

New clinical diagnostic procedures for cystic fibrosis in Europe

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on CF diagnosis

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Abstract

In the majority of cases, there is no difficulty in diagnosing Cystic Fibrosis (CF). However, there may be wide variation in signs and symptoms between individuals which encourage the scientific community to constantly improve the diagnostic tests available and develop better methods to come to a final diagnosis in patients with milder phenotypes. This paper is the result of discussions held at meetings of the European Cystic Fibrosis Society Diagnostic Network supported by EuroCareCF. CFTR bioassays in the nasal epithelium (nasal potential difference measurements) and the rectal mucosa (intestinal current measurements) are discussed in detail including efforts to standardize the techniques across Europe. New approaches to evaluate the sweat gland, future of genetic testing and methods on the horizon like CFTR expression in human leucocytes and erythrocytes are discussed briefly.

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1. Introduction

Sweat test, Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) mutation analysis and *in vivo* or *ex vivo* CFTR bioassays are the core diagnostic tests currently used in the cystic fibrosis (CF) clinic [1].

CFTR bioassays measure the voltage potentials created by epithelial ion fluxes at the mucosal surface. These assays thus

provide a direct assessment of physiology at the cellular and the ion channel levels. Nasal potential difference (NPD) and intestinal current measurements (ICM) are not widely available. Most assays are adaptations of the original techniques described by Knowles et al. [2] and Middleton et al. [3] for the measurement of NPD and Veeze et al. [4] and Mall et al. [5] for ICM. During the EuroCareCF project, techniques used in Europe were inventoried and steps taken towards standardization of test performance.

The easiest, cheapest, most sensitive and specific test to diagnose CF continues to be the determination of the chloride (Cl^-) concentration in sweat collected after stimulation of the sweat gland by pilocarpine. Also for infants detected via CF newborn screening it is advised to confirm the diagnosis of CF

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by the sweat test. This brings new challenges for an old test: defining the cut-off for abnormal test results and the urgent need in many institutions to replace old equipment by certified equipment appropriate for safe testing of infants, assuring strict quality control for this crucial diagnostic test. Several consensus reports summarize current recommendations [1,6–9]. New developments in the evaluation of the sweat duct are however, underway. In section 3, we discuss briefly these developments.

More than 1600 *CFTR* mutations have been described in CF patients and/or subjects with CF like symptoms. *CFTR* gene analysis is a useful diagnostic test, but the difficulty remains that genotype analysis cannot be used to exclude CF, as complete sequencing of the coding sequence of the *CFTR* gene is not widely available. Which mutations to include in the diagnostic panel as well as the sensitivity and specificity of the panel used will depend on the ethnic and geographic origin of the subject tested. In Northern European countries the situation is relatively simple with up to 90% of mutations being detected using a panel of about 30 mutations [10]. The majority of *CFTR* mutations are rare and each account for much less than 1% of the global population of CF patients. It is therefore difficult to raise the sensitivity of the diagnostic test further. Next generation sequencing technology may provide a more powerful approach to CF genetic testing, but the interpretation of the test result will remain the limitation. Indeed criteria have been proposed to define a CF mutation, but the ‘pathogenic significance’ of many missense mutations is not known [6,11,12].

Finally, some non genetic blood tests with potential diagnostic value are discussed in section 5.

2. *CFTR* bioassays in nasal epithelium, rectum and sweat gland

2.1. Nasal PD

2.1.1. Physiologic rationale

The measurement of nasal transepithelial potential difference (TEPD), also known as nasal potential difference (NPD), provides a direct and sensitive evaluation of sodium (Na^+) and chloride (Cl^-) transport in nasal epithelial cells by assessment of their bioelectric properties [2,3,13]. This serves not only as a diagnostic aid in difficult cases where *CFTR* dysfunction is suspected [14,15], but also as an endpoint in clinical trials to measure the restoration of *CFTR* function [16].

The basic premise is that the bioelectric abnormality of the CF nasal epithelium reflects the ion transport defects observed in the lower airways and is due to mutations in the *CFTR* gene. Because epithelia have an electrical resistance across them, the active secretion or absorption of ions such as Na^+ and Cl^- causes a potential difference (PD) or voltage [17]. Different epithelia have different ion transport characteristics and the magnitude of PD varies, depending on the site of measurement. PD can be measured by using a high impedance voltmeter between two electrodes, one on the inside (serosal side) and one on the outside (mucosal side) of

the epithelium. The electrode on the outside (the exploring electrode) rests against the surface of the target epithelium. The internal electrode (the reference electrode) can be in any internal compartment of the body, although generally the subcutaneous tissue of the forearm is used (Fig. 1). On paper this appears straightforward, however in practice, some skill and experience are required to achieve accuracy and repeatability with this method.

2.1.2. Global description of methods

The nasal cavity is accessible which makes it a good site to examine the ion transport characteristics of airway epithelia. From two to three centimeters within the nose, the squamous (“skin type”) epithelium becomes a ciliated pseudostratified columnar epithelium also characteristic of the more distal airways. Employing the NPD technique, Knowles and his colleagues demonstrated that Na^+ absorption was the primary ion transport activity in normal airway epithelia [13]. The resulting PD on the airway surface is negative with reference to the interstitium and in normal subjects is generally -15 to -25 mV. This measurement is known as the basal or baseline PD. Following insertion of the exploring electrode, the PD often gradually changes over the first few minutes, termed “stabilization of the PD”. The measurement continues with sequential perfusion of compounds that inhibit the inwardly directed Na^+ conductance or augment the outwardly directed Cl^- conductance (Fig. 2). Amiloride, the epithelial Na^+ channel (ENaC) blocking agent, is perfused and as Na^+ is not being absorbed by the cells, this causes the lumen to become less negative thus causing the PD to veer towards zero. After this a low (or zero) Cl^- solution is perfused, which causes electrogenic transport of Cl^- out of the cell, through *CFTR* Cl^- channels. In non-CF epithelia, this results in a rapid and often large hyperpolarisation of the PD, which in the presence of amiloride is thought to represent Cl^- secretion. This increase in negative PD can be further enhanced pharmacologically by the addition isoprenaline, which augments *CFTR*-mediated Cl^- secretion. Finally, ATP is perfused which activates Cl^- secretion through alternative, non-*CFTR*, Cl^- channels and serves as a marker that the epithelium is viable.

2.1.3. Comparison of methods across Europe

Following the initial description of the nasal PD by Knowles et al. [18] using a PE-50 tube, an alternate approach was developed by Alton et al. [19] using a modified Foley 8 Fr urinary catheter. However, the modified Foley catheter did not allow the measurement of drug and electrolyte solution responses, so this in turn was changed to a double lumen system [3]. Over the last few years, international collaboration has grown and discussion on the use of NPD for diagnostic purposes has focused on the comparability of results across centres. Moreover, when using NPD as an outcome measure in international trials aimed at correcting *CFTR* function, results should be comparable between centres. In Europe, the application of the NPD method as a diagnostic tool has been implemented by individual CF centres without centralised

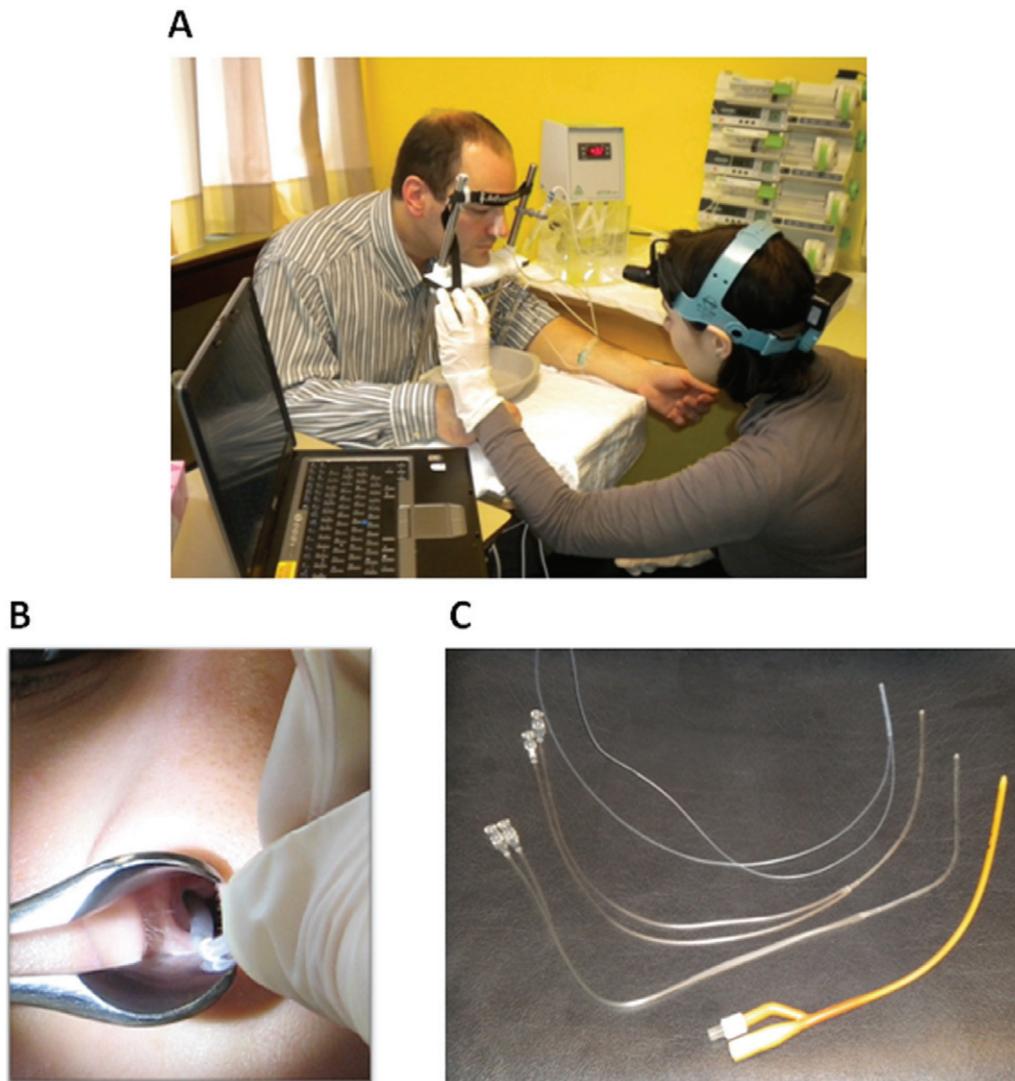


Fig. 1. Typical NPD setup: (A) Multiple pumps for perfusion of the solutions, thermostatic bath for warming of the solutions, double-lumen nasal catheter taped after visualisation of the nostril with a frontal light and a nasal speculum, agar-filled subcutaneous needle on the forearm connected to the reference Calomel electrode. Laptop computer with PowerLab for recording. (B) Position of the catheter under the inferior turbinate. (C) Different nasal catheters in use: from top to bottom PE50/PE90, Marquat with end-hole, Marquat with side-hole, modified Foley catheter.

procedures. This has led to the present situation in which different CF centres have customized certain protocols with marked variability between centres. During the EuroCareCF project, we summarised the NPD procedures performed across Europe (Table 1) by collecting operating procedures, including a description of the set up and pictures of the equipment used. We received information from 9 European countries and 2 non-European countries (Israel and Australia) who are participating in discussions of the European Cystic Fibrosis Society-Diagnostic Network Working Group (ECFS-DNWG).

There are differences in the equipment used including the voltmeter (battery charged voltmeters, digital current powered voltmeter, PowerLab), the registration device (chart recorder, printer, computer) and the electrodes (calomels, Ag/AgCl ECG electrodes, Ag/AgCl micropellet electrodes). Connections with the serosal side are made via subcutaneous needle or via skin abrasion. Different types of catheters are

used (PE tubing, adapted urinary double lumen catheter, umbilical catheter) and conductance is realized by agar, ECG electrode cream or perfusion with NaCl solution. Most operators use an otoscope to inspect the epithelium and find the site of most negative voltage, but in some CF centres the location is found without visualisation. Large catheters are associated with measurements on the nasal floor; the smaller PE50 tubing allows measurements under the inferior turbinate. Catheter position is fixed or kept in place manually during the complete measurement. Exact solution composition (e.g. low or zero chloride, pH buffer used), solution temperature, as well as the method used for warming varies between different CF centres.

2.1.4. NPD as a diagnostic test for CF: reference values

Nasal PD examines different aspects of transepithelial ion transport by the nasal epithelium. In CF, this ion transport profile is abnormal and the nasal PD measurement

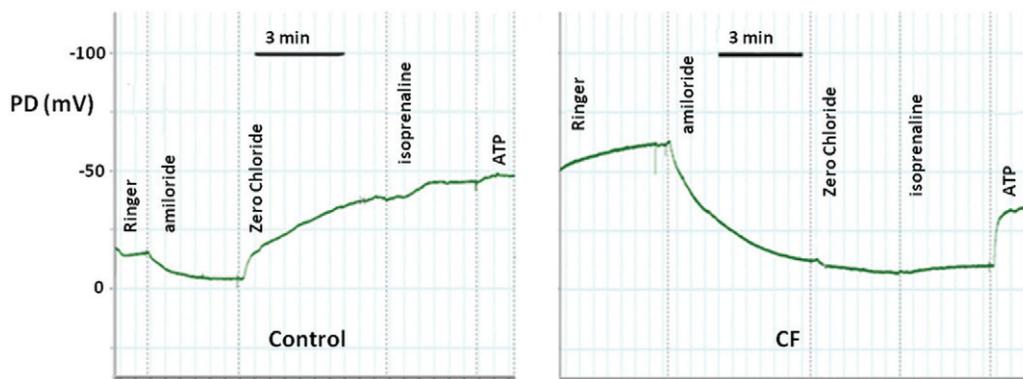


Fig. 2. NPD recordings from normal subject (left) and an individual with CF (right): When compared to normal subjects, individuals with CF have a more negative basal PD, a larger PD change after perfusion of the nasal mucosa with amiloride to inhibit ENaC and very little change after perfusion with a low chloride solution or isoproterenol in the presence of amiloride. ATP, a known stimulant of alternative chloride channels induces a large, but transient change in PD in CF subjects. See text for more details.

Table 1
Differences in equipment and method used for NPD in CF centres

Site	Skin bridge	Reference bridge	Sensing bridge	Electrodes	Exploring catheter	Fluid warmer	Temp. solut.	Otoscope	Voltmeter	Recorder	Site
Israel (Jerusalem)	s.c. butterfly	Agar in butterfly	Custom syringe with agar bridge	Calomel	PE-50	Water bath	34°	Complete reading	Battery	PowerLab	Under turb.
Netherlands (Utrecht, Rotterdam)	s.c. needle	Agar in PE-90	Agar in PE-90	Ag/AgCl pellet	Umbilical	No	Nd	To position	PowerLab	PowerLab	Under turb.
Belgium (Brussels, Leuven)	Abrasion	Electrode cream	Electrode cream	Ag/AgCl ECG	Urinary	Water bath	36.9°	To position	Battery	Computer	Along floor
Italy (Verona)	s.c. needle	NaCl in PE+3way	NaCl in PE+3way	Ag/AgCl pellet	PE-50	Water-jacketed tube	37°	To position	US, battery	PowerLab	Under turb.
Germany (Hannover)	s.c. needle	Agar in syringe	Agar in PE-90	Ag/AgCl pellet	Umbilical	Water bath	33°	Sometimes	US, battery	Chart	Under turb.
Sweden (Stockholm)	Abrasion	Electrode cream	Electrode cream	Ag/AgCl ECG	PE	No	Nd	No	Battery	Printer	Along floor
UK (Liverpool)	s.c. needle	Agar in butterfly	Agar in PE	Calomel	Custom multilumen +PE	No	Nd	To position	Battery	Chart	Under turb.
France (Paris)	Abrasion	Electrode cream	Electrode cream	Ag/AgCl ECG	Cust. double lumen	Humidifier	36°	To position	Cust.	Computer	Under turb.
Australia (Sidney)	Abrasion	Electrode cream	Electrode cream	Ag/AgCl ECG	Urinary	Tried, no difference	Nd	No	Battery	Manually	Along floor

Key: Skin bridge, connection between skin and reference bridge; Reference bridge, connection between skin bridge and reference electrode; Sensing bridge, connection between sensing electrode and exploring catheter; Temp solute, temperature of solution at tip of exploring catheter; Site of measurement: nasal floor or under the inferior turbinate.

distinguishes CF and non-CF [14,20,21]. In CF, there is a much more negative basal PD due to uninhibited ENaC activity transporting Na^+ ions into the cell. As a result, CF subjects have an increased reduction in PD following perfusion with amiloride. CF subjects are also distinguished by the absence or markedly reduced responses to perfusion with low Cl^- and isoprenaline solutions. All steps differ between CF and controls, but the different responses to low Cl^- and isoprenaline are the best discriminator. The addition

of ATP or other purines to the low Cl^- solution leads to a large hyperpolarisation in CF tissues, presumably by non-CFTR-mediated Cl^- secretion. The initial recognition of this response resulted in the examination of purinergic agents as potential therapies for CF [22].

The line delineating CF from non-CF generally runs around a basal PD of -30 mV and a Cl^- free/isoprenaline response of -5 to -10 mV, though many classical CF subjects will have basal values of -50 mV, and combined Cl^-

responses of 0 mV, whereas non-CF subjects will have values of -20 mV for basal PD and -30 mV for the combined Cl^- responses (Fig. 3). More recently, groups of patients with mild/atypical/forme fruste CF have been described with intermediate values, which are in keeping with sweat Cl^- values showing a gradual blurring of the boundaries for individuals with mildest disease.

2.1.5. Factors known to influence NPD results

Over the last few years, a number of different systems, with varied perfusion rates and compositions have been used across Europe (Table 1). Regrettably, there have been few attempts to compare the results obtained with new systems with existing versions. However, standard reference values for (i) basal PD and (ii) low Cl^- /zero Cl^- plus isoprenaline responses are comparable between different CF centres (Fig. 3).

Measurements obtained with the exploring catheter positioned along the nasal floor do not differ significantly from measurements obtained with the catheter positioned under the inferior turbinate [23]. Zero chloride results in a larger NPD response compared to low chloride solution [24]. More detailed studies are needed to better compare results obtained with the different techniques such as type of electrode and subcutaneous needle versus a skin abrasion. The best discriminating and most reproducible technique needs to be identified.

Initially, the Knowles et al. [2] technique determined manually the average PD before and after solution change using paper strip – chart recordings. Similarly, the initial Middleton et al. [3] technique used manual recording from a voltmeter with the average value recorded every 15 s. More recently, electronic recording (Powerlab) has been utilized. However, some electronic recording systems only measure to the nearest mV, unlike the previous systems which measured to an accuracy of 0.1 mV. Furthermore, the optimal data

sampling rate (every second/5 seconds/15 seconds) has not been clarified.

Routinely, experimental protocols are performed on both nostrils of a test subject and then averaged to reduce extraneous drift in the recordings. However, recently some authors [25,26] have proposed that the nostril of a test subject with the largest basal PD should be selected for drug and electrolyte responses. Although the obvious advantage of this new approach is to shorten the total duration of the test protocol, other recent data argue that there is little correlation between the magnitude of the basal PD and the Cl^- secretory response in an individual nostril [27].

Fluid composition has also changed between the initial choice of Knowles et al. [2] using lactated Ringer and Middleton et al. [3] using HEPES-buffered Krebs solution. Importantly, removal of calcium (Ca^{2+}) and magnesium (Mg^{2+}) alters the Cl^- secretory response [28], while changes in the concentration of saline also modifies ion transport in the airways [29].

Solution changes are generally performed when the response of the nasal epithelium has reached a maximum, using the term “stable” though the exact definition of that stability varies between a change of < 1 mV in 30 or 60 s. A less subjective alternative is to use fixed perfusion times.

The influence of perfusate temperature has been examined in a multi-centre trial which showed considerable variability between recordings at “room temperature” and “body temperature” with differences of up to 20 mV. On average, the body temperature responses to low chloride + isoproterenol perfusion were ~ 2 mV higher [30]. Direct comparison of body temperature versus room temperature is currently underway. Perfusate flow is generally 4–5 ml per minute although Southern et al. [31] recommend lower flow rates, especially in neonates and infants. The advantage of faster flow rates is that washout of the previous solution is more likely to be complete

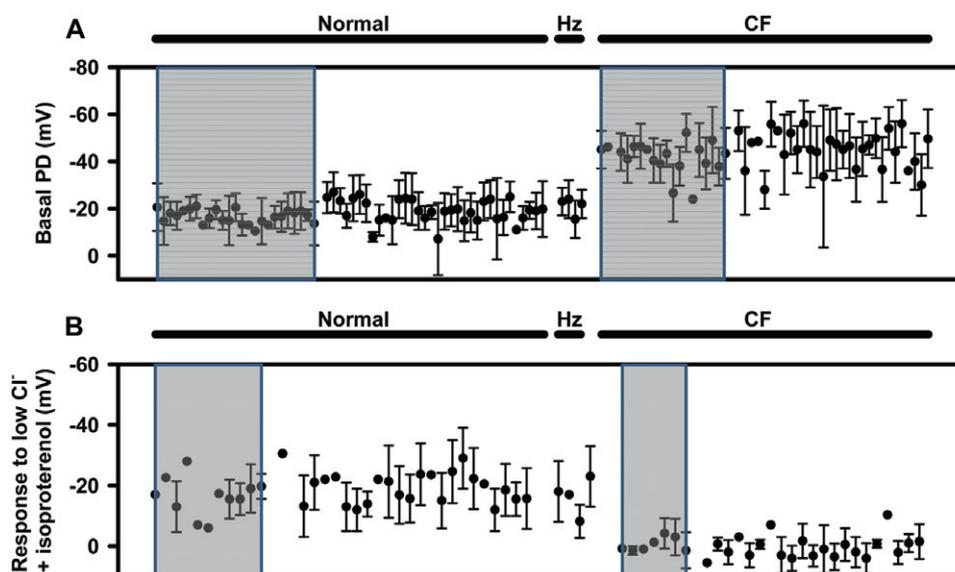


Fig. 3. Comparison of published NPD values: Compilation of basal NPD measurements (A) and the response to zero/low chloride solution and isoproterenol (B) in normal subjects, individuals with CF and heterozygotes. Shaded area: nasal floor method, other authors measured NPD under the turbinate. Data are means \pm SD. Full references are available on request.

within 10–15 s with less mixing than would occur with lower flow rates.

Patient issues are also important. In addition to the differentiation of groups of CF subjects from non-CF subjects, the nasal PD has been used to study subjects with congenital bilateral absence of the vas deferens (CBAVD) and CF heterozygotes [32–34]. It is generally accepted that previous nose or sinus surgery (e.g. for nasal polyps), current upper respiratory tract infection and even mild mechanical trauma (e.g. using a cotton bud) will remove the surface epithelium, preventing the generation of a reproducible PD. The menstrual cycle [35] and cigarette smoking [36] are known to alter nasal PD. Although most protocols involve the use of isoprenaline to stimulate Cl^- secretion, the effect of nebulised salbutamol treatment on nasal PD has not been assessed. Topical hypertonic saline markedly alters nasal PD [29,37,38], but the effect of therapy with hypertonic saline on subsequent measures of nasal PD has not been examined. Interestingly, the effects of high altitude on nasal PD [39] have even been investigated. If the observed changes reflect underlying hypoxia, they will have important implications.

It is generally accepted that the most important variable which affects nasal PD measurement is the appropriate intervention of the operator during the actual test. The catheter should be positioned in an area with good contact such that any gradual drifts in the recording over 15–30 s are minimized. The subject needs to sit comfortably without moving to keep the exploring electrode in a particular spot within the nasal cavity during the test. Especially at higher flow rates, the perfusate should exit the nasal cavity easily to avoid fluid pooling at the site of measurement. In NPD recordings where these technical aspects are not optimal, the entire recording should be rejected. Finally, a word of caution: good NPD recordings can be achieved from the squamous epithelium at the anterior end of the nose, but there is little PD response to amiloride or a low Cl^- solution [27].

2.1.6. Future information needed

Further studies are necessary to delineate the mechanisms which underlie the control of nasal PD, in particular whether hormones or other factors alter the nasal PD response.

It will be important to establish uniform normal NPD values and intra subject variability for CF patients and non-CF controls after European CF centres have adopted a uniform standard operating procedure. In addition collaboration with the Therapeutics Development Network (TDN) of the Cystic Fibrosis Foundation (Bethesda, USA) (CFF-TDN) is ongoing for the use of NPD as an outcome parameter for clinical trials aiming to correct CFTR dysfunction.

A number of studies have attempted to determine the strength of any relationship between ion transport abnormalities and the severity of lung disease. Some studies demonstrate statistical correlations between the Cl^- response and lung function [40], others show correlation with Na^+ transport [26] and yet other report no correlation with either Na^+ or Cl^- transport [41,42]. However, the strength of any relationship is quite weak suggesting that measured

ion transport abnormalities may not impact on the outcome for that individual subject. The importance of this argument becomes apparent when recent clinical trials have used nasal PD as a surrogate end point for correction of the CFTR defect in CF. Unfortunately, the clinical relevance of a 5 mV change in Cl^- conductance is not known, nor is the effect of decreasing basal PD by 4 mV. Although both changes are in the “right” direction, it is not clear whether changes such as these will result in any demonstrable clinical benefit. Large, multi-national trials are thus required to determine whether clinical benefit for the patient can be correlated with changes in Cl^- or Na^+ responsiveness.

2.2. Intestinal current measurement

2.2.1. Physiologic rationale and global description

CF is characterised by abnormal chloride (Cl^-) and bicarbonate (HCO_3^-) transport across epithelial tissues reflecting defects in the expression, localisation or function of CFTR [43]. The CFTR protein is highly expressed in the apical membrane of intestinal epithelial cells, including crypt cells of human distal colon, where it serves as the major if not sole cAMP-dependent electrogenic efflux pathway for Cl^- and HCO_3^- [44,45]. In the intestine, calcium (Ca^{2+}) agonists (e.g. carbachol, histamine) act synergistically with cAMP agonists (e.g. forskolin, 3-isobutyl-1-methylxanthine (IBMX)) to determine the magnitude of CFTR-mediated Cl^- secretion. Concomitant with the opening of CFTR Cl^- channels in the apical membrane by cAMP-dependent phosphorylation, the activation of basolateral membrane potassium (K^+) channels by an increase in the intracellular Ca^{2+} concentration creates a favourable electrical gradient for apical Cl^- efflux by limiting cell depolarisation [46,47]. In the apical membrane of epithelial tissues from CFTR knockout mice, a compensatory upregulation of a 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS)-sensitive, Ca^{2+} -activated Cl^- conductance (CaCC) has been described [48,49]. Based on this observation, some test protocols monitor the DIDS-sensitivity of colonic Cl^- secretory currents [50–52]. In contrast to the respiratory tract, CaCC-mediated intestinal Cl^- secretion is absent in the intestine of non-CF individuals and is only detectable in a small subgroup of F508del homozygous patients characterised by a more severe CF phenotype [51,52].

Intestinal current measurement (ICM) on rectal suction or forceps biopsies in “micro”-Ussing chambers were introduced about two decades ago by several research groups as a novel *ex vivo* diagnostic method for CF [4,5,53–56]. Since its inception, ICM has developed into an important functional tool to aid the diagnosis of (i) CF patients with mild or sub-clinical symptoms of CF and ambiguous or borderline sweat test results and (ii) patients with unknown or rare CFTR mutations [50,57–60]. ICM has also been employed in genotype-phenotype studies to monitor the influence of modifier genes in native intestinal epithelium [51,52,61], as well as to assess the efficacy and specificity of novel therapeutics (e.g. CFTR potentiators and correctors) [62,63].

When compared with the NPD technique, the advantages

of ICM include i) easy accessibility of intestinal tissue at any age, allowing its use in CF children identified by newborn screening; ii) no or minimal tissue destruction or remodelling triggered by bacterial or viral infections; iii) the feasibility to test novel CFTR therapeutics in native human epithelium *ex vivo* without risk to the patient (provided there is a favourable balance between the time it takes for correctors to act vs. duration of tissue viability); and iv) its ability to detect very low amounts of functionally active CFTR. For example, residual amounts of correctly spliced CFTR in a homozygous 3272-26A>G CF patient (<10% of non-CF controls) escaped detection by Western blotting or immunocytochemistry, but generated ~60% of the normal Cl⁻ secretory response in ICM (De Jonge et al., unpublished results). On the other hand, at CFTR protein levels above ~20% of non-CF controls, the CFTR conductance is no longer rate-limiting for transepithelial Cl⁻ transport, implying that CFTR mutations associated with less than 80% loss of CFTR expression in the colon would escape detection by the ICM technique. Another limitation inherent to the *ex vivo* approach is the wash out of *in vivo* applied test compounds from the tissue in the Ussing chamber, resulting in underestimation of rescue efficacies of CFTR potentiators (acting acutely, but reversibly), but not of CFTR correctors.

2.2.2. ICM methods across Europe

ICM in Ussing chambers can be used to study CFTR dysfunction *ex vivo* in human rectal biopsies. Several European CF centres have acquired comprehensive experience with different chamber setups and protocols (50, 56, 57). Currently,

there are two types of micro-Ussing chambers in use for ICM studies in Europe: the recirculating chamber [50,60,64] and the continuously perfused chamber [5,54,56,59,65] (Fig. 4). Other major technical variations are the use of suction *versus* forceps biopsies, short circuit (zero-voltage clamping) conditions *versus* the application of open circuit (i.e. monitoring transepithelial voltage, V_{te} , and resistance), and the use of different test protocols. In the original “Rotterdam” protocol, cholinergic stimulation (carbachol) precedes stimulation by cAMP agonists and histaminergic stimulation (with a DIDS inhibitory step in between) [50], whereas in the “Freiburg” protocol cAMP agonists are surrounded by several additions of a Ca²⁺ agonist (carbachol) [56] and no inhibitory step using DIDS is required. For detailed descriptions of both techniques and protocols, we refer the reader to previous publications [50,56,64].

The aim of present ongoing work performed within the ECFS-DNWG and Workpackage 3 of EuroCareCF is to describe updated practice and differences in ICM methodology and to compare results by a multicenter survey. Therefore, we invited all European ICM centres to provide details of their methodology and evaluation protocol. Because the survey results highlighted significant differences in ICM practice (e.g. biopsy technique, Ussing chambers, buffers, readout parameter and evaluation protocols), it will be necessary to standardise the ICM technique before it can be used as an outcome parameter for clinical trials aiming to rescue CFTR dysfunction.

Major advantages, limitations and pitfalls of the two main techniques currently employed, i.e. short-circuit current

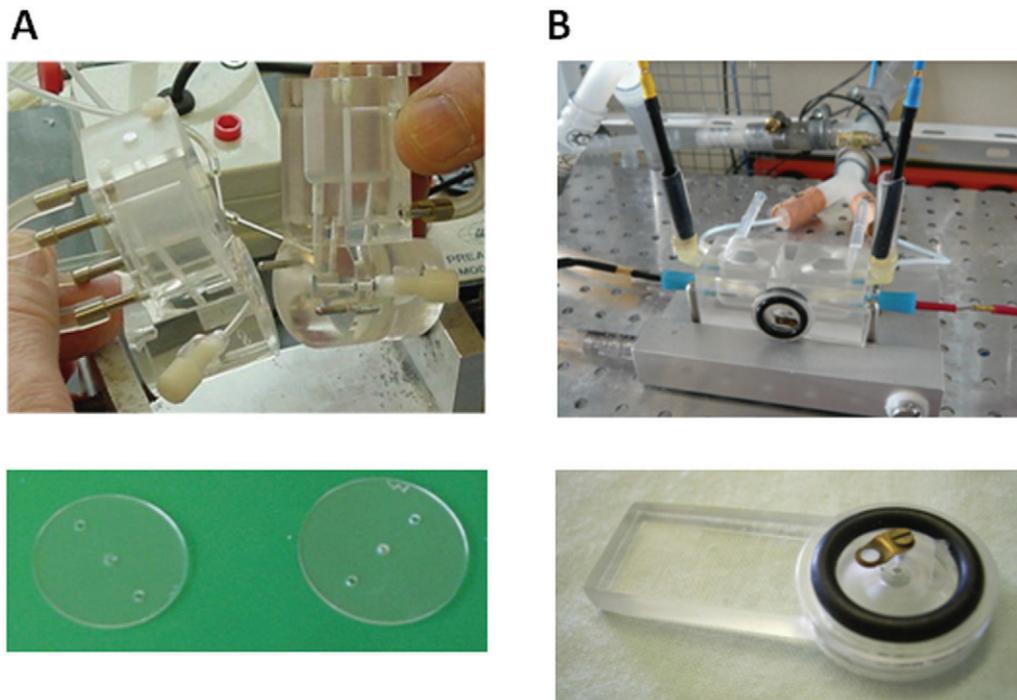


Fig. 4. Mini-Ussing chambers for rectal biopsy studies in Europe: (A) Recirculating chamber, buffer volume ~1.5 ml/side, gassed 95% O₂/5% CO₂. The rectal biopsy is mounted between two discs with exposed tissue area of 1.13 mm². (B) Perfused chamber, buffer volume ~1.5 ml/side, not gassed. The rectal biopsy is mounted on a removable insert with exposed tissue area of 0.95 mm². Both custom-made chamber designs include a water-jacketed heating system to keep tissue-surrounding buffer at 37°C.

(I_{sc}) measurements in recirculating Ussing chambers *versus* voltage and resistance measurements under open-circuit conditions in continuously perfused Ussing chambers have been summarised previously [50,56]. An intrinsic complication in interpreting the electrical response of the rectal tissue is the simultaneous activation of Ca^{2+} -activated apical membrane K^+ channels by Ca^{2+} -linked secretagogues. We refer the reader to the work of Sørensen et al. [66] for information about the molecular identity of these apical membrane K^+ channels. As no highly specific and effective inhibitors of either CFTR or the apical membrane K^+ channels are currently available for use in native tissues, the net current response to carbachol or histamine is a summation of Cl^- and K^+ secretory currents and does not solely reflect the activity of CFTR Cl^- channels. Attempts to use CFTR_{inh}-172 [67,68] in native human rectal tissue have been only partially successful [63], while reasons for missing effects on I_{sc} in a subgroup of biopsies and the best protocol for specific CFTR inhibition remain to be worked out. Therefore, a small amount of residual CFTR activity might be masked by overriding K^+ secretion generating current in the opposite direction. This potential complication is modified, but not abolished by using cAMP-mediated responses as the primary readout of CFTR function, considering the recently reported ability of cAMP to enhance intracellular free Ca^{2+} levels adjacent to the apical membrane through a protein kinase A-independent mechanism involving the cAMP receptor protein Epac [69].

Furthermore, in continuously perfused Ussing chambers extensive depletion of endogenous prostaglandins in the presence of the cyclooxygenase inhibitor indomethacin might eventually lead to a false-positive, CF-like current response to Ca^{2+} -linked agonists in control biopsies [44,54,65]. Addition of agonists increasing cAMP restore the normal, non-CF response which is why the “Freiburg protocol” contains a third application of carbachol in the presence of forskolin/IBMX. Interestingly an apparent inhibition of the response to Ca^{2+} agonists by indomethacin has never been observed using the recirculating chamber technique [50]. Finally, as predicted by equivalent electrical circuit models, the measurement of I_{sc} is less affected by changes in paracellular leak pathways, whereas V_{te} measurements are susceptible to changes in paracellular ion flow, which lead to a fall in shunt resistance. A concomitant measurement of R_{te} is therefore mandatory. By contrast, if the series resistance of the subepithelial tissue is relatively large compared with that of the epithelium, incomplete short-circuiting will occur and result in underestimation of the transcellular current [70]. In this situation, the potential drop across the epithelium might not be very different from the open-circuit situation.

2.2.3. ICM as a diagnostic tool for CF: reference data

To date, ICM had been mainly used for research purposes to determine CFTR function in genotype-phenotype studies [51,57,59,61,71]. These studies highlight the applicability of ICM as a diagnostic tool for CF, including the identification of individuals presenting with milder forms of CF (e.g. CFTR mutations associated with pancreatic sufficiency (PS)).

By contrast, obligate heterozygotes and controls are not distinguishable by ICM [72]. Several European CF centres introduced ICM into their diagnostic workup of patients with questionable CF [50,56,58,73], based on individual experience and preliminary reference data mainly obtained in pancreatic-insufficient (PI) CF patients and healthy controls (Fig. 5). ICM was discussed, but not included in the present diagnostic algorithm and consensus criteria [1,12,74].

Recently, the diagnostic reliability of a standardised ICM protocol [50] was prospectively investigated in a large cohort of PS-CF and PI-CF patients, healthy controls and individuals with questionable CF, presenting with mild symptoms and equivocal results in standard diagnostic tests [60]. For additional validation, extensive CFTR genotype analysis was performed in all subjects with questionable CF. This study was the first to describe the cumulative value of the Cl^- secretory responses ΔI_{sc} carbachol, ΔI_{sc} cAMP/forskolin and ΔI_{sc} histamine (I_{sc} carb+cAMP+hista) as the best diagnostic ICM marker with a clear cut off value of $34 \mu A/cm^2$ between PS-CF and control subjects [60]. It also summarised reference values from previous studies obtained by the CF centres in Rotterdam and Hannover.

ICM can be performed without sedation in patients of all ages starting at the time of CF newborn screening. Moreover, the applicability of ICM to young children is a notable advantage over the NPD technique. Further reference data for different standardised evaluation protocols, registration modes and the whole phenotypic range including subjects with PS-CF and CFTR-related diseases will help to establish the ICM test in the diagnostic workup of individuals in whom the diagnosis of CF is difficult.

2.2.4. ICM outcome protocols for evaluation of CFTR modulating drugs

The “Rotterdam” ICM protocol for CF diagnosis [50] has been successfully used for more than a decade. However, application of this protocol in recirculating Ussing chambers on biopsies from healthy controls results in a variable and often rather high level of basal current, concomitant with a low or occasionally absent response to 8-Br-cAMP/forskolin (controls: $7.1 \pm 8.2 \mu A/cm^2$; CF: $3.0 \pm 2.9 \mu A/cm^2$; means \pm SD; $n = 55$) which compares unfavourably with the more reproducible and discriminative I_{sc} response to carbachol (controls: $43.9 \pm 18.9 \mu A/cm^2$; CF: $-4.6 \pm 7.4 \mu A/cm^2$; $n = 55$). To interpret these findings, we assume that in fresh biopsies, maintained in optimal condition, the cAMP pathway for CFTR activation is partially pre-activated by endogenous secretagogues, despite the use of the PGE₂ synthesis inhibitor indomethacin. This is apparently not the case for the carbachol/ Ca^{2+} pathway, which acts mainly by opening of K^+ channels in the basolateral membrane to enhance the electrical driving force for Cl^- exit through CFTR Cl^- channels in the apical membrane. Another explanation might be that the continuous presence of carbachol in the recirculating chamber leads to a persistent activation of the tissue leading to a somewhat blunted response to other agonists. In the perfused Ussing chamber using the “Freiburg” protocol, the response

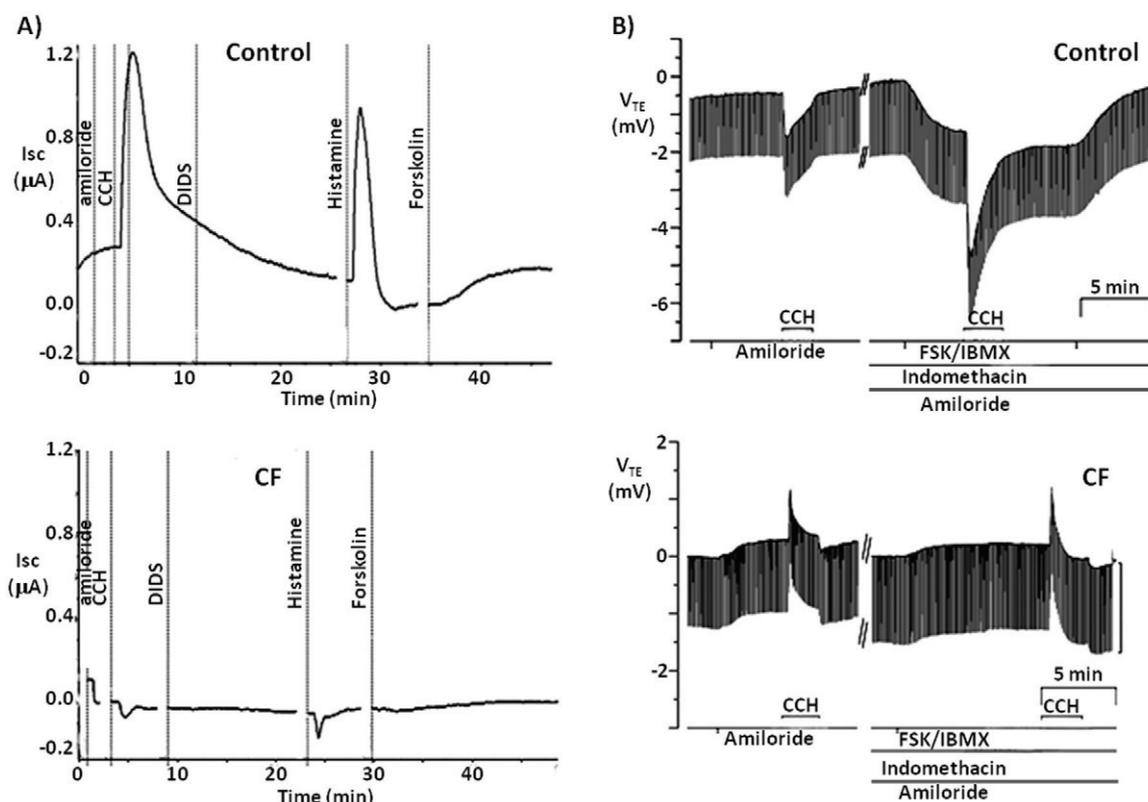


Fig. 5. Bioelectric measurements on rectal biopsies from normal subjects and individuals with CF: (A) Short-circuit current (I_{sc}) recordings in recirculating Ussing chamber in the presence of indomethacin (10 μM added to the basolateral (BL) bathing solution before the start of the measurement) and amiloride (100 μM added to the luminal (L) bathing solution). Chloride secretion is stimulated with carbachol (100 μM BL), histamine (500 μM BL) in the presence of DIDS (200 μM L) and 8-Br-cAMP (1 mM L+BL)/forskolin (10 μM BL). Transepithelial resistance (R_{te}) is measured directly before and after the I_{sc} recording. (B) Open-circuit (V_{te}) and periodical transepithelial resistance recordings (ΔV_{te} upon short 0.5 μA current impulses, indicated by the vertical bars at the end of the recording) in perfused Ussing chambers. The horizontal bars below the recording indicate the addition of i) carbachol (100 μM BL) in the presence of amiloride (10 μM L), ii) forskolin (5 μM , BL)/IBMX (100 μM BL) in the presence of amiloride and indomethacin (1 μM BL), and iii) carbachol (100 μM BL) in the presence of amiloride, indomethacin and forskolin/IBMX. R_{te} is continuously calculated using Ohm's law ($R_{te} = \Delta V_{te}/0.5 \mu A$).

to cAMP correlates well and is reproducible with the effect of carbachol.

The lack of a prominent cAMP effect using recirculating Ussing chambers is a major drawback for the introduction of ICM as an outcome parameter in clinical trials of CFTR potentiators and correctors. A modest drug-induced increase in carbachol-induced secretion could be interpreted as an increase in CFTR activity, induction of CaCCs, and/or a stimulation of Ca^{2+} -activated K^+ channels in the basolateral membrane. If the drug treatment additionally promoted forskolin/cAMP-induced secretion, this would virtually exclude effects on CaCCs and Ca^{2+} -activated K^+ channels, and would enhance the CFTR specificity of the assay.

The Rotterdam group has therefore introduced a new ICM "outcome" protocol (Table 2) which better accentuates forskolin-induced current responses in comparison with the "diagnostic" protocol (5x increase), and further improves the response to carbachol by ~ 1.5 fold. This goal was reached by recurrent washings of the tissue with fresh perfusion buffer, and extending the equilibration time prior to the washings. However, the amiloride/carbachol addition was maintained in the pre-washing phase to allow discrimination of the correct orientation of the biopsy, and its reactivity. For example, if both the pre-equilibration amiloride and carbachol responses

Table 2

Example of new ICM outcome protocol for evaluation of CFTR pharmacotherapy in a recirculating Ussing chamber system (de Jonge): Compounds are added cumulatively, unless washing out and refreshing of medium is performed by pipette as indicated. Basolateral bathing solution always contains indomethacin

Additions*	Concentration (μM)	Time of addition (min)
–	–	0
Amiloride (L)	10	5
Carbachol (L+BL)	200	15
Wash (2x fast)	Refresh medium (L+BL) (~2ml each side)	35
Wash (1x)	Idem	45
Wash (1x)	Idem	55
Amiloride (L)	10	65
Forskolin (L+BL)	2	75
Potentiator: test compound or genistein (L+BL)	Variable (test compound) 50 (genistein)	85
Carbachol (L+BL)	200	85
CFTR _{inh} -172 (L+BL)	20	100
		120

*L = luminal; BL = basolateral.

were absent, the biopsy was considered unsuitable for further experimentation. Although experience with this new protocol is still limited, the results so far demonstrate a considerable

improvement in both the I_{sc} responses to forskolin + genistein (ΔI_{sc} forskolin+genistein: controls, $34.1 \pm 11 \mu A/cm^2$; F508del homozygotes, $-1.9 \pm 9.3 \mu A/cm^2$; $n = 5$) and carbachol (ΔI_{sc} carbachol) in the presence of forskolin+genistein: $66.5 \pm 17.0 \mu A/cm^2$; F508del homozygotes $-12.4 \pm 4.4 \mu A/cm^2$; $n = 5$) as compared with the historical data obtained with the diagnostic protocol. A similar “outcome” protocol has been developed by the Hannover group for the ex vivo analysis of CFTR potentiators and correctors by I_{sc} measurements in the perfused Ussing chamber [63].

2.2.5. Standardisation of the ICM technique

The European multicenter efforts to improve the ICM method presented in this document were used to start the harmonisation of the ICM technique in Europe. Hereby, we will focus on further evaluation of ICM as a diagnostic test for questionable CF and possibly CFTR-related diseases [34] which will be promoted within the ECFS-DNWG. Refinements in methodology and more standardised measurements according to a common consensus protocol will help to facilitate the harmonisation process. In close collaboration between the ECFS-DNWG, the ECFS-Clinical Trials Network and the CFF-TDN, we aim to develop and validate a standard operating procedure for the use of ICM as an outcome parameter for clinical trials aiming to correct CFTR dysfunction.

2.2.6. Further information needed

At this stage an in-depth evaluation of the reproducibility of the ICM technique (intra- and interassay variation), improved knowledge of the possible dependency of the colonic Cl^- secretory capacity on gender and age, and more insight into the feasibility to discriminate between Cl^- and HCO_3^- secretory currents is urgently needed. Another basic question, triggered by the commonly observed poor sensitivity of the Cl^- secretory response in rectal biopsies to current CFTR inhibitors, is whether the anion secretory current in human colon is carried almost exclusively by CFTR, as suggested by the absence of current in most CF patients, or whether it is generated, in part, by other types of Cl^- channels that are functionally dependent on CFTR and/or are down-regulated in CF patients. The recent expansion of the ICM technique to multiple CF centres in Europe and to CFF-TDN centres in the USA will be of great help in resolving such questions.

3. Sweat duct potential difference and skin conductance

With increasing diagnostic challenges and new candidate drugs for CF, there is a clear need for new, reliable and simple biomarkers of defective ion transport. Thus, the CF sweat duct is now being evaluated using assays other than the standard sweat test for measuring Cl^- concentration [9]. The increased negative bioelectric potential in the CF sweat duct can be assessed by transductal voltage measurement of stimulated sweat glands at the skin surface as described by Gonska et al [75]. The sweat gland PD was measured during 30 min as the voltage between 2 electrodes, one taped over an area of skin previously stimulated by iontophoresing pilocarpine,

and the other inserted subcutaneously by needle puncture. Sweat gland PD was similar to sweat Cl^- concentration in distinguishing between control and CF subjects. Sequential stimulation of sweating with cholinergic (by pilocarpine) and β -adrenergic agonists further improved the diagnostic performance of the test. The sweat duct can also be explored by active electrophysiology, i.e. measurement of electrochemical skin conductance after application of a low direct current voltage as described by Hubert et al. [76]. A low voltage is applied between 2 nickel electrodes on which the patient has put their hands or feet. This low voltage generates a current due to ion movements that in the skin originate from sweat duct pores. This current is measured and electrochemical skin conductance calculated. The test is painless and takes less than 5 min. When control and CF subjects are studied, the assay provides a diagnostic specificity of 1 and a sensitivity of 0.93. To date, proof-of-concept of these two new simple and practical real-time assays has only been shown in adults. However, results with these new tests appear promising and justify further development as outcome parameters in clinical trials and as diagnostic tests for CF.

4. Highly parallel sequencing

In current, routine CFTR mutation testing, mutation-specific tests are used, i.e. the presence or absence of (a) given mutation(s) is tested. More than 1600 mutations have been identified in the CFTR gene. Screening all these mutations by mutation-specific tests in an individual is unrealistic. Fortunately, in most populations screening for a set of about 30 mutations achieves a combined sensitivity of 85–92% of detecting a CF-disease-causing mutation in a mutant CFTR gene. These mutations are screened for in most (commercial) mutation-specific CFTR tests. A negative genetic test result therefore does not exclude the presence of a CFTR mutation. A higher mutation detection sensitivity is obtained when the complete coding region, and exon/intron junctions, of the CFTR gene is scanned or sequenced for the presence of mutations. These techniques are laborious and expensive, and in most populations only used in individuals when selected clinical indications are met.

In fact, many clinicians favour screening for a set of well-characterized mutations only. Indeed, complete sequencing may identify mutations whose pathological consequences are unclear, i.e. are these mutations CF-causing, innocent polymorphisms, or mild mutations that cause CFTR-related disease? This especially applies to missense mutations. A further complication is that other genes than CFTR might be involved in disease in a small fraction of patients [77,78].

Sanger sequencing dominated sequencing for 30 years. In recent years, several next-generation sequencing technologies have been, and are being, developed at extraordinary speed. By 2015, it is expected that a complete human genome, including an individual's complete CFTR gene, will be sequenced for €1000. Thus, the cost of sequencing the CFTR gene alone will become equivalent or cheaper than mutation-specific CFTR tests, and from an economic point of view, very

likely become the preferred genetic test. We will then have to deal with rarer mutations or (missense) mutations of which the functional consequences are unknown and can only be determined by functional tests. This problem is being addressed, in part, by the CFTR2 (Clinical and Functional Translation of CFTR) project, which seeks to provide complete, advanced and expert-reviewed functional and clinical information on a large set of *CFTR* mutations. Fortunately, about 28% of all *CFTR* mutations identified so far are frameshift mutations, nonsense mutations or large insertions and deletions, of which the pathological consequences can be predicted without functional studies. This also applies for splice-site mutations in which the first or second nucleotide of the splice site is mutated (about 12.5% of all *CFTR* mutations are splice mutations). Identification of such a mutation after sequencing alone, even if it is a previously unknown or very rare mutation, clearly has added value. On the other hand, about 15% of the mutations in the *CFTR* mutation database are classified as sequence variations. The majority of these are missense mutations. Together with the rare CF-causing missense mutations (found in less than 5 mutant *CFTR* genes worldwide), they will thus remain a challenge in genetic counselling. A possible (temporary) solution is that *CFTR* sequencing is performed on the condition that the detection of rare missense mutations with unknown functional consequences is not reported.

The human genome, and its phenotypic expression, is so complex that a genetic test with 100% sensitivity will likely remain illusive and thus can only be approximated. In the end, the main challenge of the use, interpretation, understanding and communication of *CFTR* genetic test results will be the understanding of their limitations.

5. Diagnostic value of non genetic blood tests

Genotyping is complex and it is frequently difficult to interpret the identified mutations. At present *in vivo* CFTR bio-assays are time consuming, cumbersome and technically demanding. Therefore, they are only available in selected CF centres. There is thus great demand for non genetic blood tests that distinguish between CF and non CF subjects. As an example we highlight approaches.

5.1. Measurement of CFTR expression and function in human leucocytes and erythrocytes

Verloo et al. [79] were the first to identify a CFTR-like linear chloride conductance in the plasma membrane of human erythrocytes that was activated upon *Plasmodium falciparum* infection and defective in CF patients. Stumpf et al. [80] explored the presence of functional CFTR in human erythrocytes as a diagnostic test. The principle behind this test is the hemolysis of erythrocytes induced by gadolinium (Gd^{3+}) ions. Erythrocytes from CF patients are more resistant to Gd^{3+} -induced hemolysis than erythrocytes from healthy donors. In another approach, the authors demonstrated the differential Zn^{2+} sensitivity of Gd^{3+} -induced hemolysis for non-CF and CF erythrocytes.

Another approach recently proposed is based on the observation that leucocytes express detectable levels of CFTR [81]. Using immunoprecipitation and flow cytometry, Sorio et al. [82] provided preliminary data on CFTR expression and function *ex vivo* in leucocytes from healthy subjects and CF patients. Flow cytometry, Western blotting and cell membrane depolarization was evaluated by single-cell fluorescence imaging using the potential-sensitive probe bis-(1,3-diethylthiobarbituric acid) trimethine oxonol (DiSBAC₂). The authors detected higher levels of CFTR expression in monocytes and lymphocytes compared to polymorphonuclear cells [82]. The cell membrane depolarization assay demonstrated functional response only in monocytes from healthy controls and, to a lesser extent, from obligate CF heterozygotes. Of relevance, monocytes from CF patients showed a completely different pattern of response permitting the correct classification of healthy and diseased subjects [82]. As a reference, the authors measured NPD in selected subjects in parallel to the monocyte assay and always obtained consistent results. These findings suggest that evaluation of CFTR expression and function in monocytes by flow cytometry and optical techniques, respectively, might represent new approaches to diagnose CFTR dysfunction and evaluate the effects of drugs on CFTR expression and function.

These tests might also be of additional help to better diagnose CF.

6. Conclusion

The CF diagnostic armamentarium is increasing and is being applied not only for diagnosis, but also as an outcome parameter in trials with CFTR modulators.

NPD and ICM are being evaluated extensively. There is a clear need to establish common standard operating procedures for these techniques. The EuroCareCF project greatly facilitated this evolution. Also the sweat gland is being studied in a new way. Genetic analysis is accelerating, but linking genetic and clinical data will be even more important to understand the significance of (minor) changes in the DNA sequence of the *CFTR* gene. Finally, CFTR expression in leucocytes and erythrocytes raises the possibility of developing non genetic blood tests to diagnose CF and evaluate new therapies for the disease.

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Conflict of interest

Harry Cuppens holds patents from KULeuven for diagnostic tests using next-generation sequencing.

References

- [1] De Boeck K, Wilschanski M, Castellani C, et al. Cystic fibrosis: terminology and diagnostic algorithms. *Thorax* 2006;61(7):627–35.
- [2] Knowles MR, Paradiso AM, Boucher RC. In vivo nasal potential difference: techniques and protocols for assessing efficacy of gene transfer in cystic fibrosis. *Hum Gene Ther* 1995;6(4):445–55.
- [3] Middleton PG, Geddes DM, Alton EW. Protocols for in vivo measurement of the ion transport defects in cystic fibrosis nasal epithelium. *Eur Respir J* 1994;7(11):2050–6.
- [4] Veeze HJ, Sinaasappel M, Bijman J, Bouquet J, de Jonge HR. Ion transport abnormalities in rectal suction biopsies from children with cystic fibrosis. *Gastroenterology* 1991;101(2):398–403.
- [5] Mall M, Bleich M, Schurlein M, et al. Cholinergic ion secretion in human colon requires coactivation by cAMP. *Am J Physiol* 1998;275(6 Pt 1):G1274–81.
- [6] Rosenstein BJ, Cutting GR. The diagnosis of cystic fibrosis: a consensus statement. Cystic Fibrosis Foundation Consensus Panel. *J Pediatr* 1998;132(4):589–95.
- [7] LeGrys VA, Yankaskas JR, Quittell LM, Marshall BC, Mogayzel PJ, Jr. Diagnostic sweat testing: the Cystic Fibrosis Foundation guidelines. *J Pediatr* 2007;151(1):85–9.
- [8] Multi-Disciplinary Working Group. Guidelines for the Performance of the Sweat Test for the Investigation of Cystic Fibrosis in the UK. 2003; Available from: <http://www.acb.org.uk/docs/sweat.pdf>.
- [9] Gibson LE, Cooke RE. A test for concentration of electrolytes in sweat in cystic fibrosis of the pancreas utilizing pilocarpine by iontophoresis. *Pediatrics* 1959;23(3):545–9.
- [10] WHO. The molecular genetic epidemiology of cystic fibrosis. Report of a joint meeting of WHO/ECFTN/ICF(M)A/ECFS (<http://www.who.int/genomics/publications/en/>) and WHO Geneva, Human Genetics Programme WHO/HGN/CF/WG/04.02. 2004.
- [11] Castellani C, Cuppens H, Macek M, Jr., et al. Consensus on the use and interpretation of cystic fibrosis mutation analysis in clinical practice. *J Cyst Fibros* 2008;7(3):179–96.
- [12] Farrell PM, Rosenstein BJ, White TB, et al. Guidelines for diagnosis of cystic fibrosis in newborns through older adults: Cystic Fibrosis Foundation consensus report. *J Pediatr* 2008;153(2):S4–14.
- [13] Knowles MR, Carson JL, Collier AM, Gatzky JT, Boucher RC. Measurements of nasal transepithelial electric potential differences in normal human subjects in vivo. *Am Rev Respir Dis* 1981;124(4):484–90.
- [14] Wilschanski M, Famini H, Strauss-Liviatan N, et al. Nasal potential difference measurements in patients with atypical cystic fibrosis. *Eur Respir J* 2001;17(6):1208–15.
- [15] Goubau C, Wilschanski M, Skalicka V, et al. Phenotypic characterisation of patients with intermediate sweat chloride values: towards validation of the European diagnostic algorithm for cystic fibrosis. *Thorax* 2009;64(8):683–91.
- [16] Kerem E, Hirawat S, Armoni S, et al. Effectiveness of PTC124 treatment of cystic fibrosis caused by nonsense mutations: a prospective phase II trial. *Lancet* 2008;372(9640):719–27.
- [17] Gatzky JT. Bioelectric properties of the isolated amphibian lung. *Am J Physiol* 1967;213(2):425–31.
- [18] Knowles M, Gatzky J, Boucher R. Increased bioelectric potential difference across respiratory epithelia in cystic fibrosis. *N Engl J Med* 1981;305(25):1489–95.
- [19] Alton EW, Hay JG, Munro C, Geddes DM. Measurement of nasal potential difference in adult cystic fibrosis, Young's syndrome, and bronchiectasis. *Thorax* 1987;42(10):815–7.
- [20] Delmarco A, Pradal U, Cabrini G, Bonizzato A, Mastella G. Nasal potential difference in cystic fibrosis patients presenting borderline sweat test. *Eur Respir J* 1997;10(5):1145–9.

- [21] Wilschanski M, Dupuis A, Ellis L, et al. Mutations in the cystic fibrosis transmembrane regulator gene and in vivo transepithelial potentials. *Am J Respir Crit Care Med* 2006;174(7):787–94.
- [22] Olivier KN, Bennett WD, Hohneker KW, et al. Acute safety and effects on mucociliary clearance of aerosolized uridine 5'-triphosphate +/- amiloride in normal human adults. *Am J Respir Crit Care Med* 1996;154(1):217–23.
- [23] Vermeulen F, Proesmans M, Feyaerts N, De Boeck C. Nasal potential measurements on the nasal floor and under the inferior turbinate: does it matter? *Pediatr Pulmonol* 2011;46(2):145–52.
- [24] House HH, Middleton PG. Impact of different chloride and glucose solutions on nasal potential difference. *Pediatr Pulmonol* 2009;44(7):645–8.
- [25] Yaakov Y, Kerem E, Yahav Y, et al. Reproducibility of nasal potential difference measurements in cystic fibrosis. *Chest* 2007;132(4):1219–26.
- [26] Leal T, Fajac I, Wallace HL, et al. Airway ion transport impacts on disease presentation and severity in cystic fibrosis. *Clin Biochem* 2008;41(10–11):764–72.
- [27] Middleton PG, House HH. Measurement of airway ion transport assists the diagnosis of cystic fibrosis. *Pediatr Pulmonol* 2010;45:789–95.
- [28] Middleton PG, Pollard KA, Donohoo E, Wheatley JR, Geddes DM, Alton EW. Airway surface liquid calcium modulates chloride permeability in the cystic fibrosis airway. *Am J Respir Crit Care Med* 2003;168(10):1223–6.
- [29] Middleton PG, Pollard KA, Wheatley JR. Hypertonic saline alters ion transport across the human airway epithelium. *Eur Respir J* 2001;17(2):195–9.
- [30] Boyle MP, Diener-West M, Milgram L, et al. A multicenter study of the effect of solution temperature on nasal potential difference measurements. *Chest* 2003;124(2):482–9.
- [31] Southern KW, Noone PG, Bosworth DG, Legrys VA, Knowles MR, Barker PM. A modified technique for measurement of nasal transepithelial potential difference in infants. *J Pediatr* 2001;139(3):353–8.
- [32] Osborne LR, Lynch M, Middleton PG, et al. Nasal epithelial ion transport and genetic analysis of infertile men with congenital bilateral absence of the vas deferens. *Hum Mol Genet* 1993;2(10):1605–9.
- [33] Pradal U, Castellani C, Delmarco A, Mastella G. Nasal potential difference in congenital bilateral absence of the vas deferens. *Am J Respir Crit Care Med* 1998;158(3):896–901.
- [34] Bombieri C, Claustres M, De Boeck K, et al. Recommendations for the classification of diseases as CFTR-related disorders. *J Cyst Fibros* 2011;10(Suppl 2):86–102.
- [35] Swezey NB, Smith D, Corey M, et al. Amiloride-insensitive nasal potential difference varies with the menstrual cycle in cystic fibrosis. *Pediatr Pulmonol* 2007;42(6):519–24.
- [36] Cantin AM, Hanrahan JW, Bilodeau G, et al. Cystic fibrosis transmembrane conductance regulator function is suppressed in cigarette smokers. *Am J Respir Crit Care Med* 2006;173(10):1139–44.
- [37] Davies MG, Geddes DM, Alton EW. The effect of varying tonicity on nasal epithelial ion transport in cystic fibrosis. *Am J Respir Crit Care Med* 2005;171(7):760–3.
- [38] Hebestreit A, Kersting U, Hebestreit H. Hypertonic saline inhibits luminal sodium channels in respiratory epithelium. *Eur J Appl Physiol* 2007;100(2):177–83.
- [39] Mairbaurl H, Weymann J, Mohrlein A, et al. Nasal epithelium potential difference at high altitude (4,559 m): evidence for secretion. *Am J Respir Crit Care Med* 2003;167(6):862–7.
- [40] Thomas SR, Jaffe A, Geddes DM, Hodson ME, Alton EW. Pulmonary disease severity in men with $\Delta F508$ cystic fibrosis and residual chloride secretion. *Lancet* 1999;353(9157):984–5.
- [41] Walker LC, Venglarik CJ, Aubin G, et al. Relationship between airway ion transport and a mild pulmonary disease mutation in CFTR. *Am J Respir Crit Care Med* 1997;155(5):1684–9.
- [42] Wallace HL, Barker PM, Southern KW. Nasal airway ion transport and lung function in young people with cystic fibrosis. *Am J Respir Crit Care Med* 2003;168(5):594–600.
- [43] Riordan JR. CFTR function and prospects for therapy. *Annu Rev Biochem* 2008;77:701–26.
- [44] Kunzelmann K, Mall M. Electrolyte transport in the mammalian colon: mechanisms and implications for disease. *Physiol Rev* 2002;82(1):245–89.
- [45] Greger R. Role of CFTR in the colon. *Annu Rev Physiol* 2000;62:467–91.
- [46] Barrett KE, Keely SJ. Chloride secretion by the intestinal epithelium: molecular basis and regulatory aspects. *Annu Rev Physiol* 2000;62:535–72.
- [47] Field M. Intestinal ion transport and the pathophysiology of diarrhea. *J Clin Invest* 2003;111(7):931–43.
- [48] Rozmahel R, Wilschanski M, Matin A, et al. Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. *Nat Genet* 1996;12(3):280–7.
- [49] Clarke LL, Grubb BR, Yankaskas JR, Cotton CU, McKenzie A, Boucher RC. Relationship of a non-cystic fibrosis transmembrane conductance regulator-mediated chloride conductance to organ-level disease in *Cftr(-/-)* mice. *Proc Natl Acad Sci U S A* 1994;91(2):479–83.
- [50] De Jonge HR, Ballmann M, Veeze H, et al. Ex vivo CF diagnosis by intestinal current measurements (ICM) in small aperture, circulating Ussing chambers. *J Cyst Fibros* 2004;3(Suppl 2):159–63.
- [51] Bronsveld I, Mekus F, Bijman J, et al. Residual chloride secretion in intestinal tissue of $\Delta F508$ homozygous twins and siblings with cystic fibrosis. The European CF Twin and Sibling Study Consortium. *Gastroenterology* 2000;119(1):32–40.
- [52] Bronsveld I, Mekus F, Bijman J, et al. Chloride conductance and genetic background modulate the cystic fibrosis phenotype of $\Delta F508$ homozygous twins and siblings. *J Clin Invest* 2001;108(11):1705–15.
- [53] Hardcastle J, Hardcastle PT, Taylor CJ, Goldhill J. Failure of cholinergic stimulation to induce a secretory response from the rectal mucosa in cystic fibrosis. *Gut* 1991;32(9):1035–9.
- [54] Mall M, Kreda SM, Mengos A, et al. The $\Delta F508$ mutation results in loss of CFTR function and mature protein in native human colon. *Gastroenterology* 2004;126(1):32–41.
- [55] Barreto C, Mall M, Amaral MD. Assessment of CFTR function in native epithelia for the diagnosis of cystic fibrosis. *Pediatr Pulmonol Suppl* 2004;26:243.
- [56] Mall M, Hirtz S, Gonska T, Kunzelmann K. Assessment of CFTR function in rectal biopsies for the diagnosis of cystic fibrosis. *J Cyst Fibros* 2004;3(Suppl 2):165–9.
- [57] Veeze HJ, Halley DJ, Bijman J, de Jongste JC, de Jonge HR, Sinaasappel M. Determinants of mild clinical symptoms in cystic fibrosis patients. Residual chloride secretion measured in rectal biopsies in relation to the genotype. *J Clin Invest* 1994;93(2):461–6.
- [58] Derichs N, Bronsveld I, Mekus F, et al. Intestinal current measurement (ICM) for the diagnosis of atypical cystic fibrosis. *Pediatr Pulmonol* 2000;S20:91.
- [59] Hirtz S, Gonska T, Seydewitz HH, et al. CFTR Cl^- channel function in native human colon correlates with the genotype and phenotype in cystic fibrosis. *Gastroenterology* 2004;127(4):1085–95.
- [60] Derichs N, Sanz J, von Kaenel T, et al. Intestinal current measurement for diagnostic classification of patients with questionable cystic fibrosis: validation and reference data. *Thorax* 2010;65(7):594–9.
- [61] Derichs N, Mekus F, Bronsveld I, et al. Cystic fibrosis transmembrane conductance regulator (CFTR)-mediated residual chloride secretion does not protect against early chronic *Pseudomonas aeruginosa* infection in $F508del$ homozygous cystic fibrosis patients. *Pediatr Res* 2004;55(1):69–75.
- [62] Amaral MD, Kunzelmann K. Molecular targeting of CFTR as a therapeutic approach to cystic fibrosis. *Trends Pharmacol Sci* 2007;28(7):334–41.
- [63] Derichs N, Knoll J, Hyde R, Pedemonte N, Galiotta LV, Ballmann M. Preclinical evaluation of CFTR modulators in ex vivo human rectal tissue. *Pediatr Pulmonol* 2009;44:292.
- [64] Li H, Sheppard DN, Hug MJ. Transepithelial electrical measurements with the Ussing chamber. *J Cyst Fibros* 2004;3(Suppl 2):123–6.
- [65] Mall M, Wissner A, Seydewitz HH, et al. Defective cholinergic Cl^- secretion and detection of K^+ secretion in rectal biopsies from cystic

- fibrosis patients. *Am J Physiol Gastrointest Liver Physiol* 2000;278(4):G617–24.
- [66] Sorensen MV, Sausbier M, Ruth P, et al. Adrenaline-induced colonic K^+ secretion is mediated by $K_{Ca}1.1$ (BK) channels. *J Physiol* 2010;588(Pt 10):1763–77.
- [67] Ma T, Thiagarajah JR, Yang H, et al. Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. *J Clin Invest* 2002;110(11):1651–8.
- [68] Li H, Sheppard DN. Therapeutic potential of cystic fibrosis transmembrane conductance regulator (CFTR) inhibitors in polycystic kidney disease. *BioDrugs* 2009;23(4):203–16.
- [69] Hoque KM, Woodward OM, van Rossum DB, et al. Epac1 mediates protein kinase A-independent mechanism of forskolin-activated intestinal chloride secretion. *J Gen Physiol* 2010;135(1):43–58.
- [70] Bakker R. Electrophysiology of fish intestine. In: Gilles R, editor. *Transport processes Iono and Osmoregulation*. New York: Springer; 1985. pp. 240–250.
- [71] Stanke F, Ballmann M, Bronsveld I, et al. Diversity of the basic defect of homozygous CFTR mutation genotypes in humans. *J Med Genet* 2008;45(1):47–54.
- [72] Veeze HJ. *Pathophysiological aspects of cystic fibrosis: genotypes, phenotypes and Intestinal Current Measurements*. Rotterdam: Erasmus University; 1995.
- [73] Derichs N, Schuster A, Grund I, et al. Homozygosity for L997F in a child with normal clinical and chloride secretory phenotype provides evidence that this cystic fibrosis transmembrane conductance regulator mutation does not cause cystic fibrosis. *Clin Genet* 2005;67(6):529–31.
- [74] Mayell SJ, Munck A, Craig JV, et al. A European consensus for the evaluation and management of infants with an equivocal diagnosis following newborn screening for cystic fibrosis. *J Cyst Fibros* 2009; 8(1):71–8.
- [75] Gonska T, Ip W, Turner D, et al. Sweat gland bioelectrics differ in cystic fibrosis: a new concept for potential diagnosis and assessment of CFTR function in cystic fibrosis. *Thorax* 2009;64(11):932–8.
- [76] Hubert D, Dusser D, Bry A, Fajac I. Evaluation of a new tool for measurement of sweat chloride concentration. *Pediatr Pulmonol* 2009;S32:269.
- [77] Fajac I, Viel M, Sublemontier S, Hubert D, Bienvenu T. Could a defective epithelial sodium channel lead to bronchiectasis. *Respir Res* 2008;9:46.
- [78] Azad AK, Rauh R, Vermeulen F, et al. Mutations in the amiloride-sensitive epithelial sodium channel in patients with cystic fibrosis-like disease. *Hum Mutat* 2009;30(7):1093–1103.
- [79] Verloo P, Kocken CH, Van der Wel A, et al. Plasmodium falciparum-activated chloride channels are defective in erythrocytes from cystic fibrosis patients. *J Biol Chem*. 2004;279(11):10316–22.
- [80] Stumpf A, Weners-Epping K, Walte M, et al. Physiological concept for a blood based CFTR test. *Cell Physiol Biochem* 2006;17(1–2):29–36.
- [81] McDonald TV, Nghiem PT, Gardner P, Martens CL. Human lymphocytes transcribe the cystic fibrosis transmembrane conductance regulator gene and exhibit CF-defective cAMP-regulated chloride current. *J Biol Chem* 1992;267(5):3242–8.
- [82] Sorio C, Buffelli M, Ettore M, et al. Functional evaluation of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) expression function in human monocytes. *J Cyst Fibros* 2009;8:S22.