Chloride conductance and genetic background modulate the cystic fibrosis phenotype of ΔF508 homozygous twins and siblings

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To investigate the impact of chloride (Cl^-) permeability, mediated by residual activity of the cystic fibrosis transmembrane conductance regulator (CFTR) or by other Cl^- channels, on the manifestations of cystic fibrosis (CF), we determined Cl^- transport properties of the respiratory and intestinal tracts in ΔF508 homozygous twins and siblings. In the majority of patients, cAMP and/or Ca^{2+}-regulated Cl^- conductance was detected in the airways and intestine. Our finding of cAMP-mediated Cl^- conductance suggests that, in vivo, at least some ΔF508 CFTR can reach the plasma membrane and affect Cl^- permeability. In respiratory tissue, the expression of basal CFTR-mediated Cl^- conductance, demonstrated by 30% of ΔF508 homozygotes, was identified as a positive predictor of milder CF disease. In intestinal tissue, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid–insensitive (DIDS-insensitive) Cl^- secretion, which is indicative of functional CFTR channels, correlated with a milder phenotype, whereas DIDS-sensitive Cl^- secretion was observed mainly in more severely affected patients. The more concordant Cl^- secretory patterns within monozygous twins compared with dizygous pairs imply that genes other than *CFTR* significantly influence the manifestation of the basic defect.

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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 CF transmembrane conductance regulator (*CFTR*) gene, which encodes a cAMP-regulated chloride (Cl^-) 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 for about 70% of all CF alleles worldwide (4, 5).

The heterogeneity of CF disease is partly explained by the allelic heterogeneity of the *CFTR* locus. *CFTR* gene mutations have been categorized by their resulting phenotype into four classes (6). ΔF508 CFTR has been assigned to class II, implying that the protein fails to reach the cell membrane to function as a Cl^- channel. This classification was based on earlier experiments done in heterologous model systems and immunocytochemistry of patients' sweat glands, in which deviant CFTR expression was found in ΔF508 cells (7, 8). Since clinical presentation varies significantly among patients with the same *CFTR* genotype, and among various affected organs within CF patients, it is evident that factors in addition to the *CFTR* genotype are involved in determining CF disease severity. In *cftr*$^{-/-}$ knockout mice, an alternative Ca^{2+}-regulated Cl^- conductance was detected in the airways and pancreas; it was suggested that this conductance ameliorates CF lung disease and protects the tissue from the absence of CFTR-mediated Cl^- conductance (9). In addition, Ca^{2+}-activated Cl^- 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 (11–13) might be explained by these alternative Ca^{2+}-regulated Cl^- channels (14–16).

Furthermore, recent immunocytochemical studies on intestinal, respiratory, and hepatobiliary epithelia of ΔF508 homozygous CF patients have 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). To clarify whether ΔF508 CFTR is competent to transport Cl^- across epithelial membranes in vivo, and whether the presence of Cl^- transport can influence CF disease, the relative contribution of ΔF508 CFTR and alternative Cl^- channels needs to be assessed in patients' tissues in correlation to the CF phenotype.

In this study, we investigated the basic Cl^- secretory defect in the most severely affected tissues in CF, the respiratory and intestinal tracts. Subjects were CF twins and siblings homozygous for the ΔF508 *CFTR* gene mutation. We determined the bioelectrical properties of the tissues using the nasal potential difference (NPD) measurement and the intestinal current (IC) measurement, respectively. These methods assess the cAMP and Ca^{2+}-induced Cl^- secretory pathways by adding specific secretagogues and inhibitors, with which the presence of CFTR and/or alternative Cl^- transporters can be determined. The influence of Cl^- permeability on clinical CF phenotype in specific organs was evaluated by determining the presence and origin of different Cl^- conductances in subgroups of ΔF508 homozygous CF twin and sibling pairs with disparate manifestation of CF disease. These individuals with the most extreme clinical parameters are highly informative in the investigation of factors influencing the CF phenotype (19–21).

The investigation of ΔF508 homozygous twins and siblings is the classical approach for analyzing a monogenic disease like CF, and for differentiating the relative importance of the major genetic lesion and other genetic and environmental factors (22, 23). The disease-causing *CFTR* mutation and the intragenic haplotype are standardized, and the variation in genetic background is either reduced (in dizygous pairs) or eliminated (in monozygous twins). Dizygous twins and siblings share on average half of their genes. The intrapair variation among monozygous twins, who are genetically identical in their entire genome, can be used to delineate the relative contribution of genetic and environmental factors to a trait by comparing the intrapair variability among monozygous pairs to that of dizygous pairs. CF twins and siblings share many environmental factors that are major determinants of CF disease severity, e.g., physician, therapeutic regime, living conditions, and behavioral patterns. Thus, the influence of genetic background and environmental factors on the genotype-phenotype correlation can be examined in greater depth in these subjects 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 recruited by European physicians to enroll in the European Cystic Fibrosis Twin and Sibling Study. Out of these 228 ΔF508 homozygous patients, a cohort of 98 patients (54 females, 44 males) was actually able to take part in the clinical assessment and the IC and/or NPD measurements. These 98 patients belonged to 43 pairs and 4 trios. For data comparisons, each trio was treated as 3 separate sibling pairs, result-

Table 2

	Dizygous pairs (72 individuals: 38 female, 34 male)	Monozygous pairs (26 individuals: 16 female, 10 male)
	13. (11.0 20.3 5.4 3 .3)	12.1 (10.0 20. . 31.)
%	.3 (1.2 10 .4 4. 133.2)	1.5 (. .4 . 122.1)
	3 . (1 .0 .5 0.0 100.0)	50. (20.5 .4 4.1 2.0)
	2. (1. 3. 0.0 15.)	
%	10.1 (5. 15.0 0.4 2 .4)	3.2 (1.5 .1 0.0 1 .3)
	30.3 (11. 54. 0.5 100.0)	15.1 (11.0 2 . 2. 52.)

50

ticipating patients were pancreatic insufficient, as 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.

Assessment of clinical phenotype. In order to select the most extreme phenotypes of the patient pairs, overall disease severity and intrapair discordance were quantified as reported (27). Briefly, a patient's nutritional status was characterized by the anthropometric parameter weight, expressed as predicted weight for height percentage (wfh%), using the data published by Prader et al. (28). Pulmonary status was assessed by forced expiratory volume in 1 second (FEV_1), expressed as a predicted value (FEV_1%pred) based on the formula by Knudson et al. (29). FEV_1%pred declines with age as expected for progressive lung disease in CF (30). Therefore, we used age-specific percentiles for FEV_1%pred (FEV_1Perc), calculated from the CF population database published in the report of the European Epidemiological Registry of Cystic Fibrosis (31),to correct for the age-related decline in FEV_1%pred in our cohort. Thus, wfh% and FEV_1Perc, two of the most sensitive age-independent parameters for course and prognosis, were evaluated to characterize CF disease severity in the two major afflicted organs in CF, the gastrointestinal and respiratory tracts.

The CF patient pairs with the most disparate phenotypes, i.e., the most informative pairs, were selected as follows (27): First, rank numbers for wfh% (x) and FEV_1Perc (y) were assigned to the complete cohort, whereby rank number 1 identified the most severely affected patient (Figure 1). The disease severity of patient i was determined by the distance from origin (DfO) in the plot resulting from the rank numbers x_i and y_i (Figure 1). Intrapair discordance was defined by the distance (DELTA) between the two data points, representing patients i and j of a pair (Figure 1). Thus, DfO and DELTA were defined by: $DfO_i = (x_i^2 + y_i^2)^{1/2}$, and $DELTA_{i,j} = [(x_i - x_j)^2 + (y_i - y_j)^2]^{1/2}$. With these two parameters, a computer-assisted algorithm was used (27) to segregate patient pairs composed of 2 siblings with both equally high wfh% and equally high FEV_1Perc (concordant/mildly affected; CON_{mild}), pairs consisting of 2 siblings with both equally low wfh% and equally low FEV_1Perc (concordant/severely affected; CON_{severe}), and patient pairs comprised of 1 sibling with high wfh% and high FEV_1Perc and 1 sibling with low wfh% and low FEV_1Perc (discordant pairs; DISC). CON_{mild} pairs are characterized by a low DELTA, but the sum of both DfOs (ΣDfO) is large because the rank numbers for wfh% and FEV_1Perc for both siblings are high (Figure 1). CON_{severe} pairs also display a low DELTA, but ΣDfO is small because DfOs for both siblings are close to the origin. DISC pairs are discriminated by the highest values for DELTA. Other pairs were excluded from these extreme phenotypes by the ranking algorithm.

NPD measurements. The method of studying NPD has been adapted from a method described previously (32), and measures Cl^- and Na^+ conductances as potential differences (PDs). In short, the reference bridge was formed by a subcutaneous needle in the forearm, which is isoelectric with the submucosal space of the nasal epithelium. The exploring bridge was positioned under the inferior 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. The basal PD was measured during superfusion of the nasal epithelium with a salt solution (1.7 ml/min) and was found to be lumen-negative with respect to the sub-

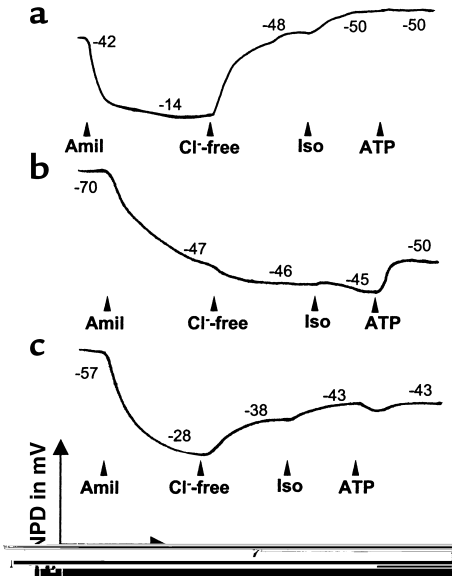


Figure 2

Table 3

	Dizygotes *(n)*	Monozygotes *(n)*
	2	2
	4	2
	11	
	5	1
	n (pairs)	*n* (pairs)
	20	
3 ( )	5	
	24	
1		1
4		2
1		1
4		

mucosal reference electrode. The salt solution consisted of (in mmol/l): 120 NaCl, 25 sodium gluconate, 5 potassium gluconate, 0.4 NaH_2PO_4, and 2.4 Na_2HPO_4. On the inferior turbinate, the spot with the maximal (most negative) stable baseline PD was selected. This baseline PD was shown to be considerably more negative in CF patients than in non-CF individuals (Figure 2). At this site, NPDs were measured in response to superfusion with solutions of different ion compositions, or containing different drugs (Figure 2). First, the catheter was perfused with amiloride (10^{-4} mol/l), a blocker of epithelial Na^+ channels, thereby inhibiting the PD by eliminating the contribution of electrogenic sodium absorption (33). To determine the basal Cl^- conductance, Cl^- was replaced with gluconate in the solution containing amiloride. Subsequently, the β-adrenergic agonist isoprenaline (10^{-4} mol/l) was added to the Cl^--free solution containing amiloride, which induces cAMP-dependent Cl^- conductance and determines the presence of CFTR (32). For the last perfusate, ATP (10^{-3} mol/l) was added to the Cl^--free solution containing amiloride and isoprenaline. ATP binds to purinergic receptors on the luminal surface, triggering the Ca^{2+}-mediated Cl^- secretory pathway by activating phospholipase C and increasing intracellular Ca^{2+} (34). 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, NPD measurements were performed in the left and right nostrils. The NPD tracing of the nostril with the highest Cl^- secretory responses, i.e., with the largest capacity to transport Cl^-, 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 (Table 3).

IC measurements in rectal biopsies. The method used to study IC has been described previously (11, 12, 35). 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 (35). The tissue was perfused with buffer solution at 37°C (composition in mmol/l: 126.2 Na^+, 114.3 Cl^-, 20.2 HCO_3^-, 0.3 HPO_4^{2-}, 0.4 $H_2PO_4^-$, and 10 HEPES; 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 application of Ohm's law. Basal I_{sc} prior to voltage clamping was calculated from the basal transepithelial resistance and the open-circuit transepithelial PD. Subsequently, the tissue was short-circuited using voltage clamps for the course of the experiment. For cell metabolism, glucose (10^{-2} mol/l) was given both mucosally and serosally. After equilibration, the following pharmaceuticals were added in a standardized order to the mucosal (M) and/or serosal (S) side: (a) amiloride (10^{-4} mol/l, M) (33); (b) indomethacin (10^{-5} mol/l, M + S), to reduce basal Cl^- secretion by inhibiting the endogenous prostaglandin formation (36); (c) carbachol (10^{-4} mol/l, S), to initiate the cholinergic Ca^{2+}-linked Cl^- secretion (37); (d) forskolin (10^{-5} mol/l, M + S) (38) together with 8-bromo-cAMP (10^{-3} mol/l, M+S), to open cAMP-dependent Cl^- channels such as CFTR and the outwardly rectifying Cl^- channel (ORCC) (39); (e) 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS;

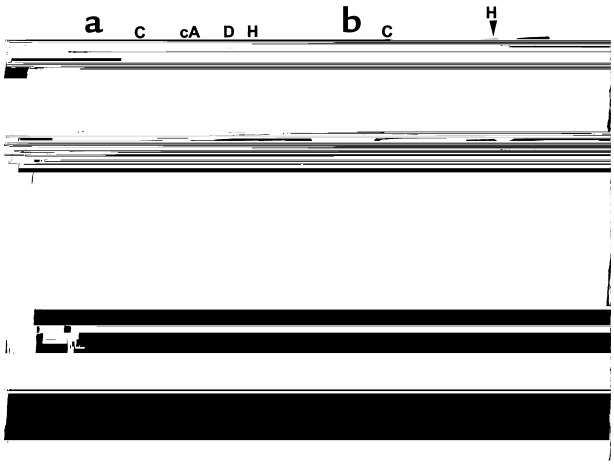


Figure 3

2.10^{-4} mol/l, M), to inhibit DIDS-sensitive Cl^- transporters like the Ca^{2+}-dependent Cl^- channels (14, 15) and the ORCC (39); and (f) histamine (5.10^{-4} mol/l, S), to reactivate the Ca

To assess the correlation between the basic defect and the phenotype in the respiratory tissue, NPD results measured within the three subgroups of extreme phenotypes (DISC, CON_{mild}, and CON_{severe}) were compared with the lung function parameter FEV_1Perc.

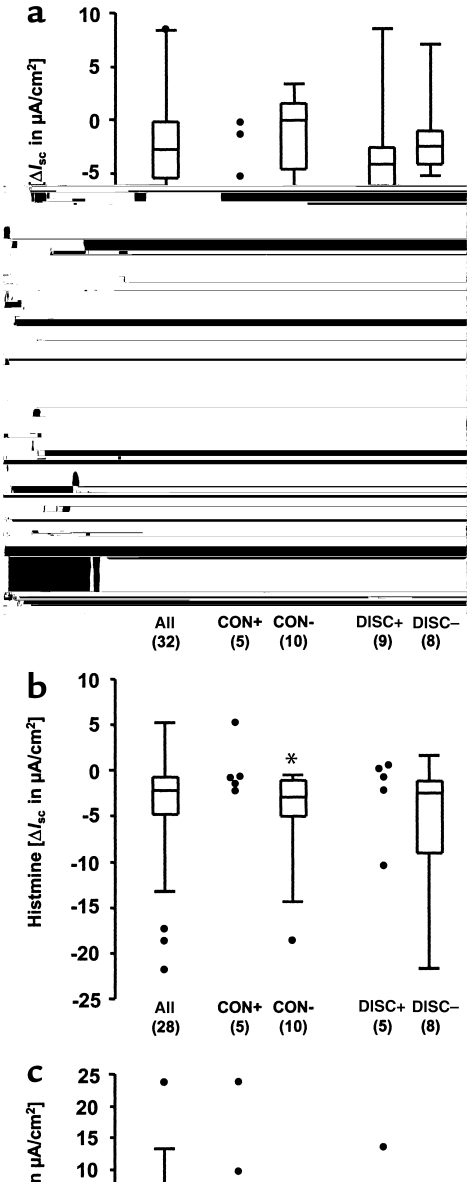
The lung functions of 8 DISC sibling pairs were plotted against their responses to superfusion with Cl^--free solution (Figure 4a). The siblings with the better lung function parameters demonstrated significantly higher (more negative) gluconate responses than their paired siblings did ($P < 0.05$, Student t test for grouped pairs), and also possessed the better wfh% of the 2 siblings (data not shown). When the lung function of patients belonging to CON_{mild} and CON_{severe} pairs was plotted against their gluconate responses, all persons (except 1) who had a gluconate response (i.e., increased negativity) belonged to CON_{mild} pairs (Figure 4b). The presence of this Cl^- secretory response upon addition of gluconate was significantly associated with the CON_{mild} phenotype ($P = 0.013$, Fisher's exact test). Only 1 patient belonging to a CON_{severe} pair showed a response to gluconate. Similarly, no responses to isoprenaline were observed in severely affected patients, i.e., patients in CON_{severe} pairs or the severely affected siblings of DISC pairs.

The amiloride-induced depolarization observed in all patients (Figure 5), and the ATP-induced hyperpolarization (increased negativity) of PD seen in 55 of the 74 recorded NPD tracings, did not show any significant correlations with lung function.

Substantiation of the observed associations between NPD values and phenotype was provided by the following results: For each pair, the NPD values of the individual siblings 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). Summations calculated for the CON_{mild} pairs were compared with those of the CON_{severe} pairs (Table 5). The intrapair summations for the basal PDs of the CON_{mild} pairs were less negative than those for the CON_{severe} pairs (–101.7 ± 18.2 versus –114.8 ± 12.9); however, this was not significant (Table 5; $P < 0.10$, Mann-Whitney U test). The $\Delta PDsib_A + \Delta PDsib_B$ values for the gluconate responses were significantly more negative for the CON_{mild} pairs ($P < 0.05$), while $\Delta PDsib_A + \Delta PDsib_B$ values for the amiloride and ATP responses did not differ between CON_{mild} and CON_{severe} pairs. The $\Delta PDsib_A + \Delta PDsib_B$ values for the isoprenaline responses within CON_{mild} and CON_{severe} pairs were not significantly different; most investigated individuals had no response, and in patients who did respond to isoprenaline, the magnitude of the response was small. However, $\Delta PDsib_A + \Delta PDsib_B$ for isoprenaline was negative in the CON_{mild} couples (–1.3 ± 2.4) and positive in the CON_{severe} couples (0.5 ± 0.8). As severe

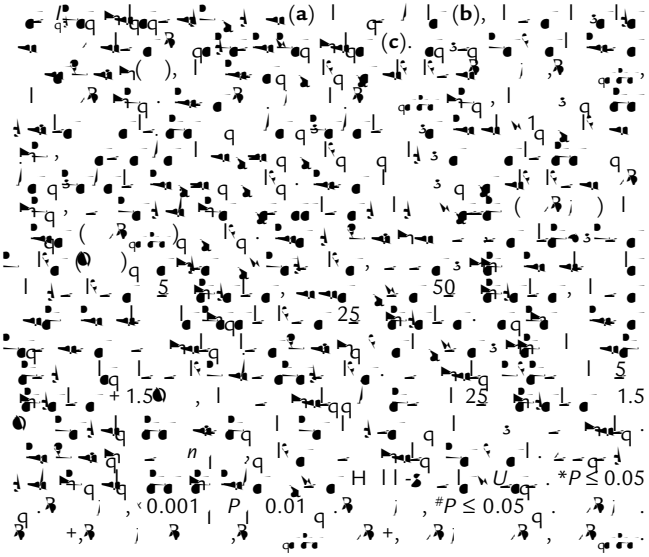
pretable responses to all three secretagogues (carbachol, 8-bromo-cAMP, and histamine) and who belonged to a pair that could be assigned to one of the extreme phenotypes (DISC, CON_{mild}, and CON_{severe}) were included in the analyses of this study. To avoid the loss of IC data points, we included results from pairs in which both siblings had complete IC measurement results, as well as IC measurements from pairs in whom only 1 sibling produced a complete IC recording. Table 7 indicates the number of patients matching the criteria of the different phenotypic groups with complete IC tracings.

To assess the impact of intestinal Cl



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 to be a processing defect involving reduced glycosylation and consequent misfolding of the CFTR protein, which prevents the protein from reaching the plasma membrane and causes it to be retained 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) have demonstrated ΔF508 CFTR localization in the plasma membrane. Moreover, functional assays of transfected cells (43), in mice (44, 45), and of human tissue specimens (18) have shown that ΔF508 CFTR is capable of transporting Cl^- 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 Cl^- secretory pathway, which is the hallmark of CFTR-mediated Cl^- transport (2, 3). Nonetheless, the magnitude of this detected Cl^- permeability is evidently insufficient to prevent manifestation of CF disease.

Figure 6



Our data confirm a correlation between better lung function and less anomalous bioelectrical properties of the nasal epithelium in the investigated ΔF508 homozygotes. The respiratory epithelium of healthier CF patients appears to be more permeable to Cl^- than that of sicker patients (Figure 4, a and b). Moreover, the presence of basal Cl^- conductance and the capacity to secrete Cl^- in response to a cAMP agonist, determined by perfusion of the nasal epithelium with a Cl^--free solution and isoprenaline, respectively, were present only in mildly affected patients (Table 4 and Figure 4). This suggests that the expression of basal Cl^- conductance and/or residual cAMP-mediated Cl^- conductance 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 from ΔF508 homozygous twins and siblings substantiate the findings in an earlier study of CF individuals with different *CFTR* mutation genotypes, in whom the expression of Cl^- conductance provided a better indication of lung function than did genotype (13). In contrast, Cl^- conductance in the amiloride-pretreated nasal epithelium that is mediated by apical purinergic receptors and a subsequent increase of intracellular Ca^{2+} (34) was not associated with better preservation of tissue function (Table 5; $P = 0.53$). Similarly, as inferred from the amiloride response, the increased Na^+ absorption in nasal epithelium that is characteristic for CF (46) and has been suggested to contribute to pulmonary disease (47, 48) was not directly related to CF disease severity (Figure 5).

In our group of investigated ΔF508 homozygous individuals, the correlation between the isoprenaline

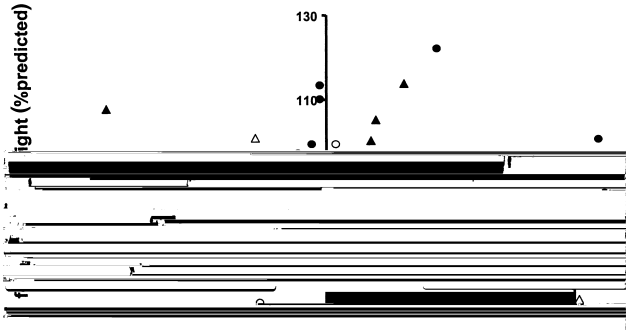
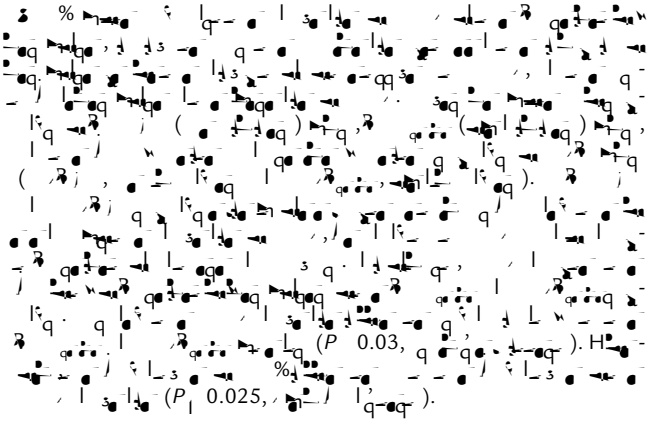


Figure 7



response and lung function was not different in females and males (data not shown); such a difference has been reported for CF adults (49). This might be due to the large number of nonresponders to isoprenaline in our investigations, as expected in a CF cohort of ΔF508 homozygotes representing all ages (32).

Although Cl^- conductance presented only in the subgroup of individuals who had better respiratory function, few patients without Cl^- conductance possessed relatively good lung function (above the 50th percentile; Figure 4b). This indicates that the capacity to transport Cl^- is not the only determinant of respiratory function. Evidently, additional factors unrelated to the basic ion transport defect are involved in CF airway performance. In short, the expression of basal Cl^- conductance and the response to isoprenaline in the respiratory tissue were predictive for disease outcome in our cohort of ΔF508 homozygous sibling and twin pairs.

In the intestinal tissue, CFTR-related DIDS-insensitive Cl^- secretion was predominantly seen in the mildly affected patients (Figures 6 and 7). Whereas the Ca^{2+}-dependent Cl^- secretion in respiratory tissue showed no association with outcome, the DIDS-sensitive Ca^{2+}-dependent Cl^- transport pathway was more frequently observed in severely affected ΔF508 homozygotes. These data are supported by a study of the biliary tract in which an inverse relationship was observed between Ca^{2+}-dependent Cl^- efflux and cAMP-dependent Cl^- transport in homozygous ΔF508 patients (18). The outcome of both studies suggests that alternative Cl^- conductance is upregulated in the absence of CFTR activity, but fails to compensate for the lack of cAMP-activated Cl^- transport. In contrast, in *cftr*$^{-/-}$ knockout mice, the upregulation of Ca^{2+}-activated Cl^- channel function was associated with healthier tissue function (9, 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

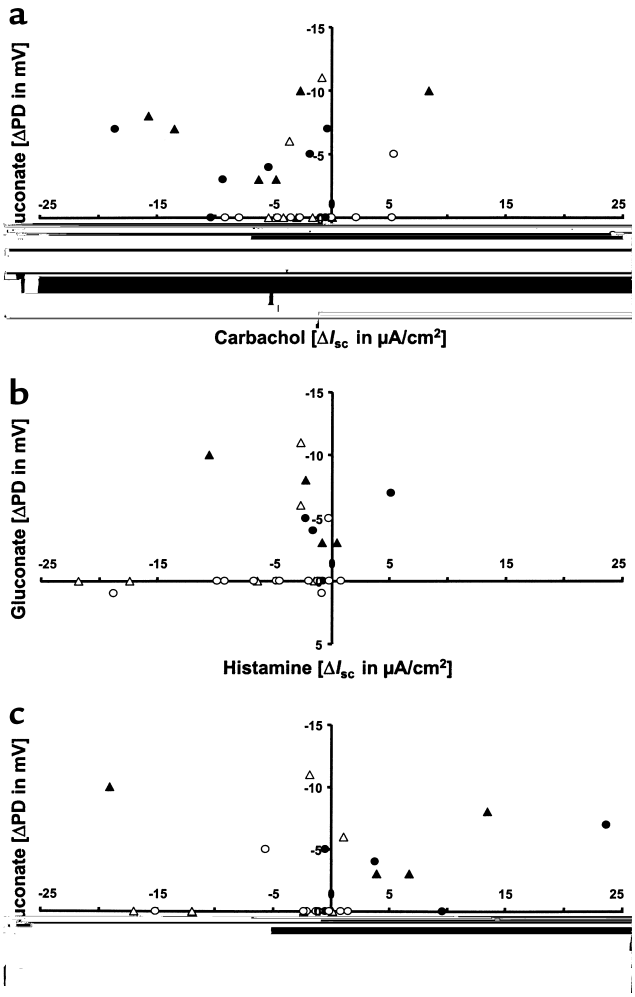


Figure 8

a ΔF508 homozygous CF patient (Figure 8) suggests that within 1 person, similar corrective mechanisms lead to maturation, processing, and residual function of ΔF508 CFTR in the major affected organs in CF disease. Furthermore, monozygous twins proved to be significantly more concordant in the electrophysiological properties of their epithelium than dizygous pairs were, especially in the intestinal tract. In addition, their clinical phenotype was even more concordant, since only 1 of the investigated monozygous twin pairs belonged to the DISC group (Table 3 and Table 7). These findings imply that genetic predisposition is important for the expression of Cl^- permeability in the respiratory and intestinal tissues (12), and for the clinical phenotype. These influencing genetic factors are located outside the *CFTR* gene, since all of the investigated sibling pairs were homozygous for the same *CFTR* lesion.

We cannot formally exclude the possibility that postzygotic events, such as genetic imprinting and/or environmental factors, play an additional role in the differences within sibling pairs. However, siblings share main environmental features such as their physician, therapeutic regimes, living conditions, and microbial contacts.

We conclude that the ability to secrete Cl^- in ΔF508 homozygous patients in the organs that are primarily involved in the course of CF disease is predictive of the CF phenotype. Basal Cl^- conductance and/or a cAMP-mediated response in the airways, together with DIDS-insensitive residual Cl^- secretion in the intestine, were associated with a positive outcome in ΔF508 homozygous CF individuals. Thus, although homozygosity for the major disease-causing genetic 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, but also to apply NPD and IC measurements to gain more insight into the patient's electrophysiological characteristics in correlation with disease severity and — possibly in the future — for stratification of clinical trials and treatment of CF disease.

Acknowledgments

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