TRANSEPITHELIAL NASAL POTENTIAL DIFFERENCE (NPD) MEASUREMENTS IN CYSTIC FIBROSIS (CF)

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Abstract
The main underlying physiologic abnormality in cystic fibrosis (CF) is dysfunction of the CF transmembrane conductance regulator (CFTR), which results in abnormal transport of sodium and chloride across epithelial surfaces. CFTR function could be tested in vivo using measurements of nasal transepithelial potential difference (PD). Nasal measurements show characteristic features of CF epithelia, including hyperpolarized baseline readings (basal PD), excessive depolarization in response to sodium channel inhibitors, such as amiloride (∆Amiloride), and little or no chloride (Cl\(^--\)) secretion in response to isoproterenol in a chloride-free solution (∆Cl\(^--\)-free-isoproterenol). PD test is applied for CF diagnosis and monitoring of new therapeutic modulations and corrections.

Key words: cystic fibrosis, nasal transepithelial potential difference (PD)

1. BACKGROUND
Cystic fibrosis (CF) is the most common severe autosomal recessive disorder among Caucasians.

CF results from mutations in the gene that encodes a protein—the cystic fibrosis transmembrane regulator (CFTR). CFTR is a chloride (Cl\(^--\)) channel located at the apical membrane of exocrine epithelial cells (1). As an ion transporter, it promotes chloride efflux and secondarily inhibits sodium influx via the epithelial sodium channel (ENaC). Its dysfunction in CF leads to increased sodium absorption and impaired chloride secretion. The resulting mucus dehydration compromises mucociliary clearance. CF airways become obstructed, more vulnerable to infection and inflammation ultimately leading to their occlusion and destruction. Obstruction of pancreatic ducts, the biliary tract, and the vas deferens can occur. CF patients typically develop persistent pulmonary infections and often have pancreatic insufficiency, diminished weight, chronic hepatobiliary inflammation, and male infertility. Respiratory failure is the most common cause of death.

At present, treatment of CF is mainly symptomatic, aimed at improving mucociliary clearance and treating infection (1, 2).

2. CF DIAGNOSIS
The diagnosis of cystic fibrosis (CF) is usually made by clinical findings or newborn screening, sweat chloride concentration and genetic analysis.

There is great heterogeneity in the clinical manifestations of cystic fibrosis (CF). Some patients may have all the classical manifestations of CF from infancy and have a relatively poor prognosis, while others have much milder disease manifestations. According to the European Diagnostic Working Group terminology patients are diagnosed with classic or typical CF if they have one or more phenotypic characteristics and a sweat chloride concentration of >60 mmol/l (3). Patients with classic CF can have exocrine pancreatic insufficiency or pancreatic sufficiency. The disease can have a severe course with rapid progression of symptoms or a milder course with very little deterioration over time. Patients with non-classic or atypical CF have a CF phenotype in at least one organ system and a normal (<30 mmol/l) or borderline (30-60 mmol/l) sweat chloride level. In these patients confirmation of the diagnosis of CF requires detection of two disease causing mutations or direct quantification of CFTR dysfunction by nasal potential difference measurement (PD). Non-classic CF includes patients with multiorgan or single organ involvement. Most of these patients have exocrine pancreatic sufficiency and milder lung disease.

Most men with CF are infertile due to obstructive azoospermia, which in its severest form presents as congenital bilateral absence of the vas deferens (CBAVD) (4, 5). CBAVD also occurs in 1 to 2% of infertile males who are otherwise healthy, the majority of whom carry CFTR gene mutations on one or both alleles (6, 7). Higher frequency of CFTR gene
mutations have been also observed in patients with other single organ diseases, like idiopathic chronic pancreatitis, chronic rhinosinusitis, disseminated bronchiectasis (8). These are clinical entities associated with CFTR dysfunction named CFTR-related disorders (CFTR-RD), where the diagnosis of CF cannot be unambiguously established. They are all listed on WHO diagnostic list for single organ disease phenotypes associated with CFTR mutations (9). It is likely that this classification will need further revision in the future as our knowledge and understanding of these conditions increase.

**Mutations analysis**

The heterogeneity of cystic fibrosis (CF) disease is partially explained by this high number of different mutations in the CFTR gene (10). More than 1900 sequence variations have been reported in the CFTR gene including missense, small deletion or insertion, frameshift, and nonsense mutations, often with geographic or ethnic variations in frequency (2). The classification of CFTR gene mutations in five classes according to their functional effects on CFTR protein production and function is based on functional studies: severe vs non-severe.

When patients who are homozygous, or compound heterozygous for a class I-III CFTR mutation, are compared to patients who carry at least one class IV-V CFTR mutation, the latter group tends to have less severe disease (11).

However, it is only partly predictive of individual outcomes, because many CFTR mutations have different functional consequences and cannot be assigned to one particular class and only a limited number of mutations have been studied. The CF phenotype is affected by the CFTR genotype as well as by other genetic and environmental factors.

Extensive mutation analysis cannot guarantee detection of the two disease-causing mutations in all patients; 1-5% of alleles remain undetermined in CF patients with the classical form and even more in patients with atypical presentations.

The limitations of genetic testing include an inability to detect mutations in the noncoding and promoter regions of CFTR.

Mutation analysis is not the answer to every diagnostic dilemma.

**The sweat test** has been used for more than 50 years for the diagnosis of cystic fibrosis (CF) and remains an important diagnostic test in the genomic era. It has been documented that sweat chloride is related to the abnormal function of CFTR and shows good discrimination for the diagnosis of CF (12). However, most studies did not include a ‘healthy’ control group, were performed prior to the availability of the CFTR gene mutation analysis and did not comply with the currently accepted sweat test method. There is no definitive study that quantitates the sweat chloride and sodium concentration in a truly normal population. Further work is required to re-establish sweat electrolyte reference intervals, using the current standardized sweat test, CFTR gene mutation analysis to exclude carriers, and correct statistical analyses in healthy participants with ages spanning from infancy to adulthood.

**Intestinal current measurements (ICM)** is one of the alternative methods for demonstration of defective ion transport. It can be carried out on suction biopsy tissue obtained from the colonic mucosa. Biopsy tissue is mounted in an Ussing chamber, the transepithelial electrical activity, expressed as the short-circuit current (SCC), measured and the response to secretagogues tested. The normal intestine generates a rise in SCC, a reflection of increased Cl secretion, when secretagogues are added, but this response is impaired in CF tissues (13, 14).

**Nasal potential difference (NPD) measurement** is an electrophysiological test that was developed by Knowles et al. (15) and is a part of the criteria for CF diagnosis according to the CFF consensus statement, also in European consensus cited above (16). This test assesses CFTR activity by the change in the transepithelial potential difference as a reaction to superinfusion of the nasal mucosa by several solutions (17).

It is used to demonstrate abnormal function of the CFTR protein, establish a diagnosis of CF in patients with atypical presentation and as a surrogate outcome measure for new therapies that correct the airway ion transport defect. CF nose and lower airway demonstrate similar abnormalities of ion transport.

### 3. PRINCIPLES OF THE PD MEASUREMENTS

Patients with CF have a characteristic pattern of bioelectric properties which reflects defective cAMP-mediated (CFTR) Cl− secretion and accelerated rate of Na+ transport across ENaC. Increased sodium absorption is reflected by a basal hyperpolarization of the nasal mucosa (a large negative PD) together with an increased response to amiloride, which blocks ENaC. The defect in chloride transport is demonstrated by a reduced or abolished repolarization in response to both an electrochemical gradient favorable to chloride efflux during perfusion of the mucosa with chloride free solution and addition of isoprenaline, a cAMP agonist (18).

Five aspects of bioelectric properties in the nasal epithelium usually tested include: 1) the basal PD; 2) the inhibition of PD with perfusion of amiloride; 3) the basal chloride conductance, as indexed by the chloride diffusion PD in response to perfusion of solution containing amiloride but no chloride; 4) the increase in the chloride diffusion PD with the addition of isoproterenol to the perfusate; and 5) the hyperpolarization associated with the addition of ATP to the perfusate (19).

**Technical aspects**

Nasal potential difference can be measured by a high impedance voltmeter between two electrodes: an exploring electrode placed on the surface of the epithelium and a reference electrode (subcutaneous needle or abrasion) in contact with interstitial milieu.

A fluid-filled bridge or a solid agar bridge were used, referenced to either a subcutaneous electrode or an abraded skin electrode system. However, recent studies showed superior reliability of the agar nasal catheter
approach compared to the perfusion nasal catheter method. Measurements under the inferior turbinate and the floor of the nasal cavity are valid, repeatabilities of both methods are similar (20). The region under the inferior turbinate or on the floor is explored for the site of most negative voltage and the probe is stabilized at that point, manually or physically with tape. The solutions are then sequentially perfused over the nasal mucosa usually through the second lumen of a double-barreled catheter and changed after a minimum of 3 min. With each solution, there is an attempt to obtain a steady, noise-free voltage plateau for the terminal 30 sec.

**Results**

Baseline voltage is -18.2±8.3 mV (mean ± SD) for normals, and -45.3±11.4 mV for CF patients. There is a lack of response to perfusion with zero chloride +isoproterenol (+3.2 ±3.5 mV) vs that in normals (-23.7±10.2 mV) (17).

Perfusion with agents that increase cAMP (like isoproterenol) are then likely to induce a greater degree of Cl⁻ secretion. Consequently, the larger changes in PD may then allow clearer quantification of the CF bioelectric defects (18). Taken together, the net response to zero-chloride plus isoproterenol provides the clearest discrimination between CF and normal subjects; the Cl⁻ conductance is absent (or very low) in CF patients.

There is overlap of the baseline values and in the responses to amiloride between CF and normal subjects.

**Method limitations**

There are many variations between the protocols used in different centers, like: warming of the solutions, type of catheter, perfusion speed, composition of solutions.

Method is time consuming and need experience operator, which limits larger use of this awaited insight into CFTR function. Variability of NPD measurements can also reduce the power of studies using NPD as outcome measures. It might be caused by the following: the material used (catheters, electrodes); the offset of the electrodes; the positioning and fixation of the catheter; stability of the measure, duration of the experiment. However, when performed in a single center, NPD could be a reproducible test for CF patients.

The basal PD can be lowered by nonspecific damage to the tissue, such as abrasion or inflammation, f.ex. rhinitis.

**4. AREA OF PD APPLICATION**

Airway ion transport abnormalities in CF can be assessed in vivo by measuring the transepithelial nasal potential difference (PD) (21).

This technique may be useful in assisting in the diagnosis of cystic fibrosis, as well as for monitoring the effect of pharmacological agents and gene transfer approaches to correct the abnormalities of ion transport (22).

**NPD and pathophysiology of CF**

The relation between the number and severity of CFTR gene mutations and the degree of CFTR-mediated dysfunction of transepithelial transport was evaluated (23). This comprehensive, prospective study of healthy males and men with a variety of CFTR-associated disorders demonstrated a wide spectrum of CFTR-mediated abnormalities of transepithelial transport, which showed a relation to the number and functional severity of CFTR gene mutations. The men with CBAVD showed a wide range of transepithelial measurements, which correlated with the number of identified CFTR mutations and overlapped with those in the healthy control subjects.

A number of different studies have examined the relationship between the extent of ion transport abnormality and the severity of disease in CF (21, 24, 25).

Studies of Fajac and Leal showed a correlation of NPD with clinical picture.

Fajac et al. study has shown that in adult CF patients with basal nasal PD in the normal range, respiratory function was less impaired than in CF patients with more negative nasal PD (24). Moreover, among CF patients with two “severe” mutations, a similar relationship between nasal PD and respiratory function was found: in patients with normal nasal PD, respiratory function was less impaired than in patients with higher nasal PD. Nasal PD in a given patient had good reproducibility.

However, there was no significant relationship between nasal potential difference and the severity of the genotype. Nasal epithelial ion transport in cystic fibrosis was linked to the clinical expression of the disease. The pancreatic function appeared to be mostly related to the defect in epithelial chloride secretion whereas the respiratory status was mostly related to abnormal sodium transport (24).

Leal et al. data also indicated that nutritional status, lung function, age, and chronic *P. aeruginosa* infection were co-dependent variables related to both regulatory (sodium channel) and primary (chloride channel) CFTR function (21). Multiple inter-relationships between ion transport defects and clinical variables were confirmed in this study.

From a practical point of view these findings encourage the measurement of nasal PD not only for diagnostic purposes in difficult cases but also to gain more insight into the degree of residual chloride conductance and of sodium abnormality in correlation with severity of the disease.

**NPD as diagnostic technique**

The nasal PD test is a delicate in vivo procedure used to help diagnosis of CF in the presence of atypical clinical features and equivocal sweat test results.

It has already established position as a part of the criteria for CF diagnosis according to the American and European consensus statements (16).

It is used to demonstrate abnormal function of the CFTR protein, establish a diagnosis of CF in patients with atypical presentation and in asymptomatic patients suspected to have CF in CF Newborn Screening Programmes (25, 26, 27).

The test is not diagnostic on its own, but is a very helpful indicative tool.
Therapeutic modulations and correction monitoring

Using nasal PD measurements researchers showed, that distinct components of the CF ion transport profile were associated with characteristic phenotypic expression. A better understanding of the mechanism of dysfunction had direct relevance to therapeutic strategies aimed at improving the CF phenotype by either restoring reduced/absent chloride transport or by normalizing exacerbated sodium transport.

The test has been used as a surrogate endpoint for clinical trials on therapeutic modulation of CF basic defects.

Gene therapy clinical trials in CF patient included viral and non-viral gene transfer to both the nasal and bronchial airway epithelium (28).

Most early trials focused on the nasal epithelium as a surrogate for the lung to allow for easy access and sampling, and, importantly, to ensure safety. Once an acceptable safety profile had been established, gene transfer agents were administered directly into the lung (29).

PD was one of the endpoint assays in the assessment of gene transfer. Partial (approximately 20% of non-CF) correction of chloride transport but not of the sodium, has been reported in some studies. However, it is still unclear, if gene transfer efficiency using currently available vectors is sufficient to change more clinically relevant endpoint assays such as lung function, inflammation or bacterial colonisation (29).

**CFTR** nonsense mutations are mutations causing a premature termination signal resulting in the formation of truncated or unstable protein and subsequently producing little or no CFTR chloride channels (30). The aminoglycoside antibiotics, in addition to their antimicrobial activity, can increase the frequency of a readthrough of the premature stop codon, thereby permitting protein translation to continue to the normal end of the gene (31).

The aim of Wilchanski and coworkers study was to determine if gentamicin can, in vivo, induce expression of **CFTR** in CF patients carrying stop mutations (30). They used the nasal potential difference (PD) to measure sodium and chloride transport before and after topical application of gentamicin drops on the nasal epithelium. Correction of the abnormal PD suggested that gentamicin can correct the primary defect among patients carrying stop mutations. However, the inconvenience of parenteral administration and the potential for serious toxic effects preclude long-term systemic use of gentamicin for suppression of nonsense mutations.

Clancy and coworkers study demonstrated that not all premature stop mutations are equally sensitive to suppression in vivo (32). These results with first-generation suppressive agents suggested the need for improved drug delivery methods and/or more potent suppressors of nonsense mutations to confer **CFTR** correction in subjects with CF heterozygous for nonsense mutations.

PTC124 – Ataluren is an orally bioavailable, nonaminoglycoside compound that was developed in a high throughput screening to induce ribosomes to read through premature stop codons, but not normal stop codons (33).

This phase II prospective trial recruited adults with cystic fibrosis who had at least one nonsense mutation in the **CFTR** gene. The primary outcome had three components: change in **CFTR**-mediated total chloride transport; proportion of patients who responded to treatment; and normalization of chloride transport. Nasal PD was used to assess whether PTC124 could overcome the effects of a nonsense mutation by restoring the functional activity of **CFTR** and increasing total chloride transport. The results showed that patients responded to treatment with PTC124, as assessed by an increase in total chloride transport, indicated by a change of –5 mV or more electrically negative and other clinical improvements.

In many patients, PTC124 shifted total chloride transport into the normal range.

These trials exemplified the concept of direct assessment of protein function in vivo and a use of nasal PD as a sensitive method to assess the activity of full-length, functional CFTR protein on epithelial cell surfaces, and **CFTR**-mediated chloride ion transport in nasal mucosa as a surrogate for lower airway epithelium. The link between changes in NPD towards normal values and clinical improvement still need to be demonstrated.

### 4. SUMMARY

Thanks to NPD is possible to observe pathophysiological events leading from **CFTR** mutation to CF phenotype. It correlates to some extent with genetic abnormalities and disease severity.

It is helpful as diagnostic test and as an endpoint of new disease modifying therapies.

There are still a lot of practical issues to be solved, like standardization and large variability of measurements (34).

There is an unresolved question of correction which is needed for clinical effect as the biggest challenge for this interesting technique still remains our understanding of the impact of potential changes on clinical course of the disease.

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### REFERENCES


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