel for routine use in central Argentina, we compared our data with those from the neighboring Cordoba province with a similarly sized population. The study of Ramirez [5] can be used for comparison based on a similar cohort of patients (78 vs 83) and the use of a second-line molecular analysis. The population tested by Ramirez et al. can also be considered as representative of the province. A Venn diagram (Fig. 2) we built to illustrate common and different points between the two cohorts showed 14 mutations common to both provinces. A larger diversity, including the two newly identified mutations, was observed in the Santa Fe province. Interestingly, four mutations were exclusively observed at least twice in the Santa Fe province. The fact that c.1585-1G > A has been observed in Western Europe, that c.2051_2052delAAinsG has been detected in Central Europe, that p.Ser4* is considered as from Slovenian origin and that p.Trp1282* is frequently associated with Ashkenazi Jewish origin [27] reflects a high degree of miscegenation of the Santa Fe population. Considering the development of a panel presenting a high detection rate to be used in both provinces, we suggest to insert three additional mutations (p.Arg1066Cys, c.1680-886A > G and c.3528delC) found at least twice in the Cordoba province [5]. The proposed panel would include the reduced number of 21 mutations (in bold in Fig. 2), but it would allow reaching a good efficacy of 84% detection rate for the Argentinian provinces representing 6.4 million inhabitants, almost a fifth of the whole country. Among the 21 mutations, three p.Ser589Ile (0.9%), p.Ser4* (0.6%) and c.3873+1G>A (0.6%) are not included in any first-line test. Finally, using comprehensive molecular analyses, this study is in line with a heterogeneous ethnic origin of Argentina. It allowed characterization of more than 91% of CFTR mutations affecting children suspected of CF from a central Argentinian area. Meta-analysis with data from a comparable neighboring province allows designing an optimized panel with a reduced number of 21 mutations and a good 84% detection rate.

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Clinical and morphological characteristics of sporadic genetically determined pancreatitis as compared to idiopathic pancreatitis: higher risk of pancreatic cancer in CFTR variants.

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The starting point of this paper was a question asked by the UCL Gastroenterology Department: “Is it possible to predict, from clinical data, a genetic factor in patients with idiopathic pancreatitis (excluding an alcoholic origin)?”. And a second question was: “Are patients with pancreatitis and carrying at least one *CFTR*, *PRSS1* or *SPINK1* mutation more frequently affected than non-carriers?”.

To answer the questions and to address the third objective of the thesis project, *i.e.,* to study the clinical manifestations, including the occurrence of pancreas cancer, in patients with sporadic idiopathic pancreatitis who display mutations in the *CFTR*, *PRSS1* and *SPINK1* genes, I thus collected, from the files of the Department of Genetics, the retrospective molecular data since 1999 (obtained by me since 2001) of patients from departments of Gastroenterology of the whole of Belgium. This constituted a database of 351 patients with idiopathic pancreatitis and genetic testing. Out of this cohort, a group of 68 patients carried at least one mutation in the *CFTR, PRSS1* or *SPINK1* genes. From the database of 351 patients, the UCL Gastroenterology Department then constituted a non-carrier control group matched for gender and age, and compared the clinical characteristics between the two groups. The results of the comparison are given in the following paper.

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Clinical and Morphological Characteristics of Sporadic Genetically Determined Pancreatitis as Compared to Idiopathic Pancreatitis: Higher Risk of Pancreatic Cancer in CFTR Variants

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Key Words
Pancreatitis · Pancreas cancer · *CFTR* · *SPINK1* · *PRSS1*

Abstract
Background/Aims: Idiopathic pancreatitis is considered to be a multigenic and multifactorial disease. Genetically determined pancreatitis is associated with mutations in the *PRSS1*, *SPINK1* and *CFTR* genes. This study aimed at examining the clinical and morphological characteristics of patients diagnosed with genetically determined sporadic pancreatitis. ***Methods:*** Inclusion criteria were the presence of *PRSS1*, *CFTR* or *SPINK1* gene mutations in patients with idiopathic recurrent or chronic pancreatitis. Patients with hereditary pancreatitis were excluded. Age- and sex-matched patients with idiopathic pancreatitis and negative genetic testing served as controls (n = 68). ***Results:*** Genetic testing was performed in 351 probands referred to our centre since 1999. Sixty-one patients (17.4%) carried at least 1 detected mutation in 1 of the 3 tested genes (34 *CFTR*, 10 *PRSS1* and 13 *SPINK1* mutations), and 4 patients showed a combination of mutations. Follow-up has been currently extended to a median of 5 years (range 1–40). Similar clinical features were noted in the case and matched groups except for an earlier age of onset of pancreatic symptoms and a higher incidence of pancreatic cancer in the case group and in patients with *CFTR* mutations compared to the control group ($p < 0.05$). The standardized incidence ratio, the ratio of observed to expected pancreatic cancers, averaged 26.5 (95% confidence interval 8.6–61.9). All pancreatic cancer patients were smokers. ***Conclusion:*** Clinical parameters of patients with sporadic idiopathic pancreatitis and gene mutations are similar to those of age- and sex-matched patients without gene mutations, except for the age of pancreatic disease onset. A significantly higher occurrence of pancreas cancer was observed in the case group, particularly in those patients carrying *CFTR* mutations. We therefore suggest to include patients with CFTR variants presenting with risk factors in a screening and surveillance programme and to strongly advise them to stop smoking.

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Introduction

Chronic pancreatitis is a syndrome characterized by chronic inflammation of the pancreas, with variable pain, calcifications, necrosis, fatty replacement, fibrosis and scarring and other complications. Variability in susceptibility to recurrent acute and chronic pancreatitis is now clearly shown to be related to genetic differences between patients [1, 2]. The estimated incidence of chronic pancreatitis in adults is 3.5–10 cases per 100,000. In developed countries, 60–70% of patients with chronic pancreatitis have a history of excessive alcohol intake, while in 30% of cases the disease is regarded as idiopathic [3, 4]. Recent research has identified gene mutations associated with chronic pancreatitis [2, 5–11]. It has long been recognized that chronic pancreatitis may segregate in selected families, thus suggesting the influence of a genetic background. In 1996, Whitcomb et al. [7] identified the first mutation associated with hereditary pancreatitis, namely the R122H mutation in the cationic trypsinogen gene *(PRSS1)*. Several other mutations (A16V, K23R, N29I, N29T, R122C) and triplication as well as duplication of the PRSS1 locus have been subsequently described [4, 12–18].

The cystic fibrosis transmembrane conductance regulator (CFTR) protein conducts both chloride and bicarbonate and is essential for normal bicarbonate secretion by pancreatic duct cells. To date, more than 1,500 mutations of the *CFTR* gene have been described. In 1998, Sharer et al. [19] and Cohn et al. [20] in their respective studies were able to demonstrate a strong association between *CFTR* mutations and idiopathic pancreatitis. The effect of mutations in the pancreatic secretory trypsin inhibitor gene, which is known as the serine protease inhibitor Kazal type 1 *(SPINK1)* gene, on the onset of pancreatitis was reported in 2000 [21, 22]. The most frequent *SPINK1* mutation is p.N34S. Several other mutations, including p.M1, p.L14P, p.L14R, c.[1–215G>A;194+2T>C] and p.R67C, are possibly associated with chronic pancreatitis [3, 14, 23–26]. More recent contributions have confirmed the strong association between SPINK1 and CFTR [27, 28].

In clinical practice, physicians regularly encounter patients with recurrent acute or chronic pancreatitis, with no history of hereditary pancreatitis, therefore so-called 'sporadic' cases. Natural history in patients with sporadic idiopathic disease is less well documented in the literature, making it difficult to define the prognosis and the follow-up of these patients [26, 29, 30]. Most of the papers on this subject focus on the hereditary aspects of pancreatitis with or without *PRSS1* mutations [29, 31], while fewer contributions report on so-called 'sporadic' genetic pancreatitis [32, 33].

Therefore, this study aims at assessing genetic, clinical and morphological characteristics of patients diagnosed with genetically determined sporadic chronic or recurrent pancreatitis and comparing these features to those recorded from a control group of patients with idiopathic pancreatitis.

Patients and Methods

Data Source

Up to 1,725 patients have been followed up for chronic pancreatitis or recurrent acute pancreatitis in our gastroenterology department since 1999. In all, 351 of them (20.3%) had idiopathic pancreatitis and were submitted to genetic testing. All clinical data were obtained from the Cliniques universitaires St-Luc electronic patient file and through contact with the patients, their general practitioners and/or their gastroenterologists. The study was approved by the local Ethics Committee of the Université Catholique de Louvain Medical School.

Inclusion Criteria

Case Group

Patients were included in this group if they had non-hereditary (sporadic) idiopathic recurrent acute pancreatitis or idiopathic chronic pancreatitis associated with at least 1 detected mutation in 1 of the 3 tested genes, i.e. *PRSS1, CFTR* or *SPINK1*. Patients were excluded if they had only genetic polymorphism and if they had hereditary pancreatitis, defined as having one or more relative with a history of acute, recurrent acute or chronic pancreatitis.

Control Group

Patients were included in this group if they had idiopathic recurrent pancreatitis or idiopathic chronic pancreatitis with no detected mutation by *PRSS1, CFTR* and *SPINK1* gene testing. Other causes of chronic pancreatitis were searched for depending on the clinical context, in particular with regards to alcohol intake. Patients were also excluded from this group if they had one or more relative with a history of acute, recurrent acute or chronic pancreatitis.

Patients of this group were matched with those of the case group for sex and age.

Definitions

Acute pancreatitis was defined by acute abdominal pain with threefold increased serum pancreatic enzymes levels and typical acute pancreatitis on imaging. Chronic pancreatitis was defined by typical chronic pancreatitis imaging (M-ANNHEIM pancreatic imaging criteria) [34]. Exocrine pancreatic insufficiency was diagnosed in the presence of steatorrhoea. Diabetes mellitus was diagnosed if the whole venous blood fasting glucose concentration was ≥126 mg/dl.

Patients were categorized for their smoking status as smokers if they had smoked for at least 2 pack-years and otherwise as non-smokers [29]. The M-ANNHEIM score was calculated for all pa-

tients. This scoring system determines the severity of chronic pancreatitis by grading the presence of abdominal pain, therapeutic approaches to pain control, pancreatic surgical interventions, exocrine and endocrine insufficiency, morphological status of the pancreas and the occurrence of severe organ complications. Diagnostic and therapeutic features are appropriately scored. Score points are recorded together and their sum is used to categorize disease severity as minor, increased, advanced, marked or exacerbated [34].

The follow-up period was defined as the interval between the date of the first symptom or sign of pancreatitis and the date of the last visit.

Genetics Data

CFTR Gene (NM_000492)

The 71 most frequent *CFTR* mutations in European populations were screened using an xTAG Cystic Fibrosis 71 Kit v2 (Luminex, Austin, Tex., USA). When a single mutation was detected, characterizing a heterozygous status, double-strand direct sequencing of all 27 exons, numbered from 1 to 27, was carried out by Dye Terminator Sequencing v3.1/ABI 3130xl (Applied Biosystems, Foster City, Calif., USA). In parallel with *CFTR* exonic sequencing, detection of large deletions/duplications in the *CFTR* gene was carried out by means of Multiplex Ligation-dependent Probe Amplification (MRC-Holland, Amsterdam, The Netherlands). When one 5T allele was detected with the xTAG method, double-strand direct sequencing of the end of intron 8 was carried out by Dye Terminator Sequencing v3.1/ABI 3130xl. This last manipulation defined the combination between the 5T allele and the TG repeats.

In this paper, mutation names are reported according to the CF Mutation Database (*CFTR* guidelines).

PRSS1 Gene (NM_002769)

Double-strand direct sequencing of all 5 PRSS1 exons was performed by Dye Terminator Sequencing v3.1/ABI 3130xl.

SPINK1 Gene (NM_003122)

Mutational analysis of exon 3 detecting the most frequent N34S mutation was performed by double-strand direct sequencing using Dye Terminator Sequencing v3.1/ABI 3130xl.

Statistical Analysis

Univariate comparisons for frequency counts were made with the χ^2 test or Fisher's exact test, as appropriate. Kaplan-Meier curves of cancer-free, diabetes-free and complication-free survival were constructed and compared between groups using the log-rank test. Statistical analysis was performed with PASW Statistics 18.0.0. The standardized incidence ratio (SIR), the ratio of observed to expected pancreatic cancers, was calculated using StatsDirect statistical software (version 2.7.7). The reference incidence ratio was obtained from Cancer Mondial, the section for cancer information of the International Agency for Research on Cancer (http://www-dep.iarc.fr/). More specifically, the age-specific incidence rate for Flanders (without Limburg) from CI5 volume VIII was used. The 95% confidence interval for the SIR was calculated by assuming that the observed cases of cancer followed a Poisson distribution.

Pathologic Examination

For endoscopic ultrasound-guided fine needle aspiration specimens or endoscopic retrograde cholangiopancreatography brushings, from 1999 until 2002, direct smears were made by immediate immersion of specimens in methanol, and retrieved tissue fragments, if present, were fixed in Bouin's fluid to make a cell block. In May 2002, the Thermoshandon's Papspin® monolayer technique was introduced into the pathology laboratory. For surgical specimens, after 10% formalin fixation, the pancreatic tissue was processed in 5-mm-thick sections, and every macroscopically visible lesion was included in paraffin. Sections stained with haematoxylin and eosin were reviewed by two senior pathologists (A.J.-M., B.W.) blinded to the clinical status of the patients.

Results

Genetic Characteristics of the Cohort

Sixty-one out of 351 patients (17.4%) with idiopathic pancreatitis included in the genetic testing displayed at least 1 detected mutation in 1 of the 3 tested genes; 34 patients (9.7%) had a disease-causing mutation in *CFTR*, 10 patients (2.8%) carried a *PRSS1* mutation and 13 (3.7%) had a *SPINK1* mutation (fig. 1–3). Four patients (1.2%) showed a combination of mutations; 3 patients had a *SPINK1* mutation associated with a *CFTR* mutation and 1 patient carried a *PRSS1* mutation and a *SPINK1* mutation. The genetic characteristics of the patients are summarized in table 1.

Clinical Features

Case Group

Clinical features recorded in the control and case groups are shown in table 2. The median age at onset of pancreatic symptoms and at genetic testing was 29 years (range 3–70) and 38 years (range 3–83), respectively. The median age at first symptom in the case group of 29 years (range 3–70) was significantly earlier than in patients of the control group (37 years, range 1–73; p = 0.01). Median follow-up was 5 years (range 1–40). All patients had pancreatic pain. Pancreatic disease led to hospitalization in most of the patients (55/61, 88.5%); the median number of hospital stays averaged 3 (range 0–15), and the median length of stay was 15 days (range 0–107). Endocrine and exocrine insufficiency occurred in 16.4% (10/61) and 26.2% (16/61), respectively. Among patients with diabetes mellitus, 80% required insulin and 20% oral drugs only. The onset of symptoms was significantly earlier in those patients carrying a *PRSS1* mutation as compared with other genetic variants.

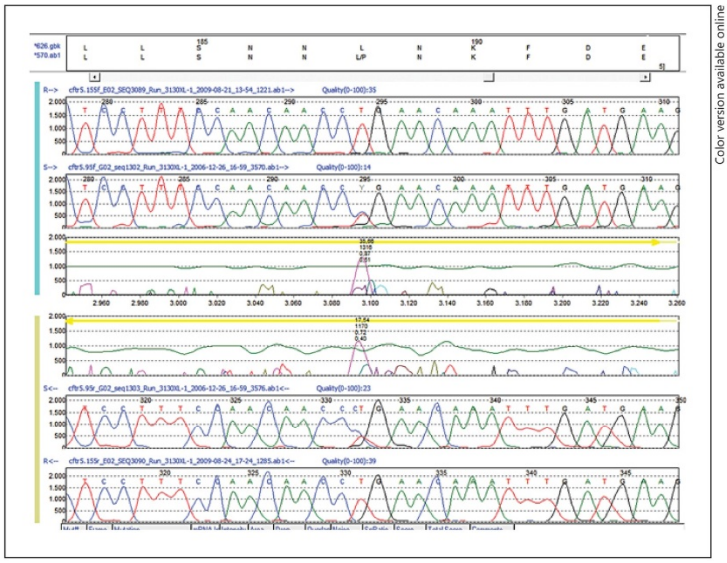

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Fig. 1. Electropherogram of the new L188P mutation of the *CFTR* gene (Sanger sequencing and analysis by Mutation Surveyor 4.0 software). The protein structure from codon 186 to 193 is given in line 1. The forward and reverse sequences are shown in lines 2–4 and 5–7, respectively. Lines 2 and 7 illustrate results from control DNA. Lines 3 and 6 illustrate results from patient DNA. Lines 4–5 illustrate the difference of the sequencing raw data between control and patient.

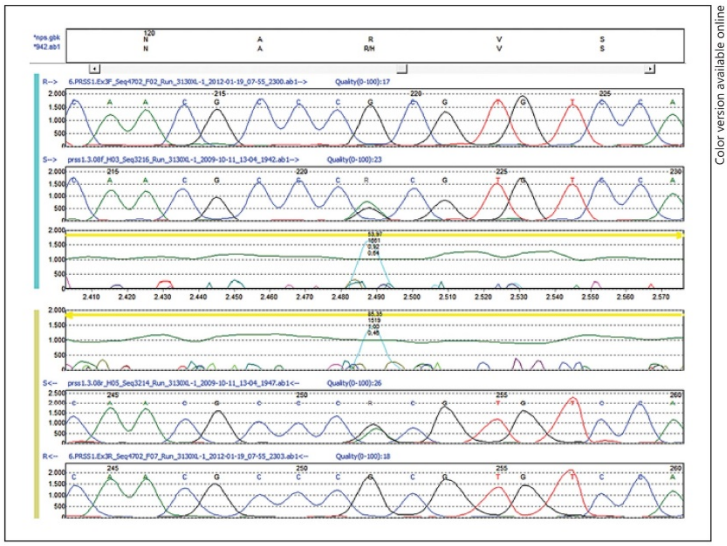

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Fig. 2. Electropherogram of the R122H mutation of the *PRSS1* gene (Sanger sequencing and analysis by Mutation Surveyor 4.0 software). The protein structure from codon 120 to 124 is given in line 1. The forward and reverse sequences are shown in lines 2–4 and 5–7, respectively. Lines 2 and 7 illustrate results from control DNA. Lines 3 and 6 illustrate results from patient DNA. Lines 4–5 illustrate the difference of the sequencing raw data between control and patient.

Age- and Sex-Matched Group

Clinical features of the 68 patients with recurrent or chronic pancreatitis and no detected mutation, matched for age and sex with the case group, are also shown in table 2. Follow-up extended to a median of 4.5 years (range 1–27). Clinical characteristics were similar between case and control groups except for the age of onset of pancreatic symptoms. No statistically significant difference was observed for between-group comparisons of age at genetic testing, number of hospitalizations or

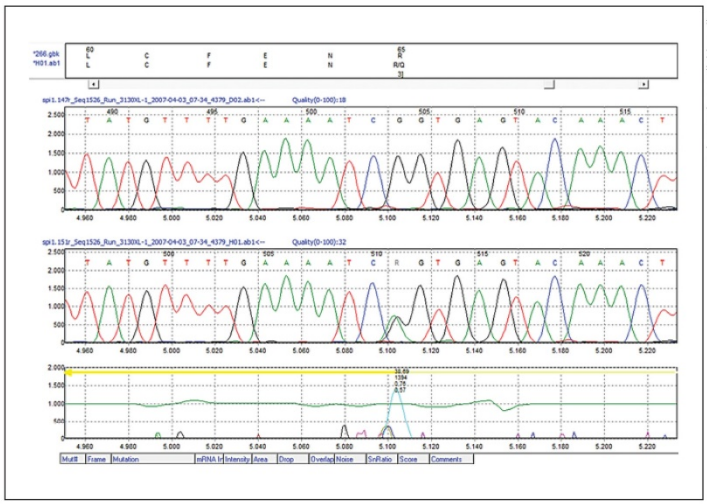

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Fig. 3. Electropherogram of the R65P mutation of the *SPINK1* gene (Sanger sequencing and analysis by Mutation Surveyor 4.0 software). The protein structure from codon 60 to 65 is given in line 1. The forward sequences are shown in lines 2–3. Line 2 illustrates results from control DNA. Line 3 illustrates results from patient DNA. Line 4 illustrates the difference of the sequencing raw data between control and patient.

endocrine and exocrine insufficiency. All patients of the control group suffered from pancreatic pain, and pancreas disease was responsible for hospitalizations in most of the patients (94%). Among patients with diabetes mellitus, 62.5% required insulin and 37.5% oral drugs only.

Morphological Characteristics

Morphologic features are summarized in table 3. No statistically significant differences in morphological features, complications and the M-ANNHEIM severity index could be detected between case and control groups. However, a trend for earlier development of calcifications ($p = 0.06$) and pancreatic cysts ($p = 0.07$) was observed in the patients of the case group carrying a *PRSS1* mutation. The complication-free survival is given in figure 4.

Treatments

Endoscopic treatment, including procedures such as endoscopic sphincterotomy and cystogastrostomy, lithiasis extraction and pancreatic duct stenting, was performed in 49.6% of the whole group (cases and controls). Surgical treatment was conducted in 9 patients (14.7%) carrying a genetic mutation and in only 3 patients (4.4%) from the control group. Pancreatic surgery included pancreaticoduodenectomy (n = 3), splenopancreatectomy (n = 4), pancreatic derivation (n = 6) and surgical cystogastrostomy (n = 1). Endoscopic retrograde cholangiopancreatography brushings and endoscopic ultrasound-guided fine needle aspiration or surgical specimens were examined by cytology or histology. Details of treatments are summarized in table 4.

Pancreatic Cancer

Five patients from the case group developed pancreatic adenocarcinoma; *CFTR* mutations (L997F, G542X, G542X/L997F, L997F/D1312G) were observed in 4 patients (3 male, 1 female), and a *PRSS1* mutation was detected in a patient who had pancreaticoduodenectomy performed for suspicion of intraductal papillary mucinous neoplasm and the presence of high-grade pancreatic ductal dysplasia in the main pancreatic duct. All pancreatic cancers were histologically confirmed. The median age at diagnosis of cancer was 54 years (range 31–67). None of the matched patients developed cancer during the follow-up. The occurrence of pancreatic cancer was statistically different between the two groups ($p = 0.02$) and between patients carrying *CFTR* mutations and patients carrying no genetic mutation ($p = 0.01$). The SIR, the ratio of observed to expected pancreatic cancers, was calculated as 26.5 (95% confidence interval 8.6–61.9). The cancer-free survival is depicted in figure 5.

Table 1. Genetic characteristics of 61 patients with idiopathic pancreatitis

Legacy name	cDNA name[a]	Protein name[a]	n	Patient No./gender
CFTR variants (NM_000492.3; chromosome 7q31.2)				
DF508/R117H-7T	c.[1521_1523delCTT(;)350G>A]	p.[Phe508del(;)Arg117His]	1	26F
G542X/*L997F*[b]	c.[1624G>T(;)2991G>C]	p.[Gly542X(;)Leu997Phe]	1	2M
L997F/D1312G	c.[2991G>C(;)3935A>G]	p.[Leu997Phe(;)Asp1312Gly]	1	1M
R75Q/L997F	c.[224G>A(;)2991G>C]	p.[Arg75Gln(;)Leu997Phe]	1	14F
G542X/*L188P*[b, c]	c.[1624G>T(;)563T>C]	p.[Gly542X(;)Leu188Pro]	1	21M
L206W/*S1235R*[b]	c.[617T>G(;)3705T>G]	p.[Leu206Trp(;)Ser1235Arg]	1	32M
N1303K/*2789+5G>A*[b]	c.[3909C>G(;)2657+5G>A]	p.[Asn1303Lys(;)?]	1	4F
DF508/WT	c.[1521_1523delCTT];[=]	p.[Phe508del];[=]	11	6F, 7M, 10M, 11F, 17F, 18M, 24M, 25F, 27M, 28M, 30M
R117H-7T/WT	c.[350G>A];[=]	p.[Arg117His];[=]	5	5M, 12M, 13M, 23F, 34M
L997F/WT	c.[2991G>C];[=]	p.[Leu997Phe];[=]	2	19F, 22M
G542X/WT	c.[1624G>T];[=]	p.[Gly542X];[=]	2	3M, 9M
3659delC/WT	c.[3528delC];[=]	p.[Lys1177SerfsX15];[=]	1	8F
A1009T/WT	c.[3025G>A];[=]	p.[Ala1009Thr];[=]	1	31F
TG12-5T/WT	c.1210-35TG[12]+c.1210-12T[5]	?	5	15F, 16M, 20F, 29M, 33F
PRSS1 variants (NM_002769.4; chromosome 7q34)				
R122H/WT	c.[365G>A];[=]	p.[Arg122His];[=]	5	36F, 39F, 41F, 42F, 45M
E79K/WT	c.[235G>A];[=]	p.[Glu79Lys];[=]	4	38F, 44F, 43M, 37F
A16V/WT	c.[47C>T];[=]	p.[Ala16Val];[=]	1	40F
SPINK1 variants (NM_003122.3; chromosome 5q32)				
N34S/N34S	c.[101A>G];[(101A>G)]	p.[Asn34Ser];[Asn34Ser]	1	59M
N34S/WT	c.[101A>G];[=]	p.[Asn34Ser];[=]	11	48F, 49M, 51F, 52F, 53F, 55M, 56M, 57F, 58F, 60F, 61F
R65P/WT	c.[194G>A];[=]	p.[Arg65Pro];[=]	1	46M
Combined variants				
PRSS1 D100H/WT; SPINK1 P55S/WT	[PRSS1:c.298G>C]; [SPINK1:c.163C>T]	[PRSS1:p.Asp100His]; [SPINK1:p.Pro55Ser]	1	35M
SPINK1 N34S/WT; CFTR DF508/WT	[SPINK1:c.101A>G]; [CFTR: c.1521_1523delCTT]	[SPINK1:p.Asn34Ser]; [CFTR:p.Phe508del]	1	47M
SPINK1 N34S/WT; CFTR L997F/WT	[SPINK1:c.101A>G]; [CFTR: c.2991G>C]	[SPINK1:p.Asn34Ser]; [CFTR:p.Leu997Phe]	1	54M
SPINK1 403+2T>C/WT; CFTR TG12-5T/WT	[SPINK1:c.194+2T>C]; [CFTR:c.1210-35TG[12]+c.1210-12T[5]]	?	1	50M

[a] According to the Human Genome Variation Society nomenclature (http://www.hgvs.org/mutnomen/). [b] Mutation detected by *CFTR* sequencing. [c] Newly identified mutation submitted to the CFTR1 database by Pepermans et al. (http://www.genet.sickkids.on.ca/MutationDetailPage.external?sp=2046).

Discussion

Our study compared clinical and morphological characteristics, including complications and endoscopic and surgical treatments, in a case group of patients with sporadic genetically determined pancreatitis with those of an age- and sex-matched group of patients with idiopathic recurrent or chronic pancreatitis but with no mutation detected. Clinical characteristics were similar between case and matched patient groups except for the age of onset of pancreatic symptoms and the higher occurrence of pancreatic cancer in the case group. Very few studies have

Table 2. Clinical features

Characteristic	Control group (n = 68)	Case group (n = 61)	*CFTR*	*PRSS1*	*SPINK1*
Male, %	51.5	52.4	61.7	27.3	50
Age at first symptoms, years	37 (1–73)*	29 (3–70)*	36 (3–67)	16 (7–49)	27 (7–70)
Age at genetic testing, years	41 (14–76)	38 (3–83)	43 (3–77)	28 (10–51)	33 (11–83)
Follow-up, years	4.5 (1–27)	5 (1–40)	8	8	3
Acute pancreatitis bouts, n	3 (1–16)	4 (1–20)	4 (1–20)	5 (2–15)	4 (1–8)
Hospitalizations, n	2 (0–13)	3 (0–15)	3 (0–7)	3 (2–15)	2 (0–15)
Length of hospital stay, days	15 (0–133)	15 (0–107)	15 (0–107)	21 (6–87)	8 (0–45)
Smokers, %	35.3	31.1	38.2	18.2	25
Diabetes, %	11.8	16.4	14.7	18.2	25
Age at diagnosis, years	40 (16–65)	42 (21–67)	44 (44–67)	30 (23–38)	37 (21–57)
Exocrine insufficiency, %	29.4	26.2	26.5	9.1	37.5
Age at diagnosis, years	46 (5–65)	38 (4–75)	40 (4–67)	31	38 (28–75)

Values represent medians with range in parentheses, except where indicated otherwise. * p = 0.01.

Table 3. Morphological features

Characteristic	Control group (n = 68)	Case group (n = 61)	*CFTR*	*PRSS1*	*SPINK1*
Pancreatic calcifications, %	33.8	42.7	35.3	63.6	43.7
Age at calcifications, years	50 (15–65)	39 (10–77)	49 (17–77)	23 (10–48)	41 (30–71)
Pancreatic cysts, %	29.4	22.9	26.5	27.3	12.5
Age at diagnosis, years	39 (4–74)	41 (20–82)	47 (21–66)	20 (20–27)	65 (48–82)
Cancer, n	0	5*	4*	1	0
Age at cancer onset, years	–	54 (31–67)	54 (44–67)	31	–
Complications, %					
Ascites	19.1	8.2	11.7	9.1	0
Bile duct stricture	5.9	0	0	0	0
Splenic vein thrombosis	2.9	3.3	2.9	0	6.25
SMV thrombosis	1.5	0	0	0	0
Portal vein thrombosis	0	3.3	5.9	0	0
Duodenal stenosis	0	0	0	0	0
Fistula	2.9	0	0	0	0
Pancreas divisum	11.8	9.8	5.9	18.2	12.5
M-ANNHEIM score, %					
Minor	39.7	30	38.2	9.1	25
Increased	42.6	36.7	32.3	64	31.2
Advanced	13.2	20	8.8	9.1	43.7
Marked	2.9	6.7	8.8	9.1	0
Exacerbated	1.5	6.7	8.8	9.1	0

Values represent medians with range in parentheses, except where indicated otherwise. * p < 0.01. SMV = Superior mesenteric vein.

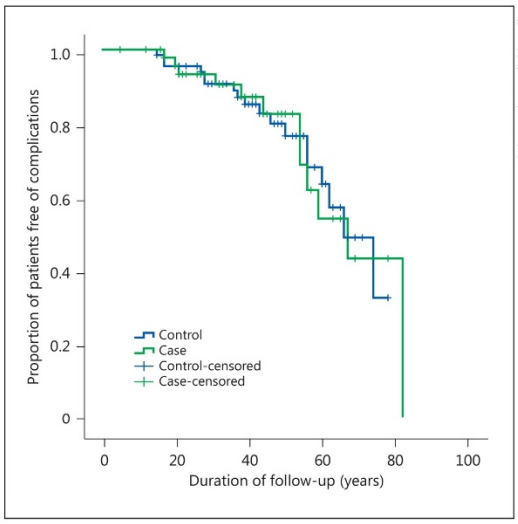

Fig. 4. Kaplan-Meier curves representing complication-free survival. Control subjects are shown in blue and cases in green. The vertical marks on the curve represent censored individuals.

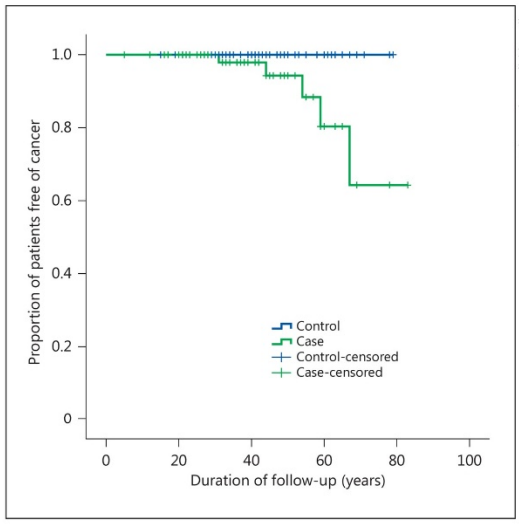

Fig. 5. Kaplan-Meier curves representing cancer-free survival. Control subjects are shown in blue and cases in green. The vertical marks on the curve represent censored individuals.

Table 4. Treatments

Treatment	Control group (n = 68)	Case group (n = 61)	*CFTR*	*PRSS1*	*SPINK1*
Endoscopic treatment, %	50	49.2	38.2	63.6	62.5
Lithiasis extraction, %	19.1	27.8	20.6	27.3	43.75
Stent, %	41.2	37.7	32.3	5.4	37.5
Cystogastrostomy, %	4.41	0	0	0	0
Age[a], years	41 (5–76)	35 (8–77)	49 (17–77)	28 (8–39)	34 (8–71)
Surgical treatment, %	4.4	14. 7	11.7	27.3	12.5
Age[a], years	63 (37–64)	32 (21–67)	49 (21–67)	31 (21–44)	31 (30–32)

[a] At time of treatment (median and range).

compared the clinical characteristics between true idiopathic pancreatitis and genetically determined pancreatitis [30]. Joergensen et al. [30] reported that patients with hereditary pancreatitis had a significantly earlier median age at presentation and a significantly higher rate of endocrine and exocrine insufficiency. This might be explained by their inclusion criterion of age less than 30 years (younger than our case and control population). Duration of follow-up was not mentioned in this series [30], and curiously, a low rate of endocrine (12%) and exocrine (9%) insufficiency in the non-genetically determined idiopathic pancreatitis group was reported. We report rates of endocrine and exocrine insufficiency of 16.4 and 26.2%, respectively, in the case group and 11.8 and 29.4%, respectively, in the control group. The median age at diagnosis of endocrine and exocrine insufficiency was higher than 30 years, with follow-up extending to 27 and 40 years in control and case groups, respectively.

The most relevant finding in our study is the clear-cut link between the risk of development of pancreatic cancer and the genetic background. Indeed, even though the clinical characteristics showed minor differences between groups, pancreatic cancers seems to be predominantly associated with mutations in the *CFTR* gene rather than in *PRSS1,* a gene more typically related to an increased risk for pancreatic cancer [31, 35]. Even though it seemed to be lower than that reported in other series, the increased incidence ratio of pancreatic cancers we observed in our study is in agreement with that reported by other groups. Indeed, a SIR of 67, which is more than twice as high as that observed in our work, has been reported in patients with PRSS1-associated hereditary pancreatitis [31], and a SIR of 53 has been recorded by the International Hereditary Pancreatitis Study Group [35]. The differences in the risk of cancer between our PRSS1 and CFTR cohorts seemed to be mainly related to smoking habits, male sex (more frequent in the CFTR group) and their older age during follow-up. Smoking and diabetes mellitus were shown to be the main associated risk factors [29, 37]. In our case group, patients who developed pancreatic cancer were smokers, and the mean age of developing cancer was 54 years. Smoking may indeed lower the age of onset of this aggressive cancer by approximately 2 decades, as previously reported [38]. However, the length of follow-up was similar between the 2 cohorts. It might be expected that with a longer follow-up, individuals with PRSS1 variants at an older age may also be at risk for cancer, especially male smokers [39, 40].

Whether germ-line mutations in the CFTR gene may lead to pancreatic adenocarcinoma is still discussed [41]. Carrying a disease-associated mutation in the *CFTR* gene has been recently linked to a modest increased risk for developing pancreatic cancer [42]. Another report [43] does not support the view that *CFTR* mutations and the 5T allele conferred a higher risk of pancreatic cancer, although a review [44] has concluded a modest but consistent increased risk of pancreatic cancer in CF patients. Recent studies proposed mechanisms to link CFTR mutations to cancer. It is postulated that cells lacking functioning CFTR display defective control of apoptosis by dysregulation of the cell glutathione concentration [36]. It has also been suggested that wild-type CFTR suppresses nuclear factor-κB-driven inflammatory signaling. Recent evidence indicates that nuclear factor-κB and the signalling pathways that are involved in its activation are also important for tumour development [45]. Finally, a recent study has demonstrated a previously undefined tumour-suppressing role of CFTR and its involvement in regulation of miR-193b in prostate cancer development [46].

CFTR channels control the bulk of pancreatic fluid secretion in pancreatic ducts. Dysfunction of these channels limits the ability of the pancreas to quickly flush digestive enzymes out of the pancreas, especially after trypsin activation, when the risk of autodigestion and pancreatitis is high. Mutations in the CFTR gene lead to recurrent acute or chronic pancreatitis. Inflammation increases the risk of neoplastic transformation. Indeed, many factors associated with chronic inflammation appear to increase genomic damage and cellular proliferation by causing the loss of tumour suppressor genes such as CDKN2A or TP53, and by stimulating oncogene expression such as KRAS2 [41, 47]. Moreover, patients with CFTR mutations and exocrine pancreatic insufficiency may develop deficiencies of antioxidants such as selenium and vitamin E, which may offer some protection from cancer [48].

Fewer mutations in the *CFTR, PRSS1* and *SPINK1* genes were detected in our series as compared with previous reports. The gene mutation detection rate we observed in idiopathic pancreatitis in the present study is lower than that recorded by others. Comparison with studies also focusing on the same 3 selected genes [3, 18, 33] is unfortunately difficult. In a Danish study [30], the contribution of mutations of the same 3 genes was searched for in a large cohort of 5,000 patients, with 122 patients suffering from idiopathic acute and chronic pancreatitis. Two main differences can be highlighted between this [30] and our report, as follows: they had an inclusion criterion concerning the age of patients which limited patients to those younger than 30 years old, while in our study there was no restriction in this regard, and hereditary cases were not excluded in the Danish series while they were in ours. A French report [31] based on a National register of hereditary pancreatitis only focused on *PRSS1* gene mutations. The fact that in the present study we selectively targeted sporadic cases of recurrent or chronic pancreatitis and excluded patients with a history of familial or hereditary pancreatitis may at least partly explain the lower rate of gene mutations. To the best of our knowledge, this is the first comparative study recording clinical and morphological characteristics in a large monocentric cohort of patients with sporadic 'genetic' pancreatitis and an age- and sex-matched group. Our rate of idiopathic pancreatitis (20.3%), which may be considered an internal control of the validity of recruitment and diagnosis, is similar to rates reported in the literature [49, 50].

Limitations of our work are the retrospective study design and the inherent risk of underestimation of the complication rate, the fact that familial history may have been incomplete without full genetic testing of first-degree relatives and the fact that more recently discovered disease-causing mutations were not studied, such as chymotrypsinogen mutations or other gene combinations [51–53]. However, all patients and referring physicians were personally contacted so that follow-up was globally extended, in both case and control groups, to more than 27 years. A complete familial tree, including at least 2 generations, was created for each patient.

In conclusion, our study is the first large comparison of genetic, clinical and morphological characteristics of patients with non-hereditary 'sporadic' idiopathic pancreatitis. We showed that patients with sporadic idiopathic pancreatitis with gene mutations *(CFTR, PRSS1* and *SPINK1)* had similar features as compared with age- and sex-matched patients without gene mutations, except for the age of onset of their pancreatic disease. We observed a significantly greater occurrence of pancreatic cancer between the case and age- and sex-matched groups and between patients carrying *CFTR* mutations and patients without mutations. We therefore suggest to include patients with CFTR variants presenting with risk factors in a screening and surveillance programme and to strongly advise them to stop smoking.

Disclosure Statement

None.

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Link between CFTR mutations and pancreatic cancer

Chronic inflammation has long been recognised as an important underlying condition for tumor development, accounting for approximately 20 % of human cancers (1). Despite years of extensive research, the mechanisms linking chronic inflammation to cancer development still look very complex and remain largely unresolved.

1. TNFα and NFκB, major actors in inflammation

The NFκB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) family of transcription factors plays an essential role in inflammation and innate immunity. They are strongly expressed in inflammation in response to stimulation by TNFα (Tumor Necrosis Factor α), in particular in CF (2). NFκB is considered responsible for increased expression of pro-inflammatory cytokines (such as IL-8, chemoattractants for neutrophils,...) (3). It has essential roles in the complex flux of information from transcription to regulation of RNA function and turnover, and in protein synthesis, functions and degradation (4).

2. CFTR deficiency and increased expression of NFκB

CFTR dysfunction affects innate immune pathways, generating an imbalance in the inflammatory status in epithelia in favour of pro-inflammatory markers.

Normal CFTR has been shown to stabilise several membrane receptor proteins involved in the inflammation signalling pathways. It regulates TLR4- (Toll-Like Receptor 4, the membrane receptor of LPS, *e.g.* from *Pseudomonas aeruginosa*) mediated responses in secretory epithelia, by controlling the activation of Src (Rous Sarcoma virus oncogene Cellular homolog) tyrosine kinase. When CFTR is defective, the negative regulation of Src is lost and tyrosine kinase is free to target TLR4/NFκB and increase its response to endotoxins (5). It has

also been demonstrated that the dF508 *CFTR* mutation impairs osteoblast differentiation and function as a result of overactive NFκB and Wnt/β-catenin signalling (6-7).

These observations support the view that *CFTR* acts as a tumor suppressor gene. RNA sequencing analyses of CFTR-deficient intestinal tumors confirmed an altered proinflammatory gene expression profile in normal CFTR-deficient tissues as well as in CFTR-deficient tumors (8). In the latter, CFTR deficiency results in upregulation of numerous proinflammatory chemokines, cytokines and their receptors, and members of the NFκB signalling pathway.

3. From NFκB increased activity to development of cancer

It has been suggested that NFκB, a hallmark of inflammatory responses that is frequently detected in tumors, may constitute a link between inflammation and cancer (9). Evidence is accumulating in favour of NFκB acting through its control of the apotosis/autophagy balance. Apoptosis is the broadly recognised mechanism of programmed cell death. Autophagy is a lysosomal process in which cytoplasmic constituents such as proteins, lipids, and organelles are degraded (10). It is activated in many situations of cell stress. It is a pivotal regulator of several important physiological processes including development, cell survival, differentiation and senescence. Tight regulation of both the NFκB pathway and that of the autophagy process is essential to homeostasis. Deregulation of both of these is frequently observed in cancer cells and is associated with tumorigenesis and tumor cell resistance to cancer therapies (11).

In the case of pancreas cancer, high-throughput technologies and accurate disease models now provide a comprehensive picture of the diverse molecular signalling pathways and cellular processes governing adenocarcinoma genesis. Central among these is oncogene KRAS, a mediator of cellular plasticity, metabolic reprogramming, and inflammatory and paracrine signalling required for tumor development and maintenance. Autophagy is proposed to be one

of the cellular mechanisms contributing to pancreatic carcinogenesis, particularly during initial stages in which the KRAS oncogene appears to play a key role. Pancreatic autophagy, induced during pancreatitis by the overexpression of VMP1 (Vacuole Membrane Protein 1), promotes the development of precancerous lesions when induced by the mutated KRAS (12). In addition, the treatment with chloroquine, an inhibitor of autophagic flux, reverses the effects of VMP1 in pancreatic cancer induced by the KRAS oncogene (13).

In summary, the link between CF, chronic pancreatitis and pancreas adenocarcinoma is complex and still largely unresolved. It involves several pathways and factors that can possibly balance each other. Better understanding of interactions between the CFTR protein, other membrane proteins such as receptors, and cytoplasmic transduction cascades could help orient future investigations. The structure of CFTR itself is a challenge for further research and the way to light is full of pitfalls.

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ABPA in adulthood: a CFTR-related disorder

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In 2009, Prof. P. Lebecque, then head of the clinical CF Centre at St Luc Hospital, revisited an earlier publication on frequencies of *CFTR* mutations in patients with Allergic Bronchopulmonary Aspergillosis (ABPA) (Chest; Marchand et al. 2001); this paper was based on a molecular method using a panel of 13 common *CFTR* mutations. At the time, the hypothesis was spreading in the scientific community of the possible existence of other, still undetected, *CFTR* mutations responsible for the ABPA phenotype. Accordingly, Prof. P Lebecque asked the department of Genetics to update the analysis, and I performed an up-to-date complete *CFTR* screening in the same cohort of patients in order to obtain more accurate insight on the link between CF and ABPA. Therefore, the work has been designed to address the fourth objective of the thesis project, *i.e.*, to better characterise the nature of CFTR mutations, by sequence analysis, in the same group of patients from the princeps paper. The results of the new study are described in the adjacent paper.

CORRESPONDENCE

ABPA in adulthood: a CFTR-related disorder

In Western countries, allergic bronchopulmonary aspergillosis (ABPA) in childhood is very unusual beyond the context of cystic fibrosis (CF). It is presumed to be different in adulthood, although three studies[1–3] reported an increased frequency of cystic fibrosis transmembrane conductance regulator (CFTR) mutations in adults with ABPA. Out of the 63 patients investigated in these studies, none was reported as pancreatic insufficient, all had sweat chloride values <60 mmol/l, only two carried the intron 8 splice variant 5T and a single patient was found to be compound heterozygous for two CFTR mutations. However, Miller *et al* studied only 10 patients and did not sequence all CFTR exons, while the two other reports, including one from our institution, were hampered by the small number of mutations initially looked for (n=13 and 16, respectively). Accordingly, current guidelines for diagnosis of CF do not list ABPA as a suggestive phenotype feature nor even explicitly as a CFTR-related disorder.[4]

We hypothesised that extending DNA analysis to the >1300 mutations currently considered as potential CF causing (http://www.genet.sickkids.on.ca/cftr/app) would provide more accurate insights on the link between ABPA and CFTR in adulthood.

The characteristics of the study group are detailed in the princeps paper.[2] DNA samples were no longer available for 3 out of the 21 original patient cohort, one of whom had

Table 1 Relevant data and genetic findings in 18 adult patients with allergic bronchopulmonary aspergillosis (ABPA)

Subject no.	Age (years)*	Gender	Sweat (Cl^-) (mmol/l)	BC	CFTR mutations	
					Princeps study[2] †	Current study
1	30	M	17	+	F508del	F508del/D1152H
6	30	M	32	+		L997F/L997F
7	74	M	34	–		G576A/D443Y–R668C
8	55	M	36	+		R1070W/3659delC
2	65	M	33	+	F508del	F508del
3	64	M	6	+	G542X	G542X
4	58	F	8	+	R117H	R117H
5	63	F	NA	+	1717-1G→A	1717-1G→A
9	59	F	14	+		R75Q
10	47	F	19	+		L967S
11	68	F	21	+		I177F
12	67	F	34	–		V1153E
13	46	F	NA	+		
14	60	F	37	+		
15	84	M	23	+		
16	79	F	40	+		
17	63	M	15	+		
18	58	M	13	–		

*In 1997.
†Investigated set of mutations: F508 del, G542X N1303K, R117H, 621+1G→T, R334W, ΔI507, W1282X, R553X, R1162X, 1717–1G→A, G551D, 3849+10kbC→T; BC: bronchiectasis (high-resolution CT scan).
CFTR, cystic fibrosis transmembrane conductance regulator; F, female; M, male; NA, declined sweat testing.

been found to carry the R1162X mutation. Sequencing of all 27 CFTR exons, including flanking intronic regions, and a search for large rearrangements were undertaken in the remaining 18 DNA samples. Calling all the patients back for further familial genetic studies could not be considered and we assumed that two identified mutations are located in trans. ORs were calculated and proportions were compared with prior probability using the likelihood ratio test and assuming an expected carrier rate of 1/25 (4%) in the Belgian general population.

Mean age (±SD) at the time of DNA sampling was 58.9 (±14.2) years. Bronchiectasis were present in 14/18 patients. Sweat chloride values between 40–59 mmol/l and 30–39 mmol/l were observed in 1 (5.5%) and 6 patients (33%), respectively. A total of 18 putative mutations were identified in 17/36 alleles (table 1), most of which were mild/uncommon.

CFTR mutation carrier frequency was much higher in patients with ABPA (12/18, 67%) than expected in the general population ($p < 0.0001$; OR 48.0, 95% CI 5.2 to 445.3). The probability of bearing two CFTR mutations was even more strikingly different ($p<0.0001$; OR 714, 95% CI 75 to 6797).

This study considerably extends previous findings by demonstrating a strong link between ABPA in adults and CFTR mutations. Although not altering the message, limitations of this work include the small population size which is inherent to the rarity of ABPA, the absence of DNA testing in parents and the dilemma of the clinical relevance of putative CFTR mutations. The hitherto best studied CFTR-related disorders are congenital bilateral absence of the vas deferens (CBAVD) and idiopathic chronic pancreatitis (ICP). It has been estimated that 85% of patients with CBAVD and 30% of those with ICP carry at least one CFTR mutation while ~50% and 10–15%, respectively, are compound heterozygous, with the F508del mutation and IVS8-T5 variant being most frequently detected.[5] The present study supports the concept that ABPA in pancreatic-sufficient adults is a CFTR-related disorder, with rare class IV–V mutations being mostly found and IVS8-T5 not seeming to play a significant role. Moreover, as ABPA is usually associated with bronchiectasis, a major phenotypic feature of CF, appropriate investigations to exclude milder forms of CF are warranted in these patients.

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Patient consent Obtained.

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CONCLUSION

Conclusion

This work contributes in several ways to a deeper understanding of the involvement of the *CFTR* gene in patients with CF and CF-related disorders and it sheds new light on the molecular diagnosis.

A new diagnostic screening method, xTAG (Luminex; panel of 71 mutations), was validated for routine analysis compared to the INNO-LiPA® (Innogenetics; panel of 36 mutations), used as a reference. The robustness of the method was established by using DNA samples extracted from different biological matrices, including blood samples, blood spots from Guthrie cards, chorionic villi and amniotic fluid. In this way, reproducible and concording results were obtained on the Luminex platform from all DNA Innogenetics positive samples. The new method, which uses a large panel of 71 mutations more specially designed for patients of southern European origin, significantly increases the detection rate. Since 2009, the method has been routinely used in first-line molecular diagnosis in the Human Genetics Centre of the Cliniques universitaires St-Luc.

A contribution to a better molecular diagnosis of CF was achieved in the Santa Fe province of central Argentina, which has never been studied before. In this province, studying mutations in the *CFTR* gene allowed us to answer two questions. First, in a country largely populated by immigrates from mediterranean origin, the nature and distribution of mutations largely reproduces that of the countries of origin. Second, specific mutations never observed before, c.2554dupT (p.Tyr852Leufs*44) and c.146T>C (p.Leu49Pro), were discovered. We raise the hypothesis that they may be of aborigenic origin. As a result of the study, a new panel of 21 mutations, better adapted locally, was proposed to replace the European panel presently used for the routine first line molecular diagnosis of classical CF in children.

Our work also contributed to a better understanding of the correlation between phenotype and genotype with a retrospective study of the effects of mutations in the *PRSS1*, *CFTR* or *SPINK1* genes on the severity of sporadic idiopathic pancreatitis. The results showed that clinical and morphological parameters were similar in patients carrying mutations in one of the three genes as in non-carriers, except for the age of pancreatic disease onset. However, a significantly higher occurrence of pancreas cancer was observed in the mutated group, particularly in patients carrying mutations in the *CFTR* gene. We therefore suggest that some *CFTR* variants present a risk factor for pancreatic cancer.

A better understanding of the role of the *CFTR* gene in a specific pathology was provided by updating the molecular analysis of the *CFTR* gene in patients with allergic bronchopulmonary aspergillosis syndrome (ABPA). To this end, samples from patients analysed in 2001 using a panel of 13 mutations were re-analysed in 2010 using complete exonic sequencing and large rearrangement screening. Compared to the first analysis, 8 cases were found carrying one *CFTR* mutation and 4 with two mutations. Thus, the study considerably extends previous findings by demonstrating a strong link between ABPA in adults and *CFTR* mutations and further enriches our knowledge of the phenotype-genotype relationship.

PERSPECTIVES

The molecular diagnosis of CF from past to future

Perspectives: The molecular diagnosis of CF from past to future

The story of the molecular diagnosis of CF begins in 1989

The discovery of the *CFTR* gene and of the p.Phe508del mutation took place in 1989. It created the molecular diagnosis of CF as a new tool for clinicians. At the time, it was initially accepted that the molecular diagnosis of CF was completely understood, with a single gene, a single protein and a single mutation, p.Phe508del, and the task of the molecular geneticist seemed simple. The evolution over the next two decades was by no means anticipated. I plan to give here an overview of the evolution of the molecular genetics, in parallel with the difficulties of the molecular diagnosis of the *CFTR* gene (patients with CF and CF-related disorders).

In the early 90's, the molecular diagnosis of the principal mutation, p.Phe508del, used a simple PCR with high resolution electrophoresis to detect deletion of the three base pairs. At the same time, a few additional mutations were identified using restriction enzymes. At the time, the best available technique was the Sanger sequencing method, which was very expensive and labour intensive (at least two weeks for only 200bp of length), and as all radioisotope-based methods, it used hazardous radioactive reagents. The development of the fluorescence method replaced the use of radioactive reagents, simultaneously reducing the technician time and allowing sequencing 500bp segments. A major advantage of the new method was the possibility to analyse an entire exon and to discriminate several mutations of different types, such as the p.Ile507del mutation which gave the same delta of 3bp as the p.Phe508del in the electrophoresis. Another advantage was that the method avoided uncertainties associated to the use of restriction enzymes, for example sufficient enzyme activity to discriminate mutated homozygous from heterozygous.

Ten years later

At the end of the 90's, a large number of mutations had been discovered in the *CFTR* gene. The most frequent one, p.Phe508del, was found in 40 to 95% of CF alleles in different European populations, and in the second position came a small group of mutations with frequencies between 1 and 5% of CF alleles. A third large group included rare local mutations, with frequencies less than 1%. A strategy of molecular diagnosis was then adopted (see, for example, European last best practice) (1), in which a panel of know mutations was defined; initially, it contained 13 mutations present regionally in high proportions and it was progressively extended to 71 mutations. The first paper presented above shows an application of this approach. An example of regional diversity in a given country is the distribution in France (2). Some regions, mainly outside Europe, have not been studied yet, and their local distributions are unknown. In my second paper above, I present the results of my study of distribution of mutations in the Santa Fe province of central Argentina, which had never been studied. In the study, I identified a small group of specific mutations present in high proportions, justifying development of a local panel of mutations.

Despite the discovery of more local mutations, the task of the molecular geneticist, which seemed initially simple, became substantially more complicated and a source of frustration due to the impossible task of satisfying the demands of clinicians ("Please, find two mutations!"). As a matter of facts, identifying one particular mutation in a patient required heavy logistics and time, making it practically impossible to implement.

In the early 2000's

At the beginning of the years 2000, the cost of reagents decreased drastically, allowing automation of the Sanger sequencing method with a much higher throughput in diagnostic laboratories. It became possible to sequence the complete coding DNA sequence (CDS) of the *CFTR* gene nowadays known as the second line of genetic diagnosis. In those days, for practical reasons, CF centers chose to give priority to the analysis of unsolved classical CF patients, and as a result, a large proportion of patients carrying two mutations was found.

At about the same time, following the detection of copy number variations (CNV), the idea appeared in laboratories of molecular genetics that Sanger sequencing alone was not enough, and the need arose for a parallel study to detect patients with two complete pathogenic alleles of the *CFTR* gene. The introduction of the MLPA method (3), easy to apply in routine practice, induced large hopes to help patients with CF phenotype carrying no, or only one mutation. Unfortunately, the number of mutations with large rearrangements in the *CFTR* gene represented only 1-1.5% of patients (4).

Despite the major technical advances, the problem remained of selecting the part of the gene to be sequenced due to the ever increasing number of mutations. As a consequence, the frustration of the molecular geneticist grew because no clear end of the analysis procedure could be defined.

Around 2010

In the years 2010, it was decided to explore other genes in cases of patients with a CF phenotype in whom no *CFTR* mutation had been found. One example of such genes is that of

the epithelial Na^+ channel (ENaC) family (5). Despite a strong hope to find mutations, the number of unresolved cases remained very high (6).

The most spectacular breakthrough came after 2008 with the development of the next generation sequencing (NGS) methods. The NGS strategy applied by the genetic laboratories consists in substituting the Sanger method for the complete CDS of the *CFTR* gene, thereby enormously increasing the number of samples analysed. For example, a trained technician can now process 50-100 patients in three days, which is more than the total number of new patients of a CF center in one year. However, implementing the methods requires solving great problems. The difficulty of the diagnostic laboratory is now to demonstrate locally high sensitivity, specificity, accuracy, robustness, …. of the NGS method in order to obtain the ISO15189 certification, following the guidelines of the International Organization for Standardization. (7-10).

Despite the information gathered in exploring other genes, it remained necessary to deepen the knowledge of the *CFTR* gene, because the other genes had not given useful answers in the case of an apparently monogenic disease, and because analysis of the *CFTR* gene was unfinished, since introns had not been sequenced. This is still valid today in routine diagnosis, and the frustration of the molecular geneticist remains.

The present situation in 2016

Now, a new situation has appeared during the last years. On the one hand, highly efficient diagnostic methods are available at an affordable cost; on the other hand, ever increasing and complex mutations have been identified so that the results of the genetic analysis can no longer be simply interpreted, as will be further explained below. From an initial apparently simple situation of a monogenic disease, additional gene alleles, called modifier genes, have

been recognised which directly influence the severity of the CF phenotype (11). And as a consequence, so far, no consensus gene panel in relation with CF and CF-related disorders has been agreed at an international level.

In parallel, another method, the genome-wide associated studies (GWAS), has been used, with the aim of identifying other loci, (12) but the new loci need more meta-analysis to confirm the involvement in this disease. A better categorisation of phenotypes in a parallel extensive database of "phenomics" constitutes a new challenge.

These two factors contribute to slow down the application of the most recent methods to laboratory practice.

Another difficulty comes from the increasingly frequent request from clinical departments for molecular diagnosis in the case of patients with phenotypes very closely related to classical CF, for example idiopathic pancreatitis and ABPA, as illustrated in the third and fourth papers above. These cases require resorting more and more frequently to a second line diagnosis, which is substantially more demanding, not only technically but also in time spent in the interpretation of complex alleles. Indeed, a grey zone broadens up between a deleterious mutation and a polymorphism, making a clear molecular diagnosis and the task of the molecular geneticist nearly impossible.

However, everything is not as grey. The new very broad interconnected public human genome databases (1000genome, Exome ExAC, ncbi, ensembl, uscs, ...) give access to a new control population database at a world-wide scale. For example, when a new variant, never observed before locally, is found in an Asian patient, consulting the large database allows comparing the variant to an Asian population. If this variant is present in a proportion larger than 5% in the database, it can be considered as a common polymorphism. The limit of 5%

represents an international consensus because the most common CFTR mutation, the p.Phe508del, is present in 4% of Caucasians.

A possible way of solving the difficulties related to the *CFTR* gene, as explained above, would be to sequence by NGS the complete gene, including introns. The problem does not lie in the technology but in the shear size of the gene. As a matter of fact, in a gene of 250,000 bp, 250 variants can theoretically be expected, as it is well known that on average, one variant is present for every 1,000 bp, and the majority of them are present in the introns. More *in vitro* models are needed to reveal the influence of splicing (13,14). This, however, is not the final answer, because when a partial splicing defect is found in the *in vitro* model, the consequence in the *ex-vivo* model is not fully predictable. Furthermore, when two complex variants are found, it is not known if they are present in *cis* or in *trans* if the parents' genes have not been analyzed. If they are adjacent, their possible reciprocal influence is also not known.

A view of the future by the molecular geneticist

In summary, disappointing immense initial hopes, the molecular diagnosis has not resolved all cases, despite huge technical progresses over the last half century. It is even quite the contrary. In the context of CF, the new methods of gene analysis and the added loci have increased the detection rate by only about 1% while making the interpretation considerably more difficult. The molecular diagnosis in medicine is now, and will remain in a foreseeable future, but one diagnostic tool among others, such as IRT screening, sweat test, nasal potential difference test,… to help the CF center. From the point of view of the geneticist, in many hereditary diseases, improving the categorisation of phenotypes, in other words the selection

of cohorts, seems to be the starting point to move forward in the way toward a more accurate diagnosis.

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ANNEXE

Annexe

Impact of MIF gene promoter polymorphism on F508del cystic fibrosis patients.

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This annexe includes a contribution I co-authored during my PhD programme. As a molecular geneticist, I took part in this collaborative work designed to try to determine if a polymorphism, 5-repeat allele displaying lower promoter activity, of the Macrophage migration Inhibitory Factor (MIF) is associated with disease severity in a group of Cystic Fibrosis patients homozygous for F508del *CFTR* mutation. Patients have been selected in two CF centers in Brussels and in Verona (Italy).

PLOS | ONE

RESEARCH ARTICLE

Impact of *MIF* Gene Promoter Polymorphism on F508del Cystic Fibrosis Patients

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Abstract

Macrophage migration Inhibitory Factor (MIF) is a pro-inflammatory cytokine sustaining the acute response to gram–negative bacteria and a regulatory role for MIF in Cystic Fibrosis has been suggested by the presence of a functional, polymorphic, four-nucleotide repeat in this gene's promoter at position −794, with the 5-repeat allele displaying lower promoter activity. We aimed at assessing the association of this polymorphism with disease severity in a group of Cystic Fibrosis patients homozygous for F508del *CFTR* gene mutation. Genotype frequencies were determined in 189 Cystic Fibrosis and 134 control subjects; key clinical features of patients were recorded and compared among homozygous 5-allele patients and the other *MIF* genotypes. Patients homozygous for the 5-repeat allele of *MIF* promoter displayed a slower rate of lung function decline ($p=0.027$) at multivariate survival analysis. Multiple regression analysis on age-normalized respiratory volume showed no association of the homozygous 5-repeat genotype with lung function under stable conditions and no correlation with *P.aeruginosa* chronic colonization. Therefore, only the Homozygous 5-repeat genotype at *MIF* −794 is associated with milder disease in F508del Cystic Fibrosis patients.

Introduction

Cystic fibrosis (CF) is the most common, severe, inherited disorder in the Caucasian population. It is caused by mutations in the CF Transmembrane conductance Regulator (*CFTR*) gene and mainly characterized by bronchopulmonary disease, pancreatic insufficiency and male infertility. Patients with identical *CFTR* genotypes can display markedly different phenotypic expression [1, 2] and modifier genes were previously described among the factors causing this discrepancy [3, 4]. Macrophage Migration Inhibitory Factor (MIF) is a key pro-inflammatory mediator [5]: it sustains an acute inflammatory response both directly, by inducing cytokines secretion, and indirectly, by overriding the anti-inflammatory activity of glucocorticoids [6]. MIF plays a significant role in immune and inflammatory-based diseases such as asthma [7], rheumatoid arthritis [8], acute respiratory distress syndrome [9] and septic shock [10, 11]. Although MIF is involved in the defence against severe infection, modulation of the high cytokine levels elicited by its action may prevent harmful effects during the inflammatory response. Indeed, lethal sepsis induced in mice by lipopolysaccharide (LPS) or *E. coli* causes increased mortality in the presence of recombinant MIF [12], while anti–MIF neutralizing antibodies were able to protect mice from lethal endotoxic sepsis induced by bacterial (*E. coli*) peritonitis [11]. It has also been suggested that neutralizing MIF could lead to improved resistance against *P. aeruginosa* infection, since clearance of the bacteria following tracheal instillation was improved in MIF-knockout mice [10]. Recently, Baugh et al. [13] identified a functionally significant polymorphism in the human MIF gene, consisting of a four-nucleotide CATT repeat located at position −794 of the MIF promoter (MIF-CATT). In an *in vitro* model, the 5-CATT repeat showed significantly lower transcriptional activity when compared to the 6-, 7- or 8-CATT repeat alleles. This polymorphism is reported as a TTCA insertion or deletion relative to the 6-repeats genotype in NCBI dbSNP entries rs3063368 and rs36224313 respectively, at the genomic coordinates (UCSC genome browser – hg19) chr22:24235773-24235772. Five percent of healthy subjects are homozygous for the 5-CATT repeat allele. Homozygosity for this allele was significantly associated with milder forms of rheumatoid arthritis, suggesting it may have a protective effect. In CF patients, Plant et al. [14] reported a significant decrease in both *P. aeruginosa* colonization and pancreatic insufficiency among adult patients carrying at least one 5-CATT *MIF* allele. Since many studies of modifier genes in CF have yielded conflicting results, it is essential to validate any association in a new, independent population and *MIF* gene is no exception [4]. This study aimed at clarifying and validating the association between MIF-CATT repeats and disease severity in a more homogeneous cohort of CF patients with homozygous F508del CFTR mutation. Given the biological relationship between MIF and acute inflammation suggested by the above cited literature, we chose as a primary outcome the time to the first acute episode causing forced expiratory volume (FEV1) to fall below the 60% of the predicted value. We also verified the

possible relationship between MIF and age-normalized FEV1 and chronic *P. aeruginosa* colonization under stable conditions.

Materials and Methods

Study population

One hundred and eighty-nine CF patients homozygous for the F508del mutation were recruited from two European centres (Verona: 138, Brussels: 51). All of the patients were able to perform reliable spirometry. A cohort of 134 adult Italian subjects was used as control. Healthy subjects were negative for the most common mutations of the *CFTR* gene, except for 4 heterozygous subjects (healthy carriers). This is consistent with epidemiological data about carrier frequency in Europe. This study was approved by the Ethics Committee of the University and Hospital Trust of Verona (protocol #24737); informed signed consent for DNA analysis was obtained from participants or from their parents, as required.

Genotyping

DNA was extracted from whole blood using the salting out method, then samples were genotyped for the polymorphism of *MIF* promoter (varying number of CATT repeats) at -794. DNA was amplified by Polymerase Chain Reaction (PCR) in a GeneAmp PCRsystem 9700® (Applied Biosystem, Foster City, CA, USA) as previously described [13]. Genotyping was performed by the BMR Genomics Sequencing Service (CRIBI, University of Padova, Italy). Results were analysed using GenescanView 1.2 software (CRIBI, University of Padova, Italy).

Clinical data of CF patients

Clinical data for the 189 patients were collected in electronic databases. Main characteristics and *MIF* genotype of the patients are summarized in **table 1** and extensively reported in S1 Table. Anthropometric parameters and forced expiratory volume (FEV1) were normalized using Freeman's [15] and Knudson's [16] equations respectively. Given the accelerated FEV1 decline with age in CF patients, the last value of FEV1 was expressed as FEV1 percentile using CF-specific reference equations [17]. Diabetes was defined by the need for insulin therapy. Chronic *P. aeruginosa* colonization was reported using a European consensus definition [18]. In brief, chronic colonization was defined as the isolation of at least 3 isolates in a six month period (at minimum 30 days interval) while sporadic colonization referred to the isolation of *P. aeruginosa* in the bronchial tree in presence or absence of inflammation.

Statistical analysis and study design

Given the involvement of MIF in acute inflammation, we focused on the FEV1 parameter as primary outcome variable, comparing patients with the 5-5 MIF

Table 1. Characteristics of 189 Cystic Fibrosis patients homozygous for the F508del mutation recruited from 2 different European centers.

	Brussels	Verona	p-value
Subjects n	51	138	
Female n (%)	22 (%)	78 (%)	0.14
Age, years	21.5±9.4	24.27±9.3	0.09
FEV1, Kulich*	67±24	45±31	<0.0001
BMI z-score	−0.63±1.03	−0.82±1.28	0.36
cc by PA n (%)	16 (31.4%)	87 (63.0%)	<0.0002
Diabetes n (%)	11 (21.6%)	40 (29.0%)	0.36
MIF-CATT 5-5 n (%)	4 (7.8%)	12 (8.7%)	
MIF-CATT 5-6 n (%)	12 (23.5%)	59 (42.8%)	
MIF-CATT 5-7 n (%)	7 (13.7%)	10 (7.2%)	0.11
MIF-CATT 6-6 n (%)	20 (39.2%)	43 (31.2%)	
MIF-CATT 6-7 n (%)	8 (15.7%)	12 (8.7%)	
MIF-CATT 7-7 n (%)	0 (0%)	2 (1.4%)	

Continuous data are presented as mean ± SD unless otherwise stated; categorical data are presented as counts and proportions. FEV1: forced expiratory volume in one second; cc by PA: chronic colonization by *P. aeruginosa*. The most recent FEV1 was used for each patient. MIF-CATT: *MIF* gene -CATT repeat genotype at position -794

* CF specific percentile according to Kulich *et al*, *Am J Respir Crit Care Med*, 2005.

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genotype to the rest of the cohort. The relationship between FEV1 and MIF genotype was analysed considering both the time to the first acute episode causing a FEV1 value under 60%, and the "static" FEV1 at last visit (normalised according to Kulich's CF-specific reference equations [17]).

Considering the differences in clinical parameters between patients from the two centres (table 1), the analyses were performed using multivariate techniques. Cox regression was used to analyse the time to first FEV1 under 60% and patient's origin (Verona or Brussels) was included as a covariate together with presence of *MIF* 5-5 genotype. Chronic colonization by *P. aeruginosa* and presence of insulin-dependent diabetes were not considered because only a minority of patients displayed these features before first acute episode (28.9% for *P.aeruginosa* and 5.0% for diabetes). Given the unequal size of 5-5 and X-X groups (patients number ratio =0.09), we used the R package PowerSurvEpi to calculate the power to detect an effect like the one estimated from our data (Hazard ratio =0.32) with the number of patients available. The power resulted to be 59%, while the sample size needed to get 80% power would have been 307 patients. Multiple linear regression was used to analyse last visit FEV1, including as covariates *MIF* 5-5 genotype, patients' origin, chronic colonization by *P. aeruginosa* and presence of insulin-dependent diabetes; age was not included because FEV1 values were already age-normalized according to Kulich. Variance inflation factor was used to monitor the presence of multicollinearity; its value was below 1.15 for all covariates.

Power analysis for last visit FEV1 was also used to calculate the power to detect a medium effect size (0.5, here corresponding to a difference between means of

15%, which was similar to the estimated difference in our cohort) in an unpaired t-test with our sample size and considering the unequal size of 5-5 (n=16, mean FEV1=50.2, SD=31.3) and X-X groups (n=173, mean FEV1=64.2, SD=25.5). The power calculated was 48%, while the sample size needed to get 80% power would have been 382 patients. All analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com), MedCalc for Windows, version 14.8.1 (MedCalc Software, Ostend, Belgium) and the R software (R Development Core Team, version 2.9; R Foundation for Statistical Computing, Vienna, Austria; www.R-project.org). Power analysis for multiple linear regression was performed with G*Power [19].

Results

Genotyping of the −794 CATT polymorphic repeats of *MIF* promoter (MIF-CATT)

In 189 CF patients, frequencies for 5, 6, 7 and 8 MIF-CATT repeats were 31.7%, 57.4%, 10.9% and 0% respectively. Corresponding values for the 135 healthy control subjects were similar (28.5%, 60%, 11.1% and 0.4%). In CF patients, MIF-CATT genotype frequencies were similar among children and adults, excluding a survival bias for 5-5 subjects.

Clinical status of CF patients and MIF-CATT genotype

Patients were categorized according to the −794 CATT polymorphic repeats (MIF-CATT). Anthropometric and clinical data are summarized in **Table 1 and 2**, and extensively reported in S1 Table.

Given the involvement of MIF in acute inflammation, we focused on FEV1 as primary outcome variable, comparing patients with the 5-5 MIF-CATT genotype to the rest of the cohort.

The relationship between FEV1 and MIF genotype was analysed considering both the time to the first acute episode causing a FEV1 value under 60% and, secondly, the "static" FEV1 at last visit. Considering the differences in clinical parameters between patients from the two centres and the possible effect of diabetes and *P. aeruginosa* colonization on pulmonary volume, the following analyses were performed using multivariate techniques.

MIF-CATT 5-5 genotype is associated to a later onset of acute episodes

Cox regression was used to analyse the time to the first acute episode causing FEV1 to fall under 60% predicted. MIF-CATT 5-5 genotype and patient's origin (Verona or Brussels) were included as independent variables. Chronic colonization by *P. aeruginosa* and presence of insulin-dependent diabetes were not considered because only a minority of patients displayed these features before first acute episode (28.9% for *P.aeruginosa* and 5.0% for diabetes). Both MIF-CATT 5-

Table 2. Clinical data of 187 Cystic Fibrosis patients homozygous for the F508del mutation according to the genotype for the *MIF* gene -CATT repeat at position −794.

	5–5	5–6	5–7	6–6	6–7
n	16	71	17	63	20
Age, years (95% CI)	18.2 (14.5– 21.9)	23.6 (21.3–25.9)	27.2 (23.1– 31.3)	23.7 (21.2–26.1)	22.3 (18.2–26.5)
BMI z-score (95% CI)	−0.51 (−1.10/0.08)	−0.84 (−1.17/−0.52)	−1.09 (−1.63/−0.54)	−0.71 (−0.97/−0.46)	−0.62 (−1.26/0.01)
FEV1, Kulich* (95% CI)	64.2 (50.6–77.8)	43.9 (36.6–51.2)	52.4 (37.7–67.08)	53.8 (45.7–61.8)	59.3 (44.8–73.8)
CC by PA – n (%; 95% CI)	5 (31.2%; 13.9–55.8%)	44 (62.0%; 50.3–72.4%)	11 (64.7%; 41.2–82.8%)	33 (52.4%; 40.3–64.2%)	8 (40.0%; 21.8–61.4%)
Diabetes – n (%; 95% CI)	2 (12.5%; 2.2–37.3%)	22 (31.0%; 21.4–42.5%)	6 (35.3%; 17.2–58.8%)	17 (27.0%; 17.5–39.1)	4 (20.0%; 7.5–42.2%)

Data are presented as mean and 95% Confidence Interval (95% CI). FEV1: forced expiratory volume in one second; BMI: body mass index; cc by PA: chronic colonization by *P. aeruginosa*.
* CF specific percentile according to Kulich *et al*, *Am J Respir Crit Care Med*, 2005.

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5 genotype (Hazard Ratio=0.327; 95% CI 0.121-0.884) and belonging to the Brussels cohort (Hazard Ratio=0.514; 95% CI 0.310 to 0.849) resulted to be independent predictors of a later onset of acute episodes (Table 3).

Kaplan-Meier analysis on Fig. 1 shows that age at first acute episode with FEV1 value below 60% predicted was higher in patients with 5–5 genotype compared to all the other genotypes; median time to event was 29.3 years for 5–5 subjects compared to 18.2 for the others (Hazard ratio=0.35; 95% CI 0.19–0.66; p=0.03, Fig. 1A). Applying per-allele analysis, no relevant differences emerged, consistent with a recessive effect of 5-CATT allele (Fig. 1B).

MIF-CATT 5-5 genotype shows no association with lower pulmonary function and *P. aeruginosa* chronic colonization under stable conditions

Since recent literature reported that MIF 5-CATT allele also correlates with lower FEV1 under stable conditions and higher prevalence of *P. aeruginosa* colonization [14, 20], we applied multiple linear regression to analyse age-normalized FEV1 [17] under stable conditions, including MIF-CATT 5-5 genotype, patients' origin, chronic colonization by *P. aeruginosa* and presence of insulin-dependent diabetes as covariates. The only factor not associated with FEV1 resulted to be MIF-CATT 5-5 genotype (table 4). Variance inflation factor was used to monitor the presence of multicollinearity; its value was below 1.15 for all covariates, indicating no apparent correlation between MIF-CATT genotype and *P. aeruginosa* chronic colonization.

Table 3. Cox regression analysis for age at first acute episode with FEV1 <60% of predicted value on 185 Cystic Fibrosis patients homozygous for the F508del mutation.

Variable	Value	Hazard Ratio	95% CI	p-value
MIF-CATT genotype	X-X	1	-	-
	5–5	0.325	0.120–0.878	0.027
Centre of origin	Verona	1	-	-
	Brussels	0.510	0.309–0.843	0.0090

Number of patients with acute episode =119
Number of censored patients=66
Overall significance p-value =0.0010
MIF-CATT genotype: *MIF* gene -CATT repeat genotype at position −794
95% CI =95% Confidence Interval

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Discussion

In this study, 8% of 189 CF patients homozygous for the F508del *CFTR* mutation carried the 5-5 allele combination for a functional CATT repeat polymorphism in the MIF gene promoter (MIF-CATT), which is expected to be associated with decreased pro-inflammatory activity. Patients carrying this genotype showed a later onset of acute episodes; the rate of lung function decline, as assessed by age at the first FEV1 value below 60% predicted, was lower in this group (table 3). By contrast, no apparent link was found with FEV1 and chronic *P. aeruginosa* colonization under stable conditions at multivariate regression analysis (table 4). Overall, our data support the role of MIF as a modifier gene of lung disease in CF only to a very limited extent, in contrast to what suggested by Plant *et al.* [14] and Adamali *et al.* [20], though the results of both studies differ from ours in several aspects. These authors identified MIF-CATT genotype in 167 adult white CF patients from a single centre, 11% of whom were pancreatic sufficient. Only a fraction of patients (57%) were homozygous for the F508del mutation while 35% were heterozygous for this mutation and the remaining 8% were heterozygous for other *CFTR* mutations. Patients carrying at least one copy of the 5-repeat MIF-CATT allele were found to have a decreased incidence of *P. aeruginosa* colonization (defined as the presence of bacteria in the sputum) and a significant reduction in the risk of pancreatic insufficiency.

The results of Plant *et al.* were partly reproduced (only for FEV and FVC) by Adamali *et al.* in a recent publication of the same research team [20], with a cohort of 143 patients selected from the same referral centre on the basis of a CF diagnosis and not of a F508del genotype. This work presents the same heterogeneous genetic background as in Plant *et al.*, and the same differences compared to our data. Moreover, an unspecified fraction of the patients from this latter study were also enrolled in the former. Interestingly, the *ex-vivo* part of the work by Adamali *et al.* showing differences of MIF levels in plasma and peripheral blood monocytes from CF patients, relies only on individuals with 5-5 and 6-6 MIF genotypes. In these experiments, the 5-5 MIF genotype is confirmed to be the genotype with lowest expression of the protein.

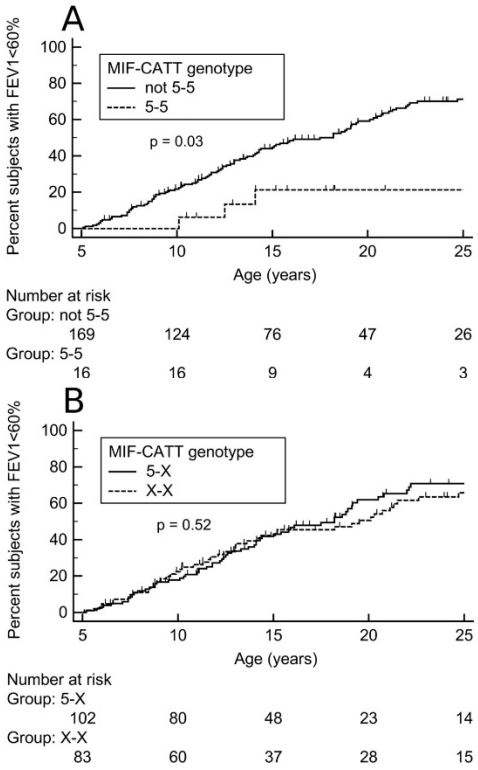

Fig. 1. Lung function decline in 185 Cystic Fibrosis patients grouped according to *MIF* -794 CATT genotypes. Kaplan-Meier plots relative to age at first acute episode with FEV1 <60% of predicted value. (A) Comparison between patients with MIF 5-5 (homozygous 5-CATT repeats) vs. not 5-5 genotype; (B) comparison between patients with at least one 5-CATT allele vs. the others. Ticks indicate censored subjects follow-up times. "Number at risk" at the bottom indicates the number of patients without acute episodes at a given time interval and whose follow- up extends at least that far into the curve.

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MIF-CATT genotype distribution in patients and controls were comparable in both studies (S2 Table). However, we found that clinical benefit was restricted to later onset of acute episodes in patients homozygous for the 5-repeats MIF-CATT allele.

Aside from these discrepancies, our results are in keeping with the observation by Baugh et al. [13] that in the context of another inflammatory disease (rheumatoid arthritis), only homozygosity for the 5-repeats MIF-CATT allele was protective against the development of severe disease.

Research into CF modifier genes has often yielded conflicting results and numerous challenges have been identified [21]; methodological issues are also

Table 4. Multiple regression analysis of FEV1 (Kulich)* data on 189 Cystic Fibrosis patients homozygous for the F508del mutation.

Variable	Coefficient	SE	r-partial	P
Constant	60.96	-	-	-
MIF-CATT genotype =5-5	7.06	7.09	0.073	0.320
Centre of origin = Brussels	15.15	4.58	0.24	0.001
CC by PA	−16.79	4.16	−0.29	0.0001
Diabetes	−19.04	4.46	−0.30	<0.0001

Overall R^2=0.274; multiple correlation coefficient =0.524
Overall significance p-value<0.0001;
FEV1: forced expiratory volume in one second; cc by PA: chronic colonization by *P. aeruginosa*. MIF-CATT genotype: *MIF* gene -CATT repeat genotype at position -794
*CF specific percentile according to Kulich *et al*, *Am J Respir Crit Care Med*, 2005.

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likely to be involved. When compared to the earlier study, several strengths of the present work can be stressed. The study population is homogenous at the CF locus, since we focused on a single *CFTR* genotype. The centres involved have been routinely using reliable CF-specific electronic databases for a substantial period of time. Additional efforts were made to normalize data, using CF-specific FEV1 predicted values and multivariate analysis to accout for centre-dependent variations. Z-score for BMI is now considered a more appropriate index expression of nutrition than percentage of ideal body weight [22] and chronic colonization by *P. aeruginosa* is clinically more relevant than airway colonization (simply referring to its presence). A large GWAS study on CF patients failed to test *MIF* as a modifier gene due to the lack of probes for this gene in the used DNA arrays; indeed the whole *MIF* gene is not covered by the Illumina 610-Quad platform used for genotyping. As for possible SNPs in linkage disequilibrium with MIF-CATT, the study used three different cohorts of patients for a total of 3467 CF patients; a sample size that, as the authors themselves stated, is several-fold smaller than the standard for GWAS studies. This implies that only strongest associations might emerge from the study. Due to the small number of MIF 5-CATT subjects and to the fact that, at least from our data, a moderate effect is suggested only in recessive homozygotes, such an effect would have probably been difficult to detect in any case [23]. Altogether the two previously available studies involved an undefined number of CF patients between 167 and 356. This might still be insufficient [24], given the wide range of FEV1 values and the low prevalence of the homozygous 5-CATT *MIF* promoter allele, so further work is encouraged. Besides clinical data, biologic plausibility of a candidate modifier gene is essential and the case of *MIF* gene is a good example. Indeed, it is a key pro-inflammatory mediator that is implicated in the pathogenesis of inflammatory diseases such as asthma, rheumatoid arthritis and acute respiratory distress. It has also been shown to sustain toll-like receptor 4 expression in murine macrophages [25]. Alveolar macrophages are believed to play an important role in regulating the local inflammatory and immune responses in the CF lung [26−29]. In addition, both macrophages [30] and their circulating precursors, monocytes

[31], have been shown to express functional CFTR, with alveolar macrophages from *CFTR* -/- mice exhibiting defective killing of internalized bacteria [30]. In Cystic Fibrosis, a vicious circle of inflammation and infection leads to destruction of obstructed airways, and it is notable that, up to now, only 4 medications - all with direct or indirect anti-inflammatory properties - are known to slow FEV1 rate of decline in this disease: high-dose ibuprofen [32], inhaled corticosteroids [20, 33, 34], macrolides [35] and DNase [36]. Conceivably, identification of modifier genes of lung disease in CF should help to provide novel therapeutic targets. In this perspective, neutralizing MIF activity by using antibodies or gene knockout, protected mice against severe sepsis by *E. coli* [11] and *Pseudomonas* and also enhanced *P. aeruginosa* airway clearance [10]. Furthermore, several powerful tautomerase inhibitors, highly selective for human MIF, have recently been identified and Adamali *et al.* showed promising *ex-vivo* results regarding their use to tame MIF-sustained immune reaction in CF patients [20, 37, 38]. In conclusion, we have provided additional independent data supporting the role of MIF as a modifier gene of lung disease in CF; the beneficial effect, however, was limited to the homozygous genotype displaying the lowest transcriptional activity. Since the protective MIF genotype is rare, most CF patients could benefit from a targeted approach aimed at reducing the powerful inflammatory response associated with high expression of this gene.

Supporting Information

S1 Table. Full dataset of Cystic Fibrosis patients used in the present study.
doi:10.1371/journal.pone.0114274.s001 (XLS)

S2 Table. Comparison of MIF -794 CATT genotype (a) or 5-CAAT allele (b) frequencies between Plant's [14] and the present study.
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Author Contributions

Conceived and designed the experiments: PM AM CS BMA TL. Performed the experiments: MO XP. Analyzed the data: PM AM PL EP XP TL. Contributed reagents/materials/analysis tools: MO XP. Wrote the paper: AM PM PL MO TL EP XP CS BMA.

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Résumé

Un large spectre de plus de 2000 mutations *CFTR* ont été associées à un phénotype clinique très variable de la mucoviscidose. Dans ce travail, nous avons analysé le spectre des mutations du gène *CFTR* chez des patients atteints de mucoviscidose et dans les troubles associés à la mucoviscidose. Le projet vise spécifiquement à étudier les points suivants:

1) Validation d'une nouvelle méthode de dépistage xTAG (Luminex, panel de 71 mutations), pour l'analyse de routine par rapport à l'INNO-LiPA® (Innogenetics, panel de 36 mutations), utilisé comme référence. Des résultats reproductibles et concordants ont été obtenus sur la plate-forme Luminex à partir d'échantillons ADN Innogenetics positifs utilisant de l'ADN extrait de différentes matrices biologiques, y compris des échantillons de sang, des taches de sang de cartes Guthrie, des villosités chorioniques et du liquide amniotique. Le nouveau panel augmente significativement le taux de détection pour les patients d'origine sud-européenne.

2) Etude des fréquences des mutations du *CFTR* dans le centre de l'Argentine, dans la province de Santa Fe, qui n'a jamais été caractérisée. Une cohorte de 83 patients sur une sélection locale initiale de 121 a été analysée. Les résultats ont été combinés avec ceux d'une étude précédente de la province voisine de Cordoba, aboutissant à la proposition d'un panel unique de 21 mutations du *CFTR* pour un diagnostic moléculaire de première ligne au centre de l'Argentine.

3) Analyse des effets des mutations dans les gènes *PRSS1*, *CFTR* ou *SPINK1* sur la gravité de la pancréatite idiopathique sporadique. Une cohorte rétrospective de 68 patients porteurs de mutations dans ces gènes a été comparée à une cohorte, pairée pour l'âge et le sexe, de patients atteints de pancréatite idiopathique avec des tests génétiques négatifs. Les caractéristiques cliniques et morphologiques des patients ont été prises en compte dans l'analyse. Les paramètres cliniques étaient similaires dans les deux cohortes, à l'exception de l'âge d'apparition de la maladie pancréatique. Une augmentation significative du cancer du pancréas a été observée dans le groupe des patients porteurs de mutations, en particulier dans le gène *CFTR*. Nous suggérons donc que les variants de *CFTR* présentent un facteur de risque pour le cancer du pancréas.

4) Mise à jour de l'analyse moléculaire du gène *CFTR* dans une cohorte de patients porteurs d'un syndrome d'aspergillose bronchopulmonaire allergique (ABPA). Les échantillons de 18 patients précédemment analysés à l'aide d'un panel de 13 mutations et rapportés dans un article publié en 2001 ont été ré-analysés en 2010 en utilisant le séquençage complet des exons. Comparativement à la première analyse, 8 cas ont été trouvés porteurs d'une mutation *CFTR* et 4 de deux mutations. L'étude a considérablement étendu les résultats précédents en démontrant une forte relation entre ABPA chez les adultes et mutations du *CFTR*.

Au total, ces études contribuent à jeter un éclairage nouveau sur le diagnostic moléculaire du gène *CFTR* dans les cas de patients atteints de mucoviscidose et de syndromes associés à la mucoviscidose.