Modifying Factors of Cystic Fibrosis Disease

Residual Chloride Secretion, Genetic Background and Epigenetics

Cover illustration: A dizygous sibling pair with identical *CFTR* mutation genotype, but presenting with different chloride conductance properties in the affected organs.

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Modifying Factors of Cystic Fibrosis Disease

Residual Chloride Secretion, Genetic Background and Epigenetics

Modificerende Factoren in Cystische Fibrose

Residuele Chloride Secretie, Erfelijkheids- en Omgevingsfactoren

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Chapter 1

Introduction

1.1 Cystic fibrosis disease

Cystic fibrosis (CF) is an autosomal recessive disease caused by genetic lesions in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. This *CFTR* gene was cloned in 1989,[1-3] and located to the long arm of chromosome 7 (7q31.2). It encodes the CFTR protein that functions as a adenosine 3',5'-cyclic monophosphate (cAMP)-regulated chloride channel in the apical membrane of exocrine epithelia,[4,5] like the sweat gland, submandibular glands, and the pulmonary, gastrointestinal, hepatobiliary, and urogenital tracts. In individuals with CF the defective chloride transport leads to abnormal ion and water transport,[6] which causes dehydration of secretions and malfunctioning of the obstructed exocrine glands, which typically results in chronic airway obstruction, pancreatic insufficiency (PI), and intestinal malabsorption. The survival of CF patients has immensely improved throughout the last century: while in 1938, 70% of babies died within the first year of life,[7] the median survival is now reported to be towards 30 years of age,[8,9] most probably due to the introduction of new therapeutic regimes, like physiotherapy, aggressive antibiotic treatment, pancreatic enzyme replacement, and proper nutrition.
CF is the most common, lethal, inherited disease in the Caucasian population.[10] There have been many reports on the incidence in Europe varying from 1 in 2000 live births in Ireland[11] to 1 in 40000 live births in Finland.[12] In the Netherlands the incidence was estimated around 1 in 3600 life births.[13] CF is found to be rare in persons from non-Caucasian origin. The most common *CFTR* gene mutation in the Caucasian population is the ΔF508 mutation, a deletion of the amino acid phenylalanine at position 508, which occurs in approximately 70% of CF alleles and 90% of CF patients. Yet, presently over 870 different *CFTR* mutations have been identified,[14] which give rise to the cystic fibrosis phenotype.

Historical overview[15]
As far back as the 17th century, reports have been found on children with symptoms as meconium ileus, pancreatic and lung disease and salt loss.[16] However, first in 1938 cystic fibrosis was recognized and described as a separate disease syndrome.[7] Chronic lung infection was recognized early as one of the major symptoms, and consequently antibiotics were introduced in the treatment of CF in the 1940s. When investigations revealed that salt loss occurred via the sweat gland and that children with CF exhibited elevated chloride and sodium concentrations in their sweat,[17] Gibson and Cooke[18] instigated the use of the diagnostic pilocarpine sweat test in 1959. In the 1980s more knowledge was gained about the molecular basis of the disease by Knowles and Boucher,[19] who described a disturbed chloride and sodium

transport in respiratory epithelia, and by Quinton and Bijman[6] who described chloride impermeability of the CF sweat gland.
After the cloning of the *CFTR* gene in 1989,[1-3] the basic defect in CF was firmly established as a dysfunctioning or mislocalization of the cAMP-mediated CFTR Cl^- channel.[4,5] Present research is addressing a large number of different aspects of the disease. With respect to the influence of the genetic background, it is interesting to investigate the effect of specific *CFTR* gene mutations on the disease phenotype, and the impact of genetic factors outside the *CFTR* gene. Other investigations focus on the possible contribution of different ion channels to disease severity, whereby the alternative chloride channels are of major interest. Additionally, it is reported that the apical sodium channel, expressed in airway and intestinal epithelium, might be hyperactive in CF and contributes importantly to the CF phenotype.[20,21] Moreover, the question arises if these channels are defective itself or if its regulation by the CFTR chloride channel might be disrupted. At the molecular and cellular level, researchers are aiming to circumvent the basic CF defect. Most fundamental, is the introduction of the normal *CFTR* gene into the affected cell by gene therapy. Other approaches are the pharmacotherapy to correct the CFTR processing and trafficking defect, or to compensate for the CFTR defect by exploiting alternative chloride channels.

1.2 Clinical description of CF[15,22-24]

CF is considered to be a monogenic disease, implicating that it is caused by mutations within a single gene. However, the clinical phenotype that is associated with this disease shows a considerable variability. Both the number of affected organs and the extent of the disease within a specific organ vary substantially between CF patients. The factors producing this variability are not well recognized, which complicates prognosis and treatment. Moreover, patients may present with little or atypical symptoms and these patients are diagnosed at adolescent or even adult age. It is assumed that at least the following factors are influencing CF disease phenotype: environmental factors (e.g., recurrent pulmonary infections, therapy, nutrition), genetic factors outside the *CFTR* gene itself, and the presence of residual chloride secretion either by the CFTR channel or by alternative chloride channels.
Clinically, typical CF is characterized by the accumulation of viscous mucus in the airways resulting in chronic obstructions, fibrosis, and finally destruction of the lung tissue.[22] The most prominent symptoms in the respiratory tract are cough, tachypnea and wheezing due to recurrent and

chronic bronchopulmonary infections. The intestinal tract shows malabsorption and pancreatic insufficiency, which causes steatorrhea and failure to thrive. Sweat glands are most consistently affected in CF and produce sweat with elevated chloride and sodium levels.
The information on the development of CF pathology is derived mostly from autopsy studies, which only report about the terminal stage of the disease. Additionally, since life expectancy has increased, organs might be affected more severely[22] and patients may present with new complications like diabetes and amyloidosis.

Respiratory tract
At birth, the macroscopic and microscopic appearance of the airways is normal, indicating that airway disease develops postnatally.[25] Over the first months of life, pathology starts in the small airways and bronchioles become plugged with mucus and bronchiolar mucosa becomes inflamed.[26,27] This mucus is very viscous with an abnormal thickness and accumulates in the airways, thus impairing mucociliary clearance.
With progressive disease this causes bronchiolitis and bronchitis, and eventually bronchiolectasis and bronchiectasis. The submucosal glands located in the proximal airways are normal at birth as well, but also become obstructed with mucus resulting in dilations and hypertrophy. Moreover, in the bronchial and bronchiolar epithelium of CF patients more mucus-containing goblet cells are found, contributing to the mucus accumulation. The naso- and oropharyngeal mucosa are also inflamed and erythematous. The onset of inflammation may occur before chronic infection is present, implying a major disease-causing role for inflammation and mucus accumulation rather than infection. In advanced lung disease, the deformations in the lung structure lead to chronic hypoxia and pulmonary arterial vasoconstriction with irreversible changes in vessel walls. This might develop into secondary pulmonary hypertension, contributing to respiratory failure. Since there are good therapies to alleviate the intestinal symptoms, respiratory failure is presently the major cause of morbidity and mortality in CF.
Usually, the first clinical symptom following from these respiratory abnormalities is intermittent coughing which becomes persistent and productive with tenacious, purulent sputum. Wheezing may occur due to the chronic inflammation of the small airways. Hyperinflation of the lungs starts early in the disease process and results in an increased thoracic index (antero-posterior diameter/transverse chest wall).[22] Most CF patients have chronic rhinitis with inflammation and irritation of the nasopharyngeal mucosa. This causes the development of nasal polyps in 15 to 20% of patients, most frequently between 5 and 14 years of age. The enlargement of

the terminal phalanges of fingers and toes, i.e. clubbing, occurs in almost all CF patients and is generally considered indicative for extensive airway disease. In addition, a recently discovered intrinsic defect in purinergic signaling in CF erythrocytes, associated with a loss of CFTR control of vascular resistance, might also contribute to a reduced oxygen supply of peripheral tissues.[28]
The progression of lung disease is associated with chronic lung infection with typical pathogens for CF disease. Typically, *Staphylococcus aureus* and *Haemophilus influenzae* are first detected,[29] and infection with *Pseudomonas aeruginosa* establishes afterwards. In advanced lung disease, *P. aeruginosa* is usually the only organism found. Unfortunately, infection with *Burkholderia cepacia*, an organism resistant to most antibiotic drugs, is increasing in CF patients.[30]
Many different origins for the chronic respiratory infection in CF have been postulated.[31] The hypothesis that mucociliary clearance in the lung would be abnormal, was proven incorrect, since the rate of mucus clearance in the central airways is normal,[32] and morphology and movement of cilia are not different from that in healthy individuals.[33,34] Another influencing factor might be the composition of the mucus itself. CF mucus contains more sulphated glycoproteins than the mucus of non-CF persons,[35,36] which makes the mucus viscous and difficult to clear. Subsequently, it was suggested that the high salt content in the airway surface liquid of the CF lung, impairs the ability to kill bacteria.[37,38] Other investigators claim that the presence of airway cells expressing CFTR increase the adherence of *P. aeruginosa* to the cells.[39,40] Thereby the organisms become internalized into the cells and removed from the mucosal surface. CF cells fail to internalize *P. aeruginosa*, giving the organism the opportunity to colonize the epithelium. In contrast, an increased adherence of *P. aeruginosa* to the CF epithelium has also been reported, owing to the up-regulation in CF cells of asialo-ganglioside (GM_1) receptors that bind *P. aeruginosa*.[41]
Although the exact mechanism of lung infection is not elucidated to date, it is clear that the accumulation of thick mucus creates a convenient environment for pathogens.

Gastrointestinal tract

Pathological changes of the mucosa of the gastrointestinal tract are minimal and consist of the dilation of ducts and acinar lumens of the Brunner glands in the duodenum due to mucus accumulation. Moreover, there is goblet cell hyperplasia, especially in the appendix, together with an increase of mucous material within the crypts and lumen of the gastrointestinal tract.[42]
Clinically, abnormalities of the gastrointestinal tract consisting of thick meconium plugs in the lumen, have been observed as early as 17 weeks of

gestation[22,43,44] both from autopsy material and *in utero* ultrasound examinations. These gastrointestinal changes are usually the first abnormalities in CF[45] and can occur without any changes in the other organs. The accumulation of meconium plugs can progress into meconium ileus (MI), the total obstruction of the distal ileum with meconium, which occurs in 10-15% of newborns with CF.[46] It results in failure to pass meconium, abdominal distension, and emesis. Meconium ileus is highly indicative of CF and over 90% of infants with MI have CF.[23] Mostly, it presents within 48 h after birth, or earlier when complications arise, like volvulus, intestinal wall perforation or meconium peritonitis.[23]
The pathogenesis of meconium ileus has been attributed to pancreatic insufficiency and the consequent indigestion of intraluminal material.[23,44] Other investigators ascribe the obstruction mainly to abnormal intestinal transport and dehydration of intestinal contents.[47,48] MI is strongly associated with pancreatic insufficiency and the presence of the ΔF508 mutation,[49] and is only rarely observed in pancreatic sufficient (PS) patients[23] (definition of pancreatic (in)sufficiency: see 'Pancreas' section below).
Some newborns are detected with meconium plug syndrome, which is obstruction by meconium in the large intestine, and is less specific for CF.[50] Later in life the terminal ileum can become obstructed as well, with voluminous intestinal contents. This occurs in about 20% of CF patients and is named 'meconium ileus equivalent' or 'distal intestinal obstruction syndrome'.[42,51] Mostly, this is provoked by large fatty meals, noncompliance with pancreatic enzyme replacement therapy, or due to intussusception or adhesions following abdominal surgery.[15]
In the duodenum, HCO_3^- secretion is necessary as a protection against the acidic secretions from the stomach. However, it has been shown that the CF condition results in defective duodenal HCO_3^- secretion. Functional CFTR is required for the cAMP-stimulated electrogenic HCO_3^- secretion, i.e. by an anion channel, as well as for the cAMP-stimulated electroneutral secretion, i.e. by the Cl^-/HCO_3^- exchanger.[52] This impairment might contribute to the gastrointestinal complaints of the CF patient.

Pancreas
Pathological changes in the pancreas may be detected in intrauterine life,[44] and exocrine pancreatic insufficiency is present from birth in the majority of CF patients.[53] Nonetheless, functional pancreatic tissue is present at birth, capable of producing pancreatic enzymes since high levels of immunoreactive trypsin-like activity (IRT) are found in the neonatal blood, implying obstruction of the secretion of pancreatic trypsinogen.[54] The pathological changes consist of dilated ducts and acini owing to thick and

inspissated pancreatic secretions. The ductal obstruction leads to destruction and loss of acinar cells and these areas are replaced by fibrous tissue and fat. Only later in the disease progress, the islets of Langerhans are affected with deformation by fibrous tissue and destruction of beta cells, which impair the endocrine pancreatic function of insulin secretion.
The obstructions in the exocrine pancreas cause decreased enzyme and bicarbonate release into the duodenum, which results in exocrine pancreatic insufficient in about 85% of CF patients. The severity of the pancreatic disorder is variable, yet it increases with age.[26,46,55] These patients secrete insufficient proteolytic and lipolytic enzymes to digest and absorb fat and protein. The subsequent steatorrhea correlates with less than 10% of normal pancreatic enzyme output, and only at this stage the pancreatic function tests become abnormal. These patients are classified as pancreatic insufficient (PI). Alternatively, about 10-15% of patients have less severe pancreatic changes with functional pancreatic tissue left, who are named pancreatic sufficient (PS). With age PS patients may develop pancreatic insufficiency, which appears to be genetically determined.[56] PS seems to correlate with mild *CFTR* gene mutations, while the PI condition is associated with more severe genetic lesions like ΔF508.
The pancreatic enzyme deficiency causes fat and protein maldigestion, with a distended abdomen and frequent, greasy, smelling stools. Fat loss in stools may be as high as 50-70% of intake, with deficiencies in (fat-soluble) vitamins in untreated patients. The enterohepatic circulation of bile acids is interrupted, since the fecal loss of bile acids is increased due to steatorrhea and binding to undigested food substances. This process aggravates the fat malabsorption, and uncorrected maldigestion results in failure to thrive. Modern therapy consists of pancreatic enzyme supplements[57] and a high caloric diet.[58] This therapy ameliorates gastrointestinal symptoms significantly, and although intestinal abnormalities still contribute substantially to CF morbidity, they are rarely life threatening. Numerous methods exist to evaluate the pancreatic function in CF patients.[23] For instance, the measurement of fecal fat excretion in 3-days-stool collection (increased with PI), the level of immunoreactive trypsin or fat-soluble vitamins in the blood (decreased with PI), or the prothrombin time (prolonged with PI). Though, the most reliable tests are the measurement of bicarbonate in pancreatic secretions after stimulation with secretin-cholecystokinin,[59,60] or the amount of elastase-1 in the stool,[61] both of which are decreased in CF patients.
The decreased secretion of insulin by the endocrine pancreas results in impaired glucose tolerance or even diabetes mellitus. The prevalence of impaired glucose tolerance is about 15% in CF patients, while diabetes mellitus occurs in about 10%, with an onset between 13 and 16 years.[62]

Hepatobiliary tract
Abnormalities in the liver are observed in up to 50% of autopsies,[63,64] although only 2 to 5% of CF patients present with symptomatic liver disease. Pathological changes in the liver structure occur in the large bile ducts, where CFTR is normally expressed, and consist of inspissated secretions, biliary duct proliferation, periportal inflammation and fibrosis.[63] These fibrotic areas surround patches of normal liver parenchyma, resulting in a lobular aspect of the liver. Symptomatic biliary cirrhosis demonstrates with hyperbilirubinemia, ascites, peripheral edema, or hematemesis due to esophageal varices.[65] The exact progression of liver disease is not known, although the high number of adults with liver disease as retrieved from post-mortem studies might point to a progression of liver disease with age. In contrast to the pancreatic involvement in CF disease, there is no association of chronic liver disease with a specific CF genotype.[66]
Although gallbladder emptying in response to meals is normal in CF, gallbladder abnormalities at autopsy are seen in a large part of the CF cases.[67] They include hypoplasia of the gallbladder, stones containing calcium and protein, and a thick, white, mucous content.

Genital tract
Anatomically the female genital tract is normal and women with CF can become pregnant. Reduced fertility might exist through reduced water content of the cervical mucus, which hinders sperm passage.[68] Other disturbing factors are chronic pulmonary sepsis initiating menstrual irregularities, and possible cysts in the ovaries.[69] Male CF patients develop the secondary sexual characteristics and sexual function is normal, yet the majority possesses abnormalities in the genital tract, and only 2 to 3% of CF males are fertile. Abnormalities include atrophic or absent vasa deferentia, and the body and tail of the epididymis, and the seminal vesicles are dilated, fibrotic, or absent as well.[70] Consequently, no spermatozoa are present in the ejaculate due to the obstruction, whereas testicular biopsies show that spermatogenesis is not affected. Male infertility originating from congenital bilateral absence of the vas deferens (CBAVD) might be the only presenting feature of CF in mild or atypical forms of CF, associated with specific mutations.[71]

Sweat glands
There are no structural abnormalities of the eccrine sweat glands[72] and production of sweat is normal upon stimulation with cholinergic drugs. However, the composition of this sweat is altered with increased chloride and sodium levels, resulting from disturbed electrolyte transport in the sweat

gland duct.[6] Moreover, the sweat production upon beta-adrenergic stimulation is absent.[73]

1.3 Electrolyte transport in non-CF and CF

The observation of excessive salt loss in sweat[17,18] and the abnormalities in exocrine secretions of CF patients[74] have led to extensive investigation of the bioelectrical properties of different epithelial tissues like the sweat glands, the respiratory tract and the gastrointestinal tract.

Sweat glands

The sweat gland consists of a secretory coil and a reabsorptive duct. In the sweat secretory coil of non-CF individuals, at least two different cell types are involved in sweat production.[75,76] One cell type is responding to β-adrenergic stimulation, which causes an intracellular increase in the second messenger cAMP, thereby activating CFTR. The other cell type is triggered by cholinergic stimulation, which increases the intracellular Ca^{2+} concentration and initiates Ca^{2+}-dependent Cl^- secretion. In this way, adrenergic and/or cholinergic agonists induce Cl^- secretion in the secretory coil, which drives the electrolyte and fluid secretion to produce sweat that is isotonic with plasma. In CF individuals, sweat production in response to β-adrenergic agonists is impaired due to the absence of CFTR, although the elevation of intracellular cAMP by adrenergic stimulation is not different between CF and non-CF.[73] The cholinergic pathway is not disturbed in CF patients, which explains the normal Cl^- secretion and production of isotonic sweat in the secretory coil[77] in response to pilocarpine, and the normal volume and rate of sweat production.[6]

In the reabsorptive duct, the electrolyte and fluid transport is driven by the basolaterally orientated Na^+,K^+-ATPase by pumping Na^+ out of the cell,[78] thus creating a low intracellular Na^+ concentration (Figure 1.1). This initiates passive Na^+ movement into the cell across the apical membrane into the cell. In non-CF individuals this Na^+ absorption is accompanied by Cl^- diffusion to maintain electrical neutrality, and since the sweat duct is impermeable to water, sweat becomes hypotonic at the end of the sweat duct. In persons with CF, however, the lack of CFTR impairs diffusion of Cl^- together with Na^+ absorption, thereby preventing NaCl reabsorption that results in increased Cl^-, and to a lesser degree, increased Na^+ concentrations in the sweat of CF patients. No other Cl^- channels are present in the sweat duct cells to compensate for the lack of the CFTR channels.

These abnormalities in electrolyte transport in CF could be assessed by measurement of the potential difference across the epithelium that is created

by the difference in charge between the apical and basolateral cell surfaces. Hence, when potential differences across the epithelium were measured, which are mainly created by Cl^- conductance and less by Na^+ conductance in normal sweat duct cells,[79] differences were observed between normal and CF sweat ducts. The PD in CF ducts was more negative (-66 mV lumen negative) than that in non-CF sweat ducts (-30 mV).[6,78] Moreover, when luminal Cl^- concentration was reduced, the PD became more negative in normal sweat ducts pointing to the ability to secrete Cl^- into the duct lumen, while the transepithelial voltage became more positive in CF sweat ducts indicating the impermeability to Cl^-, but the partial preservation of Na^+ absorption.[80] Measurements of Cl^- transport in sweat duct epithelium, demonstrated that Cl^- permeability of both the apical and basolateral cell membranes is reduced.[81,82] The Na^+ transport in the sweat gland was also investigated and was not primarily affected, though the abnormalities existed secondary to the impaired Cl^- transport.[78,81]

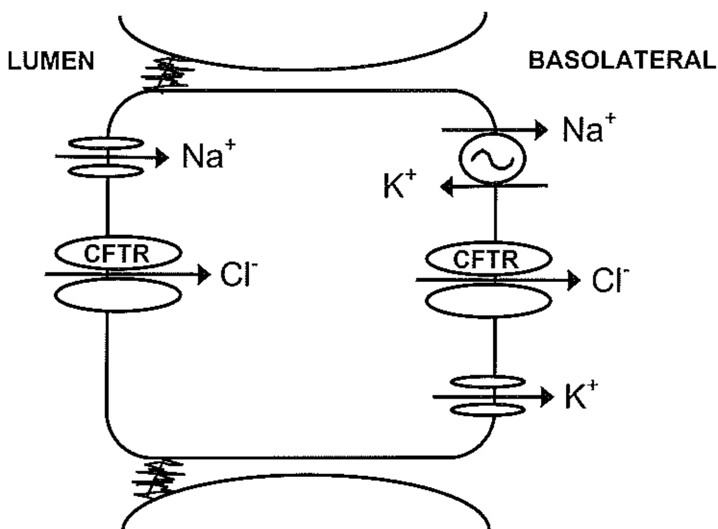

Figure 1.1 *The NaCl absorption in the reabsorptive duct of the sweat gland. Na^+ is absorbed across the apical membrane down its electrochemical gradient, and transported actively across the basolateral membrane by the Na^+, K^+-ATPase pump. Cl^- follows passively across the luminal and basolateral membrane in healthy individuals, which is impaired in CF patients.*

Respiratory tract

The apical surface of the epithelial cells of the airways contain cilia that are covered with mucus, in which pathogenic and inhaled material is

accumulated and moved out by the ciliar movement, which process is named the mucociliary clearance. The mucus is produced by the submucosal glands and goblet cells, while its hydration is controlled by transepithelial electrolyte transport. As in the sweat gland duct, fluid absorption is driven by the basolateral Na^+,K^+-ATPase[83] and the consequent low intracellular Na^+ concentration causes Na^+ ions to enter the cell across the apical membrane. Water diffusion across the epithelium follows by osmosis. When the airway surface liquid is dehydrated, apical Cl^- channels can be opened by the second messengers cAMP and/or Ca^{2+} (Figure 1.2).[84,85]

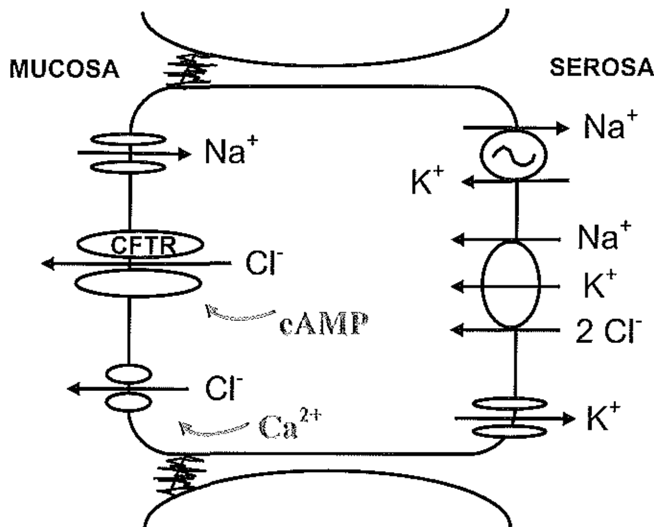

Figure 1.2 *The process of Cl^- secretion in the airway epithelium. Na^+, K^+ and Cl^- enter the cell basolaterally by the Na^+-K^+-$2Cl^-$ cotransporter. K^+ exits the cell through basolateral channels, which creates a driving force for Cl^- to exit the cell via apical channels and for Na^+ to enter the cell apically. A low intracellular Na^+ concentration is created by the basolateral Na^+, K^+-ATPase pump, and produces the electrochemical gradient for Na^+, which is needed by the Na^+-K^+-$2Cl^-$ cotransporter. In addition to CFTR Cl^- channels, Ca^{2+}-mediated Cl^- channels exist in airway epithelial cells.*

Chloride then exits the cell through these apical chloride channels down its electrochemical gradient followed by water to rehydrate the surface liquid. Na^+,K^+-ATPase preserves the low intracellular Na^+ concentration that is needed by the basolateral Na^+-K^+-$2Cl^-$ cotransporter to let chloride enter the cell basolaterally against its electrochemical gradient. In the distal airways the net result of salt and water transport by the surface epithelial cells results in secretion, like in the cells of the submucosal glands, while in the

proximal airways predominantly salt and fluid absorption takes place. When the bioelectrical properties of airway epithelium were measured in the basal condition, Na^+ absorption predominated.[86] In analogy to the sweat duct, the basal potential differences across the airway epithelium, as assessed by the nasal potential difference measurement, were higher in CF patients (-56 mV lumen negative) than in controls (-26 mV) (Chapter 2 & refs 19,86,87). Since the nasal epithelium is easily accessible and representative for proximal airway epithelium, the nasal potential difference measurement has developed as one of the diagnostic tests for CF disease.
Sodium transport in the airway also seems abnormal as can be measured by the change in potential difference upon the addition of amiloride (Chapter 2 & ref 19), which inhibits the apical epithelial Na^+ channel (ENaC). However, as was demonstrated for the sweat gland duct, this transport might be disturbed secondarily to the anion transport irregularity.[78,81]

Intestinal tract
The mechanism of fluid absorption and secretion in the intestinal tissue functions as in the airway epithelium. Apical Cl^- efflux down its electrochemical gradient causes osmotic fluid secretion into the gut lumen, whereas active Na^+ absorption through the basolateral Na^+,K^+-ATPase drives the fluid absorption. The function of the small intestine is predominantly to secrete fluid, while in the colon mainly fluid absorption occurs.
The basal potential difference in the intestine was observed to be normal in some studies,[88,89] while others demonstrated reduced baseline PD values.[90] However, defective cAMP-mediated Cl^- conductance was shown in different regions of the intestinal tract like the jejunum, colon and rectum.[91-94]
The amiloride-sensitive Na^+ transport in human rectal biopsies, as measured in our laboratory[94] and by Hardcastle et al,[95] was similar in CF and controls. However, other groups found enhanced Na^+ transport like in the airway epithelium.[21,96]
The Na^+-glucose cotransport in small intestine was increased in most previous studies.[97,98] In contrast, more recent studies in jejunal tissues of CF mice show no enhanced Na^+-coupled glucose transport.[99,100] In our own laboratories, we investigated the glucose transport in *cftr* knockout mice and their control littermates by distinguishing between the transcellular, active Na^+-glucose cotransport and the paracellular, passive glucose transport. We demonstrated that in CF the transcellular Na^+-glucose absorption is reduced by 25-30%, while the passive paracellular glucose absorption is twice as high compared to control mice.[101]

1.4 Identification of the *CFTR* gene

Since no molecular entity or any specific chromosomal region, deletions or translocations were known to be associated with cystic fibrosis disease, searching for the chromosomal localization of the disease locus only became possible with the introduction of DNA markers to perform positional or reverse genetics. This technique uses DNA markers to identify restriction fragment length polymorphisms (RFLP)[102] and variable number of tandem repeats (VNTR).[103] This was performed in families with one or more individuals affected with CF, in which crossover events between CF and other flanking DNA markers had been detected. By consequent linkage analysis of the results, DNA markers were sought that were linked to CF disease. The first important DNA markers that showed linkages with CF were D7S15,[104,105] the met oncogene (MET)[106] and D7S8,[107] the last two flanking the CF locus,[108] and localized the CF gene to chromosome 7. However, the size of the region between MET and D7S8 was large (estimated around 5000kb). Restriction maps of the CF region with respect to the linked markers were made with the use of additional DNA markers and the associated RFLPs, observation of recombination events near the CF locus, and analysis of linkage disequilibrium.[109-111] In order to clone and sequence the large DNA region, novel techniques that analyze large DNA fragments were introduced. Chromosome walking, which uses the end of one clone to re-screen a DNA library, clones DNA fragments as large as 100 kb. Chromosome jumping analyzes parts of around 500 kb, by partially digesting the DNA and circularizing it so that distant fragments of DNA are brought closer together. Pulse-field gel electrophoresis in combination with restriction enzymes can separate DNA fragments of 100-1000 kb. With these methods, the loci of the markers that were linked to CF were taken as starting points to sequence the region of interest and scan for candidate sequences. The candidate coding sequences in this cloned DNA were identified by looking at conserved stretches among different species, since these might indicate potential coding sequences. In addition, regions that contain many non-methylated dinucleotide CpG stretches may precedes human genes.[1,112] The DNA fragments that were thus selected were then used in hybridization experiments to screen cDNA libraries of various tissues. A single cDNA clone was first isolated from sweat gland of a non-CF individual. This clone was used as a probe in hybridization experiments with other epithelial cell lines. Hence, transcripts were obtained about 6.5 kb long from the T84 intestinal colonic cell line that normally expresses chloride conductance.[2] Subsequently, transcripts were also isolated from lung tissue, pancreas, nasal polyps, sweat gland, liver, parotid and placenta. Initially, no hybridization signals were found in brain adrenal gland, skin fibroblast, and

lymphoblasts.[2] This expression of the CF candidate gene was in accordance with the tissues that are mainly affected in CF disease.[2] The cDNA sequences of this gene, derived from normal and CF individuals were compared.[2] A 3 base-pair deletion was identified in the cDNA from CF individuals, but not in that of normal individuals,[2,3] implying that this would be the disease causing lesion. Thus, the CF gene was finally mapped to position 7q31.2.[1-3]

1.5 Structure of the *CFTR* gene & protein

The cloned gene is approximately 230 kb long and consists of 27 exons. The encoded mRNA is about 6.5 kb long and is translated into a protein product of 1480 amino acids.[1,113] There seem to be multiple transcription initiation sites, but the basal promotor region lies between nucleotide positions -228 and +48.[114] The encoded protein was named cystic fibrosis transmembrane conductance regulator (CFTR).[2] On the basis of the DNA sequence of the gene, a potential protein structure was postulated (Figure 1.3).
The amino acid sequence of the CFTR protein shows significant homology to the family of ATP-binding cassette (ABC) transporters, and specifically to P-glycoprotein.[115] The predicted protein structure contains 2 repeated motifs, each consisting of a membrane-spanning domain with six hydrophobic transmembrane segments and a nucleotide-binding domain (NBD) that interacts with ATP.[2] Ten of the 12 transmembrane segments contain one or more charged amino acids, and between TMS 7 en 8 are two potential glycosylation sites. A regulatory domain, the R-domain, separates the two symmetrical motifs of the putative protein. This R-domain is unique for CFTR and is not present in the other members of the ABC transporter family. This R-domain contains 9 of the 10 consensus sequences for phosphorylation by protein kinase A (PKA) and 7 of the phosphorylation sites for protein kinase C (PKC). In addition this R-domain contains many charged amino acids.[2]

1.6 mRNA and CFTR localization

Investigations with antibodies against CFTR or against specific regions of the protein, have demonstrated that CFTR is located within the cell membrane,[116] and that the R-domain and C-terminus are located intracellularly, while some of the loops between transmembrane segments are localized on the extracellular side.[117]

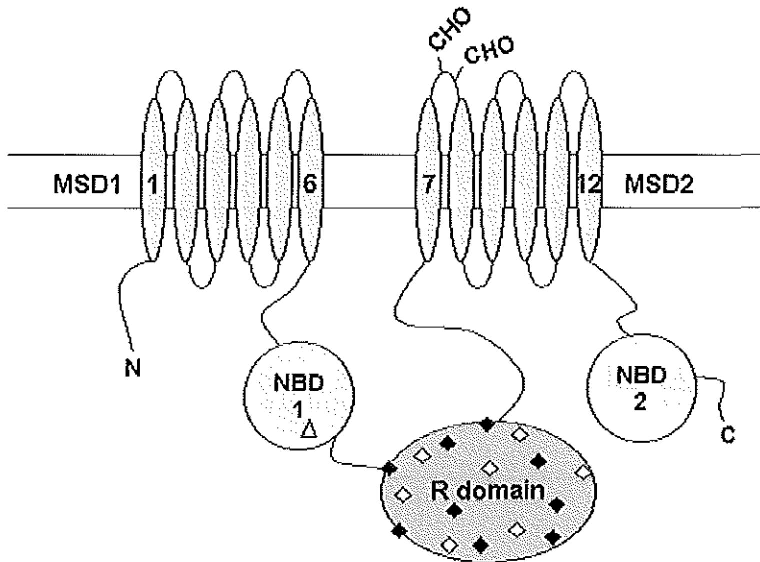

Figure 1.3 *The CFTR protein consists of two membrane spanning domains (MSD1 and MSD2), each containing 6 transmembrane segments. Subsequent to MSD1 and MSD2 the protein contains two nucleotide binding domains (NBD1 and NBD2). Between NBD1 and MSD2 lies the cytoplasmic R domain, which is the regulatory part of the protein. It contains 9 potential sites for phosphorylation by PKA (◊) and 7 for PKC (♦). Between transmembrane segments 7 and 8 are two potential glycosylation sites. The most common CFTR mutation, ΔF508, is located in NBD1 (Δ).*

The expression pattern of CFTR mRNA and CFTR protein in different organs of non-CF individuals was investigated with in situ hybridization and immunohistochemistry. The CFTR expression in organs that are mainly affected in CF are given in Table 1.1. Furthermore, CFTR was detected in parotid,[2] placenta,[118] vas deferens,[119] brain[120] and in the stomach.[120] In non-epithelial cell-lines CFTR was detected in lymphocytes,[121,122] monocytes,[123] heart cells,[124] and human endothelial cells.[125] In addition, the protein was shown on intracellular vesicles, where it may fulfill a role in regulated trafficking and/or intravesicular pH regulation.[126-130]

1.7 *CFTR* gene expression

CFTR gene transcription is increased by elevations of cAMP for more than 8 h, pointing to cAMP responsive elements in the *CFTR* gene promotor.[139] However, CFTR is already present in the apical membrane before cAMP elevation.[117] In some cell types expression is regulated by cell differentiation,[121,140] and therefore maybe important in the life-cycle, while in other cells CFTR mRNA is present throughout cellular development.[141] Down-regulation of *CFTR* gene expression is observed through phorbol esters[142] or an increase in intracellular calcium levels.[143]

Table 1.1 *Expression of CFTR mRNA and/or protein in epithelial tissues*

Pancreas[2,131-134]	exocrine pancreatic duct apical membrane not in acinar cells
Respiratory tract[2,135]	serous ducts of submucosal glands ciliated and collecting ducts little in surface epithelium apical membrane and cytoplasm
Small & large intestine[2,132-134,136,137]	(base of) crypts villus high expressing cells apical membrane not in surface epithelium of colon
Sweat gland[2,131,132]	reabsorptive duct very little in secretory coil apical and basolateral membranes
Liver[138]	bile duct
Salivary gland[132]	intralobular duct apical membrane
Kidney[118,133]	Tubules

1.8 The chloride channel function of CFTR

Before the discovery of the CF gene, functional studies were executed to determine the exact site for the abnormal electrolyte transport and to identify molecular entities responsible for the electrophysiological defect. By application of Cl^- channel blockers, Bijman et al showed that Cl^- transport in the sweat gland duct is through channels rather than paracellularly.[144] The identification of different chloride channels in the epithelial tissues of the sweat gland[145,146] and airways[147-150] supported these findings.
After the discovery of the CF gene and the resulting gene product, several studies confirmed *CFTR* as the affected gene in CF disease, and its protein

product as an epithelial cAMP-regulated chloride channel. Transfection of the *CFTR* coding sequence into cultured CF airway and pancreatic epithelial cell lines corrected the chloride permeability defect.[151,152] Normal CFTR cDNA transfection into non-epithelial cell lines resulted in plasma membrane chloride conductance that was stimulated by cAMP.[4,153,154] Since these cells normally do not show chloride conductance or express CFTR, CFTR was identified as a chloride channel itself rather than a regulator of chloride transport. Moreover, by inserting antisense DNA to CFTR into normal cells, cAMP-mediated chloride secretion was inhibited.[155] Conclusive evidence was given by Bear et al[5] by fusing purified CFTR with lipid bilayers, which resulted in the appearance of chloride channels with the characteristic properties of CFTR-associated conductances.
These properties of CFTR are a low unitary conductance (6-10 pS) and channel gating through protein kinase A mediated phosphorylation of the R-domain. In intact cells, PKA is activated by cAMP and cAMP-agonists like β-adrenergic stimuli.[156,157] CFTR shows a linear current-voltage (I-V) relationship, and does not display voltage-dependent activation or inactivation. The anion permeability sequence is $Br^- > Cl^- > I^- > F^-$. The open probability of the channel is not influenced by stilbene derivatives like 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), but influenced by 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), diphenylamine carboxylic acid (DPC), or glibenclamide.

1.9 Regulation and activation of CFTR

The CFTR chloride channel is regulated by kinases, phosphatases, intracellular ATP concentration, and hydrolysis of ATP. Activation of the channel is effected by the direct action of nucleoside triphosphates on phosphorylated CFTR.[158-160]
As mentioned above, the R domain contains 9 (8 serines, 1 threonine) of the 10 (1 more serine just before NBD1) consensus sequences for potential phosphorylation by PKA, and 7 of the binding sites for PKC.[2] Phosphorylation of these putative binding sites by PKA and/or PKC is needed to activate CFTR.[161,162] It is not the phosphorylation of just one of these binding sites that can activate CFTR, as mutagenesis of single phosphorylation sites did not alter channel activity.[159] *In vivo*, the addition of cAMP agonists mainly phosphorylates the serines S660, S737, S795, S813, and most of the decrease in open probability of the channel occurred with the mutation of these serines.[163,164] Therefore, it is suggested that these phosphorylation sites are most important for channel activity. PKC, which can be activated by the combination of increased Ca^{2+} and diacylglycerol

concentrations, is able to stimulate CFTR as well, however only up to 15% of the activation observed by PKA. When PKC was first applied to the channel with subsequent addition of PKA, the substances worked synergistic in stimulating CFTR.[165,166] In addition, cyclic-GMP-dependent protein kinases might be involved in phosphorylation and activation of CFTR in the intestine.[167,168] Both the isoenzymes cG kinase I and II can phosphorylate CFTR *in vitro* at similar sites as PKA.[168] However, only the cG kinase II isoform was able to activate CFTR in excised patches of intestinal cells.[167]
To activate CFTR the presence of PKA alone is not sufficient, as ATP is required to act as PO_4^{2-} donor to phosphorylate the R domain. This ATP does not necessarily have to be hydrolysable. As CFTR shows homology with the ABC transporters, it was predicted and subsequently demonstrated that ATP binds to the NBDs.[158,169] In this case, to activate the channel and keep it open, hydrolysable ATP is required.[158] Non-hydrolysable ATP analogs, like 5-adenylylimidodiphosphate (AMP-PNP), are unable to induce channel activity or are much less efficient.[158] This requirement for ATP hydrolysis is not fully understood, as Cl^- is transported down its concentration gradient and no energy is needed to build up an electrochemical gradient. It is hypothesized, however, that ATP hydrolysis at NBD1 might be essential to transform the protein to, or maintain it in the open conformation.[160] Phosphorylation of the R domain is not needed for ATP to bind to the NBDs.[158] However, once CFTR is phosphorylated by PKA, ATP alone can initiate channel function.
The 2 NBDs do not have a similar function, as it was demonstrated by mutagenesis of NBD2 and deletion of the R domain, that NBD2 could inactivate CFTR through interaction with the R domain.[170]
The unphosphorylated R domain of CFTR seems to regulate the channel by keeping it in the closed conformation, since deletion of part of the R domain produced Cl^- channels that are constitutively open.[170] The opening and closing by the R domain is most likely influenced by its electrical charge, given that substituting serine residues for negatively charged aspartic acid generated constitutively active channels.[164] Substitution of 6-8 phosphorylation sites was necessary to cause a similar effect.[163,164]
The membrane-spanning domains are thought to represent the pore of the channel, as mutations of specific charged amino acids in these domains resulted in changes in anion permeability.[171]
The CFTR channel is closed through dephosphorylation by certain protein phosphatases, i.e. protein phosphatase 2A, but not 1 or 2B.[162]

1.10 Mutations in the *CFTR* gene

Since the *CFTR* gene has been cloned, many different mutations have been identified, which can be grouped into pathological mutations and benign sequence variations. Pathological mutations can be separated into missense, nonsense, frameshift and mRNA splicing mutations. Missense mutations, in which a DNA nucleotide change causes an amino acid substitution in the encoded protein, represent the largest part of mutations. Deletions in the *CFTR* gene are rare, no mutations have been discovered yet in the promoter region and there is only one example of a de novo mutation, i.e. R851X (mutations are named by using the single letter code for the normal and the substituted amino acid, with the codon position of the cDNA in the middle).[172] The majority of the missense mutations affect the regions coding for the NBDs of the putative protein, mainly NBD1.[173,174] The most frequent missense mutation is a 3-bp deletion that results in the loss of the amino acid phenylalanine at position 508 in the CFTR protein product,[3] which is present on about 70% of CF chromosomes.[175] However, there is large variation in frequency between different populations, with the highest frequency of 82% in Denmark and 32% in Turkey.[175] The overall frequency of non-ΔF508 mutations is low, except for some rare alleles that segregate with a specific ethnic group. For instance, the W1282X accounts for 48% of CF chromosomes in Ashkenazi Jews[176] and 23% of French Canadian CF chromosomes carry the nucleotide transition 621+1G->T.[177,178] Other mutations with a high incidence in certain populations are A455E,[179] G542X,[179] G551D,[174] R553X,[174] and N1303K.[180] Since the frequency of most non-ΔF508 mutations in populations that are not from European origin is similar as in Caucasian CF patients, the high incidence of CF in European populations is mainly due to the presence of the ΔF508 mutation.[181] Besides, there is complete association between one specific haplotype of closely linked DNA markers,[3] or the "haplotype B" for the polymorphic markers XV-2c and KM.19,[182] and the ΔF508 mutation, indicating that ΔF508 originated from a single mutational event. This background of the ΔF508 mutation and its high frequency in the Caucasian population suggest a heterozygote advantage for the mutation itself or for another gene closely linked to the CF gene.[183]

Effect of mutations in the CFTR gene

Wild-type CFTR is glycosylated in the ER and in the Golgi, and thereafter transported to the plasma membrane.[116] The different *CFTR* gene mutations that cause CF disease are proposed to cause loss of CFTR Cl^- function by different molecular mechanisms.[184-186]

Frameshift, nonsense and splice site mutations result in defective protein production. Unstable mRNA is produced, and either a truncated, unstable protein is formed or no protein is detectable at all.[187] CFTR chloride channel function is supposed to be absent in these CF cells.
The majority of *CFTR* missense mutations, and deletions of an amino acid cause defective protein processing, like the 3-base pair deletion ΔF508. In this class of mutations, aberrant mRNA is formed but the mutant CFTR protein is unable to adopt a fully glycosylated, mature conformation.[188] Consequently, it is not exported from the ER but subsequently degraded. Therefore, most of the ΔF508 does not succeed to reach the plasma membrane.[132,188-190] However, recent immunohistochemical studies have shown ΔF508 protein localized at the apical surface membrane of epithelial tissues.[191-194] Moreover, *in vitro* (Chapter 4 & refs 195-197) and *in vivo* (Chapter 5 & ref 198) electrophysiological experiments have demonstrated that ΔF508 can exert subnormal chloride channel function, although the channel appears to remain closed longer than wild-type CFTR and its regulation by cAMP is changed.[196,197]
In the remaining *CFTR* missense mutations that do not result in partial or total defective protein processing, the protein is inserted into the apical cell membrane and can function as chloride channel, though the regulation and/or conduction of these altered proteins is abnormal. For instance, mutations in the NBDs (as G551D) cause less chloride channel activity by affecting the direct interaction between ATP and the NBD, which normally stimulates CFTR activity.[197,199] The known missense mutations that cause defective conduction of the chloride channel, e.g. R117H, are located in the membrane spanning domains that are assumed to form the channel pore. These mutations are associated with chloride currents that are regulated by cAMP and ATP similar to wild-type CFTR, but the amount of currents through these channels is reduced.[197,200]
One additional class of mutations causes reduced synthesis of normal, functional CFTR, like nucleotide alterations that result in alternatively spliced mRNA, e.g. 3849+10kbC->T, or mutations in the promoter region that reduce transcription.[186] In these cases, small amounts of normal mRNA or normally functioning protein are produced ensuing a mild phenotype.
A recent study by Moyer et al[201] suggests that the COOH-terminal amino acids of CFTR contain motifs that localize CFTR to the apical membrane. Therefore, C-terminal mutations in CFTR will result in defective apical polarization of the protein, and thus defective apical chloride transport.

Sequence variations

Nonpathological sequence variations were found within the coding region of the gene or within introns, and may or may not lead to amino acid

substitutions. For some of these polymorphisms the interpretation is difficult, e.g. F508C and R75Q. These sequence alterations have been found in CF patients but also in compound heterozygotes without symptoms. Another example is the polymorphic Tn locus in the splice acceptor site of intron 8 of the *CFTR* gene, of which three different alleles (T5, T7, T9) can be found.[202] The amount of thymidine residues at this locus determines the splicing process at the intron 8 – exon 9 junction. Alternative splicing is dependent on the length of this thymidine stretch, and the shorter the thymidine stretch the more alternative splicing occurs, resulting in CFTR mRNA transcripts without exon 9.[202,203] Transcripts that lack exon 9 are translated in CFTR proteins that do not mature, and do not result in chloride channel activity.[204,205] Individuals homozygous for the T5 allele, or compound heterozygotes for a CF mutation and the T5 allele can be observed with CBAVD, however these individuals can also be without any symptoms. Therefore, T5 is classified as CBAVD disease mutation with partial penetrance.[206] In addition, the Tn locus can influence the phenotype of the R117H *CFTR* gene mutation. The R117H mutation together with the T5 allele on one chromosome is responsible for CF, while R117H with T7 can either cause CF or CBAVD.[207] Besides the Tn locus, two other polymorphic loci have been identified to influence the quantity and quality of CFTR transcripts, i.e. (TG)m and M470V.[208,209] The number of TG repeats can vary between 9 and 13, and on a T5 or T7 background this number of TG repeats also influences the amount of transcripts without exon 9: the higher the number of TG repeats the more transcripts without exon 9. CFTR proteins with either a methionine or valine at amino acid position 470 matured differently and exhibited different electrophysiological properties. The M470 protein matured more slowly, but its open probability was about 1.7 times higher than that of the V470 protein. With the same (TG)13T5 haplotype, the M470V loci was observed to affect the phenotype being CBAVD or CF, suggesting an influence on the penetrance of the T5 allele.
These results illustrate that different polymorphisms at various loci themselves may not be disease causing, but the combination of different alleles at these loci can result in less functional or even insufficient CFTR protein.[210]

1.11 Genotype-phenotype associations

The most straightforward associations found between genetics and disease symptoms are the inherited CF mutations that determine pancreatic status of the patient. CF mutations are classified into mild and severe mutations, and CF patients carrying 2 severe mutations confer the pancreatic

insufficient phenotype, while at least one copy of a mild mutation leads to pancreatic sufficiency as mild mutations are phenotypically dominant over severe mutations.[3,179] The DNA alterations classified as nonsense, frameshift, splice-site, amino acid deletions, and most missense mutations are considered as severe mutations. Mutations found in patients with pancreatic sufficiency are G85E, G91R, E92K, R117H, R334W, R347H, R347P, A455E, G551S, P547H, S549N, and 3849+10kbC->T.[15,211,212] In these individuals the anomalous protein is presumed to reach the apical membrane and generate chloride conductance, which would confer the normal pancreatic function.[132,135,197,200] Only rarely is PS seen with ΔF508 homozygosity, and these individuals may show reduction in their pancreatic function with age, as may be observed in patients initially diagnosed as PS.[213]

Meconium ileus is not associated with any specific genotype, but it has only been recognized in pancreatic insufficient patients. The risk to develop liver disease is suggested to correlate with pancreatic insufficiency as well. In addition, a higher incidence was detected in patients with genotypes conferring severe or variable disease phenotype, while no liver disease was observed in patients with mutations related to a mild phenotype.[214]

Associations between genotype and severity of pulmonary disease were difficult to determine. The only correlation found, was a better lung function in PS patients, e.g. PS patients with the A455E mutation,[215] though it is uncertain if this is a result of the better nutritional status that is observed in PS patients when compared to the PI group.[56,216] Patients homozygous for ΔF508 have highly variable pulmonary phenotypes, and homozygosity or compound heterozygosity for mild mutations or stop-codon mutations are also not associated with better lung function.[56,217,218] The large variability in lung disease among patients with the same *CFTR* genotypes indicates the importance of other genetic factors outside the *CFTR* gene, or of environmental factors.

Abnormal sweat chloride concentrations were seen in virtually all CF patients. Patients homozygous or compound heterozygous for ΔF508, or with two non-ΔF508 mutant alleles, were observed with similar sweat chloride levels.[219] However, some investigators found less elevated sweat chloride levels in compound heterozygotes,[186] or in individuals with the 2789+5G->A or 3849+10kbC->T sequence alteration.[220] In addition, sweat test results more towards normal values are also seen in the pancreatic sufficient CF patient group.[56,221]

Another phenotypic feature that was found to correlate with the A455E mutation is a lower age of diagnosis compared to patients carrying two severe mutations.[222]

In parallel with a specific genotype that is connected with a certain phenotype, the clinical presentation of CBAVD seems to predispose for carrying *CFTR* gene mutations. About 60% of males with CBAVD carry at least one *CFTR* mutation and 10% harbor two known mutations.[223,224] Especially the T5 allele in intron 8 occurs more often in CBAVD patients compared to normal or CF populations.[225,226] (see also the above section: 'Sequence variations')

1.12 Gene therapy

The most essential elements of the CF therapeutic regime concern the patient's nutrition, antibiotics, and the physiotherapy. However, identification and cloning of the CF gene has created a new possibility for therapeutic intervention, i.e. introducing normal *CFTR* DNA into the affected cells. *In vitro* experiments demonstrated that transferring CFTR into cells carrying the CF defect restores the cAMP-dependent chloride conductance.[152] The fundamental issues determining the success of this technique, are i) targeting of the cell type that is important in pathogenesis, ii) the mechanism of bringing DNA into the cell, iii) the efficiency of the gene transfer, and iv) the expression of the normal proteins in these cells.
At present, various vehicles for gene delivery are being examined, using adenoviral vectors, adeno-associated viral vectors, DNA-liposome complexes, or receptor-mediated endocytosis. Experiments of gene delivery in CF are mainly focused on the airway epithelium, since this is the most readily accessible organ affected. For safety, the objective is to target the differentiated airway epithelial cells rather than the stem cells, since any adverse effects of the intervention will consequently be temporary. On the other hand, this will imply repeated treatment of the affected cells.
Early *in vitro* experiments already showed the possibility to use viruses as vehicle for gene transport into the target cells.[151] The most frequently exploited viral vectors that are used to investigate possible therapeutic intervention by gene transduction in CF, are the adenoviral and adeno-associated viral vectors. For this purpose viruses are made replication deficient and the advantage of these specific viruses is that they naturally infect respiratory epithelium, hence they are assumed to target many of the different types of airway cells. However, all viral vectors have the disadvantage of inducing an immune response in the host, causing inflammation of the epithelium at first application. Moreover, it can trigger antibody formation, thereby reducing the safety to use repeated doses. Therefore, one of the issues being analyzed at present is the immune response initiated by the different types of viruses,[227,228] and the possibility

to administer viral vectors to the lungs with simultaneous transfection of anti-inflammatory substances.[229,230] A trial in CF volunteers to accomplish *CFTR* gene transfer by adenoviral vectors, showed a low efficiency with undetectable levels of gene expression around 40 days after administration.[231] It appeared that the human coxsackie and adenovirus receptor (hCAR) is located basolaterally. Thus, to increase the amount of transfected cells and make adenovirus-mediated gene delivery successful, researchers suggest the use of agents that create a more permeable epithelium by opening tight junctions,[232,233] and/or by causing the cell to express hCAR apically as well.[234]
Liposomes, vesicles composed of a lipid bilayer, can be engaged to carry DNA and deliver it to the cell by fusion with the cell membrane. These liposomes are preferably positive in charge, cationic liposomes, to facilitate interaction with negatively charged DNA and enhance the merge with cell membranes, which hold a negative charge as well. In CF mice liposome-complexes have been shown to correct the CF defect.[235] The advantage of liposomes is its non-viral nature, inducing a smaller immunological reaction. However, liposome-mediated gene transduction appears less efficient than transfer by viral vectors.[229] Biological barriers that hinder delivery of the DNA into the cell by liposomes or adenoviruses are for instance the sputum layer covering the epithelium, and the plasma membrane of the airway cells itself since it does not readily bind or incorporate the different vectors.[236] In addition, it was demonstrated that lipid-mediated transfection is reduced with polarization and differentiation of CF murine airway cells.[237] The application of higher doses does not improve efficiency, since it increases inflammation and toxicity.[238,239]
Another nonviral vector system is the use of receptor-mediated endocytosis. This strategy uses receptor-specific ligands that bind to receptors on the cell membrane, to bring the desired genetic material into the cell. Obviously, a great advantage of this method, apart from its nonviral nature, is the opportunity to develop receptor-ligand combinations that target specific cell types. Studies have combined this technique with the two other abovementioned vector systems: the coupling of a receptor-targeted ligand to either a virus vector or a cationic liposome proved successful in delivering genetic material to human airway epithelial cells.[240,241]

1.13 Mouse models for CF disease

After the cloning and characterization of the mouse gene equivalent to the human *CFTR* gene,[242] mouse models could be generated to assist CF research. These murine models are of great importance to further investigate

the basic pathophysiology of CF disease and evaluate novel therapies. Coding regions of the mouse *cftr* gene showed significant sequence homology to human CFTR cDNA, particularly in the NBDs, the R domain and the potential PKA and PKC phosphorylation sites. This suggests that these sites are functionally important in both species and that cftr function in mice may be similar to its function in humans. The first mouse models were generated by disrupting the *cftr* gene in embryonic stem cells, either by creating an in-frame stop-codon in exon 10, i.e. targeted disruption,[243,244] or by introducing a duplication of part of the DNA sequence in the *cftr* gene, i.e. targeted insertional mutagenesis.[245,246] The first two models are true *cftr* knockout mice in which no cftr mRNA or protein is produced. These mice show failure to thrive, intestinal obstruction similar to meconium ileus, alterations in mucus and serous glands in the bowel, and gall bladder disease. In contrast, the mouse model created by insertional mutagenesis thrives normally and shows no clinical disease symptoms. This is presumably due to some alternative splicing producing wild-type cftr mRNA. Indeed, low levels of RNA (~10% of wild-type levels) were found by PCR experiments, resulting in a Cl^- channel rest function around 40% of controls, thereby illustrating that low levels of wild-type cftr can influence the phenotype prominently.[245]
Gross macroscopic and microscopic pathological changes are seen in the intestinal tract of the *cftr* $^{-/-}$ mice.[243,244] Obstructions were found in both small and large intestine with distended areas proximal to obstruction. In the small intestine crypts and villi are completely destroyed in many cases, and in the colon the mucous glands are dilated with consequent flattening of the epithelial surface.[243] Potential difference measurements in intestinal tissues showed reduced baseline values in all areas investigated, like jejunum, caecum, and colon, and impaired responses to agonists of the cAMP-mediated chloride secretory pathway.[245,247]
Only little pathological changes were seen in *cftr* $^{-/-}$ airways, namely dilatation of ducts of the serous glands in the nasal mucosa and an increased number of goblet cells in the proximal airways of the *cftr* $^{-/-}$ mice. However, no microscopic pathological lesions were evident,[248] pathological accumulation of mucus or pulmonary inflammation and infection were not observed in the CF mice,[243,244] and their pulmonary functions and mechanical properties of their lungs were normal.[248] Electrophysiologically, *cftr* $^{-/-}$ airway epithelial cells showed no cAMP-mediated chloride response, however ATP induced chloride secretion was preserved.[247] Baseline PDs in cultured murine nasal epithelial cells were not different,[247] however, *in vivo* measurement showed a more negative baseline nasal PD in CF animals compared to controls.[245] In summary, these *cftr* $^{-/-}$ mice do not develop severe pulmonary disease spontaneously. Contrastingly, the majority of cystic fibrosis patients suffer from progressive lung disease with chronic

pulmonary infection mainly caused by *Staphylococcus aureus, Haemophilus influenzae, Pseudomonas aeruginosa,* and *Burkholderia cepacia.* Animal models would largely facilitate the investigations into the pathogenesis of this CF lung disease. Therefore, studies have been performed in which CF mice were challenged with bacteria to incite lung infection. In one study, aerosols containing *Staphylococcus aureus* or *Burkholderia cepacia* showed a higher susceptibility in the CF mice to develop infection.[249] Other groups showed increased morbidity and mortality in CF mice infected by *Pseudomonas aeruginosa,* compared to disease severity in normal animals, due to an excessive inflammatory response that fails to kill the bacteria.[250] In addition, the pulmonary clearance of *P. aeruginosa* and the degree of inflammation was negatively influenced by malnutrition.[251] In an inbred congenic *cftr* knockout strain that was generated by backcrossing outbred $Cftr^{m1UNC}$ mice into a C57BL/6J background, early onset of progressive pulmonary disease develops spontaneously.[252] The most prominent abnormalities are the ineffective mucociliary clearance with accumulation of thick material, dilatation of acini, and interstitial thickening of the parenchyma. However, no chronic colonization with bacterial pathogens, as is associated with human CF lung disease, was observed naturally in this mouse model.[252] The administration of *Pseudomonas aeruginosa* to the airways of these animals, showed similar results as the other knockout models, namely exacerbation of lung infection, increased mortality and impaired bacterial clearance compared to control littermates.[253]

In the pancreas of $cftr^{-/-}$ mice little pathological changes were seen,[243,245] although some blockage of pancreatic ducts was seen in a few of the $cftr^{-/-}$ mice generated by Ratcliff et al.[244] Investigations into pancreatic composition of $cftr^{-/-}$ mice, however, showed reduced pancreatic weight accompanied by reduced protein content.[254] Levels of pancreatic enzymes, especially amylase and lipase, were lower compared to control littermates. Yet, it is not certain whether these changes are primary to CF disease, or due to secondary effects of malnutrition as has been seen in malnourished rats.[255] In addition, a recent study in the cAMP- and Ca^{2+}-mediated amylase secretion in isolated pancreatic acini from $cftr^{-/-}$ mice, showed no reduced responses compared to control mice.[256] The observation that exocrine pancreata of CF mice show relatively few pathological changes compared to humans, may indicate that cftr is less important in maturational development and maintaining ductal function of the pancreas, in mice. Moreover, the pancreatic ducts contain an Ca^{2+}-activated alternative secretory pathway that may compensate for the lack of cftr and protects the $cftr^{-/-}$ mouse from pancreatic abnormalities.[257]

No overt macroscopic abnormalities were seen in the reproductive tracts of female and male $cftr^{-/-}$ mice, and overall they seemed fertile. However, it is not certain if some of these mice may be subfertile, since incidentally

breeding pairs consisting of two *cftr* $^{-/-}$ mice did not produce litters within 3 weeks after the couple was formed (own unpublished observations).
Subsequent to the knockout CF mouse model, murine models carrying the ΔF508 mutation have been generated.[198,258,259] These dF/dF mice showed reduced mortality compared to the *cftr* $^{-/-}$ mice. Phenotypically, they appeared less affected than the knockout mice. Although some of the mice were observed with bowel obstruction, consequent rupture and peritonitis,[258,259] other mice showed only dilated crypts and thick mucus accumulation without complete intestinal obstruction.[198] The electrophysiological characteristics varied in the different dF mice models. While the mouse model reported by Zeiher et al[259] showed no residual cAMP-regulated chloride conductance or secretion in nasal and jejunal epithelia, in the mouse models generated by van Doorninck et al[198] and Colledge et al,[258] residual cAMP-induced chloride conductance was detected. Moreover, in the van Doorninck et al[198] mouse model these small, subnormal, residual cAMP-regulated chloride responses were seen in both nasal epithelium, intestinal tissue, and gall bladder. This supports our observations of residual chloride conductances in respiratory and intestinal tissues of a number of ΔF508 homozygous CF patients (Chapters 4 and 5 & ref 260), and the demonstration of cAMP-mediated chloride channels in gallbladder tissues of ΔF508 homozygotes.[191]
The phenotype of a mouse model for the G551D mutation, classified to cause defective regulation of the CFTR chloride channel in humans, appeared to match that of CF patients[261] in that these mice express only small amounts of chloride channel activity and suffer from prominent but less severe intestinal disease than the CF knockout mice.[262]

1.14 Additional functions of CFTR

Cystic fibrosis is not solely characterized by alterations in the Cl^- transport. In addition, other abnormalities exist like abnormal Na^+ transport, abnormal regulation of outwardly-rectifying Cl^- channels, and increased sulfation of glycoconjugates. Therefore, it is hypothesized that CFTR has more functions than just Cl^- transport, which is supported by the facts that CF is a single gene defect[263] and on the basis of the putative protein structure CFTR belongs to the ABC transporter family that is associated with transport of substrates.[264] However, another explanation would be that changes are secondary to Cl^- transport abnormality.
One of the additional functions of CFTR that has been observed is its role in cAMP-regulated membrane recycling.[265] In normal cells cAMP inhibits endocytosis and stimulates exocytosis, however this effect of cAMP is not

seen in cells lacking CFTR expression. Since membrane recycling regulates the localization of proteins on the plasma membrane, this defect may result in different expression of membrane proteins with various consequential biochemical abnormalities.
Another function of CFTR might be its involvement in the acidification of intracellular vesicles. Certain intracellular vesicles are acidified by a proton pump with Cl^- following passively into the vesicle to maintain electroneutrality.[266] If Cl^- ions are unable to follow, proton pumping will be hampered and acidification of the vesicles disturbed. al-Awqati et al[266] have suggested that the diminished chloride conductance of the membranes of these vesicles and the consequent decreased acidification might alter the activity of pH-sensitive enzymes, which influences the last steps of the biosynthesis of glycoproteins, i.e. fucosylation, sialylation and sulfation. Several studies have demonstrated abnormal compositions of glycoproteins in CF.[267,268] The compositional and/or structural anomalies might influence protein functions, and some investigators have hypothesized on increased bacterial adhesion by these altered CF glycoproteins.[269] However, Seksek et al[270] have investigated pH in the trans-Golgi and endosomal compartments, and showed no differences between CF and control, contradicting the defective acidification hypothesis.
Several investigators report that CFTR is not only a chloride channel, but able to mediate ATP permeability as well.[271,272] However, other studies did not substantiate these observations.[273-275] This issue is important to investigate since the nucleotide triphosphates are able to induce chloride conductance by interaction with apically located extracellular receptors. Consequently, differences in ATP release and purinergic signaling might be involved in CF pathogenesis.[276]

1.15 Regulation of other ion channels by CFTR

A role for CFTR has been assigned in the regulation of the ORCC. It has been demonstrated that ORCC can only be activated by PKA and ATP when the transport function of CFTR is intact.[277] Moreover, the presence of CFTR resulted in an increased open probability and altered channel kinetics of the ORCC.[278]
Another regulatory function of CFTR is demonstrated by the inhibition of the amiloride-sensitive epithelial Na^+ channel (ENaC). In some studies, the stimulation of CFTR by cAMP-agonists appears to inhibit these ENaC channels,[279] while this inhibition of sodium transport seems absent in cells co-expressing ΔF508 CFTR instead of wild-type CFTR.[280] Therefore, it is suggested that the enhanced amiloride-sensitive Na^+ conductance in CF

respiratory and possibly intestinal epithelia is mediated by the lack of CFTR, which would inhibit this Na^+ transport.[21,281] However, in another laboratory studies in nasal epithelium did not show this cAMP-sensitivety of the ENaC channel (Dr. H.R. de Jonge, personal communications).

1.16 Alternative non-CFTR Cl^- secretory pathways

The increase in intracellular cAMP that activates cAMP-dependent protein kinases (PKA), and the activation of PKC both induce chloride secretion by phosphorylation of CFTR. In normal epithelia, these processes have been demonstrated to activate other chloride channels as well.[147,150,282,283] These chloride channels have distinct properties from CFTR. They have an outwardly rectifying current-voltage relationship, with a single-channel conductance around 30 pS at hyperpolarizing voltages and around 70 pS at depolarizing voltages, and can be activated by prolonged depolarization. These outwardly rectifying chloride channels (ORCC) can be blocked by Cl^- channel blockers like DPC and DIDS. The halide permeability is different from CFTR, i.e. $I^- > Br^- > Cl^- > F^-$. It is not evident yet if these channels are the same channels as the volume regulated channels.[284]
Another apically located non-CFTR chloride conductance can be activated by a rise in the intracellular Ca^{2+} concentration. Ca^{2+}-mediated chloride secretion was observed in human colonic epithelial cell lines,[285] and by other investigators in cultured airway epithelia or cells.[156,286] Anderson et al[156] also showed that these channels are distinct from CFTR, and that CFTR itself is not sensitive to activation by Ca^{2+}. These Ca^{2+}-dependent channels expressed in secretory epithelia show a linear I-V relationship with a single-channel conductance of 15 – 20 pS, the halide permeability sequence is $I^- > Br^- > Cl^- > F^-$, and these channels are inhibited by DIDS, DPC, and NPPB.[287-289] Anderson et al[156] detected no Ca^{2+}-dependent chloride secretion in human intestinal cell lines. In parallel, Clarke et al[290] demonstrated the presence of Ca^{2+}-stimulated Cl^- conductance in the airways, but not in intestinal stripped mucosa of their CF knockout and control mice, and therefore suggested a beneficial role for this Cl^- secretory pathway. In contrast, more recent studies on another type of CF knockout mouse, demonstrated Ca^{2+}-regulated chloride secretion in the intestinal tissues of a subgroup of their CF mice. These CF knockout mice with residual Ca^{2+}-dependent chloride conductance showed a relatively mild intestinal pathology compared to their more severely affected littermates.[291]
Studies on the presence of Ca^{2+}-mediated Cl^- conductance in intestinal biopsies from CF patients show discrepancies, in that some studies did not detect Ca^{2+}-induced chloride secretory response.[92,95] In contrast, our studies

demonstrated Ca^{2+}-initiated responses in CF intestinal tissue of compound heterozygotes,[222] and in a subgroup of ΔF508 homozygotes,[260] that were not seen in controls. This residual chloride secretion was also observed in intestinal cell lines.[292,293]

The agonists that increase intracellular calcium levels may stimulate Ca^{2+}-dependent chloride secretion either by direct action of Ca^{2+} on the transport protein, or by activating calcium/calmodulin-dependent protein kinases.[289,294-296] Extracellular application of nucleotides induces the increased Ca^{2+} concentration as well. When interacting with purinergic receptors on the apical cell membrane, these substances increase the phosphoinositide metabolism, thereby mobilizing intracellular Ca^{2+} stores and stimulate Ca^{2+}-regulated chloride channels.[297]

Swelling-induced chloride channels[289] have been detected in secretory epithelia and appear to be unaffected in CF.[284]

Several other chloride channels have been identified, like the chloride channels belonging to the ClC family of Cl^- channels,[298] of which function, localization, and impact or association with CF disease are being investigated, but are not clear for all of the channels identified at present.[299] One of the ClC channels, ClC-2, has been shown to mediate regulatory volume decrease.[300]

Table 1.2 summarizes the different Cl^- secretory pathways observed in human epithelia.

The investigation of i) alternative pathways to induce chloride secretion and ii) the presence of alternative, non-CFTR chloride channels is important since these entities are suggested as modifiers of the CF disease phenotype.

Table 1.2 *Regulation of Cl^- secretion in epithelial organs of normal and CF tissues*

	Normal	CF	References
Sweat gland coil	cAMP	Defective	73,77,301
	Ca^{2+}	Ca^{2+}	6,73,77,290
	not by PKC	not by PKC	77
Sweat gland duct	cAMP	defective	302-304
Respiratory tract	cAMP	defective	150,156,282,286,305-307
	Ca^{2+}	Ca^{2+}	286,294,296,308,310
	PKC	defective	150,286,309
Intestinal tract	cAMP	defective	91,92,94,95,311
	Ca^{2+}	Ca^{2+}	293,312
			*91,*94,*260
		defective	47,92,95,311
	PKC	PKC	*47,*94,*260
	cGMP		168

NOTE. *In these studies, the indicated conductance pathway is present in CF epithelia, however, in subnormal amounts compared to control tissues.

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Scope of this thesis

Cystic fibrosis (CF) is a disease with a highly variable phenotype even among patients with identical genotype for the disease causing mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The basic defect that is produced by mutations in this gene, is aberrant or absent chloride transport in exocrine epithelial tissues. Although tremendous discoveries have been made in the past decades on the pathophysiology of the disease, substantial gaps persist in our knowledge. The European CF Twin and Sibling Study has been initiated to extend the knowledge on the relative impact of factors influencing CF phenotype. This thesis focuses predominantly on this Study, in particular on the effect of residual chloride membrane permeability on CF phenotype, and the importance of genetic background in expressing this residual chloride conductance. For this purpose, European monozygous and dizygous twins and siblings, homozygous for the most common *CFTR* gene lesion ΔF508, were recruited (Chapter 3). Studying affected patient pairs is the classical method to assess the relative impact of genetic and environmental factors in a monogenic disease like cystic fibrosis. In such an approach, individuals with extreme phenotypes are expected to be most informative on associations between genotype and phenotype. Therefore, we investigated the twin and sibling pairs belonging to one of the following three classes: discordant pairs, involving one mildly affected and one severely affected sibling; concordant mild pairs, composed of two siblings with mild disease; concordant severe pairs, in which both siblings are severely affected. By investigating monozygous and dizygous sibling pairs homozygous for the major disease causing lesion, ΔF508, the impact of genetics and epigenetic factors on disease phenotype can be assessed.
The presence of residual chloride secretion appeared relatively variable in epithelial tissues of different CF patients. Thus, it has been detected in patients with diverse genotypes, like ΔF508 homozygotes but also in patients carrying stop-codon mutations. Both these CF genotypes are considered to result in the absence of CFTR proteins in the apical plasma membrane, causing the lack of chloride channel function at this site. However, the contrasting observation of chloride secretory responses in these individuals, points to the presence of chloride conductance(s). The fact that certain stop-codon patients also exhibited these responses, in which patients CFTR chloride channels can certainly not be present, suggests the existence of alternative, non-CFTR-mediated chloride conductance(s) in their epithelial tissues.
The objectives of this thesis are to investigate the frequency and amount of residual chloride secretion in intestinal tissues of CF individuals, and

segregate the different pathways of chloride secretion (Chapter 4). This is achieved by using the intestinal current measurements on rectal suction biopsies, which challenges chloride secretion through CFTR chloride channels by addition of cAMP-agonists, and activates possible alternative, non-CFTR chloride conductances by Ca^{2+}-agonists.
In addition, this thesis aims to assess the presence, frequency and origin of residual chloride conductance in the nasal epithelium by the method of nasal potential difference measurement (Chapter 2 and 5).
When ICM and NPD results are obtained, expression and origin of residual chloride secretion in intestinal tissue can be judged against expression and origin in the respiratory tissue (Chapter 5). Moreover, the impact of these electrophysiological characteristics on respective phenotypes in these organs can be addressed.
A further objective of this thesis is to evaluate the influence of genetic background versus epigenetics on the expression of the various chloride conductances, by comparing the concordance within monozygous twins to that within dizygous sibling pairs (Chapter 4 and 5).
The association between genotypes and the expression of residual chloride conductance in CF patients that are not ΔF508 homozygous, but have nonsense, compound heterozygous, or mild genotypes, are reported in Chapter 6.
The heterogeneity of the CF phenotype is illustrated by two atypical presentations of CF disease in Chapter 7.

Chapter 2

Methods of Nasal Potential Difference and Intestinal Current Measurement

Electrophysiological characterization of respiratory and intestinal tissues
Electrophysiological techniques as Ussing chamber measurements on intestinal biopsies and nasal potential difference measurements principally determine the conductance properties for sodium and chloride ions of the specific tissues. In early reports, typical CF patients with severe phenotypes diagnosed by abnormal sweat tests, presented with clearly abnormal ion transport characteristics in the epithelia most drastically affected in CF.[1-4] In these studies, the basal potential difference (PD) of the respiratory tissue and its reaction to amiloride, and to application of a chloride free solution were abnormal,[1,2] while in jejunal and rectal tissues secretagogues like acetylcholine and prostaglandin were unable to induce chloride secretion.[3-5] Both these techniques have developed into methods that are not only used for diagnostic purposes,[2,6-11] but are widely applied for scientific investigations to elucidate the relationship and mechanisms between the basic ion transport defect and the clinical symptoms in CF disease (Chapter 5 & refs 12-14).

Nasal potential difference measurement (NPD)
The set-up to measure the nasal potential difference is depicted in Figure 2.1. The potential difference across the nasal epithelium is measured by a catheter positioned against the inferior turbinate in the nose, with a subcutaneous needle in the forearm functioning as reference electrode.

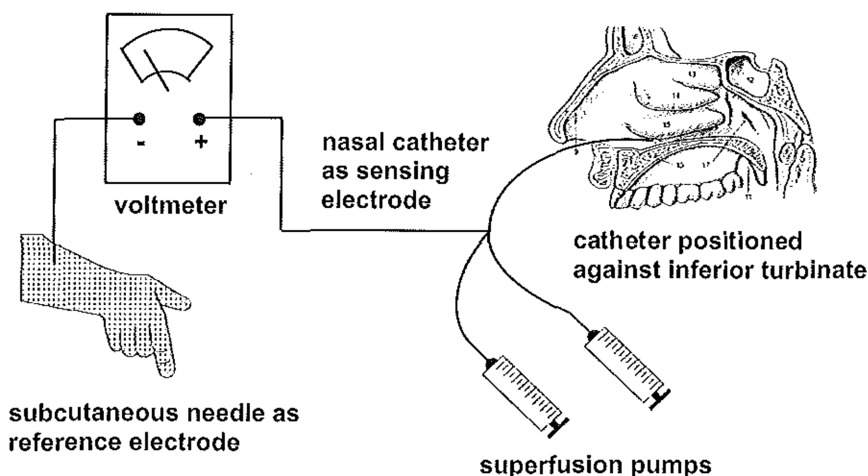

Figure 2.1 *Set-up of the nasal potential difference measurement.*

Both the exploring catheter and the subcutaneous needle are connected to a high-impedance voltmeter by Ag/AgCl electrodes. The exploring bridge consists of different PE-50 tubings to allow the independent superfusion of different solutions.
Firstly, measurement of the basal potential difference across the nasal epithelium occurs by superfusion of the epithelium with a physiological salt solution (Figure 2.2a).

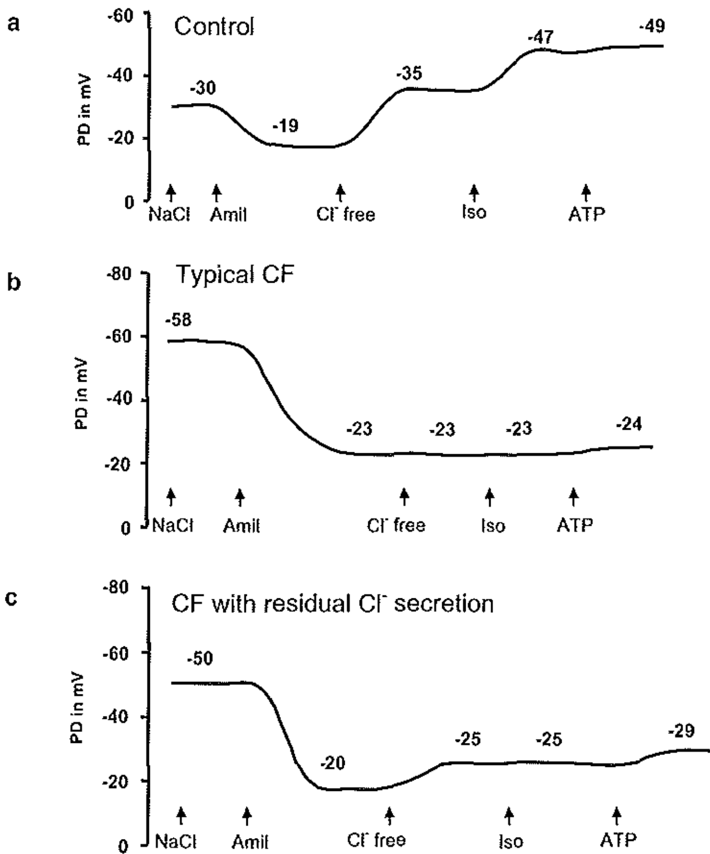

Figure 2.2 *Tracings of nasal potential difference measurements for a control person (**a**), with large responses to the Cl-free solution (Cl–free), and isoprenaline (Iso); for a typical CF patient (**b**), with no significant responses to the Cl-free solution or isoprenaline; for a CF patient exhibiting residual Cl- secretion (**c**), with a small response to the Cl-free solution, but no response to isoprenaline. Note that in these cases the ATP responses are not distinguishing CF patients from control. Amil: amiloride.*

This luminal basal PD is negative compared to the subcutaneous reference electrode. Thereafter, amiloride is applied which inhibits the Na^+ transport by the epithelial ENaC channel and consequently makes the PD less negative. Chloride conductances are identified by the subsequent addition of a Cl^--free solution, isoprenaline, and ATP, all in the presence of amiloride. The Cl^--free solution identifies the spontaneous chloride conductance, which is generated by chloride channels that are already in the opened state. In non-CF individuals the PD becomes more negative. With the β-adrenergic agonist isoprenaline, cAMP-mediated chloride channels are stimulated, and like the chloride free solution generates a more negative PD in non-CF individuals. Extracellular ATP interacts with the purinergic P_{2U}-receptors in the apical membrane with one of the consequences being an increased phosphoinositide metabolism and hence mobilization of intracellular calcium,[12] thereby inducing alternative Ca^{2+}-dependent chloride conductances. In control tissues ATP responses of variable magnitudes were observed (Table 2.1).

Table 2.1 *NPD responses for CF versus controls (mean ± SD)*

	CF patients		Range	Controls		Range
	PD or ΔPD [mV]	*n*		PD or ΔPD [mV]	*n*	
Investigated persons		98			24	
No cooperation		6			-	
(Chronic) rhinitis		18			-	
Basal PD	-56.0 ± 9.8	74	-76 – -36	-24.4 ± 10.8	24	-47 – -8
Amiloride	27.9 ± 8.6	74 (100%)	8 – 53	10.0 ± 5.8	24	3 – 29
Gluconate (Cl^--free)	-6.0 ± 2.9	22 (30%)	-12 – -1	-15.0 ± 9.6	24	-31 – 0
Isoprenaline	-2.6 ± 1.3	7 (9%)	-5 – -1	-7.8 ± 3.6	24	-17 – -1
ATP	-4.4 ± 2.7	55 (74%)	-15 – -1	-0.6 ± 3.0	24	-8 – 8

NPD tracings of non-CF

In Figure 2.2b the NPD tracing of a typical CF patient is shown. Although there is overlap, the basal potential difference of CF individuals is more negative compared to non-CF. While the mean basal PD of controls is around –25 mV, in CF the mean value lies around –55 mV (Table 2.1). Compared to controls, amiloride inhibits the PD more extensively in CF patients. The chloride free solution and addition of isoprenaline exert no effect in the severe CF condition. CF patients may present with a large ATP response

compared to controls, though this response varies considerably between CF patients.
The NPD tracing of a CF patient with residual chloride conductance is seen in Figure 2.2c. This individual shows a response to isoprenaline pointing to the presence of chloride membrane conductance. Although this is not seen in this particular patient, some CF patients might exhibit a small response to isoprenaline. The mean values for the NPD responses for a group of CF patients versus controls are depicted in Table 2.1. While the mean values differ between CF and non-CF, some responses show overlap between the two groups. We have demonstrated that the diagnosis of CF can be more easily and reliably made by the interpretation of the complete set of responses including the basal PD, rather than on any of the individual responses.[9]

Intestinal current measurement (ICM)
In the intestinal current measurement, a rectal suction biopsy is mounted in an Ussing chamber and the voltage across the rectal tissue is clamped to 0 mV. This enables the measurement of changes in ion currents, expressed as short-circuit currents (I_{sc}) in reaction to the application of specific drugs. After the stabilization of the short-circuit current, amiloride is applied to inhibit electrogenic, ENaC-mediated Na^+ transport (Figure 2.3a), which reduces the short-circuit current. Thereafter, indomethacin addition inhibits formation of endogenous prostaglandin synthesis and reduces cAMP formation. In some control biopsies but not in others, a reduction in I_{sc} is seen in response to indomethacin. Subsequently, carbachol is applied which causes chloride secretion by inducing intracellular calcium mobilization and activating protein kinase C. 8-Br-cAMP and forskolin is then used to stimulate the cAMP-regulated chloride secretion, which generates a sustained increase in I_{sc}. The tissue is then incubated with 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), which inhibits non-CFTR chloride channels. Histamine is added 10 minutes after DIDS to exert a similar action to carbachol, namely activation of the Ca^{2+}/PKC pathway, and in controls histamine causes a similar transient chloride secretory peak as seen in response to carbachol (Figure 2.3a). Carbachol and histamine interact with muscarinic[15] and H_1 receptors,[16] respectively, which are located on enterocytes. This causes increased cytosolic calcium levels, thus inducing chloride secretion. Both secretagogues increase intracellular Ca^{2+} levels by initiating the metabolism of phosphoinositides with the consequent formation of inositol (1,4,5) triphosphate, which releases calcium from intracellular stores.[17-19] Some differences between the effects of carbachol and histamine on the inositol phosphates metabolism have been identified, e.g. in the duration of the

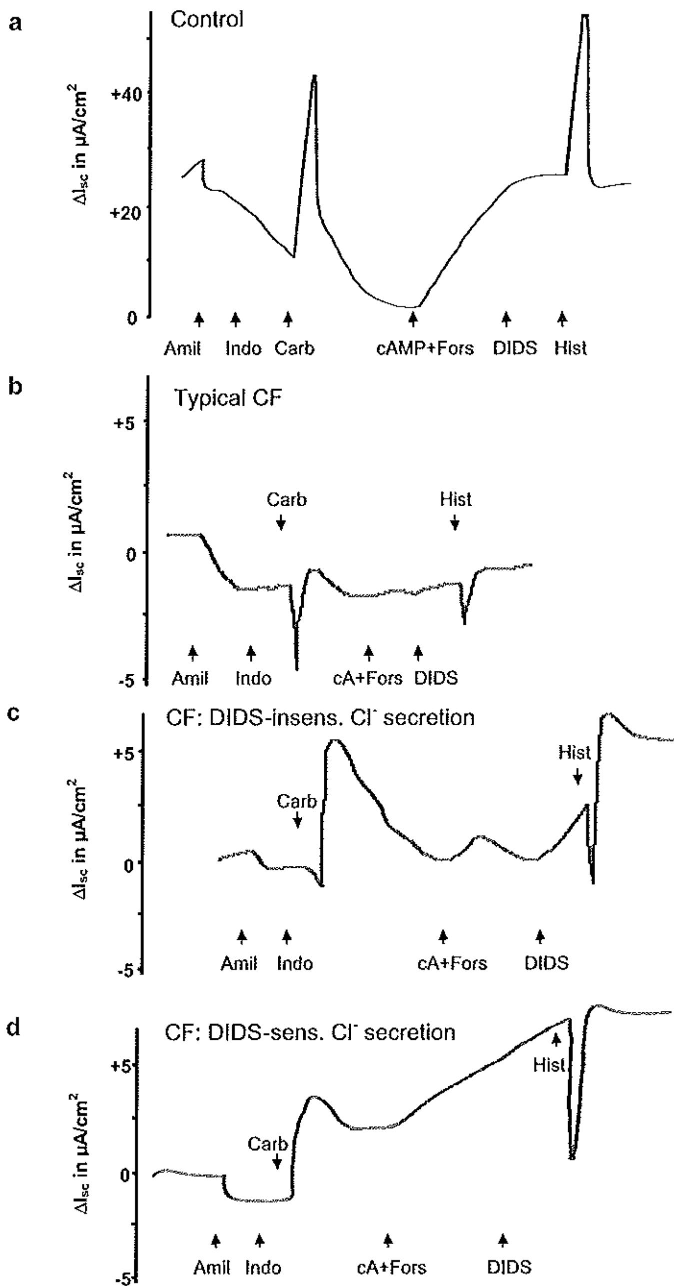

Figure 2.3 *Tracings of intestinal current measurements for a control person* ***(a)****, a typical CF patient* ***(b)****, a CF patient with residual Cl⁻ secretion that is insensitive (insens.) to DIDS* ***(c)****, and a CF patient with DIDS-sensitive (sens.) Cl⁻ secretion* ***(d)****.*

increases in concentration of inositol (1,3,4) triphosphate and inositol tetrakisphosphate,[18] however, in control non-CF tissues our ICM studies show chloride secretory responses of similar magnitudes when carbachol or histamine are applied. This is also found when the rectal tissue is incubated with DIDS after carbachol, and subsequently histamine is added. Since in control tissues DIDS-sensitive chloride conductances do not significantly contribute to PD or chloride currents, the carbachol response before the incubation with DIDS and the histamine response after the addition of DIDS, remain of similar magnitude (Figure 2.3a). With this knowledge, we introduced this protocol in ICM measurements of CF patients. By comparing carbachol and histamine responses in CF tissues, and calculating the difference we were able to segregate the presence of DIDS-sensitive non-CFTR chloride conductances from DIDS-insensitive CFTR chloride channels. The ICM tracing of a typical CF patient is seen in Figure 2.3b. Carbachol and histamine cause reversed responses in the opposite direction to the responses seen in control tissues, while cAMP-agonists do not induce any response. The reversed responses upon addition of carbachol and histamine have been investigated by several researchers, and are most likely due to apical K^+ efflux stimulated by carbachol and histamine, i.e. an increase in intracellular calcium concentration.[21]
With our protocol we were able to identify CF patients with intestinal residual chloride currents (Figures 2.3c and d). In a CF patient with DIDS-insensitive secretion, the chloride currents induced by carbachol are also induced by histamine and are not inhibited by the incubation of the tissue with DIDS. DIDS-sensitive residual chloride conductance is recognized by an ICM pattern as in Figure 2.3d, with a chloride secretory current in reaction to carbachol, which is inhibited by the incubation of the tissue with DIDS, which results in a reversed histamine response without any chloride secretory current. The responses to carbachol and histamine in CF individuals appear to consist of two components. One lumen-positive current that is most likely caused by the apical K^+ efflux through Ca^{2+}-sensitive K^+ channels,[20] since this I_{sc} response can be inhibited for about 70% by bariumchloride.[21] The second component is a lumen-negative current, caused by apical chloride secretion.[10,13,14] In ICM measurements of non-CF individuals the apical K^+ efflux component in reaction to carbachol and histamine is not observed (Figure 2.3a), since it is masked by the much larger Cl^- conductance which results in a great unidirectional, transient, lumen-negative chloride secretory response. In CF patients with a two-directional response, e.g. the histamine response in Figure 2.3c, the net response is quantified by the summation of the negative reversed response and the positive chloride secretory response.

When we compare the mean values for a group of controls versus a group of CF individuals, we see that none of the values of the responses to carbachol, cAMP, and histamine overlap between the two groups (Table 2.2).

Table 2.2 *ICM responses for CF versus controls (mean ± SD)*

	CF patients		Range	Controls		Range
	Δ I_{SC} [μA/cm²]	*n*		Δ I_{SC} [μA/cm²]	*n*	
Investigated persons		98				
No cooperation		6				
Technical disturbance		16				
Carbachol	-4.60 ± 7.36	76	-35.24 – 8.43	43.9 ± 18.9	103	11.9 – 110.2
cAMP	3.03 ± 2.90	67	0 – 13.28	7.1 ± 8.2	76	0.0 – 30.5
Histamine	-4.50 ± 5.48	59	-21.84 – 5.10	38.5 ± 19.0	69	8.3 – 80.0

In conclusion, when utilizing these protocols for NPD and ICM measurements in CF disease, significant distinction can be made between the electrophysiological characteristics of the respiratory and intestinal tissues of non-CF versus CF individuals, and can aid and facilitate the diagnostic process. Moreover, since chloride conductances of different origin can be segregated with these electrophysiological methods, ICM and NPD techniques can be used to further investigate the basic defect of abnormal CFTR-mediated chloride transport, the importance of alternative non-CFTR-dependent chloride conductances, and their influence on CF disease severity.

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Chapter 3

Categories of ΔF508 homozygous cystic fibrosis twin and sibling pairs with distinct phenotypic characteristics

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Abstract

Cystic fibrosis (CF), the most common severe autosomal recessive trait among Caucasians, is caused by molecular lesions in the cystic fibrosis transmembrane conductance regulator gene (*CFTR*). The course of the multi-organ disease CF is highly variable suggesting the influence of environmental factors and/or modulating genes other than *CFTR* on the disease phenotype. To evaluate the cause of CF disease variability, the *European CF twin and sibling study* collected data on two clinical parameters most sensitive for course and prognosis of CF, i.e. wfh% (representative for the nutritional status) and FEVPerc (representative for the pulmonary status) for a cohort of 277 sibling pairs, 12 pairs of dizygous twins and 29 pairs of monozygous twins. Of these 318 CF twin and sibpairs, 114 were reported to be homozygous for the most frequent CF disease causing lesion, ΔF508. Intrapair discordance was assessed by the intrapair differences in wfh% and FEVPerc and by DELTA, a composite parameter defined by linear combination of wfh% and FEVPerc in order to describe discordance with respect to the overall disease severity. Monozygous twins had a significantly lower DELTA than dizygous twins ($P = 0.05$) indicating that CF disease severity is modulated by an inherited component besides the *CFTR* gene itself. Extreme phenotypes are considered to be more informative for the analysis of any quantitative trait. Thus, we aimed to quantify disease-severity and intrapair discordance in order to select pairs with the extreme phenotypes DIS (discordant patient pairs), CON+ (concordant and mildly affected patient pairs) and CON- (concordant and severely affected patient pairs). The algorithm reliably discriminated between pairs DIS, CON+ and CON- among the cohort of ΔF508 homozygotes. The selected pairs from these categories demonstrated non-overlapping properties for wfh%, FEVPerc and the intrapair difference of both parameters.

Introduction

Cystic fibrosis (CF) is known as the most common severe autosomal recessive disease within the Caucasian population, exhibiting an incidence of 1 in 2500 births.[1] The symptoms of the disorder are caused by an impaired function of exocrine glands in many organs, but major manifestations involve the respiratory and the gastrointestinal tracts.[1] The disease is caused by mutations in both chromosomal copies of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene.[2] The course of CF is highly variable when comparing unrelated patients with identical *CFTR* mutation genotypes,[3,4] or even CF siblings who carry the same *CFTR* alleles and share several

environmental factors, such as socioeconomic status, general living condition and therapeutic measures. This indicates the impact of factors other than the *CFTR* genotype on the CF disease phenotype. By studying affected patient pairs, the European CF Twin and Sibling Study pursues a classical approach to address the relative impact of the *CFTR* gene, other inherited factors and environmental effects on CF disease.
Approximately 70% of CF alleles in central European populations bear the same *CFTR* mutation ΔF508.[5] Consequently, half of all CF patients are homozygous for the same *CFTR* lesion which enables analysis of the disease severity in a group with a homogeneous mutation genotype in the major disease-causing gene. Due to the prevalence of one mutation genotype in a so-called monogenic disease that follows an autosomal recessive trait, CF is the only inherited disorder where a relatively large number of patient pairs can be selected who carry the same mutation genotype in the disease-causing gene. The search for disease modulating factors of CF equals an assessment of CF disease severity as a quantitative trait whereby the phenotype under investigation - the CF disease severity - assumes a continuous distribution. Under this condition, individuals with extreme phenotypes are likely to have a large number of functional alleles at most loci determining the quantitative trait and therefore, extreme phenotypes are generally considered to be most informative.[6-9] Based on the phenotype of an individual, three categories of patient pairs with extreme phenotypes can be distinguished: concordant/mildly affected patient pairs (CON+), composed of two siblings with mild disease, concordant/severely affected patient pairs (CON-), comprised of two severely diseased siblings and discordant pairs (DIS) wherein one sibling is mildly affected and the other is severely affected. With the intention to identify these most informative pairs, we aimed at a quantitative description of disease severity and intrapair discordance for CF patients. The evaluation was based on two clinical parameters most sensitive to course and prognosis of CF disease, i.e. weight expressed as weight predicted for height (wfh%) — such as to assess the nutritional status of the CF patient — and values of forced expiratory volume in 1s (FEV1) expressed as age and gender normalised parameter — such as to assess the pulmonary status of the CF patient.[10] As a result, the CF disease phenotype was rated accounting for both major afflicted organs, i.e. the respiratory and the gastrointestinal tracts.

Methods

Patients and clinical parameters

CF patient pairs were enrolled from 158 CF clinics from 14 European countries. With a one-page evaluation form, information on gender, *CFTR* genotype, actual weight, height and forced expiratory volume in 1 s (FEV1) and the zygosity status of twin pairs was enquired. From these data, two clinical parameters most sensitive to course and prognosis[10] were calculated: nutritional status was assessed by weight predicted for height (wfh%) on the basis of age and gender corrected centiles for weight and height by Prader et al.[11]. Pulmonary status was assessed by FEV1%pred which are predicted values referring to the non-CF population based on the data by Knudson et al.[12]

Among CF patients, FEV1%pred declines with age[13] (Figure 3.1b) as expected for this progressive lung disease. To correct for the CF specific age decline of FEV1%pred, age corrected centiles for the CF population for FEV1%pred, called FEVPerc, were calculated based on the European CF registry (ERCF) report of 1996[14] that compiles lung function data of FEV1%pred from 25667 CF patients from Austria, Canada, Denmark, France, Germany, Ireland, The Netherlands, Sweden, United Kingdom and USA.

Consistent with data from other cross-sectional studies, the centiles were age-independent for wfh% [15] (Figure 3.1a) and for FEVPerc (Figure 3.1c) within the cohort of CF twin and sibling pairs.

Evaluation of mono- and dizygosity status of CF twins

If DNA was available, the zygosity status of twin pairs was assessed to confirm the information provided by the CF center using the AmpF*L*STR Profiler Plus™ typing kit on an ABI Prism 377 (Perkin Elmer Applied Biosystems)[16] or by oligonucleotide fingerprinting of simple repeats applying in situ gel hybridisation of *Mbo*I or *Hinf*I genomic digests.[17]

Definition of composite parameters

To assess the overall CF disease severity and the intrapair discordance, the two clinical parameters describing a patient's nutritional and pulmonary status, i.e. wfh% and FEVPerc, were combined. Rank numbers x_i for wfh% and y_i for FEVPerc were assigned within the complete patient cohort whereby a rank number of 1 delineated the most severely affected state. The disease severity of patient i was characterised by the distance form origin (DfO) in the plot of x_i versus y_i (Figure 3.2). The intrapair discordance was quantified by the distance between two data points representing two patients i and j of a pair

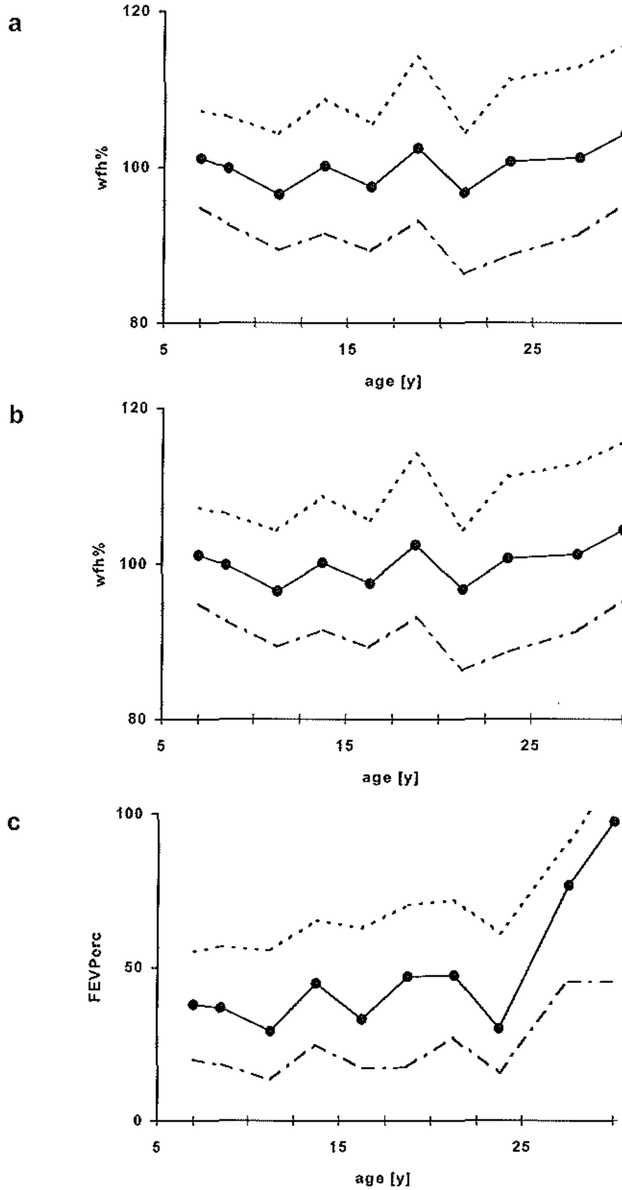

Figure 3.1 *Age dependence of wfh% (**a**), FEV1%pred (**b**) and FEVPerc (**c**). The solid line indicates the median, the dotted lines the inner quartiles. The [number of patients] within each age class are: <7y [36], 7y-10y [75], 10y-12.5y [66], 12.5y-15y [75], 15y-17.5y [84], 17.5y-20y [76], 20y-22.5y [48], 22.5y-25y [50], 25y-30y [58], >30y [68].*

within the same diagram (DELTA). Thus, disease severity and intrapair discordance were defined by:

$$DfO = \sqrt{x_i^2 + y_i^2} \quad (3.1)$$

$$DELTA = \sqrt{(x_i - x_j)^2 + (y_i - y_j)^2} \quad (3.2)$$

Analysis of intrapair rank number difference (IRND) distributions
Intrapair similarity of CF twins and siblings was characterised by comparison of the patient pair cohort to a set of unrelated couples. To assess the intrapair similarity of the complete cohort, the distribution of intrapair rank number differences (IRND) was analysed.
The IRND distribution expected for unrelated couples was derived as follows: for a cohort of n individuals, or n/2 pairs, IRNDs between 1 and (n-1) are possible. The minimal IRND of m = 1 is obtained if two individuals from a couple occupy rank numbers (n-1) and n. (n-1) rank number combinations of two individuals result in an IRND of 1, but there is only one possibility to obtain the maximal IRND of m = (n-1), by occupying rank numbers 1 and n, respectively. In general, the probability f_m for any IRND m in a cohort of n individuals is given by the normalised expression

$$f_m = \frac{n-m}{\sum_{m=1}^{n-1}(n-m)} = \frac{2}{n} \cdot \frac{n-m}{n-1} \quad ; \sum_{m=1}^{n-1} f_m = 1 \quad (3.3)$$

To test whether the IRND distribution observed among the CF twins and siblings differed from a random IRND distribution, classes of IRNDs

$\sum_{m=i}^{j} f_m$ were defined whereby the boundaries were chosen as such that each

class was occupied with the same probability in a random IRND distribution:

$\sum_{m=i}^{j} f_m = const$. The size of the classes was set to an expectancy value

$E = n \cdot \sum_{m=i}^{j} f_m$ of E = 20, 30 or 50 couples per class.

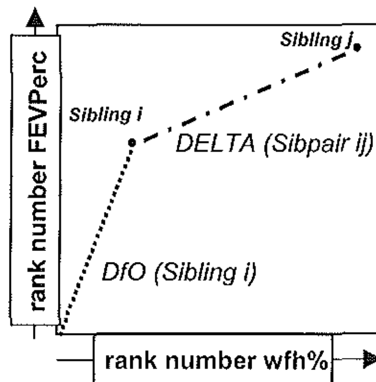

Figure 3.2 *Definition of composite parameters. The two clinical parameters, wfh% and FEVPerc, describing the patient's nutritional and pulmonary status, were combined as a measure of the patient's overall disease severity. Rank numbers for wfh% and for FEVPerc were assigned to all patients. The disease severity of a patient was characterised as distance form origin (DfO) in the plot of the patient's rank number for FEVPerc versus the rank number for wfh%. The intrapair discordance was quantified through the distance between two data points representing two patients i and j of a pair within the same diagram (DELTA). For the set of 318 pairs, maximal values of DfO and DELTA as defined by Equations (3.1) and (3.2) are 899 and 898, respectively.*

For the analysis of the cohort of all CF twins and sibs, rank numbers were assigned to wfh% for 467 pairs (n = 934, corresponding to 24 (E = 20), 16 (E = 30) and 9 (E = 50) IRND classes) and to FEVPerc for 318 pairs (n= 648, corresponding to 16 (E = 20), 11 (E = 30) and 7 (E = 50) IRND classes). Within the cohort of ΔF508 homozygous twins and siblings, rank numbers for wfh% and FEVPerc were assigned to 114 pairs (n= 228, corresponding to 6 (E = 20), 4 (E = 30) and 2 (E = 50) IRND classes). Observed occupancy of IRND classes was compared to expectancy values by χ^2 statistics.[18]

Comparison of disease severity and intrapair discordance

Unless stated otherwise in the results section, all comparisons were carried out using the non-parametric Mann-Whitney rank test.[19]

***Table 3.1** Genotype and gender of 318 CF twin and sibling pairs*

	Sibpairs	Twins	
		DZ	MZ
mm	75	3	14
ff	72	2	15
mf	130	7	0
non-ΔF508/non-ΔF508	88	3	2
non-ΔF508/ΔF508	94	5	12
ΔF508/ΔF508	95	4	15
total:	277	12	29

NOTE. non-ΔF508: all CFTR alleles other than ΔF508, including *CFTR* alleles with unknown mutation, m: male, f: female.

***Table 3.2a** Distribution of age at day of evaluation of CF twin pairs (median [inner quartiles; range])*

monozygous	(29 pairs)	14.9 years [8.8 – 21.8; 6.8 – 37.2]	*n.s.*
dizygous	(12 pairs)	14.6 years [11.0 – 17.9; 6.1 – 31.3]	
non-ΔF508/ΔF508	(17 pairs)	15.1 years [12.2 – 22.9; 6.8 - 37.2]	*n.s.*
ΔF508/ΔF508	(19 pairs)	12.7 years [8.8 – 17.3; 6.1 - 30.3]	

***Table 3.2b** Distribution of age at day of evaluation of 277 CF sibling pairs (median [inner quartiles; range])*

all siblings	(277 pairs)	17.2 [12.1 – 23.5 ; 5.9 – 59.1]
ΔF508/ΔF508	(95 pairs)	16.9 [11.2 – 20.3 ; 6.0 – 38.1]
P = 0.005		

***Table 3.3** Home country of CF twin pairs*

Number of pairs (%) recruited from:	FRA	GB&EIRE	GER	ITA	OTHER
monozygous	4 (14%)	5 (17%)	7 (24%)	7 (24%)	6 (17%)
dizygous	2 (17%)	2 (17%)	3 (25%)	3 (25%)	2 (17%)
non-ΔF508/ΔF508	3 (18%)	4 (24%)	2 (12%)	6 (35%)	2 (12%)
ΔF508/ΔF508	3 (16%)	3 (16%)	5 (26%)	2 (11%)	6 (32%)

NOTE. Number of pairs from FRA: France, GB&EIRE: Great Britain and Eire, GER: Germany and ITA: Italy, respectively, OTHER: summarizes number of pairs recruited from The Netherlands, Sweden, Poland, Austria and Switzerland.

Results

Clinical data on 318 CF twin and sibling pairs
Data on wfh% was obtained for both patients in 467 pairs. Complete clinical data, i.e. wfh% and FEVPerc, could be calculated for 318 CF patient pairs (Tables 3.1, 3.2b and 3.5). 114 pairs thereof were reported to be ΔF508 homozygous. FEVPerc was lower in our patient pair cohort than expected from the ERCF report (Table 3.5a and Figure 3.1c). This systematic shift reflects different modes of data collection and coincides with the well-known difference between best and average annual values of FEV1%pred that could also be demonstrated by the average 8.2% difference between best and mean annual FEV1%pred value for
the patient population at the CF clinic Hannover (646 entries). The EUCFR registry recorded the best FEV1%pred within a two-year-period, whereas in our study the questionnaire asked for the most recent lung function data.

Clinical data on monozygous and dizygous CF twins pairs
Zygosity status could be determined or was reliably reported by the CF center for 41 twin pairs with wfh% and FEVPerc available (Tables 3.1, 3.2a and 3.4). ΔF508 allele frequency was 0.67 which is consistent with population genetic data for central Europe.[5] Average age of DZ twins was slightly lower than that of MZ twin pairs and ΔF508 homozygous twins were younger at the day of evaluation compared to ΔF508 heterozygous twins, but the differences in age were not significant (Table 3.2a). There was no bias between MZ and DZ twins with respect to the country of origin (Table 3.3). However, while ΔF508 homozygous twins were recruited from a variety of European countries, pairs from Italy were overrepresented among ΔF508 heterozygous pairs reflecting the lower ΔF508 frequency in southern European countries[5] (Table 3.3). Comparing MZ and DZ twins, the groups were indistinguishable in wfh% but FEVPerc was significantly lower for DZ twins than for MZ twins ($P = 0.02$; Table 3.4a).
Intrapair discordance was assessed by the intrapair difference in wfh% (representative for the nutritional status), the intrapair difference in FEVPerc (representative for the pulmonary status) and DELTA (composite parameter describing discordance with respect to the overall disease severity, Figure 3.2). Regarding CF twin pairs with all *CFTR* genotypes, MZ patient pairs had a significantly lower DELTA than DZ twin pairs, but intrapair differences in wfh% and FEVPerc were comparable for MZ and DZ twins (Table 3.4b).

Table 3.4a *Disease manifestation (median [inner quartiles; range]) of CF twins*

	monozygous (58 patients)	dizygous (24 patients)	P
wfh%	98.8 [91.9 - 109.4 ; 72.0 – 136.7]	98.7 [92.6 - 109.6 ; 84.2 – 125.7]	0.43
FEVPerc	49.6 [30.6 - 74.6 ; 0.5 – 111]	28.4 [16.0 - 55.0 ; 0.1 – 114]	0.02

Table 3.4b *Intrapair discordance (median [inner quartiles; range]) of CF twins*

	monozygous (29 pairs)	dizygous (12 pairs)	P
intrapair differences in:			
wfh%	5.8 [3.0 - 9.3; 0.4 – 23.9]	6.6 [3.8 - 11.7; 0.7 – 21.0]	0.48
FEVPerc	13.8 [6.0 - 23.9; 0.0 – 69.8]	27.8 [5.6 - 49.8; 1.7 – 92.6]	0.14
composite parameter:			
DELTA	145.1 [78.2 - 213.6; 17.1 – 366.0]	179.1 [135.6 - 215.3; 70.4 – 510.1]	0.04

Table 3.5a *Disease manifestation (median [inner quartiles; range]) of CF siblings*

	all CF siblings (277 pairs)	ΔF508/ΔF508 (95 pairs)	P
wfh%	100.1 [91.0 – 109.2 ; 54.5 – 175.8]	98.7 [89.5 – 105.5; 54.5 – 145.2]	0.002
FEVPerc	43.8 [21.2 – 74.7 ; -3.0 – 120]	34.6 [16.5 – 60.6; -3.1 – 115]	< 0.0001

Table 3.5b *Intrapair discordance (median [inner quartiles; range]) of CF siblings*

	all CF siblings (277 pairs)	ΔF508/ΔF508 (95 pairs)	P
intrapair difference in:			
wfh%	11.3 [5.7 – 18.5 ; 0.1 – 61.4]	10.2 [6.1 – 15.6 ; 0.3 – 53.4]	0.21
FEVPerc	23.4 [11.8 – 41.7 ; 0.0 – 96.9]	24.1 [11.9 – 42.7 ; 0.0 – 96.9]	0.41
composite parameter:			
DELTA	244.8 [145.0 – 349.1 ; 8.1 – 771.6]	253.3 [179.0 – 347.9 ; 46.1 – 694.8]	0.13

Table 3.6 *P values of χ^2 test comparisons of IRND distributions of CF twin and sibling pair cohorts to IRND distributions expected for a cohort of random couples*

		E = 20	E = 30	E = 50
all pairs				
wfh%	(647 pairs)	$P < 0.001$	$P < 0.001$	$P < 0.001$
FEVPerc	(318 pairs)	$P < 0.001$	$P < 0.001$	$P < 0.001$
ΔF508 homozygotes				
wfh%	(114 pairs)	$0.025 < P < 0.05$	$0.025 < P < 0.05$	$0.025 < P < 0.05$
FEVPerc	(114 pairs)	$0.9 < P < 0.95$	$0.7 < P < 0.9$	$0.9 < P < 0.95$

NOTE. E: number of pairs expected within each IRND class. See methods for details

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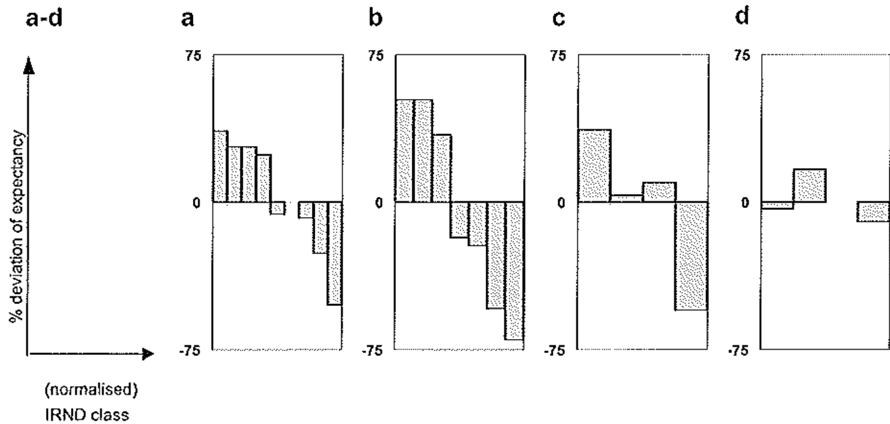


Figure 3.3 *Differences of intrapair rank number difference (IRND) distributions comparing a cohort of CF twins and siblings to a similarly sized cohort of random couples: (**a**) for wfh% and patient pairs with various CFTR genotypes (467 pairs, 9 IRND classes, 50 pairs expected within each IRND class; E = 50); (**b**) for FEVPerc and patient pairs with various CFTR genotypes (318 pairs, 7 IRND classes, 50 pairs expected within each IRND class; E = 50); (**c**) for wfh% and (**d**) FEVPerc of ΔF508 homozygous pairs (114 pairs, 4 IRND classes, 30 pairs expected within each IRND class; E = 30). Bars representing the 9, 7, 4 and 4 IRND classes in (**a-d**) are ordered according to the magnitude of the IRNDs from low IRND to high IRND. To allow comparison of data, the scale within plots (**a-d**) is normalised such as to display a 75% deviation from the expectancy value E with E=50 (**a** and **b**) and E=30 (**c** and **d**). In plots (**a**) and (**b**), +75 corresponds to an occupation of an IRND class by 88 pairs and –75 corresponds to an occupation of an IRND class by 13 pairs in contrast to the expected 50 pairs. Analogously, in plots (**c**) and (**d**) +75 corresponds to an occupation of an IRND class with 52 pairs and –75 corresponds to an occupation of an IRND class with 8 pairs in contrast to the expected 30 pairs.*

Intrapair rank number difference distribution within a cohort of CF twins and siblings

To characterise the cohort of CF twins and sibs in terms of the intrapair similarity, the distribution of intrapair rank number differences (IRND) of the patient pair cohort for wfh% and FEVperc was compared to the IRND distribution of a set of randomly assigned couples (see Eq. 3.3). The IRND distribution of the CF patient pairs differed significantly from a random IRND distribution (Table 3.6 and Figure 3.3), i.e. the average IRND was significantly smaller in CF twin and sibpairs than in unrelated couples. Likewise, the subgroup of ΔF508 homozygous twins and sibpairs was significantly more concordant in their IRND distribution of the nutritional parameter wfh%

Table 3.7a *Example for two pairs with similar high DELTA, but different intrapair difference in DfO*

	Data [rank number] of Sibling A				Data [rank number] of Sibling B			
	age	wfh%	FEVPerc	DfO	age	wfh%	FEVPerc	DfO
Example I	25y	119.5 [578]	1 [12]	578 [502]	21y	120.0 [582]	82 [526]	784 [588]
DELTA = 514								
DiffDfO = 206								
Example II	20y	103.1 [389]	75 [490]	625 [432]	16y	94.2 [206]	1 [27]	207 [115]
DELTA = 497								
DiffDfO = 418								

NOTE. The graphic representation of disease severity and intrapair discordance of these two pairs is shown in Figure 3.9l, pair 1 (example I) and Figure 3.9h, pair 7 (example II).

Table 3.7b *Example for three concordant patient pairs with similar DELTA, but different intrapair sum of DfO*

	Data [rank number] of Sibling A				Data [rank number] of Sibling B			
	age	wfh%	FEVPerc	DfO	age	wfh%	FEVPerc	DfO
Example I	8y	134.3 [618]	100 [624]	878 [634]	9y	115.9 [550]	87 [543]	772 [574]
DELTA =	105							
DiffDfO =	106							
Σ DfO =	1650							
Example II	18y	94.9 [222]	66 [446]	498 [273]	12y	98.4 [284]	79 [515]	588 [360]
DELTA =	92							
DiffDfO =	90							
Σ DfO =	1086							
Example III	14y	92.2 [176]	14 [121]	281 [107]	6 y	93.9 [197]	2 [33]	199 [112]
DELTA =	90							
DiffDfO =	14							
Σ DfO =	412							

NOTE. The graphic representation of disease severity and intrapair discordance of these three pairs is shown in Figure 3.9d, pair 1 (example I), Figure 3.9m, pair 3 (example II) and Figure 3.9a, pair 5 (example III). DELTA and DfO: composite parameters as defined in Figure 3.2. DiffDfO: intrapair difference in DfO. Σ DfO: intrapair sum of DfO. Rank numbers were assigned to wfh%, FEVPerc and DfO within the cohort of 318 patient pairs.

(Table 3.6 and Figure 3.3). In contrast, the IRND distribution of FEVPerc in the ΔF508 homozygous pairs was indistinguishable from that of randomly assigned couples. The ranges of intrapair differences in wfh% or FEVPerc were similar in the whole cohort of CF patient pairs and the ΔF508 homozygous subgroup (Table 3.5b).

Properties of discordant CF patient pairs
The age-independent clinical parameters wfh% and FEVPerc were linearly combined to define the composite parameters DfO (Eq. 3.1 and Figure 3.2) as a measure of the overall disease severity based on an equal weight for both, the anthropometric and the lung function parameter. The parameter DELTA defined as the absolute distance between the DfO values of a twin or sibpair (Eq. 3.2) was taken as the indicator of intrapair difference of disease severity (Figure 3.2). As shown in Figure 3.4, the value of DELTA did not correlate with the intrapair age difference of sibpairs. Discordant pairs, indicated by high values of DELTA, were observed at similar frequency in sibpairs with high and low age differences.

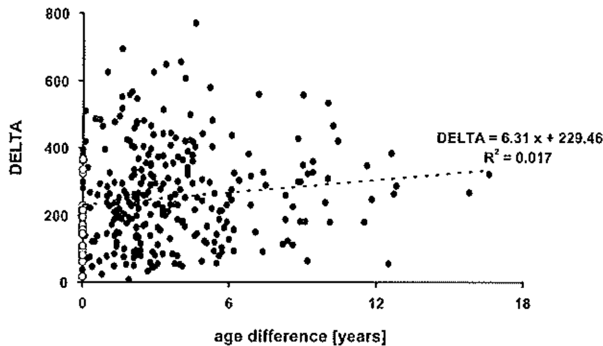


Figure 3.4 *Composite parameter DELTA and intrapair age difference. DELTA was defined as indicated in Figure 3.2 based on rank numbers for wfh% and FEVPerc to quantify intrapair discordance. Closed circles: dizygous CF patient pairs. Open circles: monozygous CF twin pairs.*

For 318 patient pairs carrying various *CFTR* genotypes, the maximum rank number that can be assigned to wfh% or FEVPerc values is by definition 636. There was no correlation between the intrapair rank number differences for wfh% and FEVPerc (data not shown): patient pairs were observed to be discordant for both parameters, or only discordant for wfh%, but concordant for FEVPerc and vice versa. Among monozygous twins, the highest value for the composite parameter DELTA was 366. Sixty-four dizygous patient pairs had values for DELTA>366. These extremely discordant pairs could be compiled into three cohorts as indicated in Figure 3.5: 15 pairs were concordant in wfh% but discordant in FEVPerc (cohort I), 25 pairs were concordant in FEVPerc but

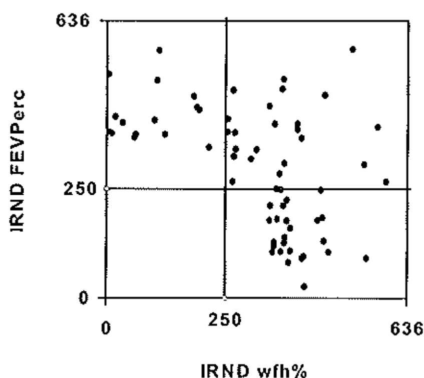


Figure 3.5 *Characteristics of the most discordant patient pairs.*
Within this plot, 64 pairs with values of DELTA = 366 or higher are divided into three cohorts with the following characteristics:

	I upper left	II lower right	III upper right
IRND wfh%	< 250	> 250	> 250
IRND FEVPerc	> 250	< 250	> 250
No of pairs	15	25	24
thereof ΔF508 homozygous	7 (47%)	7 (28%)	7 (29%)
ΔF508 heterozygous	5	9	12
Average values for:			
age [y]	20.2	17.7	21.5
wfh%	104	102	99
FEVPerc	45	50	41
DELTA	441	429	537
Average values for intrapair differences in:			
age [y]	2.7	2.8	2.6
wfh%	7	28	29
FEVPerc	71	25	67
DfO	278	556	625

discordant in wfh% (cohort II) and 24 pairs were discordant for both parameters (cohort III). These three phenotypes were neither discriminated by the patient's absolute values for age, wfh% or FEVPerc nor by the intrapair age difference (legend of Figure 3.5). There was a trend towards an overrepresentation of ΔF508 homozygotes in cohort I compared with cohorts II and III ($P = 0.15$; Fisher's exact test).[20]

The average value for DELTA was highest in cohort III and average values for intrapair difference in DfO were lower for cohort I (legend of Figure 3.5) than for cohort II and III. The intrapair difference in DfO (DiffDfO) differentiated pairs who are discordant (II, III) and who are not discordant (I) in wfh%. Defining a discordant pair (category DIS) as a pair composed of one sibling with low DfO and one sibling with high DfO, pairs from cohort I could be distinguished from pairs belonging to the category DIS by taking the intrapair difference in DfO (DiffDfO) into account. Table 3.7a displays clinical data from two ΔF508 homozygous patient pairs with similar high DELTA but different DiffDfO to illustrate their phenotypic differences.

Properties of concordant CF patient pairs

For the identification of concordant pairs two characters, i.e. the pair's concordance and their disease severity, had to be combined in order to discriminate between concordant pairs with mild phenotype and concordant pairs with severe phenotype. In Table 3.7b, data from three ΔF508 homozygous patient pairs representative for the phenotypes "concordant/mildly affected" (category CON+, example I), "concordant/moderately affected" (example II) and "concordant/severely affected" (category CON-, example III) are shown. These three pairs all have similar low values for DELTA and intrapair difference in DfO (DiffDfO) indicating their concordance. In concordant pairs, the intrapair sum of DfO (ΣDfO) is a measure of disease severity: mild: high ΣDfO with both siblings displaying wfh% and FEVPerc values above the 75th centile (example I), moderate: intermediate ΣDfO with both siblings displaying wfh% and FEVPerc values close to the 50th centile (example II) and severe: low ΣDfO with both siblings displaying wfh% and FEVPerc values below the 25th centile (example III).

Definition of rank numbers

Based on DELTA, the intrapair sum of DfO and the intrapair difference in DfO (DiffDfO), five rank numbers were calculated (Table 3.8): $DISC_{DELTA}$ defined the pair's position in the sequence of discordant pairs whereby the discordance was quantified solely on the basis of DELTA. The most discordant pairs were recognised by low $DISC_{DELTA}$. Rank numbers within the sequence of concordant pairs were assigned by linearly combining a parameter describing the disease severity of a pair with a parameter describing the pair's discordance. For instance, in a diagram wherein the rank number for DELTA was assigned to the x-axis (whereby the rank number 1 corresponded to the lowest DELTA, i.e. to the most concordant pair) and the rank number for ΣDfO was assigned to the y-axis (whereby the rank number of 1 corresponded to the

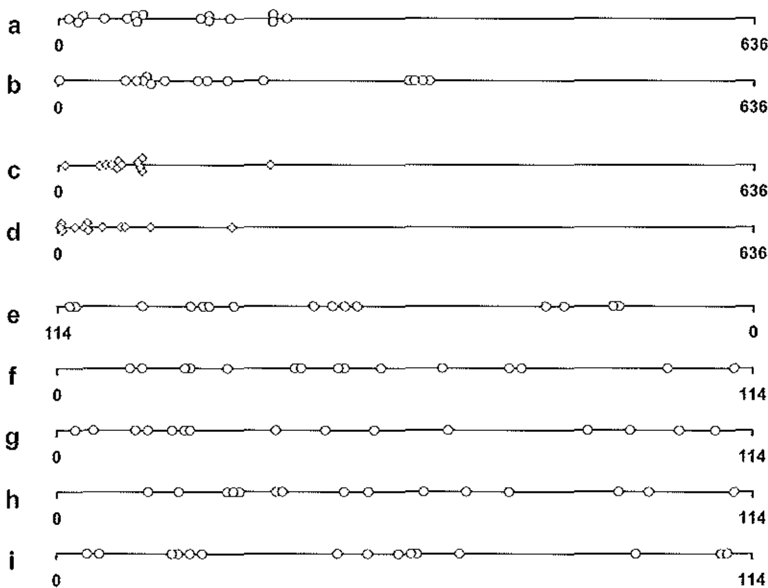


Figure 3.6 *Intrapair discordance (**a-d**) and rank numbers (**e-i**) for monozygous twin pairs. Intrapair differences of rank numbers are shown for wfh% (**a, c**) and FEVPerc (**b, d**) for monozygous ΔF508 homozygous twins (open circles in **a, b**) and monozygous twins with other CFTR genotypes (open squares in **c, d**). The maximal intrapair rank number difference of 636 is displayed for a total of 318 patient pairs.*
*Rank numbers (Table 3.8) for ΔF508 homozygous monozygous twins obtained within the cohort of 114 ΔF508 homozygous patient pairs are displayed in (**e – i**): (**e**) rank DISC$_{DELTA}$ (**f**) rank CON+$_{DELTA}$ (**g**) rank CON-$_{DELTA}$ (**h**) rank CON+$_{DiffDfO}$ (**i**) rank CON-$_{DiffDfO}$. Except for rank DISC$_{DELTA}$, no significant differences were found comparing the rank numbers between the 15 monozygous and the 99 dizygous ΔF508 homozygous patient pairs: (**e**) rank DISC$_{DELTA}$; P = 0.05 (**f**) rank CON+$_{DELTA}$; P = 0.17 (**g**) rank CON-$_{DELTA}$; P = 0.07 (**h**) rank CON+$_{DiffDfO}$; P = 0.29 (**i**) rank CON-$_{DiffDfO}$; P = 0.17*

highest value for ΣDfO, i.e. the most mildly affected pair), the data set closest to the origin defined the most concordant/mildly diseased pair employing these criteria. Accordingly, the rank number for the distance from origin in this diagram was used to define CON+$_{DELTA}$. In an analogous manner, CON-$_{DELTA}$, CON+$_{DiffDfO}$ and CON-$_{DiffDfO}$, were defined as indicated in Table 3.8. In other words, the four rank numbers for CON+$_{DELTA}$, CON-$_{DELTA}$, CON+$_{DiffDfO}$ and CON-$_{DiffDfO}$ defined a pair's position in the sequences CON+ and CON- wherein discordance was defined via the composite parameter DELTA and the pair's position in the sequence CON+ and CON- wherein discordance was defined via DiffDfO.

In Figure 3.6, rank numbers $DISC_{DELTA}$ (Figure 3.6e), $CON+_{DELTA}$ (Figure 3.6f), $CON-_{DELTA}$ (Figure 3.6g), $CON+_{DiffDfo}$ (Figure 3.6h) and $CON-_{DiffDfo}$ (Figure 3.6i) are graphically displayed for monozygous twins. Intrapair differences of rank numbers for wfh% and FEVPerc were lower for ΔF508 homozygous monozygous twins (Figures 3.6a and b) and monozygous twins with other genotypes (Figures 3.6c and d) ($P = 0.0005$ for wfh% and $P = 0.01$ for FEVPerc). Rank numbers for $DISC_{DELTA}$ were significantly lower for ΔF508 homozygous monozygous twins than for dizygous ΔF508 homozygotes ($P = 0.05$, Mann-Whitney *U*-test, Figure 3.6e). In contrast, rank numbers for $CON+_{DELTA}$, $CON-_{DELTA}$, $CON+_{DiffDfo}$ and $CON-_{DiffDfo}$ which were defined by a linear combination of a parameter describing the disease severity and a parameter describing the intrapair concordance, were not significantly different between monozygous and dizygous ΔF508 homozygotes (Figures 3.6f-i). This observation indicates that monozygous ΔF508 homozygous twins express concordant mildly, concordant moderately and concordant severely affected phenotypes and consequently, rank numbers for $CON+_{DELTA}$ and the three similarly derived rank numbers did not segregate with the zygosity status of the patient pair.

Categorisation of CF patient pairs

The interrelation of the five rank numbers $DISC_{DELTA}$, $CON+_{DELTA}$, $CON-_{DELTA}$, $CON+_{DiffDfo}$ and $CON-_{DiffDfo}$ enabled the discrimination of 6 different categories of patient pairs (Table 3.9): for a discordant patient pair (category DIS, example I in Table 3.7a), a low rank number for $DISC_{DELTA}$, but high values for the other four rank numbers were expected. Pairs ranking low in $DISC_{DELTA}$ and in $CON+_{DiffDfo}$ or $CON-_{DiffDfo}$ were distinguishable from the category DIS. These pairs were summarized as discordant/concordant mild disease (DC(1); example II in Table 3.7a) and discordant/concordant severe disease (DC(2)), respectively. Concordant/mildly affected patient pairs (category CON+, example I in Table 3.7b) had low rank numbers for $CON+_{DELTA}$ and $CON+_{DiffDfo}$, but high values for the other three rank numbers. Analogously, concordant/severely affected patient pairs (category CON-, example III in Table 3.7b) were expected to have low values for $CON-_{DELTA}$ and $CON-_{DiffDfo}$, but high values for the other three rank numbers. Concordant/moderately affected pairs were summarized as non-discordant (ND, example II in Table 3.7b). ND pairs are expected to have similarly low rank numbers for $CON+_{DELTA}$, $CON-_{DELTA}$, $CON+_{DiffDfo}$ and $CON-_{DiffDfo}$ as by definition for each of these rank numbers intrapair concordance and disease severity was weighed equally. Consequently, pairs characterised by definite intrapair concordance but average disease severity were ranked comparably low in each of these sequences. Thus, the ND pairs were

discriminated from pairs categorised as CON+ and CON- by their low difference between the corresponding rank numbers {$CON+_{DELTA}$ — $CON-_{DELTA}$} and/or {$CON+_{DiffDfo}$ — $CON-_{DiffDfo}$}.

In order to determine the sequence of pairs within each of the categories in an unambiguous manner, a pair's position within any of the sequences had to be described using the same algorithm on all CF patient pairs (Figure 3.7).

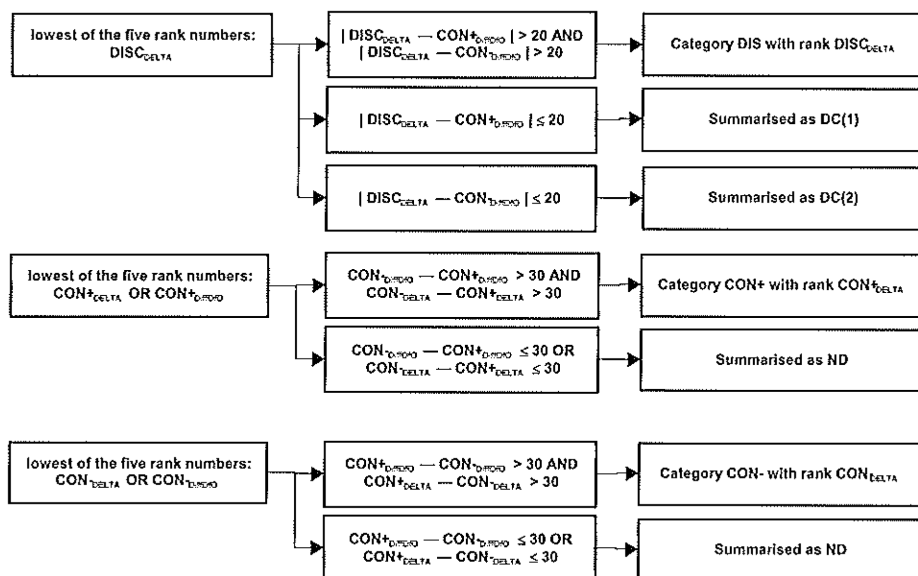


Figure 3.7 *Flow chart for the assignment of 114 ΔF508 homozygous twin and sibling pairs to the categories DIS, CON+, CON-, ND, DC(1) and DC(2) based on 5 rank numbers derived from composite parameters (see Table 3.8 for definition and text for details).*

Categories of ΔF508 homozygous CF twin and sibling pairs

The ranking algorithm (Figure 3.7) was applied to 114 ΔF508 homozygous CF twin and sibling pairs. The outcome is displayed in Figures 3.8 and 3.9. As indicated in Figure 3.8b, 59% of the ΔF508 homozygous pairs were sorted into the categories DIS, CON+ and CON- while the remaining 41% were summarized in the categories with intermediate phenotypes ND, DC(1) and DC(2).

To identify pairs from the three categories DIS (discordant pairs), CON+ (concordant/mildly diseased pairs) and CON- (concordant/severely diseased pairs), we sorted the cohort of patient pairs as such that subsequently ranked pairs possess the qualities of the respective category in declining

Table 3.8 *Definition of rank numbers DISC $_{DELTA}$, CON+ $_{DELTA}$, CON+ $_{DiffDfO}$, CON- $_{DELTA}$ and CON- $_{DiffDfO}$*

SEQUENCE OF DISCORDANT PAIRS: rank number derived from one parameter

DISC $_{DELTA}$ rank number for DELTA

rank number for DISC $_{DELTA}$ = 1: highest DELTA ≡ most discordant pair

SEQUENCES OF CONCORDANT PAIRS: rank number derived from combination of two parameters

all rank numbers are defined as distance from origin in a plot whereby the following parameters are assigned to:

	x-axis	y-axis
CON+ $_{DELTA}$	rank number for DELTA rank number for DELTA = 1 ≡ lowest DELTA ⇒ most concordant pair closest to origin	rank number for ΣDfO rank number for ΣDfO = 1: highest ΣDfO ⇒ mildest affected pair closest to origin
CON+ $_{DiffDfO}$	rank number for DiffDfO rank number for DiffDfO = 1 ≡ lowest DiffDfO ⇒ most concordant pair closest to origin	rank number for ΣDfO rank number for ΣDfO = 1: highest ΣDfO ⇒ mildest affected pair closest to origin
CON- $_{DELTA}$	rank number for DELTA rank number for DELTA = 1 ≡ lowest DELTA ⇒ most concordant pair closest to origin	rank number for ΣDfO rank number for ΣDfO = 1: lowest ΣDfO ⇒ most severely affected pair closest to origin
CON- $_{DiffDfO}$	rank number for DiffDfO rank number for DiffDfO = 1 ≡ lowest DiffDfO ⇒ most concordant pair closest to origin	rank number for ΣDfO rank number for ΣDfO = 1: lowest ΣDfO ⇒ most severely affected pair closest to origin

NOTE. DELTA and DfO: composite parameters as defined in Figure 3.2. DiffDfO: intrapair difference in DfO. ΣDfO: intrapair sum of DfO.

fashion: the most discordant pair (defined by rank 1 within the category DIS) is followed by the second most discordant pair (defined by rank 2 within the category DIS) and so forth. In a likewise manner, ranking of pairs within the categories CON+ and CON- was intended. This gradient is visible in the clinical data for the patient pair cohorts defined in Figure 3.8c: Discordance decreased with increasing rank number in the category DIS. This was observed with respect to DELTA as well as the intrapair differences in wfh% and FEVPerc (Figures 3.8d, e and f). In the categories CON+ and CON-, DELTA and the intrapair difference in FEVPerc raised with increasing rank number (Figures 3.8d and f). Intrapair differences for wfh% were lower within the categories CON+ and CON- than within the category DIS. The average disease severity of pairs from the category DIS was intermediate compared to patient pairs ranked CON+ or CON- (Figures 3.8g, h and i).

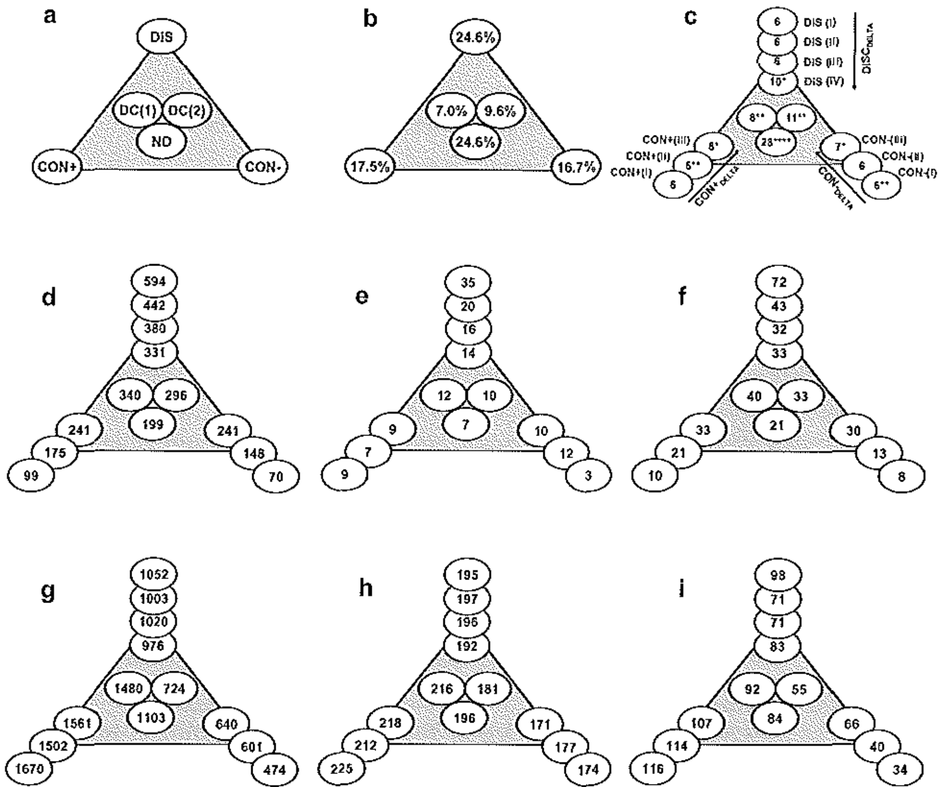


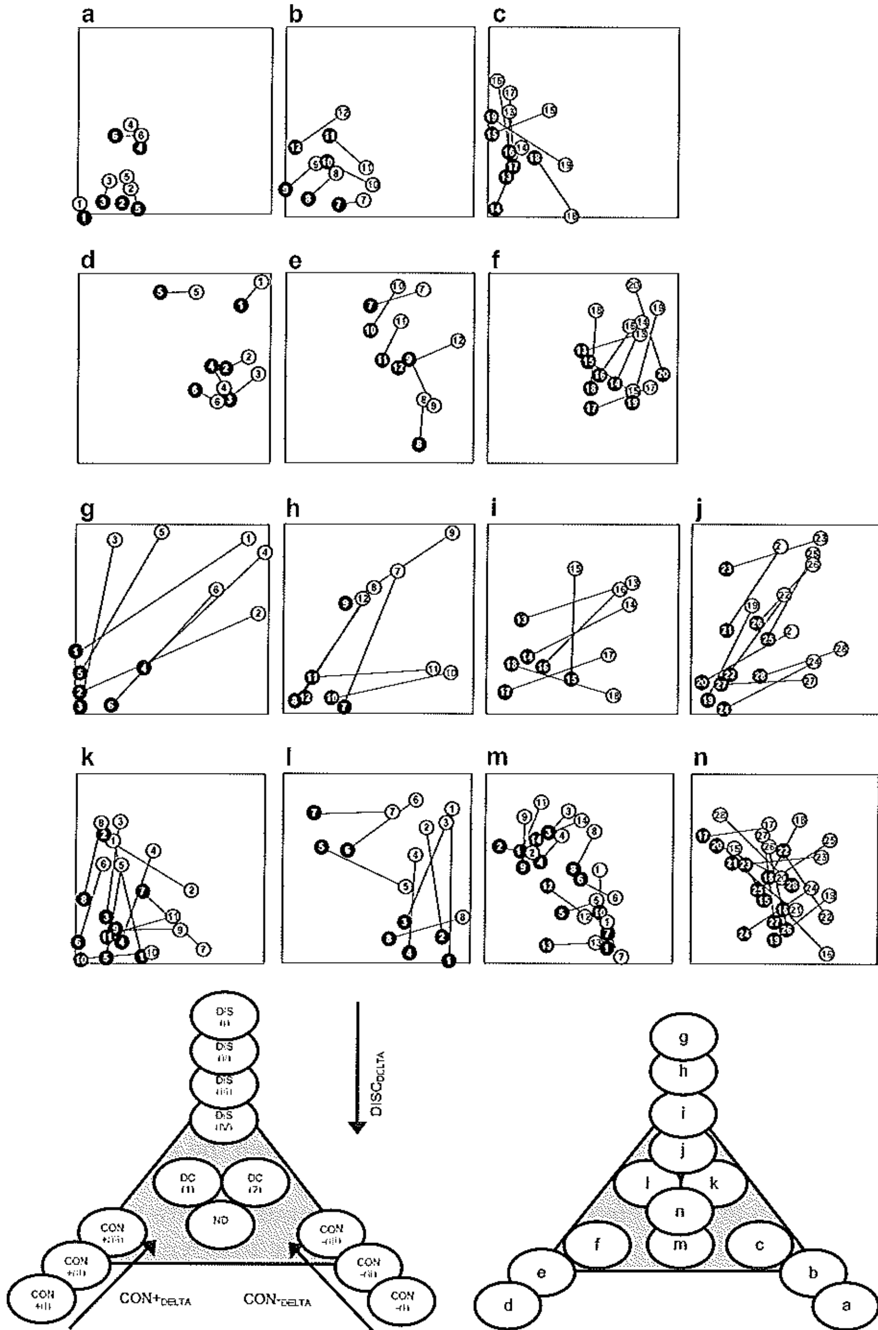
Figure 3.8 *Disease severity and intrapair discordance of 114 ΔF508 homozygous twin and sibling pairs assigned to the categories DIS, CON+, CON-, ND, DC(1) and DC(2). (**a**) Layout for (**b-i**) and proposed relation of the three extreme phenotypes DIS, CON+ and CON- to the intermediate phenotypes ND, DC(1) and DC(2). (**b**) Occupancy of the categories whereby 100% represents the total number of 114 ΔF508 homozygous CF twin and sibling pairs. (**c**) Definition of cohorts with decreasing rank numbers in the category and number of pairs per cohort. Monozygous twins are indicated by *. (**d-f**) Intrapair discordance as defined by average values within the cohorts for DELTA (**d**), intrapair difference of wfh% (**e**) and intrapair difference of FEVPerc (**f**). (**g-i**) Disease severity as defined by average values within the cohorts for the intrapair sum of DfO (**g**), wfh% (**h**) and FEVPerc (**i**).*

The dissimilar, non-overlapping character of patient pairs from the categories CON+ and CON- is evident from the dissimilar values of the average intrapair sum in DfO, wfh% and FEVPerc (Figures 3.8g, h and i). Analogously, these

observations are visible in Figure 3.9 wherein rank numbers for wfh% and FEVPerc for all 114 ΔF508 homozygous twin and sibling pairs are depicted in a plot employing the same axes assignment as for definition of the composite parameters DELTA and DfO (Figure 3.2). CON- pairs are found in the lower left area of that diagram as intended due to their low DfO (Figures 3.9a, b and c) while CON+ pairs are located in the upper right area of the diagram indicating their high DfO (Figures 3.9d, e and f). Both cohorts of concordant pairs occupy distinct, non-overlapping areas within the diagram whereby patients from pairs summarized as ND are located in the space between the two extreme concordant phenotypes (Figures 3.9m and n).
Adjacent to the cohorts CON+(III) (Figure 3.9f) and CON-(III) (Figure 3.9c), more discordant pairs with high DfO respective low DfO are summarized as DC(1) (Figure 3.9l) and DC(2) (Figure 3.9k). In most of the pairs from the category DIS (Figures 3.9g, h, i and j), the sibling with the better phenotype is characterised by the better wfh% together with the better FEVPerc as indicated by a positive slope of the line connecting both data points of a discordant pair. In contrast, pairs summarized as DC(1) or DC(2) are observed to be concordant in wfh% as well as discordant in FEVPerc or discordant in wfh% albeit concordant in FEVPerc or the sibling with the better wfh% exhibits the lower FEVPerc. Thus, most data points from DC(1) and DC(2) pairs are connected by a line parallel to the y-axis, parallel to the x-axis or with a negative slope, respectively.

Discussion

The clinical phenotype of the monogenic disease CF is characterised by a broad spectrum of disease severity and variation of the clinical course between patients with the same mutation genotype in the disease-causing gene *CFTR*.[3,4]
For the multi-organ disease CF, the anthropometric parameter wfh% and the lung function parameter FEV1 are instrumental for the follow-up of CF patients to monitor growth, development, gastrointestinal and pulmonary disease.[10] By studying affected patient pairs, i.e. CF twins and siblings, we have taken a classical approach to assess the influence of inherited versus environmental factors on the clinical parameters wfh% and the FEV1 derived FEVPerc.
The prominent role of the *CFTR* gene in CF is evident from the mode of inheritance of this autosomal recessively transmitted disease.[1]
The more than 800 reported CF associated *CFTR* mutations have been reliably classified as to whether they confer exocrine pancreatic sufficiency or insufficiency[21,22], but the association of *CFTR* mutation genotype with CF disease manifestation is less straightforward for parameters describing the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nutritional status or CF pulmonary disease. The same range of disease manifestation is observed in wfh% and FEVPerc among ΔF508 homozygotes, ΔF508 compound heterozygotes and patients with non-ΔF508/non-ΔF508 genotypes (Table 3.5a), so that the *CFTR* genotype - CF phenotype association becomes ambiguous.

In order to evaluate the influence of the *CFTR* mutation genotype on the intrapair disease variability, we evaluated the intrapair rank number difference (IRND) distribution among CF patient pair cohorts. When the whole cohort with various *CFTR* genotypes was analysed, the two members of a twin or sibpair were on the average significantly more similar in both wfh% and FEVPerc than unrelated patients (Table 3.6 and Figure 3.3) demonstrating the impact of shared *CFTR* genotype on the CF disease phenotype. However, among ΔF508 homozygous pairs representing a cohort normalised for the genotype in the major disease-causing gene, any deviation of the observed IRND distribution to the distribution expected for unrelated couples cannot be based on the *CFTR* genotype. ΔF508 homozygotes differed in their IRND distribution from random couples in wfh%, but not in FEVPerc (Table 3.6 and Figure 3.3). Although more subtle effects not evident in a hundred pairs would probably show up with increasing sample size, the global picture is clear-cut: The IRNDs were apparently randomly distributed for FEVPerc, but significantly skewed to low numbers for wfh%.

Figure 3.9 *(see previous page) Graphical representation of rank numbers for wfh% and FEVPerc for 114 ΔF508 homozygous categorised patient pairs. Each patient pair is depicted as a set of a black and a white numbered data point within a diagram of the rank number FEVPerc (y-axis) plotted against the rank number for wfh% (x-axis) as defined in Figure 3.2. Both axes of all diagrams within this Figure are set to the maximal rank number of 636 that can be obtained for 318 pairs. For each pair, the data point closer to the origin indicated by the black colour corresponding to the more severely affected patient. The line connecting both data points of a pair represents the composite parameter DELTA describing the intrapair discordance. The pairs shown in Figures* ***(a)*** *to* ***(n)*** *correspond to the cohorts defined in Figure 8c as is explained in the two triangular diagrams at the bottom of this Figure.*
(a-c) *19 pairs categorised as CON- ordered by increasing rank number for CON-$_{DELTA}$*
(d-f) *20 pairs categorised as CON+ ordered by increasing rank number for CON+$_{DELTA}$*
(g-j) *28 pairs categorised as DIS ordered by increasing rank number for DISC$_{DELTA}$* ***(k)****: 11 pairs summarized as DC(2) and* ***(l)****: 8 pairs summarized as DC(1) ordered in a sequence with decreasing DELTA* ***(m)****,* ***(n)****: 28 pairs summarized as ND ordered in a sequence with increasing DELTA.*

Table 3.9 *Definition of patient pair categories DIS, CON+, CON-, ND, DC(1) and DC(2) by rank number characteristics*

Category	DISC $_{DELTA}$	CON+ $_{DELTA}$	CON- $_{DELTA}$	CON+ $_{DIFDIO}$	CON- $_{DIFDIO}$	in Fig. 3.9
CON-	high	high	low	high	low	(a), (b), (c)
CON+	high	low	high	low	high	(d), (e), (f)
ND	high	low	low	low	low	(m), (n)
DIS	low	high	high	high	high	(g), (h), (i), (j)
DC (1)	low	high	high	low	high	(l)
DC (2)	low	high	high	high	low	(k)

NOTE. CON+: concordant/mildly affected. CON-: concordant/severely affected.
ND: non-discordant (concordant/moderately affected). DIS: discordant pair.
DC(1): discordant and concordant/mildly affected.
DC(2): discordant and concordant/severely affected.

Such a skew is only observed if the shared factors significantly outweigh the individual genetic and epigenetic factors.
An overrepresentation of shared alleles in sibs compared to unrelated subjects should account for their more similar wfh% values, because anthropometry has a strong inherited component.[23, 24,25] However, weight predicted for height is in CF influenced by eating habits and lifestyle,[26,27,28] the mode of and adherence to a high-calorie diet,[26,27,28] the administration of pancreatic enzymes and fat-soluble vitamins to treat exocrine pancreatic insufficiency and maldigestion of nutrients[29] and the frequency and severity of respiratory infections.[30] All investigated patient pairs but a few adults shared homes, family life and CF physician with each other so that they were exposed to the same nutritional lifestyle and medical expertise. Common medical treatment and living conditions certainly contributed to the significantly lower intra- than interpair variance in wfh%. The therapeutic regime aimed at the maintenance of a normal weight is reflected by the average values for wfh% near 100% among CF patients (Tables 3.4a and 3.5a & ref 15).
As outlined above, in contrast to the nutritional status, individual rather than shared factors determined the sibs' lung function. Pulmonary disease in CF is characterised by a vicious cycle of infection,[31,32] inappropriate host defence,[31,32] tissue disintegration and remodelling[33] and irreversible loss of pulmonary function.[13,34,35] Although the sibpairs' airways were typically infected with the same bacterial strain (data not shown), the differential host response seems to be more important for progression of pulmonary disease than shared environmental and genetic factors. The generation of immune responses by gene rearrangements and somatic mutation[36,37,38] and the high degree of

polymorphism in immunorelevant loci such as the HLA[39,40,41] are major reasons why siblings differ more in host defence genotypes than in any other category of expressed genotypes.
The interrelation between genes determining the individual's host defence and the challenge by immunogenic, i.e environmental, factors appears to be of substantial impact for the pulmonary status in CF as demonstrated by the observation that dizygous twins have a significantly lower FEVPerc compared to monozygous twins (Table 3.4a): an increased susceptibility to infection is known for CF[42] and nosocomial transmission of bacterial pathogens in CF is a well-known risk[43,44] that should be similar for all twin pairs irrespectively of their zygosity status. However, two monozygous twins are likely to possess equal host defence capabilities while in dizygous twin pairs, pathogens confront susceptible individuals with a different genetic repertoire of host defence.
Taken together, these findings indicate that the nutritional status in CF is modulated by few factors still detectable within the cohort of 114 ΔF508 homozygous pairs, while the pulmonary disease in CF is modulated by numerous factors. Thus, it is not surprising that the four most discordant monozygous twin pairs (Figure 3.6e) demonstrate intrapair differences in FEVPerc (Figure 3.6b), but are inconspicuous in their wfh% intrapair difference. On the day of evaluation, these pairs were 30, 16, 9 and 9 years old. Two were pairs of male, and two were pairs of female twins. Currently it remains the subject of speculation as to whether the discordance in these pairs might reflect the influence of subtle genetic differences between monozygous twins[45] such as variation in the DNA methylation pattern, the result of somatic mutations e.g. at MHC loci or differential X-inactivation in the female pairs. With equal probability, twin discordance in birth weight which has been documented among monozygous, particularly monochorionic, twin pairs[46] might give rise to differences in the twin's pulmonary status.
The comparison of intrapair discordance among monozygous (MZ) and dizygous (DZ) twin pairs is widely accepted to dissect the influence of genetic versus epigenetic factors on the individual's phenotype:[47,48] the hypothesis "a phenotypic trait is determined by inherited factors" is sustained but not proven by the observation of monozygous twin pairs being more concordant in the analysed trait than dizygous twin pairs. Within the cohort of 41 CF twins with known zygosity status, this applies to the parameter DELTA. The composite parameter describing intrapair discordance based on wfh% and FEVPerc (Figure 3.2) was significantly lower for monozygous than for dizygous twins indicating that monozygous CF twins are more concordant than dizygous CF twins (Table 3.4b). However, intrapair differences for both wfh% and FEVPerc were comparable between monozygous and dizygous CF twins (Table 3.4b). As pulmonary function and nutritional status are clinically related,[49,50] the

intrapair discordance of either parameter might be enhanced by the other. Consequently, DELTA should be more sensitive with respect to the intrapair differences than each of the individual parameters in itself. As a result, the concordance of monozygous twins detected by DELTA but not by wfh% and FEVPerc indicates the inherited component besides the *CFTR* mutation genotype that influences CF disease severity. The impact of inherited factors on CF disease is supported by the observation that DELTA is independent of the intrapair age difference in CF siblings (Figure 3.4): The smaller the age difference, the more siblings have shared environmental conditions of living. The independence of DELTA from intrapair age difference suggests a stronger impact of shared genetics than shared environmental factors on disease manifestation in CF. In other words, the shared time of exposure to environmental factors and the action of the environmental factors on sibs at a comparable stage of development, i.e. the extent of sharing patient's history and state of development is less important than age-independent factors. Given the hypothesis that CF disease manifestation is substantially influenced by genes other than *CFTR*, methods of reverse genetics may be applied to identify the loci involved. However, the success of such an approach will be determined by the selection of appropriate candidates for such a study. For the analysis of a quantitative trait extreme phenotypes are generally considered to be more informative.[6-9] Hence, a strategy to identify these most informative patient pairs was developed. As the disease phenotype had to be described in a quantitative manner, metric data was employed in order to evaluate the complex multi-organ disease CF. Using wfh% and FEVPerc, two clinical parameters most sensitive to the course and prognosis of CF[10] were combined in order to describe the overall disease severity in the two major afflicted organs, i.e. the respiratory and the gastrointestinal tracts. Moreover, the composite parameter DELTA describing the intrapair discordance was employed in the selection procedure. As has been described in detail above, DELTA was more sensible with respect to the influence of the genetic background on CF disease severity and thus the employment of this parameter for patient pair selection should facilitate the identification of subjects informative in a genetic study. To avoid equivocal scoring, a computer-assisted method was executed to rank patient pairs within the categories of patient pairs exhibiting the phenotypes concordant mild disease (CON+), concordant/severe disease (CON-) and discordant (DIS). To assure that the selected pairs represent the extremes from a continuous spectrum of phenotypes, no overlap of clinical characteristics comparing pairs from the cohorts DIS, CON+ and CON- had to be assured. As demonstrated within Figure 3.8, the algorithm employed for the ranking of the 114 ΔF508 homozygous

pairs resulted in the identification of CON+ and CON- patient pairs with non-overlapping wfh% and FEVPerc values. Likewise, discordance in both clinical parameters was distinct among pairs ranked DIS compared to pairs ranked CON+ or CON-. In conclusion, the ΔF508 homozygous twin and sibling pairs expressed various phenotypes. Three categories of extreme phenotypes — i.e. DIS, CON+ and CON- — and three categories with intermediate and/or uncommon phenotypes could be distinguished and were characterised as phenotypically distinct entities with respect to pulmonary function and nutritional state of the CF patients.

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Chapter 4

Residual chloride secretion in intestinal tissue of ΔF508 homozygous twins and siblings with cystic fibrosis

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Abstract

Background & Aims: Cholinergic stimulation of chloride secretion is impaired in the intestine of cystic fibrosis (CF) patients. However, intestinal chloride secretion has been seen in patients carrying mild CF mutations. The aim of this study was to investigate residual Cl^- secretion in intestine of ΔF508 homozygous CF patients, and examine the contribution of CFTR and alternative Cl^- conductances. Twins and siblings with identical *CFTR* genotypes were investigated to determine impact of factors other than CFTR on chloride secretion. Methods: Chloride secretion in rectal tissue was investigated by applying Ca^{2+} and adenosine 3',5'-cyclic monophosphate (cAMP)-linked agonists before and after the inhibition of alternative Cl^- conductances with 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS). Results: In 73% of patients cAMP-mediated Cl^- secretion was observed, while 20% showed DIDS-sensitive Ca^{2+}-activated Cl^- secretion. This DIDS-sensitive alternative chloride conductance was only seen in CF individuals also responding to cAMP-agonists. Chloride secretion was more concordant within monozygous twins than within dizygous pairs. Conclusions: These results suggest the presence of CFTR-mediated Cl^- secretion in a subgroup of patients, implying that a portion of ΔF508 CFTR can be processed *in vivo* and function as chloride channel in the apical membrane of intestinal cells. Moreover, a considerable number of ΔF508 homozygous patients express chloride conductances other than CFTR in their intestinal epithelium.

Introduction

Cystic fibrosis (CF) is an inherited disorder of ion transport[1] in exocrine glands that is caused by molecular lesions in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which encodes an adenosine 3',5'-cyclic monophosphate (cAMP)-regulated chloride channel found in the apical membrane of epithelial cells.[2,3] The more than 800 known *CFTR* mutations are classified as severe or mild alleles dependent upon whether they confer exocrine pancreatic insufficiency or pancreatic sufficiency. The most frequent *CFTR* mutation ΔF508, a deletion of the phenylalanine codon at position 508, occurs in about 70% of CF alleles in the Caucasian population.[4]
The diagnosis of CF can be made by applying specific and sensitive electrophysiological methods such as the nasal potential difference measurement[5] or the intestinal current measurement (ICM),[6-8] which test the conductance properties for sodium and chloride ions of respiratory and

intestinal epithelium in response to secretagogues and inhibitors. The chloride secretory response examined by ICM not only discriminates CF from non-CF, but may also reveal the presence of residual chloride secretion in affected individuals,[8] by challenging the Cl^- secretory pathways with cholinergic and β-adrenergic agonists. The chloride secretory responses that are seen in controls when applying these agonists to the intestinal tissue, are impaired in tissues from CF individuals.[7] In a previous study, residual chloride secretion has been detected in patients with CF, predominantly in pancreatic sufficient individuals who carry mild mutations such as A455E.[8] Residual chloride secretion has also been seen in biopsies from patients with stopcodon mutations (own unpublished data) suggesting the contribution of chloride channels other than CFTR. In *cftr* $^{-/-}$ knock out mice Ca^{2+}-regulated chloride channels are believed to modulate the manifestations of disease in the affected organs.[9-11] These alternative chloride channels also exist in human epithelia[12-14] and might contribute to the diversity of CF disease in individuals with the same *CFTR* genotype. Moreover, ΔF508 homozygous CF mice present with a small intestinal cAMP-activated Cl^- secretory response suggesting the presence of ΔF508 CFTR functioning as a chloride channel in the plasma membrane.[15]
In this study we addressed the issue whether CF patients with the most common severe *CFTR* genotype, i.e. ΔF508 homozygosity, express residual Cl^- secretion in the intestinal epithelium. In order to interpret the origin of this residual secretion as being either CFTR or non-CFTR-mediated, we introduce an extended protocol of the Ussing chamber experiments, in which secretagogues and inhibitors of the chloride secretory pathways are added in a specific sequence. We examined the frequency and characteristics of residual chloride secretion in ΔF508 homozygous CF twin and sibling pairs. By testing mono- and dizygous patient pairs, sharing the same homozygous *CFTR* mutation genotype, the impact of the genetic background and epigenetic factors on the amount and origin of the intestinal chloride secretion could be evaluated.

Materials and Methods

Subjects

Twin and sibling pairs with cystic fibrosis with different genotypes were enrolled for the *European Cystic Fibrosis Twin and Sibling Study*. For the study described here, only subjects homozygous for the *CFTR* gene mutation ΔF508 were selected. We invited 72 patients belonging to a dizygous twin or sibling pair, and 26 patients belonging to monozygous twin pairs. Patients

were investigated in or near their domestic countries at selected CF core centers in Hannover, Innsbruck, London, Rotterdam and Verona. Questionnaires were filled out for all 98 patients, but rectal suction biopsies were only taken from 81 patients due to restrictions appointed by the local ethics committees, or patients not approving of the ICM procedure. Individuals with interpretable responses to all secretagogues were used for further evaluation of intestinal chloride conductances (n = 55, see 'Results' section below). These 55 patients carried the following nationalities: 4 patients originated from Austria, 3 from Belgium, 1 from France, 4 from Great Britain, 24 from Germany, 5 from Italy, 11 from The Netherlands, 2 from Poland and 1 from Sweden (32 females, age range 4.2 - 38.8 yr, mean age 15.8 ± 9.4 yr; 23 males, age range 5.4 - 39.3 yr, mean age 18.1 ± 8.8 yr). Mono- or dizygosity of twins was ascertained by using the AmpF*L*STR Profiler Plus™ typing kit, analyzed on the ABI Prism 377 (Perkin-Elmer Applied Biosystems)[16] or by oligonucleotide fingerprinting of simple repeats applying in situ gel hybridization of *Mbo*I or *Hinf*I genomic digests.[17] Approval was obtained from the Hospital Medical Ethical Committees and from patients or parents by written informed consent.

Intestinal current measurement

The technique of intestinal current measurement has been described previously.[7] It measures electrogenic transport of ions across the intestinal epithelium as a short-circuit current (I_{sc}). Rectal tissue was obtained with a suction biopsy device. The biopsies were preserved in phosphate-buffered saline on ice and directly mounted in adapted micro-Ussing chambers (aperture 1.13 mm^2).[7] The tissue was perfused with Meyler buffer solution at 37°C (composition in mmol/L: Na^+ 126.2; Cl^- 114.3; HCO_3^- 20.2; HPO_4^{2-} 0.3; $H_2PO_4^-$ 0.4; Hepes 10; pH = 7.4) and gassed with 95% O_2 and 5% CO_2. Basal transepithelial resistance of the tissue was determined by measuring the voltage response to pulse currents of 1 μA and applying Ohm's law. Basal I_{sc} prior to voltage clamping was calculated from the basal transepithelial resistance and the open-circuit transepithelial potential difference. Subsequently, the tissue was short-circuited by voltage-clamps during the course of the experiment, resulting in a zero transepithelial potential difference. For maintenance of cell metabolism glucose (10^{-2} mol/L) was given both mucosally and serosally. After equilibration specific compounds (mol/L) that act on the ion conductance pathways[7,8] were added to the mucosal (M) and/or serosal (S) bathing solutions in the following order: amiloride (10^{-4}, M), indomethacin (10^{-5}, M+S), carbachol (10^{-4}, S), 8-bromo-cAMP (10^{-3}, M+S) together with forskolin (10^{-5}, S), 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) (2.10^{-4}, M, incubated for

10 minutes), and histamine (5.10^{-4}, S). For determination of interexperimental error duplicate biopsies were analyzed in a group of 27 participating patients. ICM experiments that encountered technical problems were only evaluated in their signal pattern prior to the disturbance. All drugs were obtained from Sigma Chemical Co., St. Louis, MO.

Principle of the assay: differentiation of residual chloride conductance by DIDS
The first compound added is amiloride, which blocks the Na^+ channels and thereby reduces the contribution of electrogenic sodium absorption to the I_{sc}.[18] Indomethacin inhibits endogenous prostaglandin synthesis and thereby prevents excessive cAMP production.[19] The various conductances that contribute to the chloride secretory response are then analyzed by sequential addition of carbachol, 8-bromo-cAMP + forskolin, DIDS, and histamine. Carbachol initiates the cholinergic activation of chloride secretion by increasing the intracellullar Ca^{2+} concentration through stimulation of Ca^{2+} influx and mobilization of intracellular Ca^{2+} stores.[20] This causes basolateral Ca^{2+}-dependent K^+ efflux which acts as the electrogenic driving force for apical Cl^- secretion.[21] Moreover, carbachol activates CFTR in the apical membrane by increasing the formation of diacylglycerol, thus stimulating the protein kinase C-dependent signaling pathway.[22] A large transient chloride secretory response upon addition of carbachol, i.e. an increase in short-circuit current, is characteristic for intestinal epithelium from non-CF subjects (Figure 4.1a), and is mainly caused by CFTR-mediated chloride secretion.[7,8,23] In contrast, tissues from patients with severe CF typically exhibit a transient response in the reverse direction (Figure 4.1b), presumably due to apical K^+ secretion,[24,25] unmasked by the reduction or absence of Cl^- secretion. A small group of CF patients shows a reversed response followed by a small transient change in I_{sc} in the chloride secretory direction (Figure 4.1c), or an overriding but still subnormal chloride secretory response (Figure 4.1d), both indicative of the presence of a residual chloride conductance in the membranes of the epithelial cells.[7] The cAMP-linked chloride secretion, a hallmark of CFTR,[2,3] is challenged by concurrent addition of the agonist 8-bromo-cAMP plus the adenylate cyclase activator forskolin.[26] Besides CFTR, cAMP simultaneously activates the outwardly rectifying chloride channel, the ORCC, which is known to require functional CFTR for its response to the protein kinase A agonist cAMP.[27] In controls, the addition of these two compounds causes a sustained increase in I_{sc} (Figure 4.1a). Subsequently, the tissue is incubated with the stilbene derivative DIDS,[28] which has been reported to block chloride conductances other than CFTR, such as the cAMP-stimulated outwardly rectifying chloride channel[27]

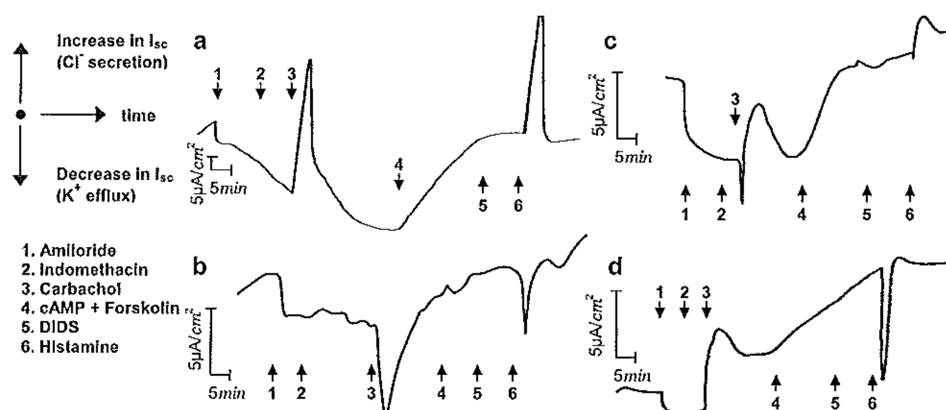

Figure 4.1 Protocol of the ICM measurement. Tracings are given for a healthy control (a), with a large increase in I_{sc} upon addition of carbachol and histamine representing apical Cl^- secretion. (b) Typical CF patient in whom neither carbachol, 8-bromo-cAMP + forskolin, or histamine is able to evoke Cl^- exchange across the apical membrane. The reversed response implies apical K^+ efflux. Note not only the difference in direction of the I_{sc} response compared to control, but also the difference in scale. (c) CF patient with residual Cl^- transport that is not influenced by the presence of DIDS: carbachol, 8-bromo-cAMP + forskolin, and histamine evoke residual Cl^- secretion. (d) CF patient with DIDS-sensitive residual Cl^- secretion, which is characterized by an increase in I_{sc} in response to carbachol and 8-bromo-cAMP + forskolin, but a decrease in I_{sc} upon stimulation with histamine in the presence of DIDS. The tracings depicted are original recordings of individuals, who express an ICM pattern representative for the subgroup of patients that all show that specific ICM response pattern.

and Ca^{2+}-activated chloride channels,[29] including the hCLCA1 expressed in enterocytes and goblet cells of the human intestine,[12] and hClC-3, recently identified as a calcium-calmodulin dependent kinase-activated Cl^- channel in the T84 human colonic cell line.[30] In addition to its action on ion channels DIDS also inhibits the apical $Cl^-/HCO3^-$ and Cl^-/OH^- anion exchangers[31] resulting in a decrease of mucosal-to-serosal chloride flux. However, cytosolic changes in pH, with possible effects on CFTR function,[32] are not generated by the DIDS concentration used in this protocol (Busche et al., personal communication). In the presence of DIDS, the Ca^{2+}-dependent signaling pathway is again activated, to increase chloride secretion driven by the transient electrogenic driving force that is generated by basolateral K^+ efflux. Since desensitization to carbachol after the first application has been reported,[33] carbachol is substituted by histamine.[34] Since man exclusively

expresses H_1-receptors, but no H_2-receptors in the colon,[35] histamine induces a chloride secretory response solely mediated by the Ca^{2+}/protein kinase C signal transduction pathway and not by the cAMP-mediated pathway.[34]
By comparing carbachol responses before the addition of DIDS to histamine responses after the incubation with DIDS, three principal ICM patterns were observed. Typical CF patients lacking any sort of residual chloride secretion showed the pattern seen in Figure 4.1b, without any significant reaction to carbachol, 8-bromo-cAMP + forskolin, or histamine. Patients with DIDS-insensitive chloride secretion, i.e. a chloride secretory response before and after the incubation of the tissue with DIDS presented a carbachol and histamine pattern as in Figure 4.1c, with a cAMP-induced response as well.

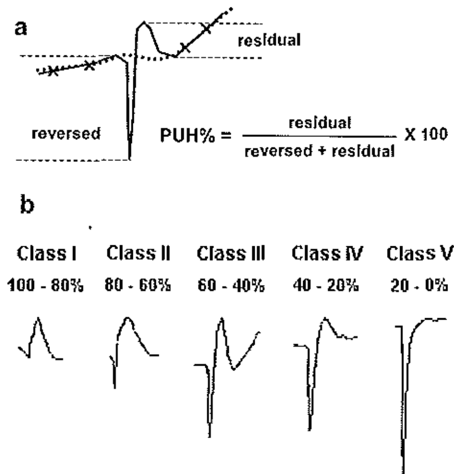

Figure 4.2 *Quantitative interpretation of the Cl⁻ secretory responses to carbachol and histamine. (**a**) Sketch of a Cl⁻ secretory response. A baseline is assigned to the response (dotted line) on the basis of two linear segments on the recording, one before and one after the secretory response (segments between crosses). With respect to this baseline the heights of the reversed and residual responses are determined (between dashed lines). The amount of residual Cl⁻ conductance ($\Delta I_{sc,Cl}$) is calculated as percentage of the total response, by dividing the height of the peak in the chloride secretory direction by the height of the total response. This is the peak up height percentage (PUH%). (**b**) On the basis of the residual Cl⁻ secretion expressed in PUH%, carbachol and histamine responses are categorized into five classes. Experimental examples of tracings are shown for each class. For comparison, the carbachol and histamine responses of the CF patients presented in Figure 4.1 are thus classified as: Figure 4.1b: carbachol class V, histamine class V; Figure 4.1c: carbachol class II, histamine class II; Figure 4.1d: carbachol class I, histamine class V.*

DIDS-sensitive chloride secretion was characterized by an ICM pattern as in Figure 4.1d, showing a chloride secretory response upon addition of 8-bromo-cAMP + forskolin and carbachol, but not upon the addition of histamine.

Interpretation of ICM patterns

Chloride secretory responses to 8-bromo-cAMP + forskolin are typically sustained, unidirectional responses, and were expressed in $\mu A/cm^2$.
To interpret the Ca^{2+}-dependent Cl^- secretory responses quantitatively, the transient responses to carbachol and histamine were analyzed with the computer program INTEG (Joachim Greipel, MHH). This program assigned a baseline to each response, which was calculated from 2 linear segments on the tracing, one prior to and one after the response, by applying a third-order polynomial spline function (Figure 4.2a). The changes in I_{sc} in response to carbachol and histamine in CF patients and most likely also in controls, seem to be the net result of two opposite currents: one component that causes a decrease in I_{sc} representing apical K^+ efflux (Figures 4.1a, c and d, downward peaks) and another component increasing the I_{sc} indicating apical Cl^- secretion (Figures 4.1a, c and d, upward peaks).[7] Hence, the scoring of the responses has to involve both components, which was calculated in the following way. The height (maximal ion flow) and area (total ion flow) below and above the baseline were computed and expressed as percentages of the total response (Figure 4.2a). Calculations with height percentages and area percentages resulted in the same classifications with comparable interexperimental error. For concise presentation, only height percentages are presented. The maximal ion flow in the Cl^- secretory direction ($\Delta I_{sc,Cl}$) was taken as a measure for residual chloride conductance and calculated as the height of the response in the residual direction divided by the total height of the response, the peak up height percentage (PUH%) (Figure 4.2a). The PUH% expresses the magnitude of the net response of the tissue into the Cl^- secretory direction in response to Ca^{2+}-linked agonists. The obtained carbachol and histamine responses were divided into five classes on the basis of this PUH% (Figure 4.2b). The residual chloride secretion $\Delta I_{sc,Cl}$, was only considered significant if it concerned class I, II or III. In rectal tissue specimens of 61 controls the measured carbachol responses were similar to the corresponding histamine responses (43.3 ± 17.6 $\mu A/cm^2$ and 39.3 ± 19.3 $\mu A/cm^2$, respectively), indicating that carbachol and histamine cause Cl^- secretory responses of comparable magnitudes, in the absence of major contributing DIDS-sensitive Cl^- conductances, as is the case in control tissues.[23] Consequently, the influence of DIDS on chloride secretion in CF patients can be determined by comparing the residual secretion before the

addition of DIDS (carbachol response) to the residual secretion after the incubation with DIDS (histamine response). The measured DIDS influences in PUH% were considered significant when carbachol and histamine responses of a patient differed by at least two classes, i.e. when the 95% confidence limits of interexperimental error of PUH% (0-37%) were exceeded. Concordance in Cl^- secretion within pairs was analyzed by comparing the carbachol and histamine responses of the two siblings of a pair. Intrapair variance was only considered significant if responses varied by at least two classes.

Statistical Analysis
Statistical differences were determined via the nonparametric Mann-Whitney *U*-test or the Fisher's exact test. *P*-values smaller than 0.05 were considered significant. Data are presented as means ± SD.

Results

Chloride secretory response in CF intestine
Of the participating patients belonging to a sibling or dizygous twin pair, 49 responses to 8-bromo-cAMP + forskolin, 56 carbachol, and 41 histamine responses could be determined. Eighteen 8-bromo-cAMP + forskolin, 20 carbachol, and 14 histamine responses from participating patients of a monozygous twin pair were collected. The mean basal transepithelial resistance of the rectal biopsy specimens of our group of ΔF508 homozygous CF patients was 27 $\Omega.cm^2$. The mean basal short-circuit current was 20 $\mu A/cm^2$.
Patients of whom a response to 8-bromo-cAMP + forskolin, carbachol, and histamine were available were taken for further evaluation (n = 41 for sibs of dizygous pairs, n = 14 for sibs of monozygous twin pairs).
The cAMP-regulated response and the Ca^{2+}-induced responses to carbachol and histamine were first analyzed separately. Of the 55 evaluated patients of dizygous and monozygous pairs, 40 responded to 8-bromo-cAMP + forskolin (ΔI_{sc} = 3.9 ± 2.9 $\mu A/cm^2$), while no cAMP-activated Cl^- secretion was detectable in 15 individuals.
The Ca^{2+}-linked Cl^- secretory responses to carbachol and histamine were classified according to their PUH% as in Figure 4.3. Eleven out of 41 carbachol responses of dizygous individuals were assigned to class I, II or III and thus clearly demonstrated Cl^- secretion in the intestinal tissue, while 9 out of 41 histamine responses were grouped as class I to III (Figure 4.3). Four out of 14 carbachol responses of persons of a monozygous twin pair

and 1 out of 14 histamine responses scored class I to III (Figure 4.3). Mann-Whitney *U*-tests on PUH% of carbachol or histamine responses revealed no significant differences between independent measurements on separate biopsies of a patient.

The influence of DIDS on chloride conductance is evaluated in Figure 4.3 by comparing the carbachol response of each patient to their histamine response. Five patients (9%) belonging to a di- or monozygous pair exhibited residual Cl^- secretion before and after DIDS (Figure 4.3, areas within black-bordered rectangle). In one of these patients that showed significant carbachol and histamine responses, the chloride secretion was partly abolished by DIDS, and in 10 other patients (totally 20%) the presence of DIDS inhibited all Cl^- secretion (Figure 4.3, dark gray areas).

Class of carbachol response	Class of histamine response: I	II	III	IV	V
I		•	•	•	•• ♦♦ 4.1d
II		4.1c	• ♦		• ♦
III		•			•••
IV			••	•• ♦♦	•••••
V	•	•	•	••••	••••••• ••••••• ♦♦♦♦♦♦ ♦♦ 4.1b

Figure 4.3 *Classification of Cl^- secretory responses to carbachol and histamine according to the peak up height percentages (PUH%) for 55 ΔF508 homozygous CF patients. The carbachol (vertically) and histamine (horizontally) responses are categorized for 41 CF individuals belonging to a dizygous pair (•), and for 14 persons of a monozygous twin pair (♦). The influence of DIDS on the chloride secretion is indicated as follows. Upper left black-bordered rectangle: patients exhibiting DIDS-insensitive residual Cl^- secretion, interpreted as the presence of residual CFTR activity; dark gray areas: patients expressing DIDS-sensitive residual Cl^- secretion, pointing to the presence of alternative Cl^- conductances; light gray areas: patients who show residual Cl^- secretion in the presence, but not in the absence of DIDS. For clarification, the ICM patterns of the three CF patients in Figure 4.1 are assigned to their corresponding carbachol and histamine classes by annotation of 4.1b, 4.1c, and 4.1d referring to the ICM patterns of Figures 4.1b, c and d, respectively.*

In 5 patients (9%) histamine, but not carbachol, caused a chloride secretory response (Figure 4.3, light gray areas) corresponding to class I, II or III. To analyze the combination of cAMP and Ca^{2+}-mediated chloride conductances within a patient, the results of the responses to cAMP + forskolin, carbachol, and histamine were combined (Table 4.1). Since the cAMP pathway, in comparison to the Ca^{2+}/protein kinase C pathway, is a relatively poor activator of apical K^+ secretion and consequently a more sensitive indicator for residual CFTR-mediated Cl^- secretion than carbachol or histamine,[7] the response to cAMP was first assessed to divide the investigated individuals into patients with (n = 40, 73%) or without (n = 15, 27%) a response to 8-bromo-cAMP + forskolin (Table 4.1).

***Table 4.1** Differentiation of chloride secretory responses in ΔF508 homozygous CF patients*

EXPERIMENTAL FINDINGS					INTERPRETATION: Type of Cl^- conductance present
Cl^- secretory response upon stimulation of:					
cAMP-med. pathway	Ca^{2+}-med. pathway				
cAMP + forskolin	Carb	Hist	*n*	%	
Yes 40 (D: 29, M: 11)	Yes	Yes	5 (D: 4, M: 1)	9	CFTR and possible other cAMP-regulated channels
	Yes	No	10 (D: 7, M: 3)	18	CFTR and DIDS-sens. alternative channels
	No	No	24 (D: 17, M: 7)	44	CFTR and possible other cAMP-regulated channels
	No	Yes	1 (D: 1, M: 0)	2	CFTR and Cl^- conductance unmasked by DIDS
No 15 (D: 12, M: 3)	Yes	Yes	Not observed	-	
	Yes	No	Not observed	-	
	No	No	11 (D: 8, M: 3)	20	No residual Cl^- conductance
	No	Yes	4 (D: 4, M: 0)	7	Cl^- conductance unmasked by DIDS

NOTE. D: number of responses observed in dizygous twins or siblings. M: number of responses observed in monozygous twins.
cAMP-med.: 8-bromo-cAMP mediated. Ca^{2+}-med.: Ca^{2+}-mediated. %, percentages of total group of 55 ΔF508 homozygotes. DIDS-sens.: DIDS-sensitive.

Subsequently, the carbachol and histamine results of these patients as evaluated in Figure 4.3 were implemented to further differentiate their Cl^- secretory patterns. The carbachol and histamine responses of 5 of the patients with a cAMP-activated Cl^- secretory response were assigned to class I, II or III and thus clearly demonstrated DIDS-insensitive Ca^{2+}-induced

residual Cl^- secretion (Table 4.1, upper part). One of these 5 patients also belonged to the group with DIDS-sensitive chloride secretion (not shown in Table 4.1), since the carbachol response was significantly reduced by DIDS, while in 10 other patients the presence of DIDS inhibited all Cl^- secretion (totally 20%). Twenty-four of the patients that exhibited a response to the cAMP-agonists, failed to react to carbachol or histamine. One patient with a cAMP-mediated chloride response presented with a histamine response, however showed no reaction to carbachol. In 15 of the 55 measurements no response to cAMP + forskolin was observed (Table 4.1, lower part). Eleven of these patients also lacked a carbachol and histamine response. Four individuals without a response to 8-bromo-cAMP + forskolin did show significant Cl^- secretion upon the addition of histamine, but lacked a carbachol response. Experimentally, as shown in Table 4.1, a DIDS-sensitive chloride conductance was only observed in individuals who also responded to cAMP ($P = 0.045$, Fisher's exact test).

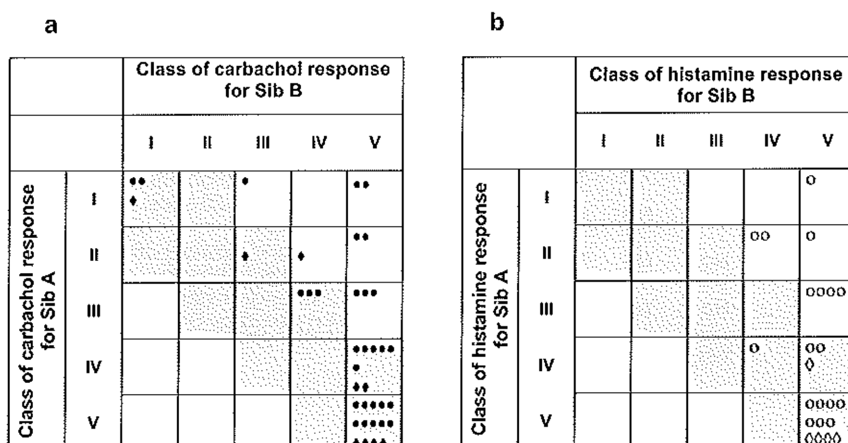

Figure 4.4 *Concordance of the Cl^- secretory responses to carbachol and histamine within patient pairs. Classification of the (**a**) carbachol responses of sib A (vertically) versus sib B (horizontally) for 29 sibling and dizygous twin pairs (•) and 9 monozygous twin pairs (♦). (**b**) Classification of histamine responses for 18 dizygous pairs (o) and 5 monozygous pairs (◊). The gray areas contain all pairs that are concordant in their Cl^- secretory response, i.e. sib A and B do not differ more than two classes in their carbachol, respectively histamine responses.*

The concordance in 8-bromo-cAMP + forskolin, carbachol, and histamine responses within the dizygous and monozygous pairs was evaluated for those pairs of which the specific response could be determined for both

siblings. Responses to 8-bromo-cAMP + forskolin for both sibling A and B were obtained for 15 pairs belonging to dizygous pairs and for 5 monozygous twin pairs. Six of the 15 dizygous pairs, but none of the monozygous pairs were discordant with respect to the presence of a cAMP-mediated Cl^- response. Carbachol responses were obtained for 29 dizygous pairs and 9 monozygous pairs. Eight of the 29 dizygous pairs and one of the 9 monozygous twin pairs differed by 2 or more classes with respect to their carbachol responses (Figure 4.4a, concordance in gray areas). Histamine responses for both sib A and B were obtained for 18 dizygous sibling pairs and 5 monozygous twins. Eight out of the 18 dizygous pairs, but none of the 5 monozygous twins differed in their histamine response (Figure 4.4b, concordance in gray areas). Intrapair variances for the responses to 8-bromo-cAMP + forskolin (expressed in $\mu A/cm^2$), and for the carbachol responses, histamine responses and DIDS influences (all expressed in PUH%), were calculated by the Mann-Whitney *U*-test. Monozygous twin pairs demonstrated to be more concordant than dizygous pairs, especially for their cAMP-mediated responses ($P < 0.001$), histamine responses ($P < 0.05$), and DIDS evoked differences between carbachol and histamine responses ($P < 0.02$).

Discussion

This study investigated the presence and frequency of chloride secretory responses in the rectal tissue of ΔF508 homozygous CF twins and siblings upon stimulation with cAMP and Ca^{2+}-linked agonists, before and after the incubation of the tissue with DIDS. Forty (73%) of the ΔF508 homozygotes expressed a cAMP-stimulated chloride secretory response in their intestinal tissue. Since cAMP-regulated chloride conductance is indicative of CFTR, [2,3,27] this finding suggests the presence of some active ΔF508 CFTR in the apical membranes of the enterocytes of these individuals. These *ex vivo* data do not concur with the current classification of the ΔF508 mutation[36] drawn from heterologous expression experiments, that ΔF508 leads to defective protein folding in the endoplasmic reticulum, which prevents its processing and targeting to the cell surface.[37] However, recent studies on CFTR expression in well differentiated human hepatobiliary, respiratory and intestinal tissues revealed that wild-type and ΔF508 CFTR were indistinguishable in their maturation and immunocytochemical localization.[13,38,39] Hence, ΔF508 CFTR can be processed *in vivo*, although its chloride channel function in response to activation by secretagogues is abolished or subnormal as is demonstrated in our study. The presence of functioning ΔF508 CFTR in intestinal cells is supported by data found in the

ΔF508 homozygous CF mouse model, which expresses a small Cl^- secretory response in the intestine, upon addition of the adenylate cyclase activator forskolin.[15]

The observation that a selection of the patients with a cAMP-dependent Cl^- secretory response showed no detectable Ca^{2+}-activated Cl^- secretion, may be explained by the fact that cAMP is a more sensitive indicator for CFTR, than carbachol or histamine (see above).[7] The lack of a Ca^{2+}-induced response in the Cl^- secretory direction in these patients may thus be caused by an overriding K^+ secretory response in the opposite direction, masking the Ca^{2+} activation of CFTR-mediated Cl^- currents.

A DIDS-sensitive chloride secretion was detected in 11 individuals (20%) of the investigated ΔF508 homozygotes, pointing to the presence of alternative Cl^- conductances that are reported to be DIDS-sensitive, such as the outwardly rectifying chloride channel[27] or the Ca^{2+}-activated Cl^- channels.[12,29,30] This number of patients possessing Ca^{2+}-stimulated residual Cl^- secretion may even be higher, as a very small Cl^- secretory response may be overruled by a larger response due to apical K^+-efflux. DIDS-sensitive Ca^{2+}-mediated residual Cl^- secretion only appeared in persons possessing cAMP-activated chloride channels. The underlying mechanism causing the observed co-expression of Ca^{2+} and cAMP-dependent chloride channels is presently not yet understood and has to be elucidated by further experiments.

It has been suggested from studies on CF mice[9,10] that the expression of an alternative chloride channel may compensate for defective or absent CFTR which attenuates the disease phenotype of organs. Our number of 11 cases with alternative, DIDS-sensitive Cl^- secretion is too low to provide a definitive answer as to whether the findings in the animal model can be extrapolated to man. However, in our cohort we did not observe a straightforward correlation between the presence of residual chloride secretion and a milder phenotype, when compared to the group that lacked any form of residual chloride secretion (data not shown). The divergent expression patterns of the members of the CLCA gene family in mouse and man, being global in mouse[11] and tissue-specific in man,[12,14] may partially account for this lack of a clear-cut association between the detection of an alternative chloride conductance and clinical status.

In a few patients (Table 4.1, 1 with and 4 without cAMP-activated secretion) a chloride secretory response in the range of class I to III was uncovered after preincubation with DIDS. This might be explained by the blockage of apical anion exchangers by DIDS,[31] probably unmasking a DIDS-insensitive serosal-to-mucosal chloride flux resulting in a larger histamine response compared to the carbachol response.

To summarize the chloride secretory data, the investigated ΔF508 homozygous CF individuals demonstrated impaired chloride secretory responses, however 40 out of 55 expressed residual chloride conductance mediated by CFTR, with additional alternative chloride conductance in 11 of these 40 patients. Though all CF patients showed impaired Ca^{2+}-mediated chloride secretion compared to controls, some ΔF508 homozygotes exhibited a subnormal carbachol response. This subnormal Cl^- secretion did in no case exceed 30% of the mean $\Delta I_{sc,Cl}$ response to carbachol seen in non-CF patients. However, this degree of Ca^{2+}-activated residual chloride secretion was previously only described for pancreas sufficient patients with CF who carry at least one mild *CFTR* mutation.[8]
The investigation of mono- and dizygous ΔF508 homozygous pairs is the adequate approach to dissect the relative impact of environmental factors, residual chloride channel activity and other genetic factors on disease phenotype. Monozygous twins proved to be more concordant in their patterns of residual chloride conductance than dizygous pairs. These findings imply that the genetic predisposition is more important for the expression of residual chloride secretion in the intestine than epigenetic factors.

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Chapter 5

Impact of chloride conductance and genetic background on disease phenotype in ΔF508 homozygous twins and siblings with cystic fibrosis

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Submitted

Abstract

To investigate impact of chloride secretion by residual activity of the cystic fibrosis transmembrane conductance regulator (CFTR) and/or by alternative chloride channels on disease manifestation in respiratory and intestinal tracts, we determined chloride secretory patterns in ΔF508 homozygous twins and siblings with cystic fibrosis (CF). We investigated most informative pairs that were either concordant for a mild or severe phenotype, or discordant in phenotype. In the majority of patients cAMP- and/or Ca^{2+}-regulated chloride conductance was detected, i.e. 84% and 73% of patients exhibited chloride conductance in the airways and intestine, respectively. Our finding of cAMP-mediated chloride conductance suggests that *in vivo*, at least some ΔF508 CFTR can reach the plasma membrane and effect chloride secretion. In respiratory tissue, the expression of basal CFTR-mediated chloride conductance demonstrated by 30% of ΔF508 homozygotes, and the response to a cAMP-agonist seen in 9% of patients, were identified as positive predictors for milder CF disease. In intestinal tissue 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS)-insensitive Cl^- secretion, indicative of functional CFTR channels, segregated with milder phenotype whereas DIDS-sensitive chloride secretion was mainly observed in more severely affected patients. The more concordant chloride secretory patterns within monozygous twins compared to dizygous pairs imply, that genes other than ΔF508 CFTR significantly influence the manifestation of the basic defect.

Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive disease in the Caucasian population, with highly variable manifestations in the pulmonary, gastrointestinal, hepatobiliary and urogenital tracts.[1] It is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which encodes a cAMP-regulated chloride channel,[2,3] that is located in the apical membrane of exocrine epithelia. The deletion of a phenylalanine residue at position 508 (ΔF508) is the most frequent of more than 900 known *CFTR* mutations and accounts worldwide for about 70% of all CF alleles.[4,5]
The heterogeneity of cystic fibrosis disease is partly explained by the broad spectrum of different mutations. *CFTR* gene mutations have been categorized by their resulting phenotype into six classes[6] whereby ΔF508 CFTR has been assigned to class II, which indicates the protein fails to reach the cell membrane to function as a chloride channel. This classification was based

on earlier experiments done in heterologous model systems and immunocytochemistry of patients' sweat glands, where deviant CFTR expression was found in ΔF508 cells.[7,8] Since clinical presentation varies significantly between patients with the same *CFTR* genotype and between various affected organs within CF patients, it is evident that more factors than the *CFTR* genotype are involved in determining CF disease severity. In *cftr* $^{-/-}$ knockout mice, an alternative Ca^{2+}-regulated chloride conductance was detected in the airways and pancreas and was suggested to ameliorate CF lung disease and protect the tissue from the absence of CFTR-mediated Cl^- conductance.[9] In addition, Ca^{2+}-activated chloride currents were observed in intestinal tissue of *cftr* $^{-/-}$ knockout mice with prolonged survival.[10]
The variable electrophysiological characteristics in human ΔF508 CF respiratory and intestinal tissue, i.e. the different degrees of residual chloride secretion observed,[11-13] might be explained by these alternative Ca^{2+}-regulated chloride channels.[14-16] Furthermore, recent immunocytochemical studies on intestinal, respiratory and hepatobiliary epithelia of ΔF508 homozygous CF patients revealed that ΔF508 CFTR may display apical distribution as is seen in control tissues, demonstrating that at least a portion of ΔF508 CFTR can be targeted to the plasma membrane.[17,18] Hence, it is important to clarify whether this ΔF508 CFTR is competent to transport chloride across epithelial membranes *in vivo*.
In this study, we investigated the basic chloride secretory defect in CF twins and siblings homozygous for the ΔF508 *CFTR* gene mutation in the most severely affected tissues in CF, the respiratory and intestinal tracts. We determined the bioelectrical properties of these tissues with the nasal potential difference measurement (NPD) and the intestinal current measurement (ICM), respectively. These methods assess the cAMP and Ca^{2+}-induced chloride secretory pathways by adding specific secretagogues and inhibitors, via which the presence of CFTR and/or alternative chloride conductances can be determined. By comparing the electrophysiological results to the phenotype in the specific tissues, the impact of residual chloride permeability on organ function could be analyzed.
The investigation of ΔF508 homozygous twins and siblings is the classical approach to analyze a monogenic disease like CF and differentiate the relative importance of the major disease causing lesion and other genetic and epigenetic factors.[19,20] The disease causing mutation and the intragenic haplotype are standardized and the variation in genetic background is either reduced (dizygous pairs) or eliminated (monozygous twins). Thus, the influence of genetic background and epigenetic factors on the genotype-phenotype correlation could be examined more profoundly than in a cohort of unrelated CF patients.

Methods

Subjects

The investigated ΔF508 homozygous patient pairs were recruited from a set of 114 CF monozygous and dizygous pairs enrolled for the European Cystic Fibrosis Twin and Sibling Study. Out of these 228 ΔF508 homozygous patients, we invited 43 pairs and 4 triplets, i.e. 98 patients. Clinical assessment, as well as NPD and ICM measurements were carried out at CF clinics in Hannover (20 pairs), Innsbruck (6 pairs), London (5 pairs), Rotterdam (12 pairs) and Verona (4 pairs). Mono- or dizygosity of twins was ascertained by using the AmpF*L*STR Profiler Plus™ typing kit, analyzed on the ABI Prism 377 (Perkin-Elmer Applied Biosystems)[21] or by oligonucleotide fingerprinting of simple repeats applying in situ gel hybridization of MboI or Hinf I genomic digests.[22] Twelve of the 47 pairs were monozygous twin pairs. Clinical data of the complete cohort of patients invited to participate in this study are provided in Table 5.1. Patients were not experiencing an episode of allergic bronchopulmonary aspergillosis or taking any systemic corticosteroids on the day of investigation, either of which could influence the performed measurements. In one pair liver disease was determined, and 3 patients all belonging to different pairs were treated with insulin for diabetes mellitus. All participating patients were pancreatic insufficient as was ascertained by the stool elastase test. Performed examinations were approved by the medical ethics committees of the local hospitals and by patients or parents by written informed consent.

Table 5.1 *Median [inner quartiles; range] for clinical data of the recruited 43 pairs and 4 triplets (n = 98 ΔF508 homozygous patients)*

	dizygous pairs (n = 74 individuals)	monozygous pairs (n = 24 individuals)
age [y]	13.8 [11.0 – 20.3; 5.4 – 39.3]	12.1 [10.0 – 20.6; 8.8 – 31.6]
wfh%	99.3 [91.2 – 106.4; 74.7 – 133.2]	91.5 [88.8 – 98.4; 78.9 – 122.1]
FEVPerc	39.6 [16.0 – 68.5; 0.0 – 100.0]	50.9 [20.5 – 78.4; 4.1 – 92.0]
intrapair difference in :		
age [y]	2.6 [1.8 – 3.8; 0.0 – 15.6]	—
wfh%	10.1 [5.8 – 15.0; 0.4 – 29.4]	3.2 [1.5 – 8.1; 0.0 – 18.3]
FEVPerc	30.3 [11.8 – 54.6; 0.5 – 100.0]	15.1 [11.0 – 26.6; 2.8 – 52.7]

Assessment of clinical phenotype

In order to select the most extreme phenotypes of 114 patient pairs, the overall disease severity and intrapair discordance were quantified, as reported.[23] Briefly, a patient's nutritional status was characterized by the

anthropometric parameter weight expressed as percentage of predicted weight for height (wfh%) using the data published by Prader et al[24]. The pulmonary status was assessed by the forced expiratory volume in 1s (FEV1), expressed as predicted values (FEV1%pred) based on the formula by Knudson et al.[25] FEV1%pred declines with age as expected for progressive lung disease in CF.[26] Therefore, we employed age-specific percentiles for FEV1%pred (FEVPerc), calculated from the CF population data base published in the report of the European CF Registry,[27] to correct for the age-decline in FEV1%pred in our cohort (Chapter 3). Thus, wfh% and FEVPerc, two age-independent parameters most sensitive for course and prognosis, were evaluated to characterize CF disease severity in the two major afflicted organs in CF, i.e. the gastrointestinal and the respiratory tracts. For ranking and selection of a patient pair composed of siblings i and j, the patient's disease severity (DfO, distance form origin) and the pair's intrapair discordance (DELTA) were defined from the rank numbers for wfh% ($x_{i,j}$) and FEVPerc ($y_{i,j}$) as defined by Equations 3.1 and 3.2.
These two terms describe the patient's disease severity DfO as a distance from the origin in the plot of x_i versus y_i and the pair's intrapair discordance DELTA as the distance between two data points representing the two siblings of the pair in the respective plot (Figure 3.2).
A computer-assisted algorithm was employed to identify patient pairs composed of two siblings with equally high wfh% and equally high FEVPerc (concordant/mildly affected; CON+), pairs consisting of two siblings with equally low wfh% and equally low FEVPerc (concordant/severely affected; CON-) and patient pairs comprised of one sibling with high wfh% and high FEVPerc and one sibling with low wfh% and low FEVPerc (discordant pairs; DIS). Other pairs were discriminated from these extreme phenotypes by the ranking algorithm, and excluded from the analyses described in this report.

Nasal potential difference
The method of studying NPD has been adapted from a method described previously,[28] and measures Cl^- and Na^+ conductances as a potential difference. In short, the reference bridge was connected to a small needle (25 gauge) inserted into the subcutaneous space of the forearm, which is iso-electric with the submucosal space of the nasal epithelium, and was filled with saline solution. The exploring bridge was formed by a PE-90 tube positioned against the nasal turbinate to apply the different perfusion solutions. Both the reference bridge and the exploring catheter were connected to a high input resistance voltmeter via 4% agar-salt bridges and Ag/AgCl electrodes. Transepithelial electric potential difference measurements were performed by positioning the exploring catheter under the inferior nasal turbinate using an otoscope to visualize the epithelium.

The basal PD was measured during perfusion of the nasal catheter (1.7mL/min) with a salt solution, and was found to be lumen-negative with respect to the submucosal reference electrode. The salt solution consisted of (mmol/L): NaCl (120), Na-gluconate (25), K-gluconate (5), NaH_2PO_4 (0.4), Na_2HPO_4 (2.4). On the inferior turbinate, the spot with the maximal (most negative), stable baseline PD was selected. This baseline PD demonstrated to be considerably more negative in CF patients than in non-CF individuals (Figure 5.1). At this site nasal potential differences were measured in response to superfusion with solutions of different ion compositions, or containing different drugs (mol/L) (Figure 5.1).

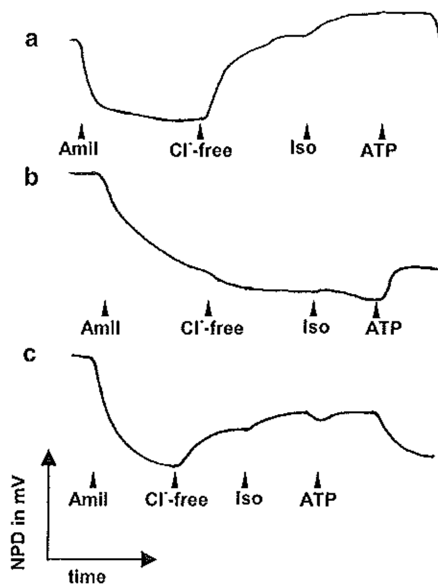

Figure 5.1 *Nasal potential difference measured upon superfusion of the nasal epithelium with amiloride (Amil), a solution in which chloride is substituted by gluconate (Cl⁻-free), isoprenaline (Iso), and ATP(ATP). Registrations are given for* ***(a)*** *non-CF control,* ***(b)*** *CF patient without a gluconate or isoprenaline response, but with a response to ATP,* ***(c)*** *CF patient exhibiting residual chloride conductance evoked by Cl⁻-free solution and isoprenaline.*

First, the catheter was perfused with amiloride (10^{-4}), a blocker of epithelial Na^+ channels (ENaC), thereby inhibiting the PD by eliminating the contribution of electrogenic sodium absorption.[29] To determine the basal Cl^- conductance, chloride was substituted by gluconate in the solution containing amiloride. In control persons this causes a hyperpolarisation

(increased negativity) of the epithelium since it creates an increased driving force for Cl^- diffusion toward the airway lumen. Subsequently, the beta-adrenergic agonist isoprenaline (10^{-4}) was added to the Cl^--free solution containing amiloride, which induces cAMP-dependent chloride conductance and evaluates the presence of CFTR.[28] For the last perfusate, ATP (10^{-3}) was added to the Cl^--free solution containing amiloride and isoprenaline. ATP binds to purinergic receptors on the luminal surface, and by activating phospholipase C and increasing intracellular Ca^{2+} it triggers the Ca^{2+}-mediated Cl^- secretory pathway.[30] All perfusion solutions were adjusted to pH 7.4. Superfusion of the nasal epithelium with the different perfusates was continued until a steady state was reached, or for at least 3 minutes. In each patient nasal potential difference measurements were performed in left and right nostril. The NPD tracing of the nostril with the highest Cl^- secretory responses, i.e. with the largest capacity to transport chloride, was assessed for the evaluations and calculations performed in this study. Tracings of patients with chronic rhinitis or a cold on the day of investigation were discarded from further evaluation.

Intestinal current measurement in rectal biopsies

The method used to study ICM has been described previously.[11,12,31] It determines Na^+ and Cl^- fluxes in the intestinal epithelium as a change in short-circuit current (ΔI_{sc}). Freshly obtained rectal suction biopsies were mounted in adapted micro-Ussing chambers with an aperture of 1.2 mm.[31] The tissue was perfused with Meyler buffer solution at 37°C (composition in mmol/L: Na^+ 126.2; Cl^- 114.3; HCO_3^- 20.2; HPO_4^{2-} 0.3; $H_2PO_4^-$ 0.4; Hepes 10; pH = 7.4) and gassed with 95% O_2 and 5% CO_2. Basal transepithelial resistance was determined by the voltage response to pulse currents of 1 μA and applying Ohm's law. Basal I_{sc} prior to voltage clamping was calculated from the basal transepithelial resistance and the open-circuit transepithelial potential difference. Subsequently, the tissue was short-circuited by voltage-clamps for the course of the experiment. For cell metabolism glucose (10^{-2} mol/L) was given both mucosally and serosally. After equilibration, pharmaceuticals (mol/L) were added in a standardized order to the mucosal (M) and/or serosal (S) side: a) amiloride (10^{-4}, M)[29];b) indomethacin (10^{-5}, M+S), to reduce basal Cl^- secretion by inhibiting the endogenous prostaglandin formation[32]; c) carbachol (10^{-4}, S), to initiate the cholinergic Ca^{2+}-linked Cl^- secretion[33]; d) forskolin (10^{-5}, M+S)[34] together with 8-bromo-cAMP (10^{-3}, M+S), to open cAMP-dependent Cl^- channels, like CFTR and the ORCC[35]; e) 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, 2.10^{-4}, M), to inhibit DIDS-sensitive Cl^- transporters like the Ca^{2+}-dependent Cl^- channels and the ORCC[14,15,35]; f) histamine (5.10^{-4}, S), to reactivate the Ca^{2+}-dependent chloride secretory pathway.[36] Two rectal biopsies were obtained

for all patients participating in the intestinal current measurements. The ICM tracing belonging to the biopsy with the highest chloride secretory responses was included in the performed computations. All drugs for NPD and ICM measurements were obtained from Sigma Chemical Co., St. Louis, MO, USA.

Interpretation of ICM patterns

By using the above mentioned ICM protocol, at least six different ICM patterns could be observed in tracings from CF individuals (Figure 5.2). To quantify the Cl^- secretory responses to carbachol and histamine the net change in I_{sc} was defined as the sum of the negative downward peak below baseline, and the positive upward peak above baseline in the chloride secretory direction. A net negative response points to no or little residual Cl^- secretion, while a net positive change in I_{sc} indicates high residual Cl^- secretion. The influence of DIDS on chloride secretion was determined by comparing the net chloride secretory response to carbachol before the addition of DIDS, to the net chloride secretory response to histamine after the incubation with DIDS.[12] Figure 5.2a shows the ICM tracing of a typical CF patient lacking any type of residual chloride secretion. Carbachol and histamine peaks are in the reverse direction to control, which is interpreted as apical K^+ secretion[37] unmasked in the absence of Cl^- secretion. These patients lack responsiveness to cAMP-analogues as well. CF patients with different degrees of DIDS-insensitive residual chloride secretion are depicted in Figures 5.2b, c and d. The presence of small amounts of DIDS-insensitive chloride secretion is only detected by 8-bromo-cAMP + forskolin (Figure 5.2b), since this is a more sensitive indicator for cAMP-mediated chloride secretion than carbachol or histamine.[11,12] In tissues with higher degrees of chloride conductance carbachol and histamine evoke chloride secretory responses as well, ranging from responses that are composed of a peak in the reversed direction followed by a small change in I_{sc} in the chloride secretory direction (Figure 5.2c), to responses that consist purely of a subnormal chloride secretory peak (Figure 5.2d). DIDS-sensitive residual chloride secretion was characterized by an ICM pattern as in Figure 5.2e or f, with a component in the chloride secretory direction in response to carbachol, which is not recurring in the histamine response since it is inhibited by the presence of DIDS.

Statistical analysis

The statistical analyses of our results were performed using the Mann-Whitney U-test, t-test for grouped pairs, Fisher's exact test, F test, or Spearman-test. P values smaller or equal to 0.05 were considered statistically significant.

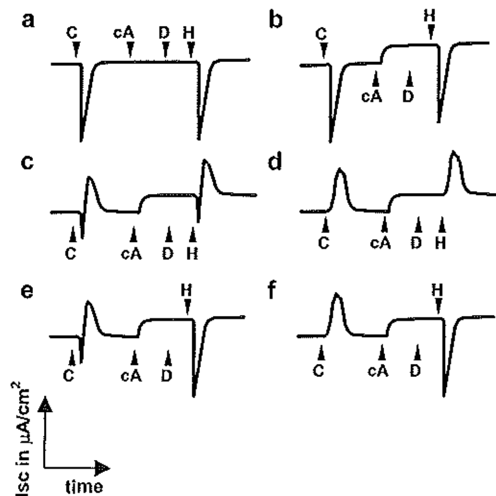

Figure 5.2 *Sketches of ICM response patterns observed in ΔF508 homozygous CF patients, upon the addition of carbachol (C), 8-bromo-cAMP + forskolin (cA), DIDS (D), and histamine (H). A downward, reversed peak upon addition of carbachol or histamine is indicative of K^+ efflux, while an upward peak corresponds to chloride secretion. (**a**) No response in the chloride secretory direction to any of the applied compounds. (**b**) cAMP-mediated residual chloride secretion only evoked by cAMP + forskolin. (**c-d**) DIDS-insensitive residual chloride secretion in the presence of a cAMP response. (**e-f**) DIDS-sensitive residual chloride secretion in the presence of a cAMP response.*

Results

According to the ranking algorithm on wfh% and FEVPerc, 29 of the 47 ΔF508 homozygous CF sibling pairs were categorized into one of the extreme phenotypes DIS, CON+ and CON- (see Methods section). Due to various reasons, electrophysiological data from NPD and/or ICM measurements were not obtained for all of these 29 pairs. Local medical ethical committees of some centers did not allow ICM measurements in minors, or prohibited a second ICM investigation if the first attempt had failed. Additionally, a number of selected patients did not accept or endure the procedures of the electrophysiological tests.

Table 5.2 *Mean [range] for clinical data of pairs ranked DIS, CON+ and CON- for which NPD data was obtained for both siblings*

	CON+ *(6 pairs)*	CON- *(6 pairs)*	DIS *(6 pairs)*	DIS *mildly affected sib*	DIS *severely affected sib*
age [y]	17.6[10.0– 34.5]	13.9[7.2– 18.3]	19.9[7.6– 38.8]	19.7[7.6– 35.4]	20.2[10.4–38.8]
wfh%	105.2[86.7–133.2]	93.5[84.6–109.3]	95.4[74.7–122.8]	103.2[83.0–122.8]	87.7[74.7–95.9]
FEVPerc	64.0[35.3–100.0]	34.3[10.5– 76.6]	41.7[4.1–100.0]	66.5[15.6–100.0]	16.8[4.1–37.3]
intrapair-difference in:					
age[y]	1.9[0.0– 5.5]	2.1[0.0– 5.5]	2.7[0.0– 5.5]	—	—
wfh%	13.0[0.4–27.7]	5.1[1.4–12.1]	15.5[3.3– 29.4]	—	—
FEVPerc	21.4[11.3–34.8]	11.9[0.5–21.8]	50.8[3.2–100.0]	—	—

NOTE. The results obtained by NPD for these pairs are displayed in Figures 5.3a and b.

Table5.3 *Mean [range] for clinical data of pairs ranked DIS, CON+ and CON- for which ICM data was obtained for at least one of both siblings*

	CON+ *(5 pairs)*	CON- *(11 pairs)*	DIS *(9 pairs)*	DIS *mildly affected sib*	DIS *severely affected sib*
age [y]	18.9[10.6– 34.5]	13.0[7.2– 20.5]	20.0[7.6– 39.3]	19.8[7.6– 36.9]	20.3[10.4– 39.3]
wfh%	105.1[89.8–122.1]	90.4[77.5–109.3]	95.3[74.7–122.8]	102.6[83.0–122.8]	87.9[74.7–100.8]
FEVPerc	65.5[35.3–100.0]	36.6[4.1– 92.0]	43.8 [4.1–100.0]	66.7[5.2–100.0]	20.5[4.1– 41.1]
intrapair-difference in:					
age[y]	1.9[0.0– 5.5]	1.4[0.0– 5.5]	2.8[0.0– 5.5]	—	—
wfh%	10.3[0.4–18.3]	7.3[0.0–23.8]	14.7[3.2– 29.4]	—	—
FEVPerc	19.2[11.0–34.8]	17.2[0.5–52.6]	52.3[3.2–100.0]	—	—

NOTE. The results obtained by ICM for these pairs are displayed in Figures 5.4 and 5.5.

Finally, NPD tracings of patients with respiratory inflammation were not included in the analysis, and ICM responses to carbachol or histamine that were not interpretable owing to technical irregularities during the experiment, e.g. air in the connecting bridges or electrical disturbances, were discarded from further evaluations. Thus, we did not obtain electrophysiological data for all of the 29 pairs who were assigned to one of the extreme phenotypes DIS, CON+, or CON-. To avoid the loss of data points we first evaluated NPD and ICM results independently and used all valid data for interpretations. Tables 5.2 and 5.3 summarize absolute values and intrapair differences of age, wfh% and FEVPerc in pairs with valid NPD tracings obtained for both siblings (Table 5.2), and for the pairs of which valid ICM tracings were obtained for at least one of the two siblings (Table 5.3).

Nasal potential difference

In our group of ΔF508 homozygous patients with a mean age of 16.3 ± 8.5 years (range 4.2 - 39.3 yrs), the absolute age or the intrapair difference in age, as well as the gender of individuals was not associated with different outcome in NPD measurements. Hence, differential NPD responses could not be ascribed to age or sex.

The basal PD measured in 57 individuals belonging to dizygous sibling pairs was -56.0 ± 10.1 mV and the decrease in PD in response to amiloride was 28.0 ± 8.9 mV (Table 5.4). In 17 patients belonging to monozygous twin pairs the basal PD was -57.0 ± 8.8 mV, while the amiloride response was 27.4 ± 7.9 mV. Eighteen individuals belonging to a dizygous sibling pair exhibited small amounts of basal Cl^- conductance measured by the superfusion with a Cl^--free solution (Table 5.4), while 5 persons demonstrated Cl^- conductance initiated by the addition of isoprenaline, and 45 patients reacted to the addition of ATP. The PD of individuals of monozygous twin pairs increased in response to superfusion with Cl^--free solution in 4 patients, while increases were seen in 2 individuals when isoprenaline was added, and in 10 persons upon addition of ATP.

The distribution of PD responses was independent of zygosity status (Table 5.4).

To assess the correlation between the basic defect and the phenotype in the respiratory tissue, NPD results measured within the 3 subgroups of extreme phenotypes, discordant (DIS), concordant mild (CON+), and concordant severe (CON-), were compared to the FEV1%predicted percentiles.

The lung functions of 8 discordant sib pairs were plotted against their responses to superfusion with Cl^--free solution (Figure 5.3a). The sibs with the better lung function parameters demonstrated significantly higher (more negative) gluconate responses than their paired siblings (t-test for grouped pairs, $P < 0.05$) and, moreover, also possessed the better weight for height parameter of the two siblings (data not shown). When the lung function of patients belonging to CON+ and CON- pairs was plotted against their gluconate responses all persons (except one) with a gluconate response, i.e. increased negativity, appeared to belong to concordant mild pairs (Figure 5.3b). The presence of this Cl^- secretory response upon addition of gluconate was significantly associated with the concordant mild (CON+) phenotype (Fisher's exact test, $P = 0.036$). Only one patient belonging to a CON- pair showed a gluconate response. Similarly, responses to isoprenaline were not observed in severely affected patients, i.e. patients of CON- pairs or the severely affected sibs of DIS pairs.

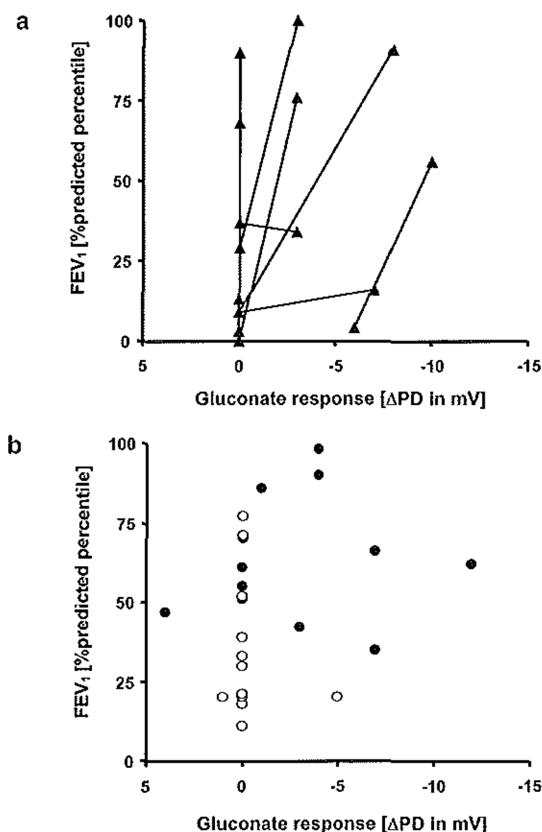

Figure 5.3 *(**a**) FEV₁ %predicted percentile versus the change in NPD upon addition of a Cl⁻-free solution (gluconate substituted for chloride) for 8 pairs that were discordant for the FEVPerc parameter. Individual sibs are indicated by triangles, sib pairs are connected by a line. The sib with the higher (more negative) response to gluconate within a pair, was also the sib with the higher lung function (P < 0.05, Student's t test for grouped pairs). (**b**) FEVPerc versus the gluconate response for 6 pairs who were ranked as concordant/mildly affected pairs (CON+, •) and 6 pairs who were ranked as concordant/severely affected pairs (CON-, o). A hyperpolarisation of the PD in response to gluconate, pointing to the presence of chloride conductance, segregated with the CON+ pairs (P = 0.001, Fisher's exact test).*

Patients in Fig. 5.3a	DIS	CON+	CON-	
pairs, dizygous	7	-	-	
pairs, monozygous	1	-	-	
data points:	16	-	-	Σ16

Patients in Fig. 5.3b	DIS	CON+	CON-	
pairs, dizygous	-	4	5	
pairs, monozygous	-	2	1	
data points:	-	12	12	Σ24

The hyperpolarisation (increased negativity) of PD seen in 55 of the 74 patients in response to perfusion of the nasal epithelium with ATP did not show any associations with lung function.
The substantiation of the observed associations between NPD values and phenotype was given by the following results. For each pair, the NPD values of the individual sibs of a pair were added, corresponding to $PDsib_A + PDsib_B$ in the case of the basal PD, and $\Delta PDsib_A + \Delta PDsib_B$ for the responses to the different solutions applied to the nasal epithelium (Table 5.5). Summations calculated for the concordant mild pairs, were balanced against those of the concordant severe pairs (Table 5.5). The intrapair summations for the basal PDs of the concordant mild sibs were less negative than those in the concordant severe couples (-101.7 ± 18.2 vs -114.8 ± 12.9), however this was not significant (Mann-Whitney *U*-test, $P < 0.10$, Table 5.5). The $\Delta PDsib_A + \Delta PDsib_B$ values for the gluconate responses were significantly more negative for the concordant mild pairs ($P < 0.05$), while $\Delta PDsib_A + \Delta PDsib_B$ values for the amiloride and ATP responses were not different between concordant mild and concordant severe pairs. The $\Delta PDsib_A + \Delta PDsib_B$ values for the isoprenaline responses within CON+ and CON- pairs were not significantly different, most likely due to the lack of a response in most investigated individuals and the small magnitude of responses in patients who did respond to isoprenaline. However, $\Delta PDsib_A + \Delta PDsib_B$ for isoprenaline was negative in the concordant mild couples (-1.3 ± 2.4) and positive in the concordant severe couples (0.5 ± 0.8, also see above: no chloride conductance induced by isoprenaline in siblings of CON- pairs).
To analyze the intrapair concordance in NPD values of monozygous pairs versus dizygous pairs, intrapair differences were calculated by absolute values $|PDsib_A - PDsib_B|$ for the basal PD, and by $|\Delta PDsib_A - \Delta PDsib_B|$ for responses to applied substances (Table 5.6). Monozygous twins (5.0 ± 3.4 mV) were more concordant than dizygous pairs (11.4 ± 8.3 mV, $P < 0.05$, Table 5.6) in baseline PD, but not any other parameter.

Intestinal current measurement

To assess the impact of intestinal residual chloride secretion on clinical outcome, responses to the application of carbachol and histamine and the influence of DIDS on Cl^- secretory responses, were compared in the CON+, CON- and DIS groups (Figure 5.4a). Carbachol responses within the mildly affected sibs, i.e. sibs belonging to CON+ pairs or the better sibs of DIS pairs, were not different from those in CON- pairs, or the sicker sibs of DIS pairs. Histamine responses, however, were less negative in the mildly affected sibs, which was significant when sibs of CON+ and CON- pairs were compared ($P = 0.05$, Mann-Whitney *U*-test, Figure 5.4b). In addition, the difference in

Table 5.4 *Means ± SD for NPD values for the group of individuals belonging to dizygous or monozygous sibling pairs*

	Dizygotes		Monozygotes	
	PD or ΔPD [mV]	*n*	PD or ΔPD [mV]	*n*
Total investigated group		72		26
No patient cooperation		4		2
(Chronic) rhinitis		11		7
Basal PD	-56.0 ± 10.1	57	-57.0 ± 8.8	17
Amiloride	28.0 ± 8.9	57 (100%)	27.4 ± 7.9	17 (100%)
Gluconate (Cl-free)	-5.9 ± 2.8	18 (32%)	-6.3 ± 3.9	4 (24%)
Isoprenaline	-2.6 ± 1.5	5 (9%)	-2.5 ± 0.7	2 (12%)
ATP	-4.3 ± 2.4	45 (79%)	-4.8 ± 3.9	10 (59%)

Table 5.5 *Intrapair summations for the NPD values of sib A and sib B, calculated for the CON+ and CON- pairs*

	CON+ (6 pairs)		CON- (6 pairs)		CON+ vs CON-
	Mean ± SD	Range	Mean ± SD	Range	*P*-value
PDsib$_A$ + PDsib$_B$					
Basal PD	-101.7 ± 18.2	-132 – -79	-114.8 ± 12.9	-136 – -100	0.07
ΔPDsib$_A$ + ΔPDsib$_B$					
Amiloride	53.7 ± 13.3	42 – 78	55.0 ± 18.3	24 – 78	0.35
Gluconate (Cl-free)	-5.7 ± 6.0	-12 – 4	-0.7 ± 2.2	-5 – 1	0.05
Isoprenaline	-1.3 ± 2.4	-5 – 1	0.5 ± 0.8	0 – 2	0.16
ATP	-6.7 ± 2.4	-11 – -4	-6.5 ± 4.7	-14 – 0	0.53

NOTE. Comparisons between CON+ and CON- data were analyzed by the Mann-Whitney *U*-test.

Table 5.6 *Intrapair differences of dizygous and monozygous pairs for the obtained NPD values*

	Dizygous pairs (29 pairs)		Monozygous pairs (6 pairs)		DI vs MONO
	Mean ± SD	Range	Mean ± SD	Range	*P*-value
|Pdsib$_A$ - PDsib$_B$|					
Basal PD	11.4 ± 8.3	38 – 0	5.0 ± 3.4	10 – 1	0.02
|ΔPDsib$_A$ - ΔPdsib$_B$|					
Amiloride	10.3 ± 8.4	34 – 0	6.7 ± 6.0	16 – 0	0.17
Gluconate (Cl-free)	2.6 ± 3.2	12 – 0	2.8 ± 3.1	8 – 0	0.33
Isoprenaline	0.4 ± 0.8	3 – 0	0.7 ± 0.8	2 – 0	0.18
ATP	2.6 ± 2.3	10 – 0	1.2 ± 1.9	5 – 0	0.06

NOTE. Comparisons between dizygous and monozygous data were analyzed by the Mann-Whitney *U*-test.

carbachol and histamine responses due to the presence of DIDS was significantly different, both when CON+ and CON- sibs were compared ($P < 0.01$) or when DIS+ and DIS- sibs were compared ($P = 0.05$, Figure 5.4c). Additionally, weight for height percentage was positively correlated to the magnitude of the DIDS influence on Cl^- secretion ($P = 0.02$, Figure 5.5, Spearman's rank test).
As previously reported,[12] monozygous twins were more concordant in their ICM patterns than the dizygous pairs, which was significant in the case of the cAMP-stimulated chloride secretory responses (Mann-Whitney U-test, $P < 0.001$), the histamine responses ($P < 0.05$), and the influence of DIDS on the chloride secretory responses ($P < 0.025$).

Nasal potential difference and intestinal current measurement
To assess differences and/or similarities in the presence and magnitude of residual Cl^- secretion between respiratory and intestinal tissues, the NPD results were compared to the ICM results. The presence of a gluconate response did not segregate with the presence of a chloride secretory component in the carbachol and/or histamine response, and there were no significant correlations between the amplitude of the gluconate response versus the amplitudes of the carbachol and histamine responses (Figures 5.6a and b). However, in this preselected cohort of extreme phenotypes, the presence of a gluconate response in the respiratory tissue and a positive DIDS influence in the intestinal tissue segregated with milder phenotype (Fisher's exact test, $P = 0.01$). Moreover, the magnitude of the gluconate response related to the magnitude of the DIDS influence on Cl^- secretion (Spearman's rank test, reaching the limit of significance, Figure 5.6c).

Discussion

The observation that disease manifestations in cystic fibrosis are highly heterogeneous even in patients with the same *CFTR* mutation genotype indicates that factors other than the *CFTR* gene itself contribute to the course of CF disease. The approach of the European CF Twin and Sibling Study of investigating mono- and dizygous ΔF508 homozygous pairs with the extreme CF phenotypes, is the optimal scenario to investigate the relative impact of environmental factors and/or inherited factors besides the *CFTR* gene, that influence the CF phenotype.[19,20,38-40] CF twins and siblings share many environmental factors that are major determinants of CF disease severity, e.g. the physician, therapeutic regime, living conditions, and the siblings' behavioral patterns.

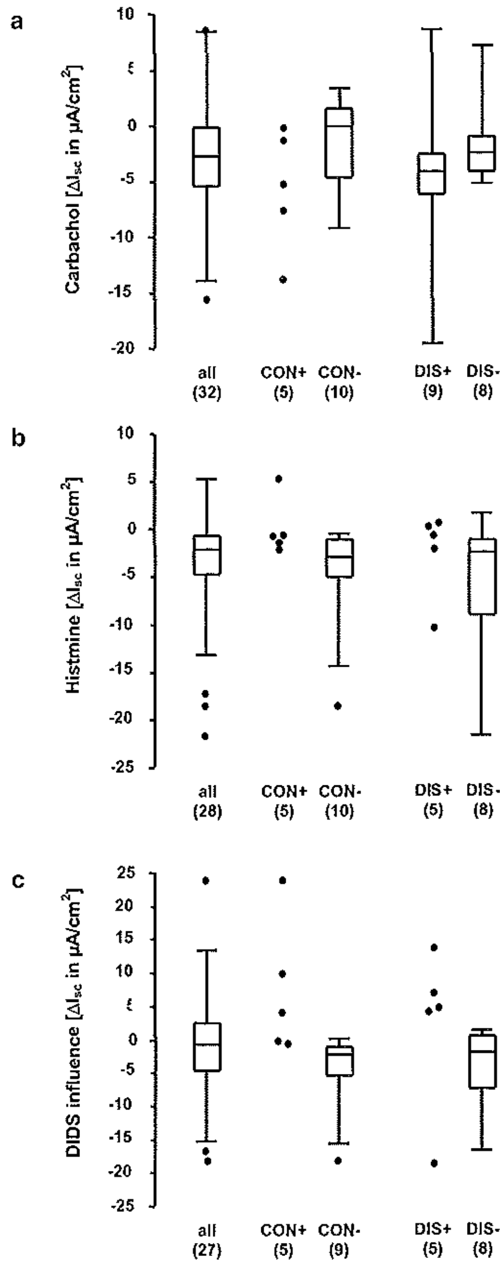
a
10
5
0
-5
-10
-15
-20
Carbachol [ΔIsc in µA/cm2]
all (32)
CON+ (5)
CON- (10)
DIS+ (9)
DIS- (8)
b
10
5
0
-5
-10
-15
-20
-25
Histmine [ΔIsc in µA/cm2]
all (28)
CON+ (5)
CON- (10)
DIS+ (5)
DIS- (8)
c
25
20
15
10
5
0
-5
-10
-15
-20
-25
DIDS influence [ΔIsc in µA/cm2]
all (27)
CON+ (5)
CON- (9)
DIS+ (5)
DIS- (8)

Figure 5.4 *(see previous page) Net I_{sc} responses to carbachol and histamine and the influence of DIDS on the chloride secretory response, given for the whole group (all), and for the sibs belonging to CON+, CON-, or DIS pairs. For the CON+ and CON- pairs, individuals were counted when only one sib of a pair had a data entry, while the mean of both sibs was included when both sibs had a data entry. For the DIS pairs, data are compared between the clinically better (DIS+) and worse (DIS-) siblings. Comparison of mildly and severely affected sibs and pairs was performed by Mann Whitney rank tests, as shown in the following Table:*

ICM parameter	Mildly affected sibs and pairs	*n*	Severely affected sibs and pairs	*n*	*P*-value
Carbachol	CON+	5	CON-	10	ns
Histamine	CON+	5	CON-	10	0.05
DIDS influence	CON+	5	CON-	9	$0.001 < P < 0.01$
Carbachol	DIS+	9	DIS-	8	ns
Histamine	DIS+	5	DIS-	8	0.177
DIDS influence	DIS+	5	DIS-	8	0.047
Carbachol	CON+ & DIS+	14	CON- & DIS-	18	$0.025 < P < 0.05$
Histamine	CON+ & DIS+	10	CON- & DIS-	18	$0.01 < P < 0.025$
DIDS influence	CON+ & DIS+	10	CON- & DIS-	17	$0.001 < P < 0.01$

By investigating ΔF508 homozygotes the major disease causing lesion of the *CFTR* gene was standardized. Apart from their identical *CFTR* mutation genotype, and an identical region around the *CFTR* gene locus, dizygous twins and siblings share on the average half of their genes, which reduces differences in genetic background. The intrapair variation among monozygous twins, who are genetically identical for their entire genome, can be used to delineate the influence of the environment on a trait by comparing the intrapair variability among monozygous pairs to that of dizygous pairs.
In this study we tested the hypothesis whether the basic defect in CF, which presents itself as aberrant chloride conductance properties, is predictive for the clinical outcome in CF, and if the manifestation of the basic defect is substantially determined by inherited factors apart from the *CFTR* gene. Therefore, we investigated the bioelectrical properties of the respiratory and intestinal epithelium that normally express CFTR, by applying the nasal potential difference measurement and intestinal current measurement, respectively. The influence of residual chloride secretion on clinical CF phenotype in the specific organs was evaluated by determining the presence and origin of residual chloride conductance in subgroups of ΔF508 homozygous CF twin and sib pairs with disparate manifestation of CF disease. These individuals with the most extreme clinical parameters are highly informative to investigate factors influencing the CF phenotype.[38-40]

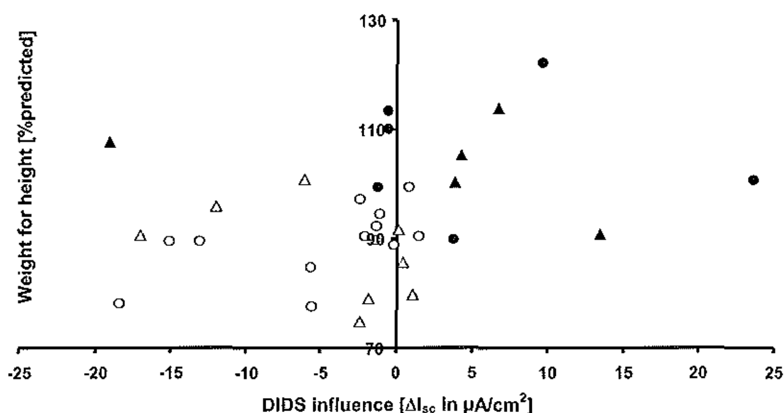

Figure 5.5 *Weight for height %predicted plotted against the influence of DIDS on the chloride secretory response, calculated as the difference between the carbachol response before the incubation of the tissue with DIDS, and the histamine response in the presence of DIDS. Values are plotted for sibs of CON+ pairs (•), CON- pairs (o), and the mildly affected (DIS+, ▲) and severely affected (DIS-, Δ) sibs of DIS pairs. All CON+ and DIS+ siblings but one, exhibited either just a small negative or even a positive influence of DIDS, meaning that DIDS did not inhibit chloride secretion in these individuals. In contrast, DIDS inhibited the chloride secretory response in the CON- and DIS- sibs, which resulted in a negative DIDS influence ($P = 0.03$, Fisher's exact test). Moreover, weight for height %predicted correlated with the DIDS influence ($P < 0.025$, Spearman test).*

Patients in Fig. 5.5	DIS	CON+	CON-	
pairs, dizygous	5	1	2	
pairs, monozygous	-	-	2	
individuals, dizygous	2	3	4	
individuals, monozygous	1	1	1	
data points:	13	6	13	Σ32

Patients in Fig. 5.6a	DIS	CON+	CON-	
pairs, dizygous	4	2	3	
pairs, monozygous	1	1	1	
individuals, dizygous	4	2	3	
individuals, monozygous	-	-	3	
data points:	14	8	14	Σ36

Patients in Fig. 5.6b	DIS	CON+	CON-	
pairs, dizygous	3	-	3	
pairs, monozygous	-	-	1	
individuals, dizygous	3	4	3	
individuals, monozygous	1	1	1	
data points:	10	5	12	Σ27

Patients in Fig. 5.6c	DIS	CON+	CON-	
pairs, dizygous	3	-	2	
pairs, monozygous	-	-	1	
individuals, dizygous	3	4	3	
individuals, monozygous	1	1	1	
data points:	10	5	10	Σ25

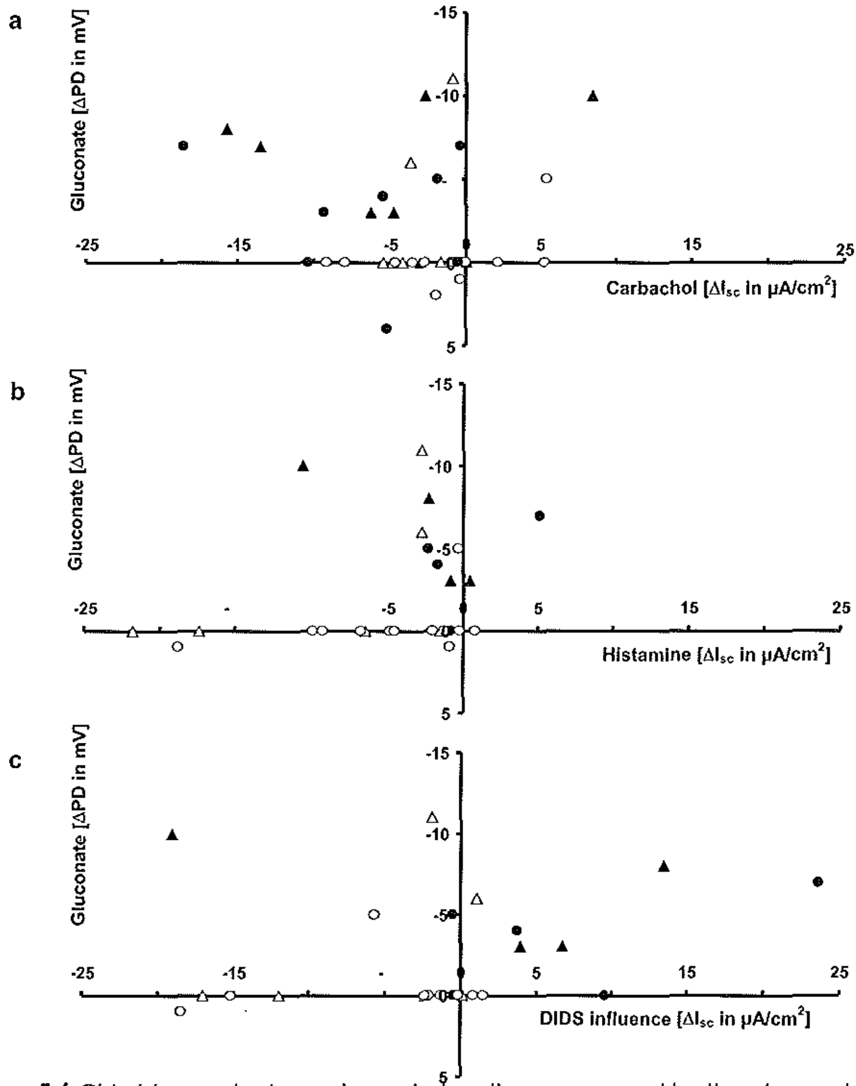

Figure 5.6 *Chloride conductance in respiratory tissue measured by the gluconate response, compared to chloride secretion in intestinal tissue. Values are given for individuals belonging to CON+ (•) and CON- (o) pairs, and for DIS+ sibs (▲) and DIS- sibs (Δ). The gluconate response measured in NPD plotted versus the chloride secretory response measured in ICM, upon the addition of (**a**) carbachol or (**b**) histamine, and (**c**) versus the influence on chloride secretion exerted by DIDS. While the gluconate response did not correlate with carbachol or histamine responses, the magnitude of the gluconate response was associated with the magnitude of the DIDS influence ($r_s = 0.31$, n.s. trend, $0.05 < \alpha(r_s) < 0.1$). A negative gluconate response pointing to chloride conductance in combination with a positive DIDS influence segregated with the CON+ and DIS+ sibs ($P = 0.01$, Fisher's exact test).*

This report and our earlier reports on intestinal chloride secretions,[11,12] demonstrate that many of the ΔF508 homozygous CF individuals express some residual chloride channel activity in both respiratory and intestinal tract. In those patients with apparent cAMP-mediated chloride secretion, i.e. an isoprenaline response and/or a DIDS-insensitive chloride secretory response, the molecular entity is most likely CFTR, indicating the presence of functional ΔF508 CFTR in the apical membranes of the epithelial cells in these organs. On the basis of studies in heterologous expression systems,[7,8] the ΔF508 *CFTR* gene mutation is considered as a processing defect by reduced glycosylation and consequent misfolding of the CFTR protein, which prevents the protein to reach the plasma membrane and retains it in the endoplasmic reticulum. However, the expression of ΔF508 CFTR *in vivo* might be different. Immunohistochemical studies performed on airway, intestinal and hepatobiliary tissues,[17,18,41,42] demonstrated ΔF508 CFTR localization in the plasma membrane. Moreover, functional assays on transfected cells,[43] in mice,[44,45] and on human tissue specimens[18] have shown that ΔF508 CFTR is capable of transporting chloride in response to cAMP-agonists. Our *in vivo* and ex vivo studies show that respiratory and intestinal tissues from ΔF508 CFTR homozygous individuals are competent to respond to agonists of the cAMP-dependent chloride secretory pathway, which is the hallmark of CFTR-mediated chloride transport.[2,3] Nonetheless, the magnitude of this detected chloride permeability is insufficient to prevent manifestation of CF disease.

The results of our study demonstrate a segregation of better lung function with less anomalous bioelectrical properties of the nasal epithelium in the investigated ΔF508 homozygotes. The respiratory epithelium of healthier CF patients appears to be less Cl^--impermeable (Figures 5.3a and b). Moreover, the presence of basal chloride conductance and the capacity to secrete chloride in response to a cAMP-agonist, determined by perfusion of the nasal epithelium with a Cl^--free solution and isoprenaline, respectively, were only present in mildly affected patients (Table 5.4 and Figure 5.3). This suggests that the expression of basal chloride conductance and/or residual cAMP-mediated chloride secretion has a beneficial influence on respiratory tissue function, most likely by increasing the hydration of the viscous airway surface mucus and increasing its clearance from the respiratory tract. These data in ΔF508 homozygous twins and sibs substantiate the findings in an earlier study in CF individuals with different *CFTR* mutation genotypes in whom residual chloride secretion provided a better indication of lung function than genotype.[13] In contrast, the chloride conductance in the amiloride-pretreated nasal epithelium that is mediated by apical purinergic receptors and a subsequent increase of intracellular Ca^{2+},[30] was not associated with a better preservation of tissue function ($P = 0.53$, Table 5.5).

Similarly, the Na^+ absorption as inferred from the amiloride response was not related to CF disease severity.
In our group of investigated ΔF508 homozygous individuals, the activation of Cl^- conductance by isoprenaline was not different in females and males, which has been reported for CF adults.[46] This might be due to the large number of non-responders to isoprenaline in our investigations, as expected in a CF cohort of ΔF508 homozygotes representative for all ages.[28]
Although residual chloride secretion only presented in the subgroup of individuals with better respiratory function, few patients without chloride conductance possessed a relatively good lung function (above the 50th percentile, Figure 5.3b). This indicates that the presence of residual chloride secretion is not the only determinant of respiratory function, and evidently additional factors unrelated to the basic ion transport defect are involved in CF airway performance.
In summary, only the expression of basal Cl^- conductance and the response to isoprenaline were predictive for disease outcome in our cohort of ΔF508 homozygous sib and twin pairs.
In the intestinal tissue, the CFTR-related DIDS-insensitive chloride secretion was predominantly seen in the mildly affected patients (Figures 5.4 and 5.5). Whereas the Ca^{2+}-dependent Cl^- secretion in respiratory tissue showed no association with outcome, the DIDS-sensitive Ca^{2+}-dependent chloride transport pathway was more frequently observed in severely affected ΔF508 homozygotes. This data is supported by a study of the biliary tract which detected the highest levels of Ca^{2+}-dependent chloride currents in the most dedifferentiated tissue samples.[18] Hence, either this alternative Cl^- conductance is not beneficial per se, or it is only up-regulated in the absence of CFTR-activity to compensate for the lack of CFTR-mediated Cl^- transport. The latter condition of Ca^{2+}-activated Cl^- channel function has been demonstrated to be beneficial for tissue function in *cftr* $^{-/-}$ knockout mice, which lack cAMP-induced chloride currents.[10]
Although all subjects were homozygous for the same *CFTR* mutation, CF was found to be heterogeneous even at the level of the basic cellular defect, which should reflect the closest link with the underlying genetic lesion. However, the consistent phenotype of CFTR expression in respiratory and intestinal tracts of a ΔF508 homozygous CF patient (Figure 5.5), suggests that within one person similar corrective mechanisms lead to maturation, processing and residual function of ΔF508 CFTR in the major affected organs in cystic fibrosis disease. Furthermore, monozygous twins proved to be significantly more concordant in the electrophysiological properties of their epithelium than dizygous pairs, especially in the intestinal tract. These findings imply that the genetic predisposition is important for the expression of residual chloride secretion.

We conclude that the ability to secrete chloride in ΔF508 homozygous patients in the organs mainly involved in the course of CF disease is predictive for CF phenotype. Basal chloride conductance and/or a cAMP-mediated response in the airways together with DIDS-insensitive residual chloride secretion in the intestine were associated with a positive outcome in ΔF508 homozygous CF individuals. Thus, although homozygosity for the major disease-causing lesion was not predictive for disease manifestation and showed a large range of disease severity, the expression of the basic defect was associated with clinical outcome. Clinicians are encouraged not only to use the sweat test, which is non-informative for outcome among ΔF508 homozygous patients, but also to apply the NPD and ICM as predictive measures for disease and prognosis and – possibly in the future – for stratification of clinical trials and treatment of CF disease.

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Chapter 6

Differential basis of residual chloride conductance in individuals with cystic fibrosis carrying null, complex or mild *CFTR* mutations

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Submitted

Abstract

Knowledge about genotype/phenotype correlations for cystic fibrosis (CF)–causing *CFTR* mutations other than ΔF508 is scarce due to the low incidence of homozygous index cases. This study reports on the manifestations of CF in homozygotes for the missense mutation E92K, the stop mutations R553X, R1162X, the splice mutations 1898+3 A-G, 3849+10kb C-T, the novel deletions CFTRdele2, CFTRdele2,3(21kb) and in carriers for the complex alleles ΔF508-R553Q and ΔF508-V1212I. The basic defect was assessed by sweat test, nasal potential difference and intestinal current measurement. Residual CFTR activity was necessary to confer lower sweat electrolyte concentrations or exocrine pancreatic sufficiency. Irrespective of the underlying genotype each patient exhibited some chloride conductance in the airways and/or in the intestine which was identified by the differential response to cAMP and DIDS as being caused by CFTR or at least two other chloride conductances. The expression of alternative chloride channels such as the calcium activatable or the outwardly rectifying chloride channels may partially compensate defective CFTR and modulate the manifestation of respiratory and gastrointestinal disease in CF.

Introduction

Cystic fibrosis (CF) is a disease of all exocrine glands and is caused by mutations in the *Cystic Fibrosis Transmembrane Conductance Regulator* (*CFTR*) gene.[1] CFTR localized in the apical membrane of epithelial cells is a multifunctional protein which confers cAMP-activatable chloride ion transport and regulates the activity of numerous other transport systems. More than 800 CF-causing lesions have been identified (Cystic Fibrosis Genetic Analysis Consortium, 1999, pers. commun.), of which the major mutation ΔF508 accounts worldwide for 70% of disease alleles.[2]
Naturally occurring mutations are a guide to dissecting the features of a gene product. The phenotypes of most *CFTR* mutations other than ΔF508 have been investigated in heterologous expression systems rather than by *in vivo* or *ex vivo* analysis of patients' material, because almost all patients with CF are either homozygous for ΔF508 or compound heterozygous for two different *CFTR* mutations. Reagents and methods are not available to differentiate the two mutant CFTR proteins in most compound heterozygotes but, even at the transcriptional level, the investigation of compound heterozygous individuals does not provide unequivocal information about the phenotype of a particular mutation *in vivo*, because stability and processing

of mRNAs transcribed from the two *CFTR* alleles does not occur independently from each other.[3]
This report describes the clinical manifestation and the basic defect of individuals with CF who are homozygous for non-ΔF508 *CFTR* mutations. These rare index cases were considered to be most informative in order to address the complex issue of genotype/phenotype correlations, in particular the association between *CFTR* mutation and defective chloride transport, the abnormality which hallmarks affected patients' tissues.[4-6] The *a priori* most severe and mildest CF-causing mutations are overrepresented in the cohort. Expression of functional CFTR was expected to vary between zero in patients who harbour two *CFTR* null alleles, and significant amounts of wild type CFTR in patients who are homozygous for mild splice-site mutations. The patients' defective chloride transport across the apical membrane of epithelial cells was measured by physiological assays employed when diagnosing CF, i.e. the Gibson-Cooke pilocarpine iontophoresis sweat test,[7] the nasal potential difference (NPD)[8] and intestinal current measurements (ICM).[9,10] The investigation of the basic defect in the individuals with complex alleles or rare homozygous *CFTR* genotypes uncovered multiple origins of apical chloride conductance in the affected epithelia *in vivo,* such as residual CFTR activity or the expression of alternative cAMP- or Ca^{2+}-activated transport systems.

Patients and Methods

Subjects

The index cases were selected from CF centers in the Czech Republic, Germany and Italy. The recruited patients are either homozygous for a non-ΔF508 CFTR mutation or carry a second sequence variation on one ΔF508 CFTR allele. Complete records since age at diagnosis were available for all participants of the study and each patient has been regularly seen by at least one of the authors.
Informed consent was obtained from all patients and in the case of patients younger than 18 years of age from at least one parental guide. The study was approved by the ethics committee of the Medizinische Hochschule Hannover.
The clinical examination at the day of assessment followed the guidelines defined at the workshop „Genotype/phenotype correlations in cystic fibrosis" held in Hannover, March 5-6, 1994.[11] The standardized questionnaire and examination form include family anamnesis, patient's history and the acute anamnesis (symptoms, current treatment, problems and complications during the last year), a physical examination, documentation of meconium

ileus, meconium ileus equivalent, hepatobiliary disease, diabetes mellitus, fertility, other diseases, anthropometry (height, weight and the derived parameters ‚body mass index', ‚centiles for height and weight' and ‚weight for height percentage', stage of puberty and onset of menarche if applicable), determination of pancreatic elastase in stool as an index for the exocrine pancreatic status, a lung function test (flow-volume curve, forced vital capacity FVC and forced expiratory volume in 1 s FEV1 given in %predicted according to Knudson et al[12]), bacteriology of sputum or deep throat swab, serum IgG and IgE and the specific IgG titer against outer membrane protein F of *Pseudomonas aeruginosa*. Relative anti-oprF titers < 1 are considered to be negative, positive titers are differentiated into three grades: 1-8, slight; 8-20, moderate; >20 strong humoral response to *P. aeruginosa* antigen.

Mutation analysis at the CFTR locus

A cascade approach was taken to identify CFTR mutations in genomic DNA from nuclear blood cells. Whenever possible, K-EDTA blood samples were taken from the patient and the parents. First, frequent CFTR mutations were directly tested by established PCR-based protocols.[13,14] Next, the 27 exons of the CFTR gene together with their flanking intron regions were amplified separately by PCR[15]. Sequence variations were sought by single strand conformation polymorphism (SSCP) analysis of restricted PCR product or denaturing gradient gel electrophoresis (DGGE).[15,16] All non-ΔF508 mutations detected by direct testing or anomalous migration behaviour in SSCP or DGGE were confirmed by direct genomic sequencing of the respective exon.[13,14] Finally, specimens which were refractory to amplification by PCR in exons 2 and/or 3 were scanned for genomic rearrangements by Southern hybridization with PCR generated genomic probes encompassing the respective exon and flanking intron sequences.[15] Hybridization was performed with 5xSSC containing 1% blocking reagent (Boehringer Mannheim), 6% (w/v) SDS and 100 µg/ml fish sperm DNA (Boehringer Mannheim).

Nasal potential difference

The technique of measuring NPD was adapted from the protocol by Knowles et al.[8] Access to the subcutaneous space was obtained by a needle filled with NaCl solution inserted subcutaneously into the forearm. The lower nasal turbinate was superfused with a polyethylene tube (PE-50) connected via syringes to the various superfusing solutions. Both the needle and the PE-50 tubing were connected to a high-impedance voltmeter via Ag/AgCl electrodes and agar/saline-filled salt bridges. By using an otoscope the tube was positioned against the lower nasal turbinate. The basal PD was measured by superfusing NaCl buffer A (120 mM NaCl, 25 mM NaGluconate, 0.4 mM

NaH_2PO_4, 2.4 mM Na_2HPO_4) over the nasal mucosa. Subsequently, the Na^+ diffusion potential was blocked with 0.1 mM amiloride in buffer A. Then the chloride conductance of the airway epithelium was determined by generating a Cl^- diffusion potential, whereby the nasal mucosa was superfused with chloride free buffer B (145 mM NaGluconate, 0.4 mM NaH_2PO_4, 2.4 mM Na_2HPO_4, 0.1 mM amiloride). Finally the responsiveness of the nasal mucosa to 0.1 mM isoprenaline in buffer B, which typically activates CFTR chloride channels, was tested.

Intestinal current measurement

Within five minutes of being obtained fresh rectal suction biopsies were mounted in a micro-Ussing chamber[9]. Potential difference across the tissue was measured through KCl-agar bridges which connected the bathing solutions to matched calomel electrodes. Calomel electrodes were connected to a voltage-clamp amplifier which recorded signals from two Ussing chambers in parallel in the open or short circuited condition. The tissue was incubated at all times at 37°C with Meyler-buffer solution (126.2 mM Na^+, 114.3 mM Cl^-, 20.2 mM HCO_3^-, 0.3 mM HPO_4^{2-}, 0.4 mM $H_2PO_4^-$, 10 mM Hepes, pH 7.4 gassed with 95% O_2 - 5% CO_2) and successive supplements. After stabilization of the basal current in the presence of 10 mM glucose for 10 to 20 min, 0.1 mM amiloride was added to the mucosal side to block sodium channels.[17] Thereafter 10 μM indomethacin was applied to both the mucosal and serosal sides in order to stop the further synthesis of prostaglandins such as PGE2.[18] Subsequently, 0.1 mM carbachol was added to the serosal side which provokes the opening of basolateral potassium channels and thereby generates the driving force for the apical secretion of chloride and, in smaller amounts, potassium ions[19,20]. Colonic tissue responds to carbachol with a singular transient response and then becomes desensitized to further action of carbachol.[21] Hence, the second chloride secretory response is evoked with histamine instead of carbachol (see below).[22] Next, 10 μM forskolin + 1 mM 8-bromo-cylic adenosine monophosphate (cAMP) were added to both serosal and mucosal sides to raise the intracellular cAMP concentration. Then the tissue was incubated with 0.2 mM 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS). DIDS is known to inhibit the human intestinal Ca^{2+}-activated chloride channel hCACL1,[23] the outwardly rectifying chloride channel ORCC,[24] a basolateral chloride/bicarbonate exchanger[25] and an electroneutral pH-sensitive chloride/butyrate exchanger in the apical membrane.[25] The presence of DIDS-inhibitable alternative Cl^- conductance was assessed by adding 0.5 mM histamine to the serosal side in the presence of DIDS. Interaction of histamine with H_1 receptors evokes a transient chloride secretory response via Ca^{2+}- and PKA/PKC-mediated signal transduction pathways.[22]

Results

Identification of large deletions in CF patients' DNA
Two DNA samples from Turkish patients of consanguineous descent were found to be refractory to amplification by PCR with intron-flanking primers of exon 2 (patients 1 and 5) and exon 3 (patient 1) of the CFTR gene, but characteristic and reproducible patterns of non-CFTR by-products, which were sensitive to the annealing temperature, were synthesized from patients' and parents' DNAs upon amplification of exon 2 (Figures 6.1a,b and c). Hybridization of Southern blots of HindIII-restricted DNA with exon 2 and 3 DNA demonstrated that patient 1 was homozygous for the out-of-frame deletion of exons 2 and 3 and patient 5 was homozygous for the in-frame-deletion of exon 2 (Figures 6.1d and e). Of the more than 800 known CFTR mutations, just seven are large deletions of more than 1,000 bp. CFTRdele2 is rare, but the 21 kb large CFTRdele2,3 deletion seems to be a rather common mutation in the Slavic CF patient population (Dörk et al., manuscript in preparation).

Clinical manifestation of CF in patients with homozygous non-ΔF508 CFTR mutation genotypes or complex ΔF508 alleles
Out-of-frame deletion. The CFTRdele2,3(21kb) homozygous index case suffers from a severe course of gastrointestinal and pulmonary CF disease with numerous complications, i.e., meconium ileus at birth, repetitive episodes of salt loss and dehydration during infancy, chronic growth retardation and underweight, late onset of menarche, and diabetes mellitus since adolescence. The Turkish parents are first-generation cousins; four of their six children died during infancy or early childhood.
Stop codon mutations. The R553X[26,27] homozygous adult German female (patient 2) and the R1162X[28,29] homozygous Italian sibs 3 and 4 have typical CF pulmonary and gastrointestinal disease.
In-frame deletion. The pancreas-insufficient (PI) CFTRdele2 homozygote has no clinical signs of lung disease. During the 10-year follow-up the lung function varied between 85% and 114% predicted (median 101%) for FVC and between 82% and 121% predicted (median 98%) for FEV1. When long-term inhalation with ß2-sympathicomimetics was initiated at the age of 16 years, lung function improved within six months to FVC of 135% predicted

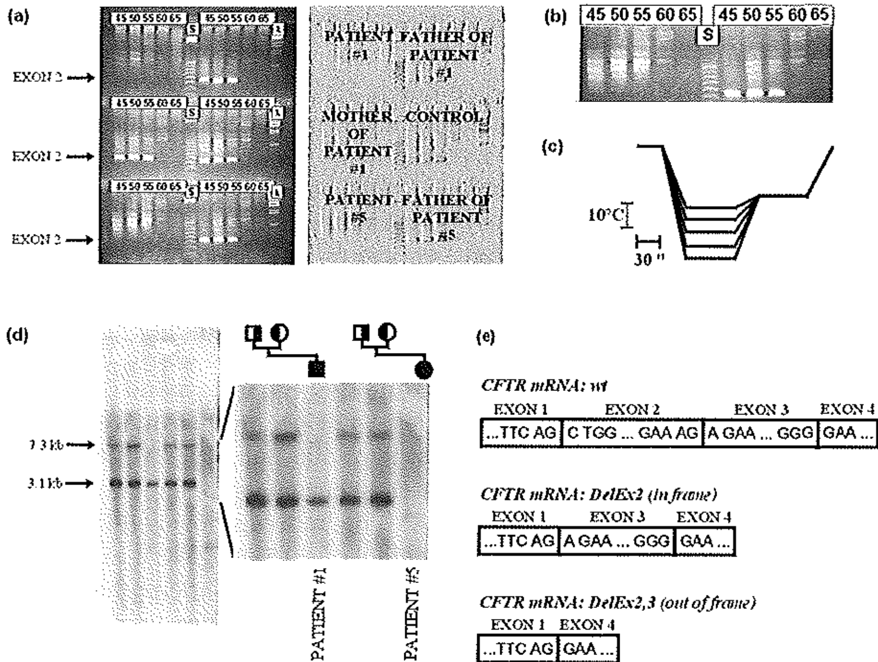

Figure 6.1 *Analysis of the deletions CFTRdele2 and CFTRdele2,3(21kb).* ***a, b.*** *DNA fragments amplified by 35 cycles of PCR from DNA of index families with exon 2 flanking intron primers (15). The annealing temperature was increased in 5°C increments from 45°C to 65°C (from left to right). Size standards: S, 100 bp-ladder, λ, BstEII digest of λ DNA. Please note that the same spectrum of by-products is synthesized in the absence (****b****, left) and presence (****b****, right) of CFTR exon2 template. (****b****) is an enlarged version of the lower panel in (****a****), (****c****) shows the temperature profile of the PCR. (****d****) Southern blot hybridization of HindIII digests of genomic DNA from index families 1 and 5 with exon 2 and exon 3 probes. Exon 2 is part of 7.3 kb HindIII fragment, exon 3 part of a 3.1 kb HindIII fragment. (****e****) Predicted mRNA sequences of CFTRdele2 and CFTRdele2,3(21kb) at exon junctions.*

and to FEV1 of 140% predicted. The Turkish parents of patient 5 are cousins who lost one of their six children by the age of 6 months because of fatal dehydration and electrolyte imbalance.

Missense mutation. The E92K[30] homozygous son of first-generation Aserian cousins was diagnosed because of repetitive episodes of severe dehydration during infancy. He has normal lung function, is pancreatic sufficient (PS), has a normal fat excretion and has never received pancreatic enzymes. In

comparison to most patients with CF, who benefit from regular exercise, physical activity rapidly leads to muscle weakness and fatigue because of excessive sweating and salt loss.

Complex alleles. The compound heterozygote for ΔF508-R553Q/R553X[31] (patient 7) exhibits the uncommon combination of an age-dependent outcome of pilocarpine iontophoresis sweat tests with gastrointestinal and pulmonary symptoms since childhood. Growth retardation, cough and wheezing were already noted in early childhood, but CF was excluded because of apparently normal chloride concentrations below 50 mmol/L in repetitive sweat tests. By the age of diagnosis at 6.8 years the sweat chloride concentrations were in the borderline range between 50-70 mmol/L. Since adolescence highly elevated sweat chloride concentrations above 100 mmol/L have been measured. The two adult ΔF508 homozygous CF siblings 8 and 9 carry the CFTR sequence variation V1212I[32] on their maternal ΔF508 chromosomes. In contrast to most PI ΔF508 homozygotes the brother received no and his sister minimal pancreatic enzyme supplementation: their serum trypsinogen levels were 15.3 and 2.0 ng/ml respectively (PI < 10 ng/ml). However, their elastase levels in stool of < 15 µg/g were in the severe PI range at the day of assessment (PI < 200 µg/g). Both patients have chronically harboured *Pseudomonas aeruginosa* in their airways from the age of 5 years. The male has moderate obstructive lung disease whereas his sister has normal lung function.

Splice-site mutations. A Kurdish married couple who are cousins passed the A-to-G transversion at the not-obligatorily conserved position +3 in intron 12 of the CFTR gene to their affected daughter. CF was suspected by the age of 4 months because of recurrent pneumonia and bronchitis and confirmed by elevated sweat chloride concentrations. Signs of CF pulmonary disease are the intermittent detection of the typical bacterial pathogens *Haemophilus influenzae, Staphylococcus aureus* and *Pseudomonas aeruginosa* in throat swabs and the presentation of slight peribronchial infiltrations in the chest roentgenogram. The pancreas sufficient girl shows no clinical symptoms of gastrointestinal disease. The cryptic splice-site mutation 3849+10kb C-T[33] in intron 19 was identified in homozygous state in two adult brothers of Polish descent. Both patients 11 and 12 are pancreas sufficient and have a normal body mass index. Numerous Gibson-Cooke sweat tests over a 10-year period yielded highly variable electrolyte concentrations of between 15 and 110 mmol/L chloride. Airway obstruction indicated by subnormal FEV1 was already noted in the younger sib by the age of 10, whereas his elder brother did not develop pulmonary symptoms until late adolescence.

Table 6.1 *Anamnesis*

Patient no.	*CFTR* genotype	sex	age at diagnosis	symptoms at diagnosis	pancreatic status	MI	colonization with *P. aeruginosa*	age at onset of *P. aeruginosa* colonization	peculiar features
out-of-frame deletion									
1	CFTRdele2,3(21kb)/ CFTRdele2,3(21kb)	f	birth	meconium ileus	PI	yes	yes	6.5 y	DM
nonsense mutation									
2	R553X/R553X	f	16 mo	steatorrhea failure to thrive	PI	no	yes	10 y	
3	R1162X/R1162X	f	5 mo	malnutrition	PI	no	yes	18.3 y	DM, atopy
4	R1162X/R1162X	m	18 d	none	PI	no	no		atopy
in-frame deletion									
5	CFTRdele2/ CFTRdele2	m	3 mo	dehydration	PI	no	no		
missense mutation									
6	E92K/E92K	m	8 mo	salt loss weak muscles	PS	no	yes	11 y 9 mo	fatigue,weak muscles,salt loss
complex allele									
7	ΔF508-R553Q / R553X	f	6 y 10 mo	growth retardation cough, wheezing	PS→PI	no	no		PI+pos. ST since adolescence,DM
8	ΔF508-V1212I / ΔF508	f	birth	none (family anamnesis)	PS→PI	no	yes	5 y	gallstones
9	ΔF508-V1212I / ΔF508	m	6 mo	recurrent bronchitis	PS→PI	no	yes	7 y	
splice-site mutation									
10	1898+3 A-G / 1898+3 A-G	f	3 mo	pneumonia	PS	no	no		salt loss, dehydration
11	3849+10 kb C-T / 3849+10 kb C-T	m	20 y 5 mo	pneumonia dyspnea, hypoxemia	PS	no	yes	26 y 6 mo	lung transplant splenomegaly
12	3849+10 kb C-T / 3849+10 kb C-T	m	11 y 2 mo	bronchitis pos. family anamnesis	PS	no	yes	12 y 3 mo	

NOTE. Sibpairs: patients 3 & 4, 8 & 9, 11 & 12. DM: diabetes mellitus. MI: meconium ileus. Pos. ST: positive sweat test.

Table 6.2 *Clinical features at the day of assessment*

Patient no.	*CFTR* genotype	age (y)	height centile	weight centile	weight pred. for height	body mass index	stool pancreatic elastase (µg/g)	FEV1 (% pred.)	FVC (% pred.)	sputum bact.	serum IgG (g/L)	serum IgE (IU/mL)	anti-*P. a.* oprF titer
out-of-frame deletion													
1	CFTRdele2,3(21kb)/ CFTRdele2,3(21kb)	17.6	3-10	< 3	0.89	17.9	< 15	55	70	*P. a.*	23	38	10
nonsense mutation													
2	R553X/R553X	24.8	25-50	10-25	0.91	19.9	< 15	43	70	*P. a.*	17	520	7
	R1162X/R1162X	23.7	50-75	3-10	0.82	17.8	15	28	52	*S. a.* *P. a.*	16	48	11
4	R1162X/R1162X	14.5	> 97	75-90	0.79	17.5	15	88	85	*S. a.*	14	< 8	7
in-frame deletion													
5	CFTRdele2/ CFTRdele2	16.0	< 3	< 3	0.96	18.6	< 15	114	109	*S. a.*	10	< 8	< 1
missense mutation													
6	E92K/E92K	11.8	90	> 97	1.23	24.6	450	102	97	*P. a.*	13	170	6
complex allele													
7	ΔF508-R553Q / R553X	17.3	25	10-25	0.96	20.0	n.d.	56	70	negative	16	< 8	2
8	ΔF508-V1212I / ΔF508	22.1	25-50	50-75	1.06	22.0	< 15	92	115	*P. a.*	12	120	3
9	ΔF508-V1212I / ΔF508	23.9	90-97	90-97	0.96	22.1	< 15	55	79	*P. a.*	18	290	7
splice-site mutation													
10	1898+3 A-G / 1898+3 A-G	5.8	25-50	25-50	1.05	(15.5)	> 500			negative	8	12	< 1
11	3849+10 kb C-T / 3849+10 kb C-T	31.9	90	90	0.98	22.0	500	88 (LTX)	74 (LTX)	*P. a.*	6	< 8	9
12	3849+10 kb C-T / 3849+10 kb C-T	20.2	75-90	75-90	0.98	21.4	> 500	43	72	*P. a.*	15	120	9

NOTE. Sibpairs: patients 3 & 4, 8 & 9, 11 & 12; n.d., not determined. *P.a.: Pseudomonas aeruginosa. S.a.: Staphylococcus aureus*.

Table 6.3a Basic defect: Sweat tests and NPD measurements (mV)

Patient no.	*CFTR* genotype	sweat Cl^- (mmol/L) DOI	prior tests (age)	basal PD	Amil	Cl^--free	Iso	residual Cl^- conductance
out-of-frame deletion								
1	CFTRdele2,3(21kb)/ CFTRdele2,3(21kb)	103	95 (10 mo)	- 60	- 38	- 48	- 48	yes
nonsense mutation								
2	R553X/R553X	96	100 (16 mo)	- 62	- 28	- 27	- 35	yes
3	R1162X/R1162X	98	110 (2 y 1mo)	- 48	- 25	- 27	- 29	yes
4	R1162X/R1162X	104	112 (1 mo)	- 39	- 9	- 9	- 9	yes
in-frame deletion								
5	CFTRdele2/ CFTRdele2	102	134 (4 mo)	- 45	- 15	- 16	- 17	no
missense mutation								
6	E92K/E92K	118	93 (8 mo)	- 52	- 32	- 32	- 43	yes
complex allele								
7	ΔF508-R553Q/ R553X	117	40 – 50 (4 - 5 y) 63 (6 y 10 mo)	- 46	- 16	- 13	- 13	no
8	ΔF508-V1212I/ ΔF508	86	94 (1 mo)	- 55	- 29	- 32	- 32	yes
9	ΔF508-V1212I/ ΔF508	104	108 (6 mo)	- 32	- 26	- 25	- 25	no
splice-site mutation								
10	1898+3 A-G/ 1898+3 A-G	73	69 (4 mo)	- 33	- 12	- 15	---	yes
11	3849+10 kb C-T/ 3849+10 kb C-T	92	64 (20 y 5 mo) 49 (28 y 4 mo)	- 44	- 14	- 24	- 26	yes
12	3849+10 k b C-T/ 3849+10 kb C-T	20	50 (11 y 2 mo)	27	- 15	- 13	- 12	no
NPD reference values								
non-CF (n = 25)				- 16±4	- 7±3	- 30±8		
CF (n = 23)				- 48±9	- 19±10	- 17±10		

NOTE. Amil: amiloride. Cl^--free: Cl^--free solution. Iso: isoprenaline. DOI: day of investigation.

***Table 6.3b** Assessment of basic defect (A): sweat test and NPD (mV)*

Patient no.	CFTR genotype	sweat test Cl^- (mmol/L) DOI	prior tests (age)	NPD (mV) basal PD	Amil	ΔPD ≠ 0 (mV) Cl^--free	Iso	residual Cl^- conductance
out-of-frame deletion								
1	CFTRdele2,3(21kb)/ CFTRdele2,3(21kb)	103	95 (10 mo)	- 60	- 38	-10	—	yes
nonsense mutation								
2	R553X/R553X	96	100 (16 mo)	- 62	- 28	—	- 8	yes
3	R1162X/R1162X	98	110 (2y 1mo)	- 48	- 25	—	- 2	yes
4	R1162X/R1162X	104	112 (1 mo)	- 39	- 9	—	—	no
in-frame deletion								
5	CFTRdele2/ CFTRdele2	102	134 (4 mo)	- 45	- 15	—	—	no
missense mutation								
6	E92K/E92K	118	93 (8 mo)	- 52	- 32	—	- 11	yes
complex allele								
7	ΔF508-R553Q/ R553X	117	40 – 50 (4-5 y) 63 (6 y 10 mo)	- 46	- 16	—	—	no
8	ΔF508-V1212I/ ΔF508	86	94 (1 mo)	- 55	- 29	- 3	—	yes
9	ΔF508-V1212I/ ΔF508	104	108 (6 mo)	- 32	- 26	—	—	no
splice-site mutation								
10	1898+3 A-G/ 1898+3 A-G	73	69 (4 mo)	- 33	- 12	- 3	—	yes
11	3849+10 kb C-T/ 3849+10 kb C-T	92	64 (20y 5 mo) 49 (28 y 4 mo)	- 44	- 14	- 10	- 2	yes
12	3849+10 kb C-T/ 3849+10 kb C-T	20	50 (11y 2 mo)	- 27	- 15	—	—	no
NPD reference values								
non-CF	(n = 25)			- 16 ± 4	- 7 ± 3	- 23 ± 8		
CF	(n = 23)			- 48 ± 9	- 19 ± 10	-1 ± 5		

However, progression of pulmonary disease was rapid and by the age of 28 years he became terminally ill with global respiratory insufficiency and Cor pulmonale (FEV1 0.6 L = 13% predicted). A successful double lung transplant re-established an almost normal life.
CF-relevant anamnestic data and clinical features of the 12 patients are summarized in Tables 6.1 and 6.2.

Assessment of basic defect
The basic defect of chloride conductance in exocrine epithelia was evaluated by determination of electrolyte concentrations in pilocarpine iontophoresis sweat test,[7] chloride permeability of upper respiratory epithelium (NPD)[8] and ion flow in rectal suction biopsies (ICM).[9]
Defective chloride reabsorption in the sweat duct was noted for all genotypes but ΔF508-R553Q and 3849+10kb C-T which demonstrated highly variable

sweat sodium and chloride concentrations on separate occasions (Tables 6.3a and b). ΔF508-R553Q was found to be associated with an age-dependent increase of sweat NaCl concentration from the normal to the unequivocal CF range within 15 years, whereas a substantial day-to-day variation without any age-dependent trend was observed for the two 3849+10kb C-T homozygotes.
The reduced or absent chloride permeability of the apical epithelial membrane in CF leads to hyperpolarisation and an increased potential difference between surface and submucosa which is a factor for diagnosing CF by nasal potential difference (NPD).[5,8] As expected for the CF condition, the basal NPD of all 12 patients was more negative than that of non-CF controls (Tables 6.3a and b). A basal PD of –27 mV within the borderline range between CF and non-CF was measured in one 3849+10kb C-T homozygote. A chloride diffusion potential in chloride free gluconate solution indicative for a residual chloride conductance was detectable in seven of the 12 patients. Three patients responded to the cAMP-activating agonist isoprenaline which, at least in the case of the R553X homozygote, has to be attributed to a chloride conductance other than CFTR. Interestingly, three of the four patients who are homozygous for a stop mutation or an out-of-frame deletion expressed an alternative chloride conductance in their upper airways as indicated by the presence of a chloride diffusion potential. All three sibpairs were discordant in the expression of residual chloride conductance in their airways, implying that there exists no strict association between CFTR genotype and chloride permeability of airways.
Three types of chloride secretory response could be differentiated by ICM in patients 3 – 12 (Table 6.4). Figure 6.2 compares ICM tracings of rectal suction biopsies of a non-CF individual with those of a CF patient who lacked any chloride secretory response. Complete absence of chloride secretion was observed in the R553X and CFTRdele2,3(21kb) homozygotes. A DIDS-insensitive transient response to carbachol and histamine was seen in specimens from the homozygotes for 1898+3 A-G, 3849+10kb C-T, E92K and the carrier for the complex allele ΔF508-R553Q (Figures 6.3 and 6.4). Patients 6, 10, 11 and 12 showed a DIDS-insensitive response to forskolin / 8Br-cAMP. This combination of signals indicates residual CFTR activity. Figure 6.3 shows the original tracings of open and closed circuit current measurements of the biopsy from the 1898+3 A-G homozygote with the largest, but still CF-typical, residual chloride secretion. Second, a DIDS-inhibitable cAMP-insensitive chloride secretory response was evoked in biopsies from the sibpairs with the ΔF508-V1212I and R1162X mutations, respectively (patients 3, 4, 8, 9, Figure 6.4).

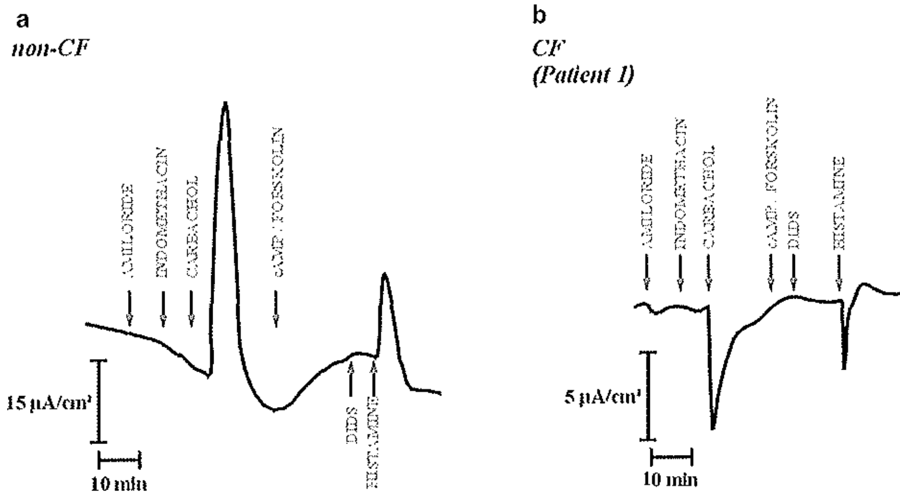

Figure 6.2 *Typical ICM tracings of a non-CF proband (**a**) and an individual with CF who shows no chloride secretory response (**b**, here: patient 1 who is homozygous for the out-of-frame deletion CFTRdele2,3(21kb)).*

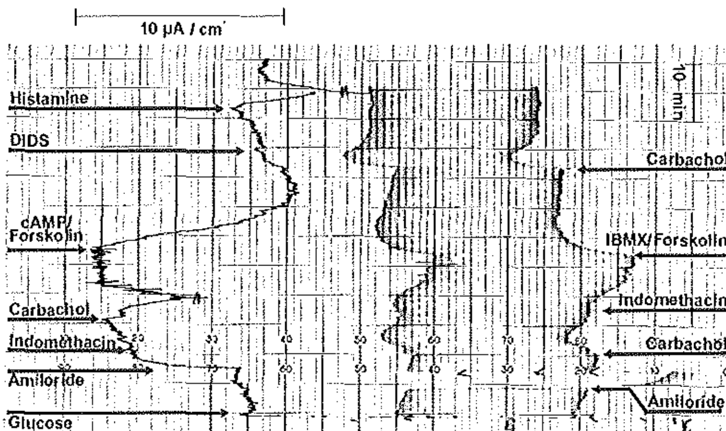

Figure 6.3 *ICM of rectal suction biopsies obtained from patient 10 who is homozygous for the splice-site alteration 1898+3 A-G. Signals were recorded as short circuit currents (left) and open circuit currents (right, pulses of 0.5 µA for 0.1 s).*

Third, a DIDS-sensitive bimodal response to cAMP/forskolin was seen in the ICM of the CFTRdele2 homozygote (patient 5, Figure 6.4). A DIDS-sensitive cAMP response was also observed in specimens from a compound heterozygote for the stop mutations R553X and L1059X (data not shown). In summary, the secretagogues employed in our protocol uncovered residual DIDS-insensitive CFTR activity and two alternative chloride conductances in CF rectal tissue which are both inhibitable by DIDS, but differentially stimulated by cAMP.

Discussion

More than 800 disease-causing lesions have meanwhile been identifed in the *CFTR* gene but, since about 90% of patients with CF are either homozygous or compound heterozygous for the most common mutation ΔF508, our knowledge about the phenotype of non-ΔF508 mutations is based mainly on the characterization of mutant CFTR transcript or protein in heterologous expression systems.[1,35] Data about the patients' phenotype with homozygous non-ΔF508 *CFTR* genotypes is scarce and scattered in more than 30 separate publications (examples in refs 27, 29, 30, 33, 34). The clinical manifestation of CF has so far been described for 25 PI and 10 PS non-ΔF508 *CFTR* mutations in about 100 homozygotes, but with the exception of patients with the PS splice-site mutations 2789+5 G-A[34] or 3849+10kb C-T[33] who were characterized by NPD, the basic defect was only assessed by the Gibson-Cooke sweat test.
In our study clinical phenotype and basic defect in sweat gland and respiratory and gastrointestinal tracts were investigated for two complex ΔF508 alleles, one missense, two splice-site and two nonsense mutations and two novel deletions (Tables 6.1 to 6.4). A chloride diffusion potential in NPD and a chloride current evoked by secretagogues in ICM are indicative for a chloride conductance in respiratory and intestinal CF epithelium (Tables 6.3 and 6.4). Since the inhibitor DIDS blocks chloride conductances other than CFTR,[23-25] a DIDS-insensitive chloride secretory response in ICM was taken as a criterion for the expression of functional CFTR (Table 6.4). Residual CFTR activity was only detected in pancreas sufficient individuals who carry a missense mutant in the first ectoplasmatic domain (E92K) or who are capable to produce some amounts of wild-type CFTR (1898+3 A-G, 3849+10kb C-T).[33] The PS status in CF corresponds with about 10-20% of normal exocrine pancreas function.[36]

***Table 6.4** Assessment of basic defect (B): ICM measurements*

Patient no.	CFTR genotype	Response to carbachol (% reverse signal)	cAMP (µA/cm²)	DIDS + histamine (% rev. signal)	Interpretation residual Cl⁻ cond.	sensitivity to DIDS	cAMP response
out-of-frame deletion							
1	CFTRdele2,3(21kb)/ CFTRdele2,3(21kb)	100	none	100	no	—	—
nonsense mutation							
2	R553X/R553X	100	none	—	no	---	—
3	R1162X/R1162X	0	none	40	yes	yes	no
4	R1162X/R1162X	45	none	90	yes	yes	no
in-frame deletion							
5	CFTRdele2/ CFTRdele2	100	5	100	yes	yes*	yes
missense mutation							
6	E92K/E92K	40	4	50	yes	no	yes
complex allele							
7	ΔF508-R553Q/ R553X	> 60	< 0.5	> 60	yes	no	(yes)
8	ΔF508-V1212I/ ΔF508	50	< 1	100	yes	yes	(yes)
9	ΔF508-V1212I/ ΔF508	0	none	80	yes	yes	no
splice-site mutation							
10	1898+3 A-G/ 1898+3 A-G	0	10	0	yes	no	yes
11	3849+10 kb C-T/ 3849+10 kb C-T	70	< 1	70	yes	no	(yes)
12	3849+10 kb C-T/ 3849+10 kb C-T	100	< 1	100	yes	no	(yes)

NOTE. *cAMP response sensitive to DIDS

***Table 6.5** Patients' patterns of residual chloride conductance in NPD and ICM*

ICM Residual chloride conductance	sensitivity to cAMP	DIDS	interpretation ion channel	NPD Residual Cl⁻ conductance yes (patient number)	no
yes	yes	no	CFTR	6, 10, 11	7, 12
yes	yes	yes	ORCC		5
yes	no	yes	CACL	3, 8	4, 9
no				1, 2	

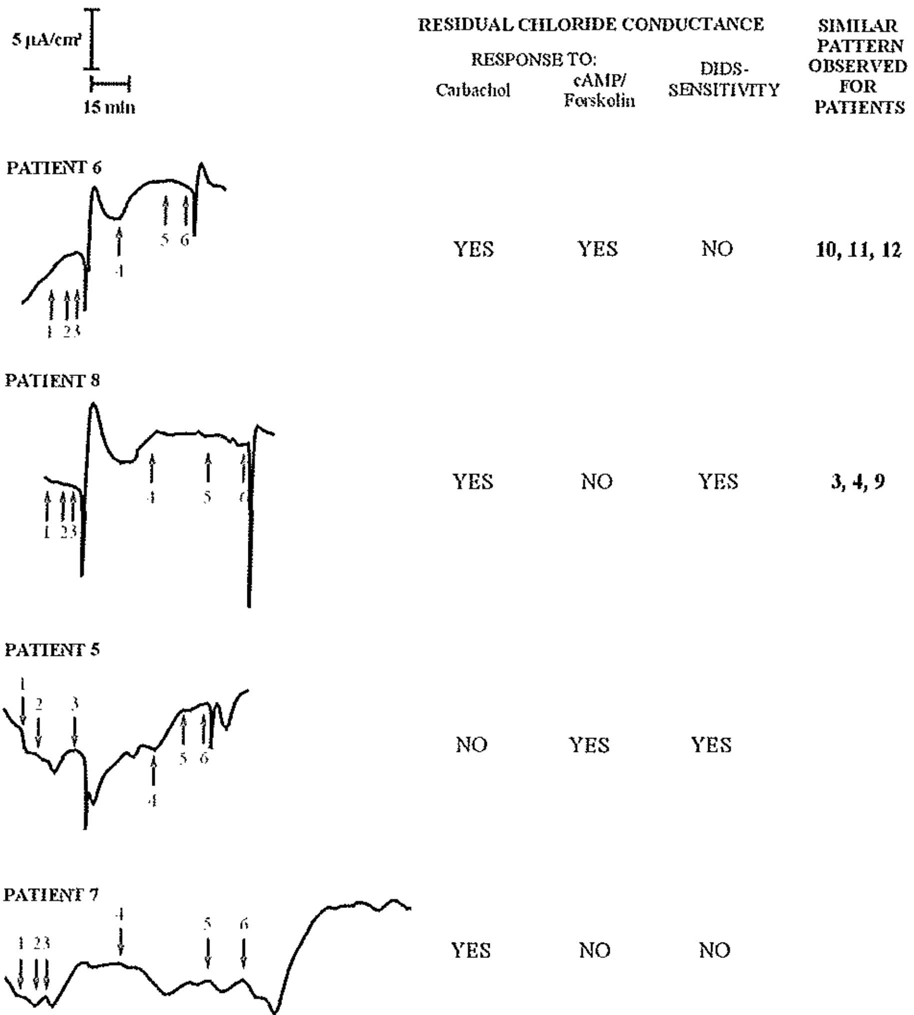

Figure 6.4 *ICM patterns of residual chloride conductance for patients 6, 8, 5 and 7. Secretagogues: 1, amiloride; 2, indomethacin; 3, carbachol; 4, 8Br-cAMP/forskolin; 5, DIDS; 6, histamine.*

Expression of chloride conductances other than CFTR found by NPD or ICM did not rescue the pancreas insufficient phenotype in accordance with the general rule that the *CFTR* mutation genotype determines the exocrine pancreatic status, whereby carriage of at least one 'PS' *CFTR* allele is sufficient to confer pancreatic sufficiency.[37] Severity of pulmonary disease was highly variable in our index cases and even discordant in one sibpair, which points to the importance of environmental factors and genetic modifiers other than CFTR on airway disease in CF, as has been seen in large cohorts of ΔF508 homo- and compound heterozygotes.[1,35,38]
A pathological outcome of sweat test, NPD or ICM is diagnostic for most CF, although some day-to-day variance of sweat electrolyte concentrations has been observed, particularly for mild splice mutations such as 2789+5 G-A (data not shown) and 3849+10kb C-T (Tables 6.3a and b). A notable exception is the complex allele ΔF508-R553Q. The singular index case ΔF508-R553Q/R553X presented an atypical manifestation and course of CF when compared with the ΔF508 homozygous and ΔF508/R553X compound heterozygous patients at our clinic.[31] She was apparently healthy during neonatal period and toddler age and developed some gastrointestinal and pulmonary symptoms compatible with CF during pre-school age. Sweat electrolyte concentrations were normal until the age of six years, and then over a period of ten years gradually increased into the borderline range and finally reached the typical CF range. The course is suggestive for an age dependent manifestation of the basic defect at least in the sweat glands, which are affected in almost all CF and do not undergo secondary pathological alterations.
The recently resolved crystal structure of the evolutionarily conserved nucleotide binding fold of a prokaryotic ABC transporter[39] suggests that phenylalanine 508 and arginine 553 are located in adjacent helices of the first nucleotide binding fold of CFTR. The sequence variant R553Q has been demonstrated in heterologous systems as rescuing the processing and chloride channel gating defects caused by the ΔF508 mutation[40] and has been classified as a disease-reverting suppressor mutation in accordance with the anomalous course of our index patient.[35,40] *In vivo*, however, the role of R553Q as providing protection from disease faded over the years. By the age of 17 years, when the index case presented typical clinical features of CF, sweat chloride concentrations were highly elevated, no chloride diffusion potential was detected by NPD, and the chloride secretory response in ICM was very subtle (Figure 6.4, Tables 6.3 and 6.4).
ΔF508-V1212I[32] is the other complex *CFTR* allele which was investigated as to its impact on the basic defect. The male index case as well as a further five ΔF508 homozygotes were selected from 228 ΔF508 homozygous adults for comprehensive analysis of the *CFTR* coding sequence, because these six

patients were clinically exocrine pancreatic sufficient and hence were suspected to carry disease-attenuating suppressor mutations. V1212I was the only detected complex ΔF508 allele in the six patients. However, ICM revealed that ΔF508-V1212I did not confer residual CFTR activity. F508 and V1212 are located in functionally divergent sites of the first and second NBF.[39] Lack of physical interaction may explain why V1212I cannot rescue the omission of ΔF508 CFTR. The index sibpair expressed no CFTR activity, but another DIDS-inhibitable chloride secretory response in their intestinal epithelium (Figure 6.4). The pharmacological profile is characteristic for the recently cloned Ca^{2+}-activated chloride channel hCLCA1.[23]

hCLCA1 is exclusively expressed in intestinal mucosa[23] and hence cannot compensate absent or defective CFTR in pancreas. In non-CF individuals CFTR accounts for almost all chloride secretion in colonic epithelium.[10,41] Although hCLCA1 plays only a minor role for intestinal chloride secretion under physiological conditions, it could attenuate the basic defect in the intestine of individuals with CF.

The members of the Ca^{2+}-mediated Cl^- channel (CaCC) family in man exhibit tissue-specific non-overlapping expression patterns.[23,42] For example, the demonstration of a Ca^{2+}-sensitive chloride conductance in patients' rectal biopsies may or may not be associated with residual chloride secretion in the airways as exhibited in the incoherent pattern of the four individuals in our study who showed a DIDS sensitive chloride conductance in the intestine. In contrast murine mCLCA1 was detected in a variety of tissues of epithelial and nonepithelial origins, including exocrine secretory respiratory and intestinal epithelia.[43] This discordant mode of expression pattern could result in a differential role of CLCA as a modifier of disease in CF mice[44] and patients and limits the validity of predictions of phenotype by extrapolation from mouse to man.

The other DIDS-sensitive alternative chloride conductance detected by ICM was stimulated by cAMP. Such a response is typical for the ORCC.[24] Although no evidence for residual chloride secretion was seen by NPD, supranormal lung function could be achieved in the index case by treatment with β_2 -adrenergic agonists which both activate ORCC and CFTR in non-CF individuals. In this patient, who is homozygous for a large deletion, no residual CFTR activity was seen which demonstrates that in principle the basic defect caused by molecular lesions in the *CFTR* gene can be bypassed by pharmacological means if alternative ion channels are expressed.

Stop codon mutations and large deletions are generally considered to be null alleles. In case of the large out-of-frame deletion[45] and the R553X mutation[46] the absence of protein or strong reduction of CFTR mRNA transcript, respectively, have been demonstrated whereas wild-type amounts of transcript have been measured for R1162X.[47,48] Three of our five

homozygotes for a large deletion or a nonsense mutation expressed a residual chloride conductance in the respiratory and/or gastrointestinal tracts. The large chloride diffusion potentials in patients 1 and 2, who definitely produce no CFTR, point to the presence of other chloride channels. Both patients suffer from severe pulmonary disease indicating that the alternative chloride channel cannot fully compensate for the loss of CFTR. On the other hand, as in CF knock-out mice,[44] the absence of CFTR may predispose to the expression of these alternative chloride channels in some but not all patients, which could explain the discrepant reports in the literature as to whether patients with two stop mutations had milder or more severe pulmonary disease than ΔF508 homozygotes.
Table 6.5 summarizes the electrophysiological findings in our cohort of patients with complex alleles or rare non-ΔF508 homozygous genotypes. The pattern of residual chloride conductance is versatile as indicated by the discordant outcome in siblings and the lack of correlation between airways and intestine. Each patient expressed some chloride conductance in at least one of the two organ systems. This data underlines the inherent complexity of genotype-phenotype associations in monogenic CF. Missense, splice-site or complex mutations can give rise to substantial, but still subnormal, CFTR-mediated chloride conductance. This residual CFTR activity is necessary to confer pancreatic sufficiency and cannot be compensated by other chloride channels such as CLCA, probably because they are not co-expressed in pancreas. However, in the respiratory and intestinal epithelia the basic defect of impaired apical chloride transport can be modulated by other chloride conductances. Their role on the disease phenotype is currently being evaluated in a study on ΔF508 homozygous twins and sibpairs with extreme phenotypes, i.e. a very mild or very severe course of disease.

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Chapter 7

Diagnoses in CF-like disease

Chapter 7a

Cystic fibrosis-like disease unrelated to the cystic fibrosis transmembrane conductance regulator

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Abstract

Cystic fibrosis (CF) is considered to be a monogenic disease caused by molecular lesions within the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene that is diagnosed by elevated sweat electrolytes. We investigated the clinical manifestations of cystic fibrosis, *CFTR* genetics and electrophysiology in a sibpair of which the brother is treated for having CF while his sister is asymptomatic. The diagnosis of CF in the index patient was based on highly elevated sweat electrolytes in the presence of CF-related pulmonary symptoms. The investigation of chloride conductance in respiratory and intestinal tissue by nasal potential difference and intestinal current measurements provided no evidence for CFTR dysfunction in both siblings who share the same *CFTR* alleles. No molecular lesion was identified in the *CFTR* gene of the brother. Findings in the investigated sibpair point to the existence of a CF-like disease with a positive sweat test without CFTR being affected. Other factors influencing sodium or chloride transport are likely to be the cause for the symptoms in the patient described.

Introduction

Cystic Fibrosis (CF) is a generalized disease of the exocrine glands affecting predominantly the gastrointestinal, hepatobiliary, reproductive and respiratory tract.[25] CF is considered to be a monogenic disease of recessive inheritance. Therefore, the detection of molecular lesions in both cystic fibrosis transmembrane conductance regulator (*CFTR*) alleles[12] is regarded as being confirmatory for the diagnosis of CF. Absent or dysfunctional CFTR leads to defective chloride transport across the apical membrane of epithelial cells[25] which is the basic principle when diagnosing CF by physiological assays, i.e. the pilocarpine iontophoresis sweat test,[9] the nasal potential difference (NPD)[13] and the intestinal current measurements (ICM).[23,24]
We report on a patient whose clinical features, *CFTR* genotype and basic defect phenotype were contradictory with respect to the diagnosis of CF. This is the first case of a CF-like disease unrelated to *CFTR* that could be substantiated by pedigree data.

Materials and Methods

Informed consent was obtained from the patient and his sister. This study was approved by the local medical ethical commitee.

CFTR haplotype analysis
Inheritance of *CFTR* alleles was traced in all members of the two generation pedigree by intragenic marker haplotype. Analysis of dimorphic markers and intron 8 splice site haplotype was carried out as previously described.[6,7] Polymorphic microsatellites were typed using a PCR protocol.[17]

Mutation analysis at the CFTR locus
Mutation analysis in the patient was performed by direct testing for frequent *CFTR* mutations as well as by single strand conformation polymorphism (SSCP) and sequencing analysis of coding and flanking intron sequences as described elsewhere.[8,26] Briefly, the region of interest was amplified by polymerase chain reaction (PCR) in presence of [α-33P]dATP and the resulting product digested by restriction enzymes to give rise to fragments of 150--250 bp. For each PCR product, two different restriction enzymes were used. SSCP samples were loaded on a high resolution gel (running conditions: length 40cm, 0.2--0.4 mm, 5--7.5% acrylamide:bisacrylamide 29:1, 5--10% glycerol, 5--7h 25W const at 10°C) which has been shown to result in a sensitivity for *CFTR* mutations detection of more than 90%.[19]

Nasal potential difference
The technique of measuring NPD was adapted from the protocol by Knowles et al.[14,22] This electrophysiological measurement is used as a diagnostic tool for CF by evaluating the Na^+ and Cl^- potential difference across the epithelium of the lower nasal turbinate representative for the airway epithelium. Access to the subcutaneous space, which is isoelectric throughout the body, was obtained by a needle filled with NaCl solution inserted subcutaneously in the forearm. The lower nasal turbinate was superfused with a polyethylene tube (PE-50) connected via syringes to the different superfusion solutions. Both the needle and the PE-50 tubing were connected to a high-impendance voltmeter via Ag/AgCl electrodes and agar saline filled salt bridges. The tubing was positioned on the lower nasal turbinate by using an otoscope. The basal PD was measured by superfusing a NaCl solution over the nasal mucosa. Subsequently, 10^{-4} M amiloride was added to block the Na^+ diffusion potential. In earlier studies amiloride was shown to inhibit approximately 25% of the basal PD in controls and 50--60% in CF patients.[2] To assess the Cl^- conductance of the airway epithelium, Cl^- was replaced by gluconate in the presence of amiloride which shows a Cl^- diffusion potential in controls but not in CF patients.[14]

Intestinal Current Measurement
Freshly obtained rectal suction biopsies were mounted in a micro Ussing chamber. After stabilisation of the basal short circuit current in the presence of glucose, amiloride (10^{-4} M) was added to the mucosal side. The endogenous prostaglandin synthesis, possibly linked in this tissue to cAMP-mediated chloride secretion, was inhibited by adding indomethacin (10^{-5} M applied both sides). Thereafter, carbachol (10^{-4} M, added to serosal side) provoked in controls an inward current reflecting transcellular Cl^- transport from serosa to mucosa. However, CF patients demonstrate an outward current suggesting K^+ secretion in the absence of Cl^- secretion.[23] Milder phenotypes may also demonstrate a small inward current, but this is significantly reduced from that observed in controls.[24] Finally the tissue was incubated with 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, 2.10^{-4}, added to mucosal side). DIDS is considered to inhibit the Ca^{2+} regulated Cl^- channels.[10] The presence of DIDS inhibitable alternative Cl^- was assessed by adding histamine 5.10^{-4} M to the serosal side in the presence of DIDS. The results were expressed as the maximal signal evoked by the used secretagogues.

Results

Clinical and laboratory findings in the patient and his sibling
Neonatal period and infancy of the male sibling were uneventful with no episodes of dehydration. Starting at the age of 6 y, he suffered from recurrent bronchitis, chronic cough and excessive sputum production that required three to four courses of oral antibiotics per year. On the occasion of a severe pneumonia at age 17 he was diagnosed as having CF by a chloride concentration of 102 mmol/l in the pilocarpine iontophoresis sweat test.[9] Following diagnosis he was treated with daily inhalation of salbutamol, oral n-acetylcystein and intermittent oral antibiotics, but received no pancreatic enzymes or vitamin supplements. Occasionally, *Staphylococcus aureus* was detected in routine sputum cultures. Clinical and laboratory data at two subsequent days of assessment are summarized in Table 7a.1. The investigations confirmed the pathological sweat test values and revealed a subnormal lung function and a reduced fertility. However, no signs of exocrine pancreatic insufficiency or chronic inflammation of the lung were detected.
The patient's sister had no signs of bronchitis, cough, increased sputum production or impaired pancreatic function.

***Table 7a.1** Actual clinical and laboratory data of the siblings*

	Index case	sister	normal range
age (years)	23	27	
Growth			
height (m)	1.73	1.71	
body mass index (kg/m^2)	26.4	27.6	19 - 25 (25)
Lung function parameters			
FVC (%-predicted)[a]	75 %	94 %	80 - 120 (26)
FEV1 (%-predicted)[a]	72 %	75 %	80 - 120 (26)
Pilocarpine iontophoresis sweat test			
sweat chloride (mmol/L)	97	36	< 60
Tests of exocrine pancreatic sufficiency			
stool chymotrypsin (U/g)	29	33	> 6
stool pancreatic elastase (μg/g)	> 500	440	> 200
serum vitamin A (μg/L)[b]	540	700	200 - 1,200
serum vitamin E (mg/L)[b]	5.8	10.1	5 - 20
Microbiology and inflammation parameters			
sputum bacteriology	normal flora	normal flora	
serum IgG (g/L)	16.0	14.7	6.6 -18.4
anti-*P. aeruginosa* OprF IgG titre	0.7	0.8	< 1
Spermiogram			
volume (mL)	2.5		2 - 6
density (10^6/mL)	1.9		20 - 200
motility	45 %		> 60 %

NOTE. [a]FVC: forced vital capacity. FEV1: forced expiratory volume in 1 s.
[b]both probands did not receive supplementation of fat-soluble vitamins.

Haplotype and mutation analysis at the CFTR locus

Eight intragenic dimorphic[6] and three polymorphic microsatellite markers[17] were used for haplotype analysis within the family. Both sibs shared the same intragenic *CFTR* marker haplotypes (Figure 7a.1) indicating that the *CFTR* alleles of both siblings are identical by descent.

No *CFTR* mutation was found when all 27 *CFTR* exons and flanking intron sequences of the index patient were screened by single strand conformation polymorphism and sequencing analysis[7,26]. He is compound heterozygous (TG)11T7/(TG)10T7 for the acceptor splice site polymorphism in intron 8.[4,5,7,22]

Electrophysiological findings

Defective chloride conductance in epithelial tissues is known to be the pathophysiological origin of CF.[25] To establish a chloride transport defect in these patients, we repeated the sweat test and extended the evaluation by measuring NPD[13] and ICM.[23,24] Sweat Na^+ and Cl^- were again found to be

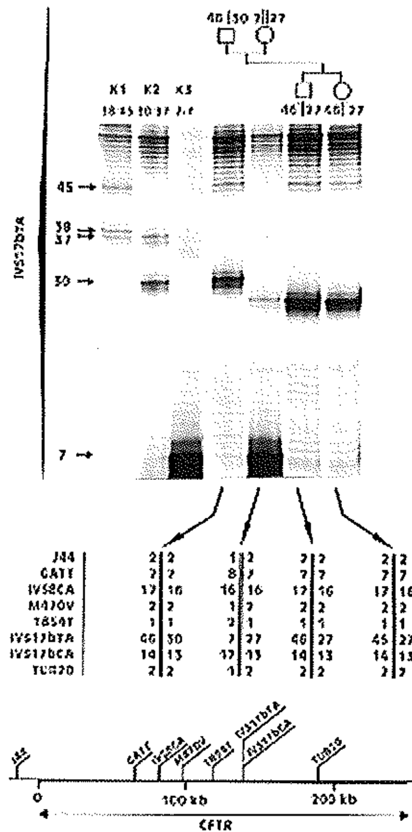

Figure 7a.1 Pedigree of inheritance of intragenic 8-marker CFTR haplotypes in the index family. The autoradiogram shows the separation of PCR-amplified intragenic microsatellite IVS17bTA alleles. Lanes K1, K2, K3 show unrelated control samples of known allele composition.

elevated in the brother and normal in the sister. However, in more specific tests i.e. NPD and ICM which both give more insight in the electrolyte transport, no abnormalities could be demonstrated in both siblings (Figure 7a.2). Although ranges of the control and CF responses do overlap in NPD, our index case and his sister can clearly be discriminated from CF by their combination of normal baseline PD and gluconate response (Figure 7a.2a).[2] In ICM, a Cl^- secretory current was evoked by Carbachol and the CF condition could not be mimicked by the use of DIDS which inhibits the Ca^{2+} activated Cl^- channels.[10] This data suggests that CFTR mediated chloride conductance in the respiratory and gastrointestinal tracts in both siblings is normal.

Differential diagnosis
The pathological sweat Na^+ and Cl^- values can be caused not only by impaired chloride reabsorption as in CF, but also by defective sodium reabsorption. Hence we searched for clinical symptoms in the patient that are compatible with known sodium transport abnormalities, i.e pseudohypoaldosteronism[3,11,21] and Liddle disease.[1,20] Both conditions were excluded by findings of normal blood pressure, serum electrolytes, plasma renin and aldosterone levels prior to and after administration of furosemide.[16]

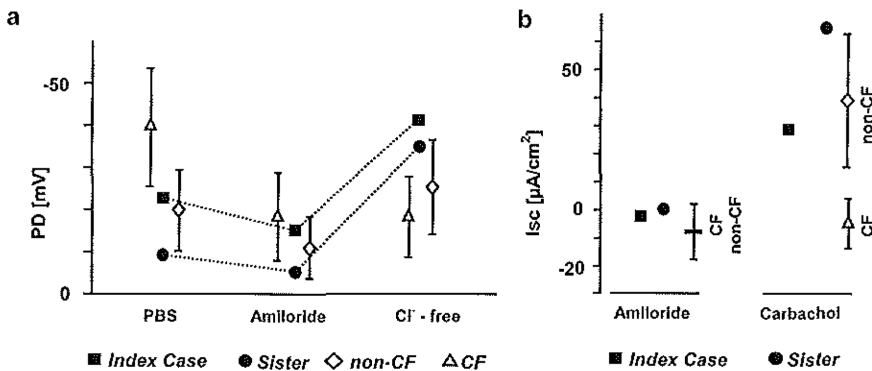

Figure7a.2 *NPD values (**a**) upon superfusion with: phosphate buffered saline (PBS), amiloride 10^{-4}M in PBS (amiloride), Cl^--free solution in the presence of amiloride 10^{-4}M (Cl^--free). For comparison, mean PD values $\pm$SD of 25 controls and 23 CF patients are plotted. ICM values (**b**) of index patient and sister after addition of amiloride and carbachol. Short circuit currents (I_{sc}, mean $\pm$SD) are given for the amiloride response in a group of controls and CF patients. I_{sc} responses to carbachol are given for a control (n=50) and a CF (n=51) group.*

Discussion

In the case described, the elements of CF diagnosis -- clinical features, genetic analysis and electrophysiological measurements -- are conflicting. Based on anamnesis, clinical investigation and the positive sweat test, the diagnosis of CF was independently made by two physicians with profound and long-standing expertise in CF care. Whereas the clinical features and the pathological sweat Na^+ and Cl^- values are compatible with mild CF, *CFTR* genetics and ICM and NPD-measurements provide no evidence for defective CFTR. Normally, ICM/NPD are sensitive assays for diagnosing CF, even in

patients with borderline sweat tests,[24] but in this case the results of the sweat test were not sustained by the other two electrophysiological assays. Instead, the patient presents a CF-like disease which is apparently not related to defective CFTR: no disease-causing lesion was found within the *CFTR* gene, no clinical signs of disease were presented by the sister who inherited the same *CFTR* alleles as the index patient, and no sign of CFTR dysfunction was found in the gastrointestinal and respiratory epithelia predominantly affected in CF.

We present the first case of a CF-like syndrome associated with a positive sweat test and mild respiratory disease which could be substantiated by both genetic and electrophysiological sibpair data. Although the highly unlikely occurrence of de novo mutations in the non-coding region of the *CFTR* gene, variable penetrance of shared sequence variation(s) and/or somatic mosaicism cannot be excluded, the shared genotype between affected and unaffected sib as well as the normal chloride transport properties in the respiratory and gastrointestinal tracts point to the non-existence of *CFTR*-caused CF in the diseased individual. The demonstrated defective NaCl reabsorption in the sweat duct could be due to an aberrant tissue-specific factor acting on CFTR or due to an anomalous sodium reabsorption. The established entities pseudohypoaldosteronism[3,11,21] and Liddle disease[1,20] were excluded. However, perturbations of sodium transport of another etiology could affect electrolyte homeostasis in both sweat glands and airways, the latter being a predisposition to respiratory infection as in CF.

Therefore it is tempting to speculate that individuals with clinical signs of CF and a positive sweat test, but no identified *CFTR* mutation, may suffer from lesions in another gene. After exhaustive screening of the promotor, all exons and flanking intron regions for sequence variations and pulsed field gel electrophoresis analysis of the CF locus, three out of 350 patients in our panel with a positive sweat test and unequivocal clinical signs of „classical“ CF are still negative for an anomaly in the *CFTR* gene. Genetic heterogeneity is the rule rather than the exception for inherited disease in man and correspondingly CF may also be a genetically heterogeneous disease, albeit loci other than *CFTR* should account for 1% or less of all cases.

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Chapter 7b

Clinical presentation of exclusive cystic fibrosis lung disease

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Submitted

Abstract

Background-The diagnosis of cystic fibrosis (CF) is based on two mutations in the *Cystic Fibrosis Transmembrane conductance Regulator* (*CFTR*) gene and on assays that measure the basic defect of perturbed chloride transport in the affected organs. However, in cases of atypical CF, not all diagnostic measures may be informative. We report on a pancreatic sufficient patient with normal electrolyte concentrations in sweat tests who suffers from severe pulmonary manifestations typical for CF such as recurrent nasal polyps, bronchitis, bronchiectasis, continuous cough, sputum production, airway infections, hypoxaemia and cachexia.
Methods-To substantiate the diagnosis of CF, the *CFTR* gene was screened for mutations and the basic chloride transport was electrophysiologically assessed by nasal potential difference and intestinal current measurement. Pancreatic function was tested by stool elastase and chymotrypsin.
Results-Our patient showed normal elastase and chymotrypsin values. All sweat tests were in the normal range. Intestinal current measurement showed normal levels of CFTR-mediated Cl^- conductance. However, the baseline nasal potential difference was pathologically elevated and there was subnormal CFTR Cl^- secretion in the nasal epithelium. Genetic analysis of the *CFTR* gene uncovered only one disease causing lesion, the sequence alteration 1898+3 A->G.
Conclusions-We present a patient with an atypical CF phenotype in which the only presenting symptom is severe CF-like lung disease, which is substantiated by an abnormal nasal potential difference measurement. Genetic analysis shows that the index case is a symptomatic heterozygote implying that one disease-causing lesion in the *CFTR* gene may be sufficient to cause CF-like lung disease.

Introduction

Typical cystic fibrosis (CF) is caused by two lesions in the *Cystic Fibrosis Transmembrane conductance Regulator* (*CFTR*) gene which give rise to a generalised exocrinopathy in respiratory, gastrointestinal, reproductive and hepatobiliary tracts.[1] The protein product of the *CFTR* gene is a chloride channel expressed in the apical membrane of epithelial cells.[2] Therefore, diagnostic tests that measure the chloride conductance in exocrine epithelia like the pilocarpine iontophoresis sweat test,[3] intestinal current measurement (ICM)[4] and nasal potential difference (NPD)[4,5] show clearly abnormal values in typical CF cases. Atypical cases of CF have a different presentation of clinical features: pancreatic sufficiency, mild bronchitis,

nasal polyposis, congenital bilateral absence of the vas deferens (CBAVD), a borderline sweat test, or ICM values which point to low residual chloride secretion in intestinal tissue either by CFTR or an alternative chloride channel.[7-10] We present a case that is characterised exclusively by severe lung disease, whereby other organs typically involved in CF are not affected and fail to show a chloride transport defect.

Methods

This study was approved by the medical ethical committees of the Dutch and German participating hospitals. Informed consent was obtained from the patient described. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, USA.

Analysis of the CFTR gene
The promotor (-3.8 to -0.3 kb) and coding regions and the exon-flanking intron sequences of the *CFTR* gene were screened for mutations by single strand conformation polymorphism (SSCP) analysis[11,12] and, in the case of the appearance of non-wild type band patterns in the high resolution SSCP gel, by subsequent sequencing.[13] Deletions in the *CFTR* gene were sought for by repeated Southern hybridizations of macrorestriction blots with CFTR cDNA probes encoding exon 1, exons 7-24 or the second nucleotide binding fold (codons 1202-1422). Preparation of unsheared genomic DNA from fresh blood, complete restriction digestions with *Apa*I, *Eag*I, *Fsp*I, *Sal*I or *Xho*I, pulsed-field gel electrophoresis, blotting and hybridization followed protocols 1, 5, 7, 8 and 9 of reference 14. The intragenic *CFTR* haplotype was determined for the sequence alterations T854T and M470V,[15,16] the splice site polymorphism in intron 8 $(TG)_mT_n$[11,17] and the microsatellites IVS8CA, IVS17bCA, IVS17bTA.[18,19]

Nasal potential difference
The method of studying NPD has been adapted from the method described previously.[5] In short, the nasal turbinate was superfused (1.7ml/min) subsequently for periods of 3 minutes with the following solutions (mol/L): 1) saline, to measure baseline PD; 2) amiloride (10^{-4}), to block Na^+ channels; 3) gluconate with amiloride, to assess spontaneous Cl^- conductance; 4) isoprenaline (10^{-4}) in solution 3, to evaluate the presence of CFTR and finally 5) ATP (10^{-3}) in solution 4, to open non-CFTR Cl^- channels. To access the submucosal space a small needle (25 gauge) was inserted into the forearm and filled with saline. Both the needle and superfusion catheter were connected to a high input resistance voltage measuring device via salt

bridges and AgAgCl electrodes. The saline solution consisted of (mmol/L): NaCl (120), Na-gluconate (25), K-gluconate (5), NaH_2PO_4 (0.4), Na_2HPO_4 (2.4). In the gluconate solution NaCl was replaced by Na-gluconate (145). All solutions were adjusted to pH 7.4.

Intestinal current measurement on rectal biopsies

The method used to study ICM has been described previously.[4] In short, freshly obtained rectal biopsies were mounted in saline solution in the Ussing chamber (apperture 1.2mm). After stabilizing of the basal short circuit current (I_{sc}) the tissue was exposed to inhibitors and secretagogues (mol/L) to the mucosal (M) or serosal (S) side: 1) glucose (10^{-2}, M+S); 2) amiloride (10^{-4}, M); 3) indomethacin (10^{-5}, M+S), to inhibit basal Cl^- secretion by inhibiting the endogenous prostaglandin formation; 4) carbachol (10^{-4}, S), a Ca^{2+} linked secretagogue; 5) forskolin (10^{-5}, M+S) + 8-bromo-cyclic adenosine monophosphate (cAMP, 10^{-3}, M+S), to open Cl^- channels by activation of a cAMP-dependent protein kinase and phosphorylation of specific membrane proteins; 6) 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, 2.10^{-4}, M), to inhibit Ca^{2+} dependent Cl^- secretion; 7) histamine (5.10^{-4}, S), to reactivate the Ca^{2+} activated pathway.

Results

Patient history

The 34-year old index case is the third child of Caucasian first-generation cousins. The first child was born preterm with meconium ileus and died at day 10. The second child died during infancy at 6 months and CF was suspected at autopsy. Our index case suffered from chronic nasal polyposis which led to 13 polypectomies between the age of 5 to 23 years. By the age of 15 years she was referred to a chest physician because of shortness of breath during sports. Clubbing, subnormal lung function and decreased exercise tolerance were noted. The diagnosis of cystic fibrosis was proposed because of the typical pulmonary manifestations, but was discarded when normal sweat electrolyte concentrations were measured. The differential diagnosis of allergy as the underlying disease was excluded by normal bioassay of specific IgE by skintest and normal levels of specific IgE in serum. Immotile cilia syndrome was excluded by normal microscopic appearance of a nasal cilia biopsy. Since adolescence she was underweight (below 3rd percentile) and had recurrent lower airway infections. Over the years increased sputum production and chronic cough became a clinical problem. A lung biopsy in 1984 showed a histological pattern consistent with CF: bronchiectasis with localised purulent bronchitis and surrounding

fibrosis. Cultures of throat swabs or sputum were often positive for *Staphylococcus aureus* (>90% of samples), but never for *Pseudomonas aeruginosa*. However, the specific anti-*P. aeruginosa* oprF IgG titer was positive, indicating that she had been exposed to *P. aeruginosa*. Bronchodilators and intermittent antibiotics were prescribed, but the patient generally discontinued medication after a few days even during acute respiratory tract infections. At the age of 23 her lung function was reduced (VC 1.5L = 48% predicted), her chest X-ray had a Chrispin-Norman score of 20, grade 3 (range 0-38), her height was at the 25th percentile and weight below the 3rd percentile. However, pancreatic function is sufficient as has been shown by normal chymotrypsin in 1986, 1992, 1995 and by normal stool elastase in 1995 (386 mg/g) and ultra sound of the pancreas. Our patient has never received pancreatic enzymes or vitamin supplements. Serum levels of vitamins (vit. A 370 µg/L, vit. E 11 mg/L), bilirubin and liver enzymes have always been in the normal range. Moreover, the sweat electrolytes in repetitive pilocarpine iontophoresis sweat tests which have been performed since she was 11 years old, were always in the normal range (sweat chloride 7 to 32 mmol/L).

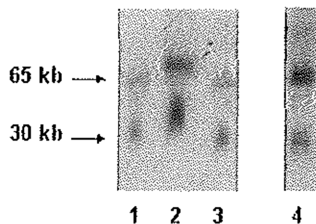

Figure 7b.1 *Autoradiogram of genomic ApaI digestions probed with CFTR cDNA (exon 7-24). No anomalous band pattern is seen for the sample from the index case (lane 1). For comparison, lane 4 shows the probe-reactive fragments from a specimen which carries a deletion in one CFTR allele. ApaI-cleaved fragments were separated in a CHEF-DR™II cell at 5.6 V/cm in 1% agarose gels (0.5 TBE buffer, 10°C). Pulse times were linearly increased in two ramps from 5-20s in 18hrs and then from 5-90s in 20hrs.*

CFTR genetics

Despite the familial consanguinity, the index case is heterozygous at the *CFTR* locus which is shown by the different number of (TA)n repeats

(n=30,34) for the microsatellite at the locus IVS17bTA, and her heterozygosity for a rare *CFTR* mutation. She is homozygous (TG)12T7-2-1 for the *CFTR* haplotype (TG)mTn-M470V-T854T. The combination of the TG12 repeat with the T7 allele and the V470 CFTR isoform are known to significantly decrease the amount and chloride channel activity of CFTR.[16] After all *CFTR* exons and flanking intron sequences and most of the promotor area up to -4 kb had been screened by SSCP one molecular lesion, the splice site consensus transition 1898+3 A->G, was identified. A pancreatic insufficient child with CF at our clinic is homozygous for this condition and exhibits the typical pulmonary and gastrointestinal manifestations of CF indicating that 1898+3 A->G is a CF-causing lesion. No anomalous bands were seen in autoradiograms of macrorestriction blots probed with CFTR cDNAs indicating that the two *CFTR* alleles of our index case do not carry any major genomic alteration (Figure 7b.1).

Table 7b.1 *Electrophysiological results of the index case. Means ± (SD) for a control group and a CF patient group are given for comparison*

	Control	Index case	CF
Nasal potential difference baseline and ΔPD (in mV) after addition of	*n*=25		*n*=23
baseline	-24 (11)	-52	-45 (10)
amiloride	+10 (6)	+21	+21 (9)
Cl^--free solution	-15 (10)	-3	-1 (5)
isoprenaline	-8 (4)	-4	-2 (3)
ATP	-1 (3)	0	-1 (3)
Intestinal current measurement ΔI_{sc} (in $\mu A/cm^2$) after addition of	*n*=50		*n*=51
amiloride	-8.7 (11)	-4.9	-8.7 (11)
carbachol	38.5 (23)	45.5	-5.3 (10)
histamine (after DIDS incubation)	33.0 (26)	19.4	-5.0 (10)

Diagnostic assessment

On the day of electrophysiological investigation at the age of 32 she was underweight, below the 3rd percentile with a body mass index of 17 kg/m2 and her lung function was severely reduced (FEV1 0.39L= 14% predicted, FVC 1.23L = 38% predicted). At this time, she was treated with continuous oxygen and showed severe clubbing. Electrophysiological measurements were carried out by assessing the presence of chloride conductance in her

airway and intestinal epithelium (Table 7b.1). The basal NPD found with saline superfusion was -52 mV (Figure 7b.2). In the presence of amiloride, blocking the sodium conductance of nasal turbinate epithelium, the PD depolarized to -31 mV, a decrease of 40%. Superfusion of gluconate in the presence of amiloride resulted in a net response of -3 mV, indicating a subnormal Cl^- conductance. With isoprenaline, opening CFTR Cl^- channels, only a small response of -4 mV was obtained which suggests that little CFTR channels are present. No ATP response, usually indicative of the presence of alternative Cl^- channels,[20] was seen in the airway tissue. In our earlier ICM studies carbachol provoked a negative, reversed I_{sc} in rectal tissue of CF patients, sometimes followed by a positive, residual response indicating the presence of residual Cl^- secretion. In our index case, the ICM showed a Cl^- secretory current in the normal range upon carbachol addition (Figure 7b.3). DIDS, which inhibits the alternative pathway of Ca^{2+} activated Cl^- currents, did not influence the Cl^- current. This suggests the presence of normal CFTR mediated chloride conductance in the intestine.

Discussion

The clinical and diagnostic features presented by this case are conflicting in terms of the expression of the basic defect in different tissues. In typical CF defective electrolyte transport in sweat gland, intestine and airway epithelium is demonstrated by a pathological outcome of sweat test, ICM and NPD. In our case, however, sweat chloride concentration and ion flow in the intestine are normal, whereas the NPD values are abnormally high. Pathologically elevated NPD values have so far only been found in CF and not in any other lung disease with related clinical manifestations like α_1-antitrypsin deficiency, immotile cilia syndrome or congenital bronchiectasis.[21] As well as a high baseline nasal PD her gluconate and isoprenaline response are also in the CF range. However, they do point to the presence of little residual CFTR Cl^- permeability in her respiratory epithelium. The clinical symptoms of our index case are typical of CF lung disease such as progressive obstructive bronchitis and bronchiectasis, sputum production, pathological bacterial flora and she has been permanently on oxygen since the age of 30. This could explain her anorexic status, since there is no sign of malabsorption or gastrointestinal disease, as confirmed by normal vitamin A and E levels, pancreatic sufficiency and a normal ICM.

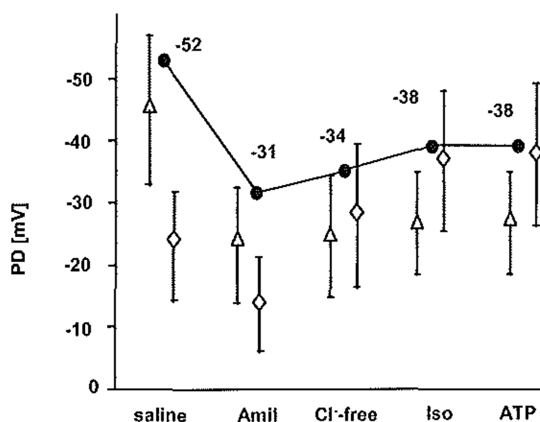

Figure 7b.2 *Nasal Potential Difference (PD) measurements of the index case (•), and mean PD values (SD) of 25 controls (◊) and 23 CF patients (Δ), upon superfusion with saline solution, amiloride (10^{-4}M) in saline solution, Cl^--free solution with amiloride, isoprenaline (10^{-4}M) in Cl^--free solution with amiloride, ATP (10^{-3}M) in Cl^--free solution with amiloride and isoprenaline.*

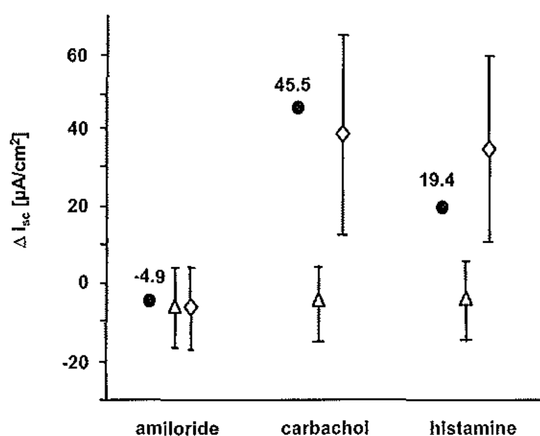

Figure 7b.3 *Intestinal Current Measurements expressed as changes in short circuit current (I_{sc}) of the index case (•), and mean I_{sc} (SD) of 50 controls (◊) and 51 CF patients (Δ), after addition of amiloride, carbachol, and after the tissue was exposed to DIDS, histamine.*

This highly discordant expression of the basic defect in different tissues is described here for the first time: CFTR function was unaffected in gastrointestinal tissue whereas in airway tissue the abnormally low Cl^- conductance can only be accounted for by defective epithelial Cl^- transport. Other cases of CF with normal sweat test and pulmonary problems, as described for patients carrying the A455E or 3849+10kb C->T mutation,[7,8,22,23] can clearly be diagnosed by an abnormal ICM, even when little or no clinical signs of gastrointestinal involvement are evident.[10]
The *CFTR* gene has been screened for disease causing lesions in all exons and flanking intron sequences: on one chromosome a sequence alteration in a donor splice site has been found (1898+3 A->G). This sequence alteration has first been described in a compound heterozygous pancreatic sufficient patient (Ferrari, *et al*; Cystic Fibrosis Genetic Analysis Consortium, pers. comm.) and has been found as well in our clinic in a pancreatic insufficient CF patient homozygous for this condition (see above). According to *CFTR* mutation analysis and the family anamnesis, our index patient is a carrier for one *CFTR* mutation and hence should not express any CF symptoms. However, our index case carries an unfavourable combination of common intragenic polymorphisms. She is homozygous $TG_{12}T_7$ for the TG_mT_n polymorphism at the intron 8 splice acceptor site. The TG_{12} repeat together with the T_7 stretch places the branchpoint nucleotide in an unfavourable position for splicing leading to 30% of exon 9- CFTR transcript which are known to be translated in CFTR proteins that do not mature.[16,24] Moreover, she is homozygous for the V470 allele in exon 10. V470 CFTR proteins have a 1.7 fold decreased intrinsic chloride channel activity compared with M470 CFTR proteins.[16] These predisposing polymorphisms in the intragenic background decrease the expression and function of CFTR. Yet, they are present in both rectal and nasal tissue and cannot account for the unimpaired function of CFTR in the intestine while nearly complete absence of Cl^- conductance in the nasal tissue was observed. However, the patient's consanguineous descent leads to a genome wide overrepresentation of homozygous genotypes. This may lead to further unfavourable combinations of factors modifying the *CFTR* expression or function in the lung. As gastrointestinal disease is not present, we propose that the genetic background predisposes to severe lung disease, caused by tissue specific regulatory elements which lead to the loss of CFTR function exclusively in the respiratory epithelium.

Acknowledgements

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Chapter 8

Discussion and future perspectives

Discussion and future perspectives

Cystic fibrosis is a disease with highly variable manifestations in the pulmonary, gastrointestinal, hepatobiliary and urogenital tracts,[1] and is caused by molecular lesions in the cystic fibrosis transmembrane conductance regulator. The heterogeneous phenotype of CF is partly explained by the large number of different *CFTR* gene mutations.[2] However, since clinical presentation varies significantly even between patients with the same *CFTR* genotype and between the affected organs within CF patients, it is evident that other factors than the *CFTR* genotype are involved in determining CF disease severity. These additional aspects involved in CF disease phenotype might consist of genetic factors outside the *CFTR* gene and/or environmental factors. An example of influencing genetic factors is the expression of non-CFTR mediated chloride secretion in the affected epithelia of the airways[3] and the intestine.[4]
In this thesis, we investigated the presence of residual chloride conductances in CF individuals, and made a distinction between residual cAMP-mediated CFTR conductances and/or the presence of Ca^{2+}-stimulated alternative chloride channels. These features were investigated in a pre-selected cohort of ΔF508 homozygous sib and twin pairs with highly concordant or highly discordant phenotypes (Chapter 3). In this group the major CF disease-causing lesion is standardized. By comparing results in the CF sib pairs who vary in genetic background, to results in monozygous twins who share identical genetic background, it is possible to dissect the importance of genetics and environment in the course of CF disease.[5,6] Moreover, analyzing individuals with most disparate phenotypes facilitates the detection of determinants of this CF phenotype (Chapter 3).[7,8] Therefore, this cohort of ΔF508 homozygous sibs and twins with most extreme disease manifestations is optimal to investigate influences on CF phenotype and compare the importance of genetic background versus epigenetic factors.
In these CF individuals, the electrophysiological methods of intestinal current measurement and nasal potential difference measurement (Chapter 2) were employed, to determine frequency and origin of residual chloride secretion in the most severely affected tissues in CF, the intestinal and respiratory tracts (Chapter 4 and 5). Moreover, the influence of the various chloride conductances that were detected in the respiratory and intestinal tracts, on the course of CF disease severity was evaluated (Chapter 5). To analyse the possible role of genetic factors outside the *CFTR* gene in the expression of residual chloride conductance, CF patients homozygous for the most common severe *CFTR* genotype, i.e. ΔF508, were investigated (Chapter 5). In Chapter 6, the chloride transport in CF patients with various missense and nonsense mutations, and compound heterozygous genotypes was

studied. The NPD and ICM patterns for the chloride conductances in the respiratory and intestinal tissues were investigated and were related to their genotype. Furthermore, CF individuals were described with atypical patterns in the NPD and ICM examinations (Chapter 7), which complicate CF diagnosis despite the application of these electrophysiological methods.

Electrophysiological characterisation of respiratory and intestinal tissues

The electrophysiological techniques of NPD[9,10] and ICM[11,12] test the conductance properties for sodium and chloride ions of the respiratory and intestinal epithelium. In clinical settings, these tests are used together with the Gibson and Cooke sweat test, for diagnostic purposes since they give distinct results between controls and CF. In the NPD measurement, the basal PD and the change in PD in response to Cl^--free and isoprenaline solutions are most different between CF and control, while in the ICM the chloride secretory responses to carbachol and histamine are most distinct between the two groups (Chapter 2). The NPD and ICM methods not only discriminate CF from non-CF, but within the group of CF patients variable responses to secretagogues and inhibitors could be observed (Chapter 4 and 5), indicating different degrees of chloride membrane permeability. In earlier ICM studies, the milder CF patients such as compound heterozygous for the mild A455E mutation appeared to exhibit a more active chloride transport than the CF patients with more severe phenotypes.[13] Moreover, in these studies chloride conductances in response to cAMP-agonists, but also in response to Ca^{2+}-inducing agonists, were detected in a minority of ΔF508 homozygous patients.[13] In this thesis, the NPD and ICM methods were effectively utilized to further evaluate the amount of chloride conductance in CF patients homozygous for the ΔF508 *CFTR* gene mutation and various other genotypes. Moreover, by using protocols for the NPD and ICM measurements in which the drugs were added in a specific sequence, different chloride conductance pathways could be segregated in respiratory and intestinal epithelia.

Presence and origin of residual Cl secretion: respiratory tract

In the investigated group of 74 ΔF508 homozygous CF individuals, we observed spontaneous chloride conductance in the NPD measurement in 22 out of 74, and 7 with chloride conductance in response to the β-adrenergic agonist isoprenaline (Chapter 5). Presently known chloride conductances that are stimulated by cAMP, are the ORCC[14] and CFTR[15]. However, the ORCC has only been observed to react to stimulation by cAMP- or PKA-agonists in the presence of functional CFTR.[16] Therefore, the finding of cAMP-induced chloride currents by isoprenaline, in the epithelial cells of a

small number of ΔF508 homozygotes suggests the presence of at least some active ΔF508 CFTR in the apical membrane.
Responses to ATP were observed in 55 of the 74 patients. ATP is reported to increase intracellular calcium concentrations by initiating the phosphoinositide metabolism and thereby inducing Ca^{2+}-dependent chloride secretory pathways.[17,18,19] Additional studies in CF and control cells have shown that the induction of chloride secretion by apical ATP is rather complex,[20,21] and might involve both the cAMP- and Ca^{2+}-mediated chloride conductance pathways. However, since we have already stimulated the beta-adrenergic pathway with isoprenaline previous to the addition of ATP, the observed additional ATP responses will be mainly induced by the Ca^{2+}-mediated pathway, and is likely to involve apical Ca^{2+}-dependent Cl^- channels.[3,4,22-24]

Presence and origin of residual Cl secretion: intestinal tract
In the ICM measurements of 55 ΔF508 homozygous CF twins and siblings selected for their extreme phenotypes (Chapter 4), the application of cAMP-agonists induced a chloride secretory response in 40 (73%) of the 55 patients of which interpretable ICM tracings were obtained. This suggests that, as in the respiratory tissue, also in the intestinal tissue residual CFTR is present and responds to cAMP-agonists.
Comparison of carbachol responses registered before the addition of DIDS, to the histamine responses recorded after the incubation of the rectal tissue with DIDS, segregates DIDS-insensitive chloride channels like CFTR,[25] from DIDS-sensitive chloride conductances, such as the outwardly rectifying chloride channel[14] or the Ca^{2+}-activated Cl^- channels (CaCC).[3,4,22-24] In 11 individuals (20%) of the investigated ΔF508 homozygotes DIDS-sensitive chloride secretion was identified, indicating the presence of these alternative non-CFTR Cl^- conductances.
The observation that DIDS-sensitive Ca^{2+}-mediated residual Cl^- secretion only appeared in ΔF508 homozygous patients possessing residual cAMP-activated chloride currents is not yet completely understood. As a possible explanation for this apparent co-expression of Ca^{2+}-dependent chloride channels and ΔF508 CFTR, one might postulate that a less stringent quality control not only causes enhanced trafficking of ΔF508 CFTR to the apical membrane, but also results in an increased expression of Ca^{2+}-dependent chloride channels. An alternative explanation, i.e. that expression of *CFTR* is directly coupled to the expression of Ca^{2+}-sensitive chloride channels, is unlikely since no DIDS-sensitive Cl^- current component was found in control patients (Chapter 4).
To investigate whether DIDS-sensitive Ca^{2+}-mediated currents also exist in tissues in which cAMP-mediated conductances are absent, Ca^{2+}-dependent

conductances should be further investigated in patients carrying two *CFTR* stopcodon mutations, and do not express any chloride conductance in response to cAMP-agonists. In *cftr* knockout mice, the expression of Ca^{2+}-dependent chloride channels is enhanced and was suggested to be beneficial for the disease phenotype.[26-28] The mechanism for this overexpression of CaCC channels in the absence of CFTR has not been elucidated, but may involve enhanced transcription of genes encoding DIDS-sensitive chloride channels. Notably, this overexpression was not observed in the intestinal tract of one of the *cftr*$^{-/-}$ knockout models,[27] despite its clear overexpression in the respiratory tract.

ΔF508 CFTR

The classifications of the different *CFTR* gene mutations[29-31] and investigation of the properties of ΔF508 CFTR in heterologous model systems categorise ΔF508 as a mutation that causes defective processing of the CFTR protein. The ΔF508 CFTR protein is misfolded and unable to form a protease-resistant mature conformation causing CFTR to be retained in the ER. Consequently, CFTR fails to traffic to the cell membrane and express chloride channel function at the correct cellular location.[32,33,34]
However, the observations reported in this thesis demonstrate that a subgroup of ΔF508 homozygous CF individuals are competent to respond to agonists of the cAMP-mediated chloride secretory pathway in both respiratory and intestinal tract, identified by a response to isoprenaline in the nasal epithelium (Chapter 5), and DIDS-insensitive chloride secretion in the intestinal tissue (Chapter 4 and 5). Since cAMP-sensitivity is the hallmark of CFTR, this suggests that at least some functional ΔF508 CFTR is present in the apical membranes of the epithelial cells in these organs and is capable to function as a chloride channel stimulated via the cAMP/PKA-dependent signaling pathway.
We suggest that the expression patterns of ΔF508 CFTR *in vivo* might be different from that in heterologous expression experiments, and that in this subgroup of CF individuals, a portion of ΔF508 CFTR is able to reach the plasma membrane. In support of our results, earlier studies have shown that ΔF508 transfected cells exhibit chloride secretion,[35] and that ΔF508 mouse models present with a residual CFTR-like chloride conductance in the intestinal[36] and respiratory[36,37] tissues. Moreover, recent studies have now observed cAMP-induced chloride currents in human biliary tissue specimens in which subnormal expression of ΔF508 CFTR was recognized.[38] In addition, immunohistochemical studies performed on human airway,[39,40] intestinal[41] and hepatobiliary tissues,[38] demonstrated apical localization of ΔF508 CFTR, in some cases indistinguishable from wild-type tissues.[41]

In conclusion, this thesis showed by application of functional *in vivo* and *ex vivo* assays that respiratory and intestinal tissues from our selected group of ΔF508 CFTR homozygous individuals are competent to respond to agonists of the cAMP-dependent chloride secretory pathway, which is the hallmark of CFTR-mediated chloride transport.
The addition of chemical chaperones to CF cells, such as glycerol[42] or trimethylamine-*N*-oxide[43] have shown to increase the targeting of ΔF508 CFTR to the cell membrane. The finding described in this thesis of residual cAMP-sensitive chloride conductance in ΔF508 homozygotes, implies that overcoming the ER quality control barrier, and enhancing ΔF508 CFTR processing to the apical membrane in CF individuals, might be a successful way to overcome the typical phenotype of ΔF508 homozygotes. Therefore, further investigations of potential approaches to increase the transport of mutated CFTR to the cell membrane, is of great importance for new therapeutic strategies in CF.

Alternative Ca^{2+}-dependent chloride secretion

The Ca^{2+}-dependent chloride conductance has been observed to be present in both normal and CF airway epithelium.[44] In intestinal tissues contrasting results have been obtained, varying from a complete absence of Ca^{2+}-dependent chloride conductances in most controls and the majority of CF individuals (Chapter 4 & ref 45), to a clear Ca^{2+}-dependent DIDS-sensitive component in a subclass of ΔF508 homozygotes.[12,13] The existence of such Ca^{2+}-dependent chloride conductances has been verified by studies in *cftr* $^{-/-}$ knockout mice. In the $Cftr^{m1UNC/m1UNC}$ knockout mouse,[27] alternative conductances were observed in the lung and pancreas, and in the $Cftr^{m1HSC/m1HSC}$ knockout mouse,[26,28] such Ca^{2+}-dependent chloride conductances were observed in the respiratory, but also in the intestinal tract.
The first detection of Ca^{2+}-dependent chloride conductance in CF individuals was predominantly in mild CF phenotypes.[13] The data presented here show that a substantial part of our selected ΔF508 homozygotes with extreme phenotypes also exhibit this Ca^{2+}-dependent chloride membrane permeability.[12] Patients with other genotypes such as patients with double stopcodon mutations and certain compound heterozygotes can also exhibit Ca^{2+}-dependent chloride transport (Chapter 6 & ref 9). Several molecular entities that might be responsible for the Ca^{2+}-dependent chloride conductance, have now been cloned and characterized. In the intestinal tissues of healthy individuals, hCLCA1 was identified, a chloride channel that is stimulated by increased intracellular Ca^{2+} concentrations, inhibited by DIDS, and is only expressed in small intestine and colon, and not in any other human organs.[4] Furthermore, a member of the ClC family of chloride

channels, hClC3, was identified and cloned in T84 cells, which is activated by calcium-calmodulin dependent kinase, and also sensitive to DIDS.[23] In the human respiratory tissue, hCLCA2 is recognized which is stimulated by Ca^{2+}-ionophores and inhibited by DIDS. This channel seems to be expressed in lung, trachea and mammary tissue.[3] Future experiments, like immunohistochemical studies, Northern blot hybridization and RT-PCRs, in tissue preparations of cystic fibrosis individuals should reveal whether the above mentioned molecular entities are expressed in the CF cells, and whether their level of expression is modified in CF. Comparison of these results with the outcome of electrophysiological tests like the NPD and ICM of these same individuals, can then verify if Ca^{2+}-dependent chloride conductances are indeed coinciding with the molecular presence of these channels.
A different study should investigate the expression patterns of the CaCC family in the respiratory and intestinal tracts within the same individual, in both controls and CF patients. More insight in the expression and function of these Ca^{2+}-activated chloride conductances is desirable, because of their possible influence on the CF phenotype.

Correlation with phenotype
The expression of cAMP- and/or Ca^{2+}-activated residual chloride conductances in individuals with cystic fibrosis disease potentially compensate for the lack of CFTR-mediated chloride transport and might ameliorate CF disease severity. Consequently, exogenous administration of agents that initiate these residual chloride secretory pathways, might be a novel therapeutic strategy in CF disease.
Up till now, there are no studies known that try to hyperactivate residual CFTR *in vivo* as therapy in CF disease. However, in 2 differently generated *cftr* $^{-/-}$ knockout mice, an alternative Ca^{2+}-regulated chloride conductance was detected in several organs and was suggested to attenuate CF disease and protect the tissue from the absence of CFTR-mediated Cl^- conductance.[26-28] Clarke et al[27] found Ca^{2+}-activated chloride currents in respiratory and pancreatic tissues in their *Cftr*$^{m1UNC/m1UNC}$ knockout mouse model, and postulated that this is the reason for the lack of lung and pancreatic disease in these CF mice. Rozmahel et al[26] and Wilschanski et al[28] found Ca^{2+}-mediated residual chloride conductance in the intestinal and respiratory tissues of a subgroup of their *Cftr*$^{m1HSC/m1HSC}$ knockout mice, and these mice had a prolonged survival compared to their CF knockout littermates. As previously mentioned, alternative Ca^{2+}-mediated chloride channels have also been identified in human epithelia.[3,4,22-24] In airways these channels are suggested to be activated by stimulation with extracellular nucleotide triphosphates.[18,46] Consequently, the induction of

chloride secretion by extracellular nucleotides has been suggested as a possible new therapeutic approach for CF airway disease.[47]
As described above, we demonstrated the presence of cAMP- and/or Ca^{2+}-mediated chloride conductances in the respiratory and intestinal tracts of ΔF508 homozygous individuals within our group of investigated CF sib and twin pairs. To evaluate the importance of the expression of residual CFTR and/or alternative Cl^- conductances in these two tissues, we compared the phenotype of airways and intestine to the observed Cl^- conductances in selected ΔF508 homozygous twins and sibs with the most disperate manifestation of CF disease: i.e. concordant pairs with relatively mild CF disease, condordant pairs with severe CF disease, and discordant pairs (Chapter 3). These individuals are highly informative to investigate modifying factors of the CF phenotype. Thus, we could analyze if the expression of cAMP- and/or Ca^{2+}-mediated Cl^- conductance are important modifiers of CF disease and if they influence the disease manifestation of CF.

Influence of residual chloride conductance on pulmonary phenotype
In the group of investigated ΔF508 homozygotes described in this thesis, a better lung function (FEVPerc) segregated with higher chloride transport properties of the respiratory epithelium, which appears to be less Cl^--impermeable (Figures 5.3a and b). Spontaneous basal chloride conductance (Cl^--free response) and the capacity to secrete chloride in response to a cAMP-agonist (isoprenaline response) were only observed in clinically better patients. These data suggest a beneficial function of basal chloride conductance and/or residual cAMP-mediated chloride secretion for respiratory tissue performance, most likely by increasing the hydration of the viscous airway surface mucus and increasing its clearance from the respiratory tract. These data of ΔF508 homozygous twins and sibs substantiate the findings of an earlier study in CF individuals with different *CFTR* mutation genotypes in whom residual chloride secretion provided a better indication of lung function than genotype.[48]
The chloride conductance mediated by apical purinergic receptors and a subsequent increase of intracellular Ca^{2+} (ATP response),[18,46,49] did not correlate with better lung function ($P = 0.53$, Table 5.5). This does not support the data suggested in CF knockout mice, that Ca^{2+}-dependent chloride conductances induced by ATP, results in better lung function.[26-28]
Clinical trials have already investigated the safety of addition of extracellular nucleotides to the respiratory epithelium, and showed that aerosolized UTP improves clearance of airway secretions in CF individuals to the basal rate of healthy controls.[50,51] Subsequently, the long-term influence of ATP and UTP on lung function should be investigated, to determine if continuous aerolization therapy with UTP is beneficial for the CF individual.

As was reported previously,[52] we observed an increased membrane potential for Na^+ in respiratory tissues of CF individuals as inferred from the amiloride response (Chapter 2, 4 and 5). However, within the cohort of CF patients the magnitude of this response was not related to CF disease severity (FEV1Perc), suggesting that the magnitude of Na^+ hyperabsorption does not influence CF symptoms in the airways.
In conclusion, in our cohort of ΔF508 homozygous sib and twin pairs the expression of basal Cl^- conductance and the response to isoprenaline are predictive for CF disease severity as expressed by the lung function parameter, FEV1Perc.

Influence of residual chloride conductance on intestinal phenotype
In the intestinal tissue, both the cAMP- and Ca^{2+}-dependent chloride currents were evaluated against the CF disease manifestation in the intestinal tract (weight for height %predicted). The CFTR-dependent DIDS-insensitive chloride secretion was predominantly seen in the mildly affected patients (Figures 5.4, 5.5 and 5.6). In contrast to the respiratory tissue, the DIDS-sensitive Ca^{2+}-dependent Cl^- secretion demonstrated to correlate with CF phenotype and was more frequently observed in the severely affected ΔF508 homozygotes. Studies in the biliary tract of ΔF508 homozygotes gave similar results in that the highest levels of Ca^{2+}-dependent chloride currents were detected in the most affected tissue samples.[38] Hence, either this alternative Cl^- conductance is not beneficial per se, or it is up-regulated in the absence of CFTR-activity to compensate for the lack of Cl^- transport and tissue damage would be even worse in the absent of this chloride conductance, as was postulated in the case of *cftr* knockout mice.[27,53]
To compare the basic defect and the presence of alternative chloride conductances in the different tissues within one individual, NPD and ICM patterns were compared. The presence of basal chloride conductance (Cl^--free response) in the respiratory tissue was not associated with the presence of a chloride secretory component in the carbachol and/or histamine response in the intestinal tissue (Fisher's exact test, Spearman rank test, Figures 5.6a and b). However, in this selected cohort of ΔF508 homozygotes which consists of individuals with disparate phenotypes (Chapter 3), the presence of a gluconate response in the respiratory tissue and a positive DIDS influence in the intestinal tissue segregated with milder phenotype (Fisher's exact test, $P = 0.01$). Moreover, the amount of basal chloride conductance detected in the respiratory epithelium was related to the magnitude of the DIDS influence on Cl^- secretion in the intestine: the expression of basal chloride conductance in the airways correlated with the DIDS-insensitive intestinal chloride currents, i.e. CFTR-related chloride conductance (Spearman test, reaching the limit of significance, Figure 5.6c).

Thus, these two most affected epithelia in CF showed similar expression patterns of the basic defect, i.e. CFTR-related chloride conductance, suggesting comparable maturation and processing mechanisms of the mutated ΔF508 CFTR in these two organ systems.
In conclusion, the identical *CFTR* genotype of ΔF508 homozygosity was not predictive for the appearance of disease symptoms, since a large variety of disease phenotypes were observed in this group. In contrast, the expression of the basic defect as measured by NPD and ICM was associated with clinical disease manifestation, which makes this group of patients with various expression of the basic defect and with extreme phenotypes, highly informative to search for genetic modulators outside the *CFTR* gene. In addition, these data show that the utilization of the NPD and ICM measurements can assist in evaluation and prognosis of disease severity within such a group of identical *CFTR* genotypes, in contrast to the sweat test which is similar within the group of ΔF508 homozygotes. For better understanding of the correlation between expression of residual chloride conductance and the manifestation of disease, follow up studies of individual CF patients would be especially interesting in which the cAMP- and calcium-stimulated chloride conductance patterns in the epithelia are determined in early disease stages and monitored over time. Although protocols for these particular studies will be complicated, the interaction between CFTR-mediated chloride permeability, Ca-dependent chloride conductance, and the resulting phenotype can be analyzed.

Importance of genetic background and environmental factors
The variable manifestation of cystic fibrosis disease even in patients with the same *CFTR* mutation genotype necessitates the investigations to determine the factors that cause this heterogeneity. The approach of the European CF Twin and Sibling Study to investigate mono- and dizygous ΔF508 homozygous pairs is especially suitable to dissect the relative impact of environmental factors, residual chloride channel activity and other genetic factors than the *CFTR* genotype on a the phenotype phenotype of a monogenic disease like CF.
By comparing dizygous pairs homozygous for the same *CFTR* gene mutation, the disease causing lesion and the intragenic haplotype were standardized and the variation in genetic background reduced. The intrapair variation among monozygous twins, who are genetically identical for their whole genome, can be used to delineate the influence of the environment on a trait by comparing the intrapair variability among monozygous pairs to that of dizygous pairs.[5,6]
Thus, to unravel the importance of genetic factors versus environmental factors for the expression of residual chloride conductance, the intrapair

concordance of dizygous sibling pairs was compared to that within monozygous pairs.
Monozygous twins proved to be more concordant in their patterns of residual chloride conductance than dizygous pairs. This was predominantly seen in the intestinal tract. The cAMP responses, histamine responses and DIDS-sensitivity of the chloride secretory response were all significantly more concordant within monozygous twins than within dizygous pairs. In the NPD measurement, however, only the basal PD was significantly more similar in monozygous pairs. Thus, genetic factors are important for the expression of residual chloride secretion in the intestinal tissue, and are apparently not obscured by possible differences in epigenetic factors. Since the investigated CF pairs were homozygous for the ΔF508 *CFTR* mutation, these genetic modifiers seem to be located outside the *CFTR* gene. The disease manifestation in the respiratory tract appears more dependent on environmental factors than genetic influences, most obviously since this organ is far more accessible for influences from the environment. These data are substantiated by many studies that failed to discover evident associations between a certain genotype and the pulmonological phenotype. One of the goals of the *European CF Twin and Sibling Study* is to further investigate possible genetic modifiers of the CF phenotype, by influencing disease manifestation either in the intestinal or respiratory tract. Therefore, part of the study is to perform a genome-wide linkage analysis by microsatellite genotyping (first starting with selected candidate genes), and association studies in patients with rare haplotypes are being executed.

Electrophysiological characterization of rare genotypes

Apart from the group of ΔF508 homozygous individuals we investigated the sweat test plus NPD and ICM patterns in a group of individuals with rare genotypes. This group consisted of homozygotes for the missense mutation E92K, for the stop mutations R553X or R1162X, for the splice mutations 1898+3 A-G or 3849+10kb C-T, the novel deletions CFTRdele2 or CFTRdele2,3(21kb) and in compound heterozygotes for ΔF508 together with the complex allele ΔF508-R553Q or ΔF508-V1212I. Sweat tests were all in the pathological range except one patient homozygous for 3849+10kb C-T, who had varying sweat test results on different occasions. And the individual with ΔF508-R553Q/ΔF508 had a normal sweat test in early life which progressed to abnormal sweat chloride levels at adolescent age.
In NPD measurements, the basal PDs were all elevated in the respiratory tissues of these CF individuals. Chloride conductance was detected upon the addition of a chloride free solution in the patient homozygous for CFTRdele2,3(21kb), 1898+3 A-G and 3849+10kb C-T, and in one of the individuals carrying the ΔF508-V1212I allele (Table 8.1). The 3849+10kb C-T

homozygote with a chloride free response also exhibited chloride conductance in response to isoprenaline, and three other patients showed isoprenaline responses: the homozygotes for R553X, R1162X and E92K. Within the 3 investigated sib pairs with CF (Chapter 6), no concordance was observed in their expression of chloride conductance.
The chloride secretory responses in the intestinal tissue as observed by ICM are depicted in Table 8.1.

Table 8.1 *Expression of Cl^- conductance in nasal and intestinal tissues of individuals with rare genotypes*

Patient nr.	Genotype	NPD		ICM	
		Cl^--free	Iso	cAMP-med. residual	DIDS-sensitive residual
1	CFTRdele2,3(21kb)/ CFTRdele2,3(21kb)	-10	-	No	No
2	R553X/R553X	-	-8	No	No
3	R1162X/R1162X	-	-2	No	Yes
4	R1162X/R1162X	-	-	No	Yes
5	CFTRdele2/CFTRdele2	-	-	Yes	No
6	E92K/E92K	-	-11	Yes	No
7	ΔF508-R553Q/ΔF508	-	-	Yes	No
8	ΔF508-V1212I/ΔF508	-3	-	Yes	Yes
9	ΔF508-V1212I/ΔF508	-	-	No	Yes
10	1898+3 A-G/1898+3 A-G	-3	-	Yes	No
11	3849+10kb C-T/3849+10kb C-T	-10	-2	Yes	No
12	3849+10kb C-T/3849+10kb C-T	-	-	Yes	No

NOTE. Patients 3&4, 8&9, 11&12 are sib pairs. Cl^--free: Cl^--free solution. Iso: isoprenaline. cAMP-med. residual: cAMP-mediated residual secretion.

The expression of residual cAMP-mediated chloride transport in the respiratory and/or nasal tissue might be attributed to residual CFTR activity in the homozygote for the missense mutation in the first ectoplasmatic domain (E92K), or in the 1898+3 A-G and 3849+10kb C-T homozygotes,[54] who are capable to produce small amounts of normal CFTR mRNA. In addition, carrying the R553Q mutation on the same allele as ΔF508 has been identified to revert the ΔF508 mutation, suggesting that CFTR-induced chloride secretion might also be possible in this individual.[55] In the case of the ΔF508-V1212I/ΔF508 siblings CFTR activity could hypothetically also be possible due to mutant CFTR as we have also seen in our ΔF508

homozygous patients who also exhibited small amounts of cAMP-dependent chloride transport (Chapter 4 and 5).
The ICM and NPD patterns in some of our investigated patients homozygous for a large deletion or stop codon mutation were remarkable. In the case of the homozygous R1162X siblings, residual chloride permeability may be ascribed to functioning CFTR, since levels of wild-type transcript have been detected. However, the out-of-frame deletion (CFTRdele2,3(21kb))[56] and the R553X stop codon mutation[57] are described as null alleles that do not produce any CFTR. Therefore, the residual chloride conductance seen in the respiratory tissue of these individuals suggests the presence of alternative non-CFTR chloride conductances.[3,4,22-24] On the other hand, these alternative chloride channels are activated by calcium, which does not explain the isoprenaline response in the R553X homozygote airway. In epithelial cells, there have only been identified 3 non-CFTR cAMP-dependent Cl^- channels. However, one is the ORCC which only conducts chloride in the presence of functioning CFTR,[16] the second was found in kidney cells but is only expressed in the basolateral membrane,[58] and the third is the ClC-2G Cl^- channel which is expressed in intestine[59] but does not contribute to the transepithelial chloride transport in this tissue in short-circuit current measurements (personal communications, Dr. H.R. de Jonge).
Calcium dependent chloride conductance was seen in the intestinal tract of at least two of our investigated individuals, homozygous for R1162X and the compound heterozygote ΔF508-V1212I/ΔF508, indicated by a DIDS-sensitive chloride secretory response. As stated earlier, such a DIDS-sensitive chloride conductance in the gut has also been detected by Gruber et al.[4]
Two of the three investigated sib pairs were observed with similar chloride secretory patterns in their intestinal tissues.
The electrophysiological identification of this group of rare genotypes shows variable chloride conductance patterns without a clear-cut correlation between residual chloride conductance expressed in respiratory and intestinal tissue. Moreover, the absence of concordance in the chloride conductance patterns in the 3 sib pairs, especially in the respiratory tract, demonstrates that other factors, such as environmental and non-CFTR genetic factors, are important determinants for the capacity to transport chloride.
In conclusion, these investigations emphasize the variable expression of the basic defect in the different tissues in patients with these rare genotypes, and the complex relationship between genotype and phenotype in cystic fibrosis disease.
Future research should be directed towards the electrophysiological characterization of larger groups of CF patients with different genotypes, to gain more insight into the capacity of these persons to express CFTR and

transport chloride relative to their type of gene alterations. The knowledge on the existence of alternative chloride channels would increase by investigating chloride conductance patterns in additional individuals carrying definite stop codon mutations in whom no CFTR mRNA levels have been identified.

Complexities in diagnosing CF

As was shown in this thesis, the methods of NPD and ICM give clear distinction between controls and CF patients homozygous for the ΔF508 mutation. The ΔF508 mutation is classified as severe and all ΔF508 homozygotes show clear clinical manifestation of CF. And although these patients show considerable variation in the expression of residual chloride secretion in both the respiratory and intestinal tracts, these amounts of chloride transport are all in the CF range, i.e. different from the control group. Moreover, all these patients exhibited pathological sweat chloride concentrations > 60 mmol/L.
However, in specific cases the diagnostic process of CF is complicated. In Chapter 7a, a CF patient is presented with recurrent pulmonary infections and pathological sweat chloride concentrations but with pancreas sufficiency, who was diagnosed with CF disease at 17 years of age. However, in *CFTR* mutation analysis no gene alterations were found. The fact that the *CFTR* mutation analysis is negative does not totally exclude the possibility of CF. Firstly, the detection rate of mutations is around 95%,[60,61] and it is estimated that about 10-20% of mutations are still unknown at present.[1] However, the intragenic *CFTR* marker haplotype in the index case was similar to that in his asymptomatic sister, making CF disease unlikely since there would be total discordance in disease manifestation both in the sweat gland and the pulmonary disease. There is the possibility of a de novo mutation in the non-coding region of the *CFTR* gene, although this event and its consequence to establish CF disease are highly unlikely. In this individual both the NPD and ICM examinations showed normal chloride transport, excluding the diagnosis of CF. So in this case in which sweat test was positive and *CFTR* mutation analysis did not give any conclusion, the diagnosis of CF could be excluded by the assistance of the NPD and ICM results. Some patients in our clinic have been observed with a borderline or positive sweat test, with normal ICM results and only single symptoms of CF disease, e.g. oligospermia or nasal polyps, in whom CF diagnosis should be doubted (unpublished observations).
In another individual in whom the diagnosis was not unequivocally provided by the exciting diagnostic tests, we found severe pulmonary disease with recurrent infections, continuous cough, and nasal polyps. However, repeated sweat tests gave normal chloride concentrations, she is pancreas sufficient, and with DNA analysis only one allele showed a sequence alteration, 1898+3

A->G. The ICM showed normal results concordant with the absence of intestinal disease while NPD gave results consistent with CF disease supporting the clinical manifestation of severe pulmonary disease. This individual is of consanguineous decent which leads to increased homozygosity for her overall genotypes. Moreover, she is homozygous for TG12T7 (intron 8) and V470 (exon 10) which cause reduced mature CFTR protein and decreased chloride channel activity, respectively.[62] We hypothesize that this genetic constitution has a negative effect on the *CFTR* expression in the lung, while this genotype does not influence the expression in the intestine, suggesting that one mutation and very unfavourable genetic background can cause a CF phenotype. The possibility that this patient might have CF is rather unlikely, since the other allele showed no sequence alterations in any of the coding regions, flanking introns, or the promotor area up to –4 kb. Thus, it could only be an unknown polymorphism in a non-coding region that would cause CF. In addition, patients with normal sweat tests and pulmonary disease in which two *CFTR* mutations were found, always showed a subnormal ICM, which was not the case in this individual, rejecting the diagnosis of CF as well.
These two atypical cases illustrate the importance of using various diagnostic tests in the diagnostic process for CF disease. The sweat test has been shown to give false positive results, for instance in neonates in whom Na^+ transport is not yet matured,[63] and false negative results occur due to specific genotypes[54] and/or in cases of mild CF.[64,65] DNA mutation analysis confirms CF disease when two mutations are found, however not all mutations are known yet. In addition, laboratories use different methods for *CFTR* genotyping and in recent studies a rather high error percentage was found in identifying the alterations in the CF alleles.[66] In individuals in whom the sweat test and the *CFTR* mutation analysis give inconclusive results, the addition of further tests is indicated. In these cases, electrophysiological examinations, such as the NPD and ICM, can certainly aid in the diagnosis and give information on the chloride transport in the specific tissue and moreover, recognize or discard the CF diagnosis (Chapter 7). Therefore, we suggest to expand the standard diagnostic process. If CF is suspected, the sweat test and the *CFTR* mutation analysis should be supplemented with the NPD and ICM assessments in the diagnostic process.

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Summary

Cystic fibrosis (CF) is considered to be a monogenic disease, implicating that it is caused by mutations within a single gene. It is an autosomal recessive disease resulting from alterations in both chromosomal copies of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. CF is the most common severe autosomal recessive disease within the Caucasian population, with an approximate mean incidence of 1 in 2500 births in Western European countries. The incidence in the Netherlands has been estimated around 1 in 3600 life births. There are over 800 known *CFTR* mutations of which a deletion of the phenylalanine residue at position F508 (ΔF508) is the most frequent *CFTR* mutation, and occurs in about 70% of CF alleles in the Caucasian population. The *CFTR* gene encodes an adenosine 3',5'-cyclic monophosphate (cAMP)-regulated chloride channel found in the apical membrane of epithelial cells. The clinical symptoms in CF are caused by the impaired epithelial chloride transport in many exocrine glands, but major manifestations involve the respiratory and gastrointestinal tracts. Typical CF is characterized by the accumulation of viscous mucus resulting in chronic obstructions and fibrosis of the pulmonary tissue with cough, tachypnea and wheezing due to recurrent bronchopulmonary infections. The intestinal tract demonstrates with malabsorption and pancreatic insufficiency, which causes steatorrhea and failure to thrive. Sweat glands are most consistently affected in CF and produce sweat with elevated chloride and sodium levels.

The abnormal chloride transport in CF can be determined by electrophysiological methods such as the sweat test, the nasal potential difference measurement (NPD) and the intestinal current measurement (ICM), which test the conductance properties for sodium and chloride ions of sweat gland, respiratory and intestinal epithelium, respectively. **Chapter 2** describes the methods of NPD and ICM, and compares the results within a group of CF individuals to that in controls. With the NPD the following values are determined: baseline PD, Na^+-transport, basal Cl^- conductance, cAMP-mediated Cl^- conductance and the ATP-stimulated Cl^- conductance. In the ICM, a unique sequence for the addition of drugs has been introduced to segregate the different Cl^- conductances that might be present in the intestinal epithelium. Consecutively measured are: Na^+-transport, the Ca^{2+}/PKC pathway of Cl^- secretion, cAMP-dependent Cl^- secretion, and finally the tissue is incubated with 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) to inhibit the alternative Cl^- conductances, after which the Ca^{2+}/PKC pathway is stimulated again. The response to cAMP assesses the presence of CFTR, while the Ca^{2+}/PKC-mediated pathway involves both CFTR and alternative Cl^- conductances. By challenging this pathway before and after the incubation of the tissue with DIDS, sensitivity of the chloride channels to DIDS could be determined. DIDS-insensitive Cl^- secretion indicates the presence of CFTR, while DIDS-sensitive Cl^- secretion indicates the presence of alternative Ca^{2+}-dependent Cl^- channels.
When NPD results of CF patients are compared to controls, the Na^+-transport is larger in CF. Moreover, the chloride free solution and addition of isoprenaline exert no effect in the typical CF condition, while controls exhibit chloride conductance in response to these solutions. The ATP response in most CF patients is larger than in controls, although this response varies considerably between CF patients.

When we compare the mean ICM values between the CF group and controls, we see that none of the mean values for the responses to carbachol, cAMP, and histamine overlap between the two groups.
In conclusion, when utilizing these protocols for NPD and ICM measurements in CF disease, significant distinction can be made between the electrophysiological characteristics of the respiratory and intestinal tissues of non-CF versus CF individuals. Therefore, these methods can aid and facilitate the diagnostic process. Moreover, chloride conductances of different origin can be segregated with these electrophysiological methods.

The *European CF twin and sibling study* has been initiated to dissect the importance of genetic and environmental modifiers of CF disease. In **Chapter 3**, data collection for a cohort of 277 sibling pairs, 12 dizygous twin pairs and 29 monozygous twin pairs is described. Of the 318 CF twin and sib pairs, 114 were reported to be ΔF508 homozygous. From these 114 pairs two clinical parameters most sensitive for course and prognosis of CF, i.e. wfh% (representative for the nutritional status) and FEVPerc (representative for the pulmonary status) were obtained. Intrapair discordance was assessed by the intrapair differences in wfh% and FEVPerc and by DELTA, a composite parameter defined by linear combination of wfh% and FEVPerc in order to describe discordance with respect to the overall disease severity. Monozygous twins had a significantly lower DELTA than dizygous twins ($P = 0.05$) indicating that CF disease severity is modulated by an inherited component besides the *CFTR* gene itself. Extreme phenotypes are considered to be more informative for the analysis of any quantitative trait. Thus, we aimed to quantify disease-severity and intrapair discordance in order to select pairs with the extreme phenotypes DIS (discordant patient pairs), CON+ (concordant and mildly affected patient pairs) and CON- (concordant and severely affected patient pairs). The algorithm reliably discriminated between pairs DIS, CON+ and CON- among the cohort of ΔF508 homozygotes. The selected pairs from these categories demonstrated non-overlapping properties for wfh%, FEVPerc and the intrapair difference of both parameters.

Chapter 4 investigates the residual Cl^- secretion in intestine of ΔF508 homozygous CF twins and siblings, and examines the contribution of CFTR and alternative Cl^- conductances to transepithelial chloride transport, by applying the ICM method. The expression of residual chloride conductances, either by CFTR-mediated or alternative chloride channels, is suggested to modify CF disease phenotype and compensate for the lack of CFTR-mediated chloride secretion.
Twins and siblings with identical *CFTR* genotypes are investigated to determine impact of factors other than *CFTR* on chloride secretion. A majority of ΔF508 homozygous CF patients (73%) shows cAMP-mediated Cl^- secretion, while 20% shows DIDS-sensitive Ca^{2+}-activated Cl^- secretion. In this cohort of investigated CF patients, DIDS-sensitive alternative chloride conductance is only seen in CF individuals also responding to cAMP-agonists. These results suggest the presence of CFTR-mediated Cl^- secretion in a subgroup of patients, implying that a portion of ΔF508 CFTR can be processed *in vivo* and function as chloride channel in the apical membrane of intestinal cells. Moreover, a considerable number of ΔF508

homozygous patients express chloride conductances other than CFTR in their intestinal epithelium.

Chapter 5 analyzes the impact of chloride secretion by residual activity of CFTR and/or by alternative chloride channels on disease manifestation in respiratory and intestinal tracts, in ΔF508 homozygous twins and siblings with CF. The most informative pairs, as selected in Chapter 3, who are either concordant for a mild or severe phenotype, or discordant in phenotype are analyzed. In a majority of patients cAMP- and/or Ca^{2+}-regulated chloride conductance is detected, i.e. 84% and 73% of patients in the airways and intestine, respectively. In respiratory tissue, the expression of basal CFTR-mediated chloride conductance demonstrated by 30% of ΔF508 homozygotes, and the response to a cAMP-agonist seen in 9% of patients, are identified as positive predictors for milder CF disease. In intestinal tissue DIDS-insensitive Cl^- secretion, indicative of functional CFTR channels, segregates with milder phenotype, whereas DIDS-sensitive chloride secretion is mainly observed in more severely affected patients. The more concordant chloride conductance patterns within monozygous twins compared to dizygous pairs, especially in the ICM results, imply that genes other than ΔF508 *CFTR* significantly influence the manifestation of the basic defect.

In **Chapter 6** the expression of residual CFTR and/or alternative Cl^- conductances is investigated in rare CF genotypes by applying the sweat test and the NPD and ICM methods. CF patients were analyzed, homozygous for the missense mutation E92K, the stop mutations R553X, R1162X, the splice mutations 1898+3 A-G, 3849+10kb C-T, the novel deletions CFTRdele2, CFTRdele2,3(21kb) and in carriers for ΔF508-R553Q or ΔF508-V1212I. Residual CFTR activity was necessary to confer lower sweat electrolyte concentrations or exocrine pancreatic sufficiency. All investigated patients exhibited some chloride conductance, either cAMP- and/or Ca^{2+}-mediated, in pulmonary and/or intestinal tract. The expression of alternative chloride channels such as the calcium activatable or the outwardly rectifying chloride channels may partially compensate for defective CFTR and modulate the manifestation of respiratory and gastrointestinal disease in CF. To determine if this is true in patients with these genotypes, larger groups will have to be investigated.

In **Chapter 7** the difficulties in confirming CF diagnosis are described by presenting two patients with atypical CF symptoms. The patient of the first case presents with a pathological sweat test and CF-like pulmonary disease. However, CF diagnosis was excluded on the basis of his normal NPD and ICM results, and the fact that he is carrying the same *CFTR* alleles as his asymptomatic sister.
The other complex patient is from consanguineous descent and presents with normal intestinal Cl^- transport as measured by ICM, and normal sweat test values. In contrast, she shows severe CF-like lung disease with recurrent nasal polyps, bronchitis, bronchiectasis, continuous cough, sputum production, airway infections, hypoxaemia and cachexia. This clinical picture was substantiated by abnormal NPD values. Genetic analysis demonstrated only one disease-causing lesion, 1898+3 A->G, but also homozygosity for an unfavorable combination of intragenic polymorphisms. In addition, her consanguineous descent leads to a genome wide overrepresentation of homozygous genotypes, causing further

unfavorable genetic background. The data of this index case suggest that one disease-causing lesion together with unfavorable genetic background is sufficient to modify CFTR expression and/or function, and cause manifestation of severe CF lung disease.

Chapter 8 discusses the findings in this thesis and its implications for further research. In short, in the investigation of the ΔF508 homozygous CF cohort described in this thesis, specific protocols are applied for the NPD and ICM measurements to discriminate between the presence of residual CFTR and/or alternative Ca^{2+}-dependent Cl^- conductances.
Moreover, in the diagnostic process of CF the methods of NPD and ICM should be employed in parallel to other diagnostic procedures like the sweat test and genetic *CFTR* mutation analysis, to determine the presence of Cl^- conductances in the respiratory and intestinal tissues. In contrast to the sweat test, which is similar in most ΔF508 homozygous patients, specific NPD and ICM results are associated with better disease parameters and predict milder manifestation of CF disease.
The reports in this thesis of the *European CF twin and sibling study* show that the investigation of twin and sib pairs homozygous for the same *CFTR* mutation genotype and with extreme disease phenotypes, creates the opportunity to dissect the influence of genetic and environmental modifiers of CF disease. For a better understanding of CF pathogenesis, it is important to further investigate these potential modifiers of CF disease.

Samenvatting

Cystische fibrose (CF), ofwel taaislijmziekte, is een monogenetische ziekte die veroorzaakt wordt door mutaties in een enkel gen. Het is de meest voorkomende levensbedreigende autosomaal recessieve ziekte in de Kaukasische populatie, die ontstaat wanneer beide chromosomen van het CF gen, de cystic fibrosis transmembrane conductance regulator (*CFTR*), aangedaan zijn. In de Kaukasische populatie is de gemiddelde incidentie 1 op 2500 geboortes, en in Nederland wordt de incidentie geschat op 1 op 3600 geboortes. Er zijn meer dan 800 mutaties bekend van het *CFTR* gen, waarvan de deletie van een fenylalanine residue op positie 508, ΔF508, de meest frequente mutatie is die op 70% van de CF allelen voorkomt. Het *CFTR* gen codeert voor een adenosine 3',5'-cyclic monophosphate (cAMP)-gereguleerd chloride (Cl^-) kanaal, dat voorkomt in de apicale membraan van epitheel cellen. De klinische symptomen in CF worden veroorzaakt door het gestoorde chloride transport in vele exocriene organen, maar de duidelijkste afwijkingen treden op in de tractus respiratorius en tractus gastrointestinalis. De ziekte wordt gekenmerkt door de produktie van taai slijm in de longen waardoor obstructie en schade van het weefsel optreedt. Klinische kenmerken zijn hoesten, het opgeven van taai slijm, een snelle ademhaling en recidiverende luchtweginfecties. In het maagdarmkanaal komt de ziekte tot uiting door aantasting van de alvleesklier met malabsorptie en diarree. De zweetklieren produceren zweet met een hoog chloride en natrium (Na^+) gehalte.

Het (abnormale) chloride en natrium transport kan in de zweetklier, het luchtwegepitheel en in de darmen gemeten worden door respectievelijk de zweettest, de nasaal potentiaal meting (NPD, nasal potential difference), en de intestinal current measurement (ICM), met behulp van het toedienen van bepaalde stoffen die het iontransport stimuleren of juist remmen. **Hoofdstuk 2** beschrijft de NPD en ICM en vergelijkt resultaten van een groep CF patiënten met een controle groep. Bij de NPD meting worden de volgende waarden bepaald: de basale PD, Na^+ transport, basale Cl^- secretie, cAMP-gemedieerde Cl^- secretie, en ATP-gestimuleerde Cl^- secretie. Bij de ICM methode worden gemeten: Na^+ transport, de Ca^{2+}/PKC-afhankelijke Cl^- secretie, cAMP-gestimuleerde Cl^- secretion, waarna het weefsel wordt geïncubeerd met 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) om de alternatieve Ca^{2+}-afhankelijke Cl^- conductances te remmen, waarna nogmaals de Ca^{2+}/PKC pathway wordt gestimuleerd. Een chloride secretie respons op cAMP duidt op de aanwezigheid van CFTR, terwijl de Ca^{2+}/PKC pathway CFTR maar ook alternatieve Cl^- kanalen stimuleert. Door deze pathway voor en na de incubatie met DIDS te meten, kan aan de hand van de DIDS-gevoeligheid, het type van de Cl^- kanalen bepaald worden. DIDS-ongevoelige chloride transport wijst, net als de cAMP respons, op de aanwezigheid van CFTR. DIDS-gevoelige Cl^- secretie wordt veroorzaakt door alternatieve Ca^{2+}-afhankelijke kanalen.
Bij vergelijking van de NPD resultaten in de CF groep met die in controles, zien we dat het Na^+ transport verhoogd is in CF. Bij het typische CF beeld zijn geen basaal Cl^- transport of een respons op cAMP aanwezig, in tegenstelling tot in controle personen. De ATP respons varieert aannemelijk binnen de CF groep, maar is meestal groter dan in controle personen. De gemiddelden voor de Cl^- secretie responsen die gemeten zijn met de ICM, zijn voor de CF groep allemaal afwijkend en

vertonen geen overlap met de waarden die gevonden zijn in de controle groep. Concluderend kunnen we zeggen dat de NPD en ICM significant onderscheid kunnen maken tussen de electrophysiologische kenmerken van het luchtwegepitheel en het darmweefsel van CF en niet-CF personen. Daarbij kan met deze methoden de aanwezigheid van verschillende Cl^- conductanties worden aangetoond.

De *European CF twin and sibling study* is opgezet om te onderzoeken welke genetische en/of omgevingsfactoren de manifestatie en het beloop van CF kunnen beïnvloeden. In **Hoofdstuk 3** wordt het cohort van de CF paren beschreven: 277 sibling paren (broer-broer, zus-zus, of broer-zus), 12 twee-eiige tweelingen, en 29 eeneiige tweelingen. Van deze 318 paren zijn 114 paren homozygoot voor de ΔF508 mutatie. Van deze 114 paren zijn de parameters voor de voedingstoestand (weight for height %predicted = wfh%) en voor de longfunctie (FEVPerc = age corrected centiles for FEV_1 %predicted) verzameld. Het verschil in ziektemanifestatie in een paar is bepaald door de verschillen tussen de wfh% en FEVPerc waarden binnen een paar te berekenen. Daarnaast is ook het vershil in DELTA binnen een paar gemeten. DELTA is een berekende waarde uit wfh% en FEVPerc, waardoor met één waarde (DELTA) het verschil in ziektemanifestatie in de longen en darmen binnen een paar kan worden aangegeven. Eeneiige tweelingen toonden een significant lagere DELTA dan twee-eiige tweelingen ($P = 0.05$).Omdat alle onderzochte paren ΔF508 homozygoot zijn, geeft dit aan dat de ernst van de ziekte niet alleen door het *CFTR* gen zelf bepaald wordt, maar ook nog door een andere genetische factor. Wanneer men zoekt naar zulke beïnvloedende factoren, blijken paren die het meest verschillen in ziektemanifestatie, het meest informatief te zijn. Met een in dit hoofdstuk beschreven algoritme zijn de volgende paren geselecteerd om verder onderzoek naar modifying factors te verrichten: discordante paren (DIS), concordante paren die mild aangedaan zijn (CON+), en concordante paren die ernstig ziek zijn (CON-). Deze geselecteerde paren hebben waarden voor wfh% en FEVPerc die niet overlappen. Ook de intrapaar verschillen voor wfh% en FEVPerc van deze groepen overlappen niet.

Hoofdstuk 4 onderzoekt met behulp van de ICM, de Cl^- secretie in het darmweefsel van ΔF508 homozygote sibling en tweeling paren. De expressie van residuele CFTR activiteit of de aanwezigheid van alternatieve chloride kanalen in CF patiënten zou mogelijk kunnen zorgen voor een minder ernstig ziektebeeld.
Door ΔF508 homozygote paren te onderzoeken, kan onderzocht worden of andere factoren dan de *CFTR* mutatie, de aanwezigheid van chloride secretie in CF bepalen. Het grootste deel van de ΔF508 homozygoten (73%) presenteert zich met cAMP-gestimuleerde Cl^- secretie, wat suggereert dat het gemuteerde eiwit ΔF508 CFTR *in vivo* in het apicale membraan terecht komt en daar Cl^- kan transporteren onder stimulatie van cAMP. Twintig procent van de ΔF508 homozygoten vertoont DIDS-gevoelige Cl^- secretie, wijzend op alternatieve Ca^{2+}-afhankelijke Cl^- kanalen.

In **Hoofdstuk 5** wordt de groep ΔF508 homozygote sibling paren onderzocht met de meest verschillende ziektemanifestaties zoals geselecteerd in Hoofdstuk 3: DIS, CON+ en CON- paren. Gekeken wordt of de expressie van CFTR Cl^- kanalen of alternatieve Cl^- kanalen in het luchtwegepitheel of het darmweefsel van deze

patiënten, gecorreleerd is aan de ernst van hun ziekteverschijnselen. In de luchtwegen van 84% en in het darmweefsel van 73% van de ΔF508 homozygoten werd cAMP- en/of Ca^{2+}-gestimuleerde Cl^- secretie gevonden. Bij de NPD metingen van de ΔF508 homozygoten toonden 30% basale CFTR-gemedieerde Cl^- secretie en 9% reageerde op cAMP. Beide deze responsen zijn geassocieerd met een mildere manifestatie van CF. Van de responsen in het darmweefsel is het hebben van DIDS-ongevoelige Cl^- secretie, i.e. CFTR, geassocieerd met een betere klinische conditie, terwijl de DIDS-gevoelige Cl^- secretie voornamelijk voorkomt in patiënten die ernstiger ziek zijn. De chloride secretie patronen, voornamelijk die van de ICM metingen, zijn concordanter in eeneiige tweelingen dan twee-eiige paren. Dit betekent dat andere genen buiten het *CFTR* gen een belangrijke rol spelen in de uiting van de ziekte.

In **Hoofdstuk 6** wordt de expressie van Cl^- secretie in zweetklier, luchtwegen en darmweefsel van zeldzame genotypes geanalyseerd. De patiënten hadden de volgende genotypes: homozygoten voor de missense mutatie E92K, de stop mutatie R553X, de stop mutatie R1162X, de splice mutatie 1898+3 A-G, de splice mutatie 3849+10kb C-T, de deletie CFTRdele2, de deletie CFTRdele2,3(21kb), en in carriers van ΔF508-R553Q of ΔF508-V1212I. Een minder afwijkende zweettest of een normal functionerende alvleesklier komt alleen voor in patiënten met residuele CFTR activiteit. Al de onderzochte patiënten presenteerden zich met cAMP- en/of Ca^{2+}-gemedieerde Cl^- secretie in de luchtwegen en/of het maagdarmkanaal. Mogelijk spelen deze chloride conductanties een rol in het ziekteproces van CF. Echter, om dat bij patiënten met deze genotypes uit te verifiëren, zullen in de toekomst grotere groepen onderzocht moeten worden.

In **Hoofdstuk 7** komen de diagnostische problemen aan de orde die optreden bij patiënten die zich met atypische symptomen presenteren. De patiënt uit de eerste casus heeft een pathologische zweettest en CF-achtige longklachten. Echter, de diagnose CF is uitgesloten op basis van normale NPD en ICM resultaten, en het feit dat hij dezelfde *CFTR* allelen draagt als zijn asymptomatische zus.
De tweede atypische patiënt, is de dochter van een eerste graads neef en nicht. Zij vertoont normaal Cl^- transport in haar darmweefsel, en heeft een normale zweettest. Daarentegen presenteert zij zich met ernstige CF-gelijkende longaandoeningen: nasaal poliepen, bronchitis, bronchiectasis, hoesten, sputum productie, luchtweginfecties, hypoxemie en cachexie. Daarbij vallen haar NPD waarden in het CF-gebied. Genetische analyse toonde één mutatie, 1898+3 A->G, maar ook homozygotie voor *CFTR* polymorfismen die de expressie van wild-type CFTR kunnen verminderen. Bovendien heeft zij door haar consanguine ouders een overrepresentatie van homozygote genotypes. De presentatie van deze patiënt impliceert dat het hebben van één *CFTR* mutatie samen met een zwakke genetische achtergrond, voldoende zijn om de expressie van *CFTR* nadelig te beïnvloeden, waardoor CF zich kan manifesteren.

Hoofdstuk 8 bespreekt de bevindingen die in deze dissertatie worden beschreven met de mogelijke vervolgonderzoeken. In het kort: bij de NPD en ICM metingen van het cohort ΔF508 homozygoten worden specifieke protocollen gebruikt voor de toediening van de pharmaca, zodat onderscheid gemaakt kan worden tussen

residuele CFTR secretie, of de aanwezigheid van alternatieve Ca^{2+}-gestimuleerde Cl^- kanalen.
In het diagnostische process van CF moeten de NPD en ICM metingen naast de zweettest en de genetische mutatie analyse worden uitgevoerd, om de aanwezigheid van Cl^- conductanties in het luchtwegepitheel en het darmweefsel te bepalen. In tegenstelling tot de zweettest, die in waarde niet veel verschilt binnen een groep ΔF508 homozygoten, zijn specifieke uitkomsten van de NPD and ICM metingen gerelateerd aan betere klinische parameters en een mildere manifestatie van CF. De resultaten in deze dissertatie over de *European CF twin and sibling study* tonen aan dat het onderzoeken van CF sibling paren en tweelingen, homozygoot voor hetzelfde *CFTR* genotype, de mogelijkheid biedt om de invloed van genetische en omgevingsfactoren op het ziektebeloop te bepalen. Voor een beter begrip van de pathogenese van CF, is het belangrijk verder onderzoek te verrichten naar de potentiële modifiers van de manifestatie van CF.

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Dank je, danke sehr, thank you,

Inez

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AIO-cursussen

Cursus proefdierdeskundige, art. 9 functionaris
Veilige laboratorium technieken
The Oxford Examination in English as a Foreign Language
Receptor and Signal Transduction
Spijsvertering / parenterale voeding

Publications

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