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REVIEW

CFTR biomarkers: time for promotion to surrogate end-point?

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ABSTRACT: In patients with cystic fibrosis, cystic fibrosis transmembrane conductance regulator (CFTR) biomarkers, such as sweat chloride concentration and/or nasal potential difference, are used as end-points of efficacy in phase-III clinical trials with the disease modifying drugs ivacaftor (VX-770), VX809 and ataluren. The aim of this project was to review the literature on reliability, validity and responsiveness of nasal potential difference, sweat chloride and intestinal current measurement in patients with cystic fibrosis.

Data on clinimetric properties were collected for each biomarker and reviewed by an international team of experts. Data on reliability, validity and responsiveness were tabulated. In addition, narrative answers to four key questions were discussed and agreed by the team of experts.

The data collected demonstrated the reliability, validity and responsiveness of nasal potential difference. Fewer data were found on reliability of sweat chloride concentration; however, validity and responsiveness were demonstrated. Validity was demonstrated for intestinal current measurement, but further information is required on reliability and responsiveness. For all three end-points, normal values were collected and further research requirements were proposed.

This body of work adds useful information to support the promotion of CFTR biomarkers to surrogate end-points and to guide further research in the area.

KEYWORDS: Clinical trials, cystic fibrosis transmembrane conductance regulator, intestinal current measurement, nasal potential difference, surrogate end-point, sweat test

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Outcome measures fall into three classes: clinical end-points, surrogate end-points and biomarkers.

Clinical end-points reflect how a patient feels, functions or survives [1, 2] and detect a tangible benefit for the patient. The improved life expectancy in cystic fibrosis has rendered survival, the gold standard clinical efficacy measure, an impossible end-point to use in clinical trials. Therefore, intermediate clinical efficacy measures, such as the frequency of respiratory exacerbation were introduced. The latter has been used in registration trials for rhDNase [3], tobramycin solution for inhalation [4] and aztreonam lysinate [5]. Clinical end-points particularly useful for young children include anthropometric measures. Quality of life as measured by the Cystic Fibrosis Questionnaire-Revised is also accepted as a measure of treatment benefit in cystic fibrosis [6, 7]; however, it is considered only an optional end-point by the European Medicines Agency [6, 8].

A surrogate end-point is a laboratory measurement used as a substitute for clinical end-points and predicts the efficacy or toxicity of therapy [1, 2]. It is an indirect measurement of effect and is used when direct measurement of clinical effect is not feasible or practical. Surrogate end-points can be used complementary to measures of treatment

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benefit and may shorten the period of follow-up required. The link between the surrogate end-point and survival, long-term prognosis or accepted measures of treatment effect (both improvement and deterioration) must be proven. Forced expiratory volume in 1 s (FEV_1) has been widely used as a surrogate end-point due to the established link with survival [9]. However, in many patients with cystic fibrosis, the rate of decline in FEV_1 has slowed [10], limiting the current sensitivity of the measure, particularly in children or in patients with mild lung disease [10, 11].

A biomarker is defined as ''a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic response to a therapeutic intervention'' (*e.g.* nasal potential difference (NPD), mucociliary clearance, inflammatory markers and sputum bacterial density) [1, 2]. These measures are mainly used in phase-I or -II clinical trials when proof-of-concept for a specific compound is explored. Biomarkers are useful for gaining information about the mechanism of action of potential drugs, for identifying treatment responders and for dose selection. Some biomarkers are currently being considered for ''promotion'' to the status of surrogate end-point. They are often used as secondary outcome measures in phase-III trials which provide data on responsiveness, confirm mechanism of action and compile information for promotion of biomarkers to surrogate outcome measure. During phase-III trials with ivacaftor, cystic fibrosis transmembrane conductance regulator (CFTR) correctors (Vertex Pharmaceuticals Ltd, Cambridge, MA, USA) and ataluren (PTC Therapeutics Inc., South Plainfield, NJ, USA) in patients with cystic fibrosis, CFTR biomarkers are being used as end-points. These new therapies address the basic defect in cystic fibrosis and may be particularly well suited for people with early or mild lung disease.

To gain acceptance by researchers and licensing bodies, an outcome measure must be assessed for clinimetric properties, such as reliability, validity and responsiveness to treatment (table 1). Reliability (*e.g.* an assessment of the consistency of a given measurement), is important, both in terms of inherent biological variation (repeatability) and also in relation to differences across different assessors and centres (reproducibility). To optimise reliability in multiple centre trials, standardised operating procedures (SOPs) and training are needed [12]. Validity refers to clinical and biological relevance; in other words, there must be a direct link with the disease process and the mechanism of action of the intervention [13]. The outcome measure should correlate with established measures of treatment benefit or a gold standard (*i.e.* concurrent validity and predictive validity) and reflect clinical severity (*i.e.* discriminate validity) [14]. When a gold standard is not available, evaluation of convergent validity can be performed (*i.e.* an outcome measure can be compared with another which measures the same attribute). Prediction of prognosis is also important, for example, the ability to predict survival (predictive validity). Responsiveness refers to the ability of the measurement to detect change due to an intervention known to alter the attribute of interest.

Also important in the development of surrogate end-points is feasibility, referring to financial, practical and ethical considerations, as well as patient and assessor acceptability [15]. A feasible end-point should be cost-effective, pose minimal risk/discomfort to the patient and should be applicable throughout the entire range of ages and disease severities. Feasibility will determine whether outcome measures gain acceptance into research practice. Clinimetric properties and feasibility are population and situation dependent.

SCOPE AND PURPOSE OF THE GUIDELINE

This guideline documents the European Cystic Fibrosis Society (ECFS) Clinical Trial Network's current agreement on aspects of CFTR biomarkers for use in clinical trials in the area of cystic fibrosis. After preparatory work over a period of 6 months, participants met twice to discuss their results and conclusions (November 17 and 18, 2010, and June 9, 2011). This resulted in a draft document that was circulated among all participants and further amended.

After a description of the CFTR biomarkers, we explore the clinimetrics and the feasibility of the chosen outcome measures, we report on their use in clinical trials and we conclude by answering the following questions. 1) Do CFTR bio-assays have the potential to become surrogate outcomes? 2) For what kind of therapeutic trial is this outcome appropriate (therapeutic aim, phase of trial, target population, trial duration, number of patients involved and number of sites involved)? 3) Within what time frame can change be expected and what treatment effect can be considered clinically significant? 4) What are the most needed studies to further define these outcome measures in patients with cystic fibrosis?

The guideline also provides an inventory of the literature on selected CFTR bio-assays. We chose to include papers published since 1980 only. It is hoped that this document will offer some guidance for pharmaceutical companies, investigators and regulatory authorities.

CFTR BIO-ASSAYS

CFTR biomarkers measure the presence and/or function of the CFTR protein in different organs. We chose to discuss the sweat chloride test, NPD measurements and intestinal current measurements (ICMs) because they are functional assays and not only document the presence of CFTR, but also its ion transport activity. As such, they are most appropriate for use in clinical trials of compounds aiming to correct the basic defect in patients with cystic fibrosis, *e.g.* gene therapy and small molecules such as CFTR potentiators, correctors, and premature termination codon suppressors [16–21]. These biomarkers of CFTR function are currently used to confirm the diagnosis of cystic fibrosis [22–25]. Since values for these biomarkers differ in cystic fibrosis *versus* non-cystic fibrosis subjects, it seems logical to hypothesise that, when treatments correct the basic CFTR defect at the protein level, the values for these biomarkers will change as well.

After stimulation of sweat production by pilocarpine iontophoresis and collection of sweat in a gauze or collector (Macroduct®; Wescor Inc., Logan, NV, USA), the sweat chloride concentration is determined by original titration with colorimetric end-point, by titration with coulometric end-point (chloridometer), by *in situ* selective electrode (Exsudose®; TemSega, Lormont, France) or by indirect potentiometry [26]. The increase in sweat chloride concentration in cystic fibrosis is the consequence of decreased chloride re-absorption *via* CFTR in the water impermeable sweat ducts [27]. NPD and ICM measure the voltage potential or electrical current, respectively, resulting from epithelial ion fluxes

TABLE 1 Definitions and justification of importance for clinimetric/psychometric properties

Clinimetric/ psychometric property	Definition	Justification of importance
Reliability	Degree to which a measurement is consistent and free from error	Important to quantify error (systematic and random) so that true changes can be discerned from changes due to normal fluctuations
Validity	Concurrent validity: degree to which a test correlates with a ''gold standard'' criterion test which has been established as a valid test of the attribute of interest	The gold standard outcome measures are often not feasible; therefore, it is important to know how an alternative outcome measure compares to the gold standard, and how different outcome measures compare
	Convergent validity: degree to which a test correlates with another test which measures the same attribute	It is important to know the ability of outcome measures to discriminate between different groups
	Discriminate validity: degree to which a test differentiates between groups of individuals known to differ in the attribute of interest	
	Predictive validity: degree to which an attribute can be predicted using the result of a predictor test/or degree to which prognosis can be predicted	
Responsiveness	Degree to which a test changes in response to an intervention known to alter the attribute of interest	Important attribute of tests used in clinical practice or research to assess treatment benefit (*e.g.* to identify improvements response to an intervention)

at the mucosal surface *in vivo* and *ex vivo*, respectively. The NPD measurement is thought to provide information on both sodium absorption and chloride secretion [28, 29]. In normal airway epithelia, sodium absorption is the primary ion transport activity so that the resulting airway surface potential difference is negative with reference to the interstitium. Perfusion of the ENaC channel blocker amiloride will lead to a less negative potential difference. Creating a chemical gradient for chloride by superfusion of chloride free solution followed by activation of the CFTR channel with isoproterenol, will lead to chloride secretion and thus again a more negative potential difference. In contrast, in cystic fibrosis subjects there is heightened ENaC mediated sodium absorption due to absent or dysfunctional CFTR [30–32]. The resultant baseline potential difference is thus more negative. The change with application of amiloride is larger, whereas minimal or no change in potential difference is seen upon stimulation of chloride secretion through CFTR dependent pathways. Recently the notion of increased sodium absorption in cystic fibrosis epithelia has been challenged by data in the newborn cystic fibrosis pig and in cultured tracheal epithelia [33, 34]. In these models, the defective CFTR chloride current seemed sufficient to explain all phases of the NPD measurement. For ICM, an intestinal (usually rectal suction) biopsy and special micro-Ussing chamber are needed for measurement of *ex vivo* transepithelial short-circuit current (Isc) as a measure of net ion fluxes across the tissue. In cystic fibrosis, the intestinal CFTR-mediated chloride secretion is impaired, while absorptive processes remain unchanged or may be enhanced. In cystic fibrosis, the normal Isc response to forskolin, an activator of CFTR, is absent or reduced. The Isc responses to carbachol and histamine consist of two components: a lumen-positive current that is most likely caused by the apical potassium efflux, and a lumen-negative current, caused by apical chloride secretion. In ICM of healthy individuals, the apical potassium efflux in reaction to carbachol and histamine is masked by the much larger chloride efflux. In cystic fibrosis, the response is reversed due to the apical potassium efflux in the absence of a chloride efflux, or biphasic due to residual CFTR-mediated chloride efflux in milder forms of cystic fibrosis [35–37].

CLINIMETRICS OF CFTR BIO-ASSAYS

For NPD, data were collected on reliability (table 2), validity (table S1, online supplement) and responsiveness (table 3). Eight studies document reliability of NPD and demonstrate that with repeated measurements, the mean results per group and the diagnostic conclusions do not differ; however, the within-subject variability is considerable. There is strong evidence that NPD has excellent discrimination validity. 25 studies consistently show a statistically significant difference in chloride and sodium conductance between patients with cystic fibrosis and healthy controls. In patients with ''questionable'' cystic fibrosis, NPD composite scores provided a highly sensitive tool to diagnose patients as ''CF-likely'' and ''CF-unlikely'', with both cohorts having significantly different disease presentation [39, 77–79]. Data from studies with ataluren, ivacaftor, the CFTR corrector VX-809 and gene therapy confirm that NPD is a responsive end-point. In the earliest gene therapy trials, the overall results were not uniformly conclusive. The low subject numbers of subjects along with the relatively low bioactivity of the agents tested may explain these non-significant results for NPD. Tables S2 and S3 in the online supplement report reference values for NPD measures in patients with cystic fibrosis and in healthy controls. The majority of the available data concerns adults.

Reliability data for sweat chloride are inconclusive as these are mainly from retrospective studies with few, or combined cystic fibrosis and non-cystic fibrosis individuals (table S4, online supplement). Data clearly establish validity of sweat chloride, which discriminates between patients with cystic fibrosis and non-cystic fibrosis individuals, between patients with cystic fibrosis and carriers (table S5, online supplement) and between patients with different disease severity (*e.g.* patients with pancreatic sufficiency and insufficiency). Individuals grouped according to their sweat chloride result had significantly different disease presentation. The sweat chloride concentration has been used as an end-point in studies of ivacaftor and VX-809 which clearly demonstrated responsiveness of this parameter (table 3). However, one study investigating ataluren demonstrated a

TABLE 2 Nasal potential difference (NPD) reliability

First author [ref.]	Subjects n	Subject type	Measurements n	Basal potential p-value	Δ amiloride p-value	Δ low chloride + isoproterenol p-value	Statistic
When NPD measurements are repeated in patients with CF, non-classic CF or questionable CF, the mean results per group do not differ							
VERMEULEN [38]	14	CF (floor)	2	ND	NS	NS	Wilcoxon
	16	CF (turbinate)	2	ND	NS	NS	
JARON [39]	17	Questionable CF	2	NS	NS	NS	Paired t-test
YAAKOV [40]	25	CF	2	NS	NS	0.07	Paired t-test
	43	Non-classic CF		0.008	NS	NS	
When NPD measurements are repeated in patients with CF, non-classic CF, questionable CF or non-CF, the individual "diagnostic" conclusion remains the same but the intra-patient variability for individual NPD parameters is considerable							
MIDDLETON [41]	6	CF	⩾4	7.5	ND	1.5 (low chloride only)	Within subject SD
	6	Non-CF	⩾4	2.1	ND	3.3 (low chloride only)	BA limits of agreement
	46	CF	2	18.8, -9.8 mV	10.3, -15.3 mV	3.1, -4.1 mV	R and L nostril difference
	40	Non-CF	2	11.2, -10.4 mV	7.6, -7.2 mV	14.0, -23.6 mV	(mean difference ±2×SD)
VERMEULEN [38]	14	CF (floor)	2	ND	ND	10.2, -11.5 mV	BA limits of agreement
	16	CF (turbinate)		ND	ND	11.5, -12.7 mV	(mean difference ±2×SD)
	34	Non-CF (floor)				21.9, -19.0 mV	
	38	Non-CF (turbinate)				17.4, -20.2 mV	
YAAKOV [40]	25	CF	⩾2	ND	ND	7, -10 mV	BA limits of agreement
	43	Non-classic CF		ND	ND	12, -12 mV	(mean difference ±2×SD)
AHRENS [42]	±10	CF	9	6.0, 8.5	7.5, 11.4	3.5, 9.3	Within subject SD (min. and max. for 4 sites estimated from fig. 1)
DELMARCO [43]	35	CF	2	6.4 %	ND	ND	CV
	18	Questionable CF		10.5 %	ND	ND	
	16	Non CF		9.5 %	ND	ND	
HOFMANN [44]	14	CF (subcutaneous)	2 (3 min)	r=0.97, p<0.05, variation 3.9%	ND	ND	Not reported
			2 (>1 month)	r=0.78, p<0.05, variation 11.3%	ND	ND	
	82	CF (epicutaneous)	2 (3 min)	r=0.92, p<0.05, variation 4.3%	ND	ND	
			2 (>1 month)	r=0.73, p<0.05, variation 14.3%	ND	ND	
ALTON [45]	7	Non-CF	6 (7 weeks)	16%	ND	ND	CV

CF: cystic fibrosis; ND: no data; NS: nonsignificant; BA: Bland–Altman; R/L: right/left; CV: coefficient of variation.

TABLE 3 Responsiveness of nasal potential difference (NPD) and sweat chloride concentration

First author [ref.]	Subject n and type	Intervention	Basal potential p-value	Δ amiloride p-value	Δ low chloride + isoproterenol p-value	Sweat chloride	Statistic
The total chloride response (low chloride + isoproterenol) improves during treatment with ataluren t.i.d. in phase-II open label trials in children and adults with CF carrying at least one nonsense mutation							
SERMET-GAUDELUS [19]	30 CF with nonsense mutation	Ataluren 4, 4, 8 mg (14 d)	NS	NS	0.04 (-4.6 mV)	ND	Paired t-test
		Ataluren 10, 10, 20 mg (14 d)	NS	NS	0.05 (-3.9 mV)	ND	(mean change)
KEREM [18]	53 CF with nonsense mutation	Ataluren 4, 4, 8 mg (14 d)	NS	NS	0.0001 (-7.1 mV)	NS	Paired t-test
		Ataluren 10, 10, 20 mg (14 d)	NS	NS	0.03 (-3.7 mV)	NS	(mean change)
Inconsistent findings whether systemic administration of aminoglycoside changes NPD or sweat chloride values in patients with CF							
Local administration of gentamycin nose drops improves NPD read-out in CF patients carrying at least one nonsense mutation							
15 d intravenous gentamycin treatment							
SERMET-GAUDELUS [46]	9 CF with Y122X mutation		NS	0.09 (20 to 15 mV)	0.04 (-0.8 to -4.6 mV)	0.03 (109 to 85 mmol·L^{-1})	Wilcoxon (mean before and after)
	4 CF with other nonsense mutation		NS	NS	NS		
	5 CF without nonsense mutation		NS	NS	NS		
15 d nasal aminoglycoside treatment							
CLANCY [47]	11 CF with nonsense mutation		NS	NS	NS	NA	Paired t-test
	18 CF without nonsense mutation		NS	NS	NS	NA	
7 d intravenous gentamycin treatment							
CLANCY [48]	5 CF with nonsense mutation		NS	NS	NS (4/5)	NS	GLM for repeat measures (number of patients with ⩾1 reading ⩾5 mV)
	5 CF without nonsense mutation		NS	NS	NS (0/5)	NS	
14 d gentamycin nose drops t.i.d.							
WILSCHANSKI [17]	11 CF homozygous nonsense mutation		0.008 (-48 to -34 mV)	0.05 (33 to 24 mV)	0.001 (0.4 to -5.5 mV)	NA	t-test/MWU p-value (mean before and after)
	8 CF heterozygous nonsense mutation		NS	NS	0.04 (-05 to -4.8 mV)	NA	
	5 CF homozygous F508del		NS	NS	NS	NA	
14 d gentamycin nose drops t.i.d.							
WILSCHANSKI [49]	9 CF with nonsense mutation		NS	NS	<0.001 (-0.6 to -10 mV)	NA	MWU (mean before and after)
Systemic administration of VX-770 to CF adults and children carrying at least one G551D mutation is associated with large drop in sweat chloride and a moderate improvement of total chloride response measured by NPD							
ACCURSO [20]	20 CF with G551D mutation	VX-770 75 mg *b.i.d.* 14 d	ND	ND	0.003 (-4.7 mV)	<0.001 (-40 mEq·L^{-1})	Paired t-test (mean change from baseline)
		VX-770 150 mg *b.i.d.* 14 d	ND	ND	0 .01 (-5.3 mV)	<0.001 (-42 mEq·L^{-1})	
		VX-770 150 mg *b.i.d.* 28 d	ND	ND	0.02 (-3.5 mV)	0.008 (-60 mEq·L^{-1})	
		VX-770 250 mg *b.i.d.* 28 d	ND	ND	0.05 (-5.5 mV)	0.02 (-38 mEq·L^{-1})	

TABLE 3 Continued

First author [ref.]	Subject n and type	Intervention	Basal potential p-value	Δ amiloride p-value	Δ low chloride + isoproterenol p-value	Sweat chloride	Statistic
Ramsey [50]	161 CF with G551D mutation	83 VX-770 150 mg *b.i.d.* 48 wks	ND	ND	ND	<0.0001 (-49 mEq·L^{-1})	MMRM (mean change from baseline through 24 wks)
		78 placebo	ND	ND	ND	NS (-0.8 mEq·L^{-1})	
Aherns [51]	52 CF (6–11 yrs) with G551D mutation	26 VX-770 150 mg *b.i.d.* 24 wks	ND	ND	ND	<0.0001 (-56 mEq·L^{-1})	MMRM (mean change from baseline through 24 wks)
		26 placebo	ND	ND	ND	NS (-1.2 mEq·L^{-1})	
Systemic administration of VX-809 to CF patients homozygous for F508del is associated with a small, dose-dependent drop in sweat chloride							
Clancy [21]	89 CF homozygous F508del mutation	VX-809 25 mg *q.d.* 28 d	ND	ND	NS	NS	Paired t-test (mean change from baseline); linear trend test p=0.0013
		VX-809 50 mg *q.d.* 28 d	ND	ND	NS	NS	
		VX-809 100 mg *q.d.* 28 d	ND	ND	NS	<0.05 (-6 mEq·L^{-1})	
		VX-809 200 mg *q.d.* 28 d	ND	ND	NS	<0.01 (-8 mEq·L^{-1})	
After treating patients homozygous for F508del with VX-809 for 14 days, the addition of ivacaftor 250 mg b.i.d. for 7 days is associated with a further small but statistically significant drop in sweat chloride							
Boyle [52]	61 CF homozygous F508del mutation	VX-809 200 mg *q.d.* 14 d	ND	ND	ND	<0.01 (-4.2 mEq·L^{-1})#	Paired t-test mean change from day 14 or baseline#
		+VX-770 150 mg *b.i.d.* 7 d	ND	ND	ND	NS (-2.2 mEq·L^{-1})	
		+VX-770 250 mg *b.i.d.* 7 d	ND	ND	ND	p<0.001(-9.1 mEq·L^{-1})	
NPD parameters detect effect of treatment in phase-II trials of various modes of gene therapy							
Konstan [53]	12 CF	Compacted DNA nano-particles in saline 0.8 mg, 2.67 mg or 8.0 mg, single dose	No change	ND	8 out of 12 subjects showed partial to complete response	NA	Descriptive
Noone [54]	11 CF	EDMPC cholesterol complexed with CFTR cDNA 0.4375 mg, 1.3 mg or 4 mg total dose	NS	NS	NS	NA	Paired t-test
Alton [55]	16 CF	pCF1-CFTR cDNA complexed with 229 mg GL-67/DOPE/DMPE-PEG$_{5000}$ single dose	NS	NS	NS	NA	MWU and Wilcoxon rank sum
Zabner [56]	9 CF	pCF1-CFTR plasmid 1.25 mg, single dose	NS	NS	p<0.05 (3 mV to -3 mV)	NA	Not reported (mean before and after)
		pCF1-CFTR plasmid 1.25 mg complexed with 2 mg GL-67:DOPE, single dose	NS	NS	p<0.05 (3 mV to 0.5 mV)	NA	

TABLE 3 Continued

First author [ref.]	Subject n and type	Intervention	Basal potential p-value	Δ amiloride p-value	Δ low chloride + isoproterenol p-value	Sweat chloride	Statistic
Porteous [57]	16 CF	400 μg pCMV-CFTR complexed with 2.4 mg DOTAP cationic liposome, single dose	NS for group 2/8 treated patients demonstrated partial correction	NS for group 2/8 treated patients demonstrated partial correction	NS for group 2/8 treated patients demonstrated partial correction	NA	Not reported
Zabner [58]	6 CF	CFTR cDNA *via* adenovirus vector, single dose					Sign rank statistic
		2×10^9 IU	NS	NS	p=0.04 (2 to -2 mV) (terbutaline)	NA	
		6×10^9 IU	NS	NS	p=0.03 (2 to -0.5 mV) (terbutaline)	NA	
Gill [59]	12 CF	CFTR cDNA *via* DC-Chol/DOPE	NS	NS	NS	NA	Not reported
Caplen [60]	9 CF	CFTR cDNA	NS	p<0.05 (+4 mV)	NS	NA	Not reported
Hay [61]	9 CF	AdCFTR cDNA *via* adenovirus vector, single dose	p=0.01 (-53 to -35 mV)	p=0.02 (37 to 20 mV)	p=0.05 (-5 to -9 mV)	NA	Paired t-test
Middleton [29]	3 CF	DC-Chol:DOPE	NS	NS	NS	NA	Not reported
No observed effect of single dose of CPX on either NPD or sweat chloride parameters							
McCarty [62]	37 CF	CPX, single dose	NS	NS	NS	NS	ANOVA
NPD total chloride response detects effect of Moli1901 (activator of alternative chloride channels)							
Zeitlin [63]	4 CF	Moli1901 (1, 3 and 10 $\mu mol \cdot L^{-1}$)	NA	NA	<0.05 for all doses	NA	Paired t-test *versus* vehicle
NPD total chloride response detects effect of CFTR activation in patients homozygous for F508del mutation							
Rubenstein [64]	10 CF homozygous F508del mutation	Sodium 4-phenylbutyrate 6, 6, 7 g (7 d)	NS	NS	p=0.0055 (5.2 to 0.6)	NS	Paired t-test (mean before and after)
Basal NPD detects effect of aerosolised sodium channel blockers							
Rodgers [65]	10 CF	Amiloride nasal spray Benzamil nasal spray	p<0.0001 (+20 mV) p<0.0001 (+21 mV)	NA	NA	NA	Two way ANOVA
Hofmann [66]	12 CF	Aerosolised amiloride	p<0.05	NA	NA	NA	Independent t-test
Hofmann [67]	41 CF	Aerosolised amiloride (10^{-3} M) (n=16)	+35 mV	NA	NA	NA	No statistics
		Aerosolised benzamil (7×10^{-3} M) (n=5)	+35 mV				

TABLE 3 Continued

First author [ref.]	Subject n and type	Intervention	Basal potential p-value	Δ amiloride p-value	Δ low chloride + isoproterenol p-value	Sweat chloride	Statistic
NPD detects effect of flavonoids on CFTR function							
Pyle [68]	12 non-CF	Quercetin 20 µg:mL single dose	NR	p<0.05 (-7 mV)	p<0.05 (-15 mV)	NA	ANOVA
Illek [69]	25 non-CF	Quercetin (n=15), genistein (n=3), kaempferol (n=3), apigenin (n=4)	p<0.05 (-3 mV)	ND	ND	ND	Paired t-test
NPD detects effect of hypertonic saline							
Middleton [70]	7 non-CF	150 mM 250 mM 500 mM 1200 mM 2000 mM	p<0.05 (6.6 mV) p<0.05 (7.6 mV) p<0.05 (10.0 mV) p<0.05 (13.1 mV) p<0.05 (14.8 mV)	ND	ND	ND	Paired t-test
NPD detects effect of fluticasone propionate on epithelial sodium absorption							
Graham [71]	6 non-CF	Fluticasone propionate	ND	p=0.03 (1.8 to 3.3 mV)	NS	NA	Paired t-test
NPD detects effect of milrinone on epithelial sodium absorption							
Smith [72]	6 CF	Milrinone (perfused during NPD)	p<0.05 (52 to 57 mV)	NS	NS	NA	MWU
Total chloride response increases in response to increased temperature							
Boyle [73]	32 non-CF		NS	NS	0.01 (-4.4 mV)	NA	Paired t-test
PD recorded at the end of Ringers (i.e. basal) and at the end of isoproterenol were more polarised when using agar catheter versus perfusion method							
Solomon [74]	26 non-CF		p<0.05 (-15.9 *versus* -14.0 mV)	NS	p<0.05 (-31.2 *versus* -24.8 mV)	NA	Paired t-test
Basal NPD and amiloride response detects effect of moderate exercise							
Alsuwaidan [75]	7 CF	Cycle ergometer exercise at 80% HR_{peak}	p<0.05	ND	ND	ND	Paired t-test
Hebestreit [76]	9 CF	Cycle ergometer exercise at 85% of VT	p<0.01 (-34 to -7 mV)	p<0.01 (+26 to +16 mV)	NS	ND	Paired t-test

CF: cystic fibrosis; NS: nonsignificant; ND: no data; NA: not applicable; GLM: generalised linear model; MWU: Mann–Whitney U-test; MMRM: mixed-effects models for repeated measurements; NR: not reported; HR_{peak}: peak heart rate, VT: ventilatory threshold. Subject type: CF subjects with a specific mutation can be homozygous or heterozygous for this mutation unless specifically stated.

significant difference in NPD but failed to show a difference in sweat chloride [18]. Subsequently, the sweat chloride test was not included as an end-point in additional phase-II trials of ataluren, but is currently being evaluated in the phase-III trial.

Few studies were found of clinimetric properties of ICM (table S6, online supplement). No studies were found on reliability. ICM has been shown to discriminate between patients with cystic fibrosis and healthy individuals [37, 80–86] and, at a group level, can discriminate between pancreatic sufficient and insufficient patients [35]. Similar to the sweat test and the NPD, patients with cystic fibrosis who were grouped according to their ICM result have been shown to differ in disease presentation: the more chloride secretion measured in the rectal mucosa, the milder the disease presentation [35, 37, 82]. These data provide evidence of sound discriminate validity. ICM has been shown to correlate well with results from CFTR mutation analysis and moderately with sweat chloride [37]. No studies used ICM as an end-point. For reference values we refer to DERICHS *et al.* [37].

FEASIBILITY OF CFTR BIO-ASSAYS

The sweat test has a long tradition and is widely available, relatively noninvasive and easy to perform, but for reliable performance rigorous adherence to standard techniques is needed [26, 87, 88]. The more recent measurements of NPD and ICM are limited to selected centres with expertise. Given the complexity of these tests, strict adherence to SOPs is important.

All three tests can be performed from infancy through to adulthood. However, NPD can be problematic in young children. NPD in infants can be done for diagnostic purposes in single centres with extensive experience in this age group [77, 89]. As such, use of NPD as an outcome measure in clinical trials in infants and preschool children has a limited role. Conversely, ICM may be better tolerated by younger children than in adults because it involves rectal sampling. Obtaining a sufficient amount of sweat can be an issue in some (mainly young) patients. Obtaining valid NPD measurements may be impossible or (temporarily) unreliable in subjects with acute upper respiratory tract infection, extensive nasal polyps or after prior sinus surgery.

The risk of infection is minimal in all three tests when care is taken to discard, disinfect or sterilise equipment as appropriate. Electrical equipment for sweat testing and NPD should be checked annually for current control and leakage. The sweat test is viewed as comfortable and very safe as it uses a low voltage electrical current produced by a battery. Some local erythema lasting a few hours is expected; skin burns can occur when sweat test equipment is not properly handled [26]. A small scab or skin scar can occur when too deep a skin abrasion is performed during NPD measurement. Rarely, a rectal bleed can occur after biopsy taking for ICM [90] which is contraindicated in patients with abnormal haemostasis or portal hypertension.

The cost for equipment is lower for the sweat test than for the NPD or ICM. The sweat test requires staff time to cover sampling and assay. The NPD requires staff time to prepare solutions and catheters/bridges and to perform the procedure. ICM requires an experienced gastroenterologist/cystic fibrosis specialist to obtain the sample, a research nurse and a technician. The time required to perform each test is approximately the same (90–120 min). The sweat test and ICM require clinical space for sample collection and laboratory space for assay. NPD requires sufficient clinical space to accommodate the equipment along with the personnel due to the *in vivo* nature of the test.

Training is required to perform each test. Dedicated laboratory personnel can easily learn sweat collection and analysis assay. NPD and ICM require more extensive training and experience in order to minimise variability of the results (for NPD, correct placement and fixation of the catheter, real time interpretation of readings, including stable baseline and end of response to solutions, and troubleshooting; for ICM, biopsy taking, mounting the tissue in the Ussing chamber, real time interpretation of readings and checking biopsy viability).

COMPARISON OF THE DIFFERENT CFTR BIOMARKERS

The advantages of using sweat chloride as outcome measure are its feasibility, availability and the assessment of CFTR function in an organ unaffected by chronic infection and inflammation. Results evaluating ivacaftor and VX-809 also suggest it is more sensitive to small changes in CFTR activity. The advantage of NPD is that it reflects CFTR function in the respiratory tract (albeit the upper respiratory tract), the organ strongly related to cystic fibrosis survival. Measurements in the lower respiratory tract can be performed bronchoscopically [91] but are too complex and invasive for use in large scale trials. Advantages of ICM include easy application in young children and the ability to measure both chloride and bicarbonate transport. It is anticipated that ICM may have a fast response to CFTR correctors because of the exceptionally high cell turnover in the intestinal epithelium that renews itself within 3–5 days).

Limitations of sweat testing and ICM include that they do not measure CFTR activity in the respiratory epithelium. Limitations of NPD include the large intra-subject variability and the difficulty of performing it in young children. Although it is a painless procedure, some adults are reluctant to undergo a rectal suction biopsy. Other limitations of the ICM are the very low number of centres with expertise and the short viability of the rectal biopsies, which precludes long-distance transport to a central laboratory for analysis.

USE OF SWEAT TEST, NPD AND ICM AS OUTCOME PARAMETERS IN CLINICAL TRIALS TO DATE

Sweat chloride is an appropriate biomarker in clinical trials for systemic therapies only. Marked changes in sweat chloride occurred after administration of the CFTR potentiator ivacaftor to cystic fibrosis subjects with the G551D mutation [20, 50]. In subjects homozygous for the F508del mutation, small changes were seen after intervention with the CFTR corrector VX-809 [21] and moderate changes during combination treatment with ivacaftor and VX-809 [52]. In patients with a nonsense mutation, ataluren improved NPD but not sweat chloride [18, 19]. Therefore, the organ specificity or efficacy might differ between drugs.

In CFTR gene therapy trials, applications of viral and synthetic vectors to the nasal epithelium have resulted in significant changes in chloride secretion on NPD. Interventions with the nonsense mutation read-through drugs (aminoglycosides and ataluren) [17–19] have also been proven to change only the chloride response and not the basal potential nor amiloride response. An improvement in chloride and sodium transport was observed with ivacaftor therapy, the latter only in the

combined data set from the two parts of the trial [20]. In patients exposed to ataluren for 84 days, both components of the total chloride response, the zero chloride response and the isoproterenol response, improved significantly, but the zero chloride improvement was larger [92]. In the ivacaftor trial where both have been measured, changes in sweat chloride concentration were more impressive than changes in NPD readout [20].

For ICM, substantial experience has been gathered in preclinical human *ex vivo* corrector studies [93, 94]. What follows can be taken into consideration when contemplating use of ICM as an outcome parameter. CFTR is the dominant, if not sole, apical chloride channel in the intestine and becomes rate-limiting for transepithelial chloride transport in rectal biopsies at CFTR protein levels below ~20% of wild-type controls. Therefore, a small gain in CFTR expression or function induced by CFTR corrector compounds (*e.g.* from 1% to 5% of wild-type values) will result in a large gain in chloride and bicarbonate secretory current (Isc) (*e.g.* from 5 to 25% of wild-type controls). In contrast to the sweat test and to NPD, ICM performed with bicarbonate rich perfusion fluid provides information on CFTR-dependent bicarbonate secretion, an important and cystic fibrosis-relevant determinant of mucus release, expansion and viscosity [95]. ICM is the only biomarker that can directly assess the beneficial effects of pure CFTR correctors, *i.e.* compounds that allow the mutant F508del-CFTR to reach the plasma membrane [96]. Rescued F508del-CFTR has major gating defects [97] that might be overcome by CFTR potentiators, *i.e.* compounds that increase the opening of the CFTR channel. Since ICM evaluates CFTR activity *ex vivo*, potent potentiators (*e.g.* genistein and ivacaftor) can be applied directly on the tissue removed from the patient under corrector treatment to assess full CFTR activity and hence membrane rescue of the mutant protein. When using sweat test or NPD as outcome measure, pure correctors can only be tested properly *in vivo* by conducting combination trials of correctors with potentiators to overcome the gating defect of the rescued mutant protein [52].

QUESTION 1: DO SWEAT CHLORIDE, NPD AND ICM HAVE THE POTENTIAL TO BECOME SURROGATE OUTCOMES?

Our view is that each of these measures has the potential to be a surrogate outcome since they are *in vivo* (sweat chloride and NPD) and *ex vivo* (ICM) markers of CFTR function. To achieve this, long-term studies with disease modifying drugs need to demonstrate that improvement in CFTR function correlates with improvement in clinically relevant outcomes (increased longevity, patient reported outcomes and decrease in pulmonary exacerbations) or surrogate outcomes (improvement in FEV_1). In patients aged 12 yrs and older, treatment with ivacaftor for 48 weeks led to large improvements in sweat chloride and clinical as well as surrogate outcome measures: a decrease of sweat chloride concentration from a mean of 100 $mmol \cdot L^{-1}$ to below 60 $mmol \cdot L^{-1}$, a mean weight gain of 3.1 kg, a 55% decrease in likelihood of experiencing a pulmonary exacerbation and a mean improvement of 10.6% predicted in FEV_1 [50]. The intermediate results of the ivacaftor trial in 52 children 6–11 yrs of age demonstrate the same improvement in all outcomes: a drop in sweat chloride concentration from a mean of 104 to 60 $mmol \cdot L^{-1}$, a large weight gain, and a mean improvement in FEV_1 of 12.7% pred or 17.4% change from baseline [51]. Concurrent overall changes in the clinical outcome, surrogate outcome and the sweat test result are expected, rather than a close correlation between the improvement in sweat chloride concentration and the improvement in clinical or surrogate outcome. Indeed, the latter are dependent on many variables (including those unrelated to disease mechanism, such as environment, adherence, and exposure to respiratory infections). The ongoing phase-III ataluren trial is expected to provide additional information, and may offer further data supporting the use of sweat test and NPD as surrogate outcome measures.

QUESTION 2: FOR WHAT KIND OF THERAPEUTIC TRIAL ARE CFTR BIO-ASSAYS APPROPRIATE (THERAPEUTIC AIM, PHASE OF TRIAL, TARGET POPULATION, TRIAL DURATION, NUMBER OF PATIENTS INVOLVED AND NUMBER OF SITES INVOLVED)?

Sweat chloride concentration and NPD are particularly well suited for phase-II trials with disease modifying therapies aimed at correcting the basic CFTR defect *via* gene therapy or strategies to rescue or potentiate CFTR protein. Power calculations need to take into consideration the moderate (sweat chloride concentration) to large (NPD) intra-subject variability (for specific values consult the online table) and the uncertainty of the effect size that should be aimed for (see further). For phase-III studies involving systemic drugs, sweat chloride concentration may be the most feasible choice. Given the complexity of the NPD technique and the large intra-subject variability even in sites with great expertise, a large, multicentre trial using NPD as outcome can be challenging and costly, but is presently in progress for the ataluren phase-III trial.

For similar therapies, ICM may be useful in phase-II clinical trials in adults, children and infants with cystic fibrosis. But more information on reliability is required before firm statements can be made. ICM has, at present, most application in preclinical drug testing of potentiators and correctors. As stated above, for ''pure corrector compounds'', only ICM is appropriate.

Cystic fibrosis is a rare disease with at present a slow lung disease progression, especially in young patients. This makes demonstration of real clinical benefit in phase-III studies extremely difficult in children. Therefore, sweat chloride and NPD, being in the causal pathway of the disease, could be considered as efficacy outcome measures in such phase-III trials with disease modifying drugs, especially if a compound has proven efficacy and safety in adults. Efficacy and further safety testing can follow during phase-IV pharmacovigilance. Using a biomarker or surrogate outcome as preliminary proof of efficacy is also suggested in the European Medicines Agency guidance for trials in small populations [98, 99].

QUESTION 3: WITHIN WHAT TIME FRAME CAN CHANGE BE EXPECTED AND WHAT TREATMENT EFFECT CAN BE CONSIDERED CLINICALLY SIGNIFICANT?

The timeline in which changes in the measurement will be detected will depend on the mechanism of action of the drug and on the rate of renewal of the epithelium studied. The kinetics of such changes in humans have not been widely evaluated. During treatment with the potentiator ivacaftor, improvements in sweat chloride concentration and NPD have been demonstrated at the earliest time point measured (3 days and 14 days, respectively) [20]. During treatment with the CFTR

corrector VX-809, alone or in combination with the potentiator ivacaftor, decreases in sweat chloride concentration were reported at day 14 to 21 [100]. Also during treatment with ataluren, changes in NPD readout were present at the first time point measured, *i.e.* 14 days [18, 19].

The magnitude of change which is of clinical significance has not been established for any of the CFTR bio-assays. The mean changes in sweat chloride concentration reported with ivacaftor were large (in the order of 50–60 $mmol \cdot L^{-1}$) [20, 50, 51]. Since, in these trials, all clinical and surrogate outcomes improved, one can conclude that such changes in sweat chloride are clinically meaningful. Further analysis of these data may help to determine if a cut-off value of improvement in sweat chloride concentration can be correlated to a change in clinical benefit. Determining the minimally clinically important difference will be an important parameter for guiding the development of further agents active towards modulating CFTR. A zero chloride plus isoproterenol response above the threshold of -5 to -7 mV is considered significant because it is the cut-off between cystic fibrosis and non-cystic fibrosis subjects in cross sectional evaluation. Prospective phase-III studies still have to provide evidence for this assumption. To assess response in an individual, the correct approach may be to monitor whether a repeated test, measured to monitor the response to an intervention, has changed beyond its natural variability [101]. In the phase-II ivacaftor trial, the improvement in NPD chloride secretion was small, *i.e.* only -3.5 mV [20]; still, the clinical benefit of this drug is very marked. In another trial, small sweat chloride changes were detected with VX-809 therapy [21], whereas no changes in NPD or lung function was observed. Therefore, the relative sensitivity of changes in different outcomes is at present unclear. Is NPD less sensitive than sweat test? Will a CFTR measurement in the respiratory tract give a better prediction of respiratory outcome than, for example, the sweat test? Will modifier drugs differ in their organ specific efficacies? For NPD we need to keep in mind that changes in basal PD and changes in amiloride response reflect sodium transport, whereas changes in zero chloride and isoproterenol response reflect chloride transport. It remains to be determined which of these is most important for disease amelioration.

Only theoretical considerations can be made regarding ICM. Because of the fast renewal rate of intestinal epithelium (3–5 days), test compounds that act by improving CFTR function through effects on *de novo* protein synthesis are expected to show full beneficial effects in less than 1 week, abolishing the need for prolonged testing.

QUESTION 4: WHAT ARE THE MOST NEEDED STUDIES TO FURTHER DEFINE THIS OUTCOME MEASURE IN PATIENTS WITH CYSTIC FIBROSIS?

For sweat test, better knowledge of reliability in genetically well-defined controls and cystic fibrosis patients is needed. For NPD and ICM, further unification of test performance and establishment of normal values for use in multicentre trials are needed. These aims are being addressed by the new ECFS NPD and ICM SOPs and the ongoing multicentre reference data validation study in the ECFS Diagnostic Network Working Group. In addition, the track record of these biomarkers in longitudinal phase-III studies is needed. We must understand which change in CFTR bio-assay is associated with long-term clinical benefit of drug therapy, and how well this associates in individual responses.

CONCLUSION

This document provides a systematic review of the clinimetric properties of CFTR biomarkers and provides supporting evidence for promoting these biomarkers to surrogate end-points. Data collected demonstrate the reliability, validity and responsiveness of NPD. Fewer data were found on reliability of sweat chloride concentration; however, validity and responsiveness are demonstrated. Validity is demonstrated for ICM, but further information is required on reliability and responsiveness. Normal values are collected for all three end-points. Further research requirements are proposed for each end-point. In particular, sweat test and ICM require further supporting data.

There is great interest in biomarkers and surrogate end-points in cystic fibrosis. It is already more than a decade ago that participants in a National Institutes of Health (NIH) workshop challenged statisticians to develop robust metrics to study relationships between surrogate end-points, clinical end-points and interventions [1]. That NIH workshop also highlighted the need to assess data from both epidemiological studies and randomised clinical trials as a source of information on biomarkers when considering promotion to surrogacy [1]. In a small population such as cystic fibrosis, it is all the more important that valuable information is shared and that centres work together to improve clinical research.

STATEMENT OF INTEREST

None declared.

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