

CFTR functional measurements in human models for diagnosis, prognosis and personalized therapy: Report on the pre-conference meeting to the 11th ECFS Basic Science Conference, Malta, 26–29 March 2014

1. Overview

Cystic fibrosis (CF) is a life-shortening genetic disease caused by loss-of-function mutations of the *Cystic Fibrosis Transmembrane conductance Regulator (CFTR)* gene that encodes an anion channel critical for epithelial ion and fluid transport [1]. Almost 2000 gene mutations have been described in *CFTR*, which can be grouped according to their disruptive mechanism on *CFTR* function, and association with residual function and disease severity [2] (www.genet.sickkids.on.ca and www.cftr2.org). These mutations range from no functional protein expression (class I), severely impaired folding and trafficking (class II), gating (class III), conductance (IV), to limited apical expression of normal functioning *CFTR* (class V) associated with significant residual function [3]. A sixth class associated with functional but unstable plasma membrane expression of *CFTR* that associates with severe disease has also been proposed [3].

Recent FDA/EMA approval of the first *CFTR* modulator drug (ivacaftor) for clinical use in patients with the G551D gating mutation and more recent FDA approval for another 8 class III mutations provided strong proof-of-concept that pharmacological manipulation of *CFTR* protein synthesis and/or function can bring clear clinical benefit to CF patients, albeit only for 4–5% of all CF patients. This opened new opportunities to apply the same approach to develop the so-called “*CFTR* protein enhancing drugs” for additional *CFTR* mutations, and so as to extend the applicability of *CFTR*-targeting pharmacotherapy to more CF patients. Nevertheless, the nearly 2000 mutations so far reported in the *CFTR* gene make this a colossal endeavor.

Therefore, CF Patients Associations foster approaches to speed up the process of bringing *CFTR* modulator therapies (including ivacaftor) to a greater number of patients. *CFTR* dysfunction as the basis to stratified or precision or even personalized medicine to treat CF was thus the proposed topic of this seminar which was organized by the Patients’ Associations from the Netherlands, Germany, and France in collaboration with ECFS as a pre-conference meeting to the

11th ECFS Basic Science Conference that took place in Malta, 26–29 March 2014.

Its purpose was to have key experts in the field reviewing and discussing how innovative approaches, including the *ex vivo* use of tissue samples from patients (rectal biopsies, intestinal organoids, cells from nasal scrapings or epithelial cells differentiated from patient-specific induced pluripotent stem (iPS) cells) can be validated for better and more accurate diagnosis, prognosis as well as in preclinical research to determine individual patients responsiveness to *CFTR* correctors and potentiators directly on his/her tissues (personalized medicine). Finally, it was also discussed how these approaches can be explored to expand drug discovery of new compounds using primary tissues for high-throughput approaches or as secondary screening models.

For the generalized usage of these innovative approaches as surrogate markers in clinical trials or in personalized medicine, however, the CF community has to move towards more standardized and accessible formats.

The possibilities arising from basic research are constantly evolving and major breakthroughs in other apparently unrelated areas (e.g. adult stem cell cultures such as intestinal organoids or induced pluripotent progenitor stem cell derivatives) help moving the field forward rapidly. Thus, as a community we should not “write things on stone”, but instead we need to be alert and open to test, validate and introduce new approaches which can be advantageous to CF patients in many respects, namely to increase the sensitivity, specificity and speed of CF diagnosis and also to identify responders to therapies. Our goal should be to diagnose and to treat 100% of CF patients, which is certainly not the case at present.

1.1. General overview of *CFTR* biomarkers by Isabelle Sermet-Gaudelus

The meeting started with an overview by Isabelle Sermet-Gaudelus describing how different existing *in vivo* bioassays have the potential to become a surrogate outcome for clinical trials of *CFTR* modulators [4]. It was highlighted that

Table 1
Comparison of the characteristics of different CFTR biomarkers in clinical use.

CFTR biomarker	Nasal transepithelial potential	Sweat Cl ⁻ test	Rectal transepithelial Cl ⁻ secretion
Target	Reflects CFTR function in the respiratory tract, the organ strongly related to CF survival	Reflects CFTR function in an organ unaffected by chronic infection and inflammation	Reflects CFTR function in an organ usually affected by the disease
Reproducibility/reliability	Not extensively assessed	Not extensively assessed	Not extensively assessed
Validation	Some correlation with respiratory clinical endpoints	Discriminates between healthy and typical CF but more challenging in CF-PS patients and CFTR-related diseases	Some correlation with clinical phenotype (exploratory studies)
Responsiveness	Seems sensitive to changes in CFTR activity, but needs further confirmation	Seems sensitive to small changes in CFTR activity	Ability to detect very low amount of active CFTR in a tissue with exceptionally high turnover (exploratory studies)
Type of study	Phase-II trials	Phase-II and -III trials	Phase-II trials Feasibility to test novel CFTR therapeutics ex vivo
Limitations	Unreliable in subjects with acute upper respiratory tract infection, extensive nasal polyps or after prior sinus surgery Potentially modified by CF-related inflammation, which decreases its specificity and sensitivity Threshold of response not defined	Does not correlate to respiratory improvements Threshold of response not defined	Threshold of response not defined
Feasibility and availability	Requires highly trained staff, available in few centers	Wide	Very low numbers of centers and short viability of rectal biopsies Some patients may be reluctant for rectal biopsy

the available CFTR modulators already allow comparing the measurements deriving from these bioassays with changes in clinical outcomes in the same patients (Table 1). This heralds a new era in CF care where therapeutic choices may be driven by patient specific biomarker responses to a bioactive compound, thus providing rationale for a personalized medicine strategy tailored for every CF patient in the very near future. Sermet-Gaudelus briefly described three CFTR bioassays, which so far have been carried out in three epithelia: sweat gland, airway epithelium and intestine. Below is a summary of the presentation.

1.1.1. Respiratory epithelium and transnasal epithelium chloride (Cl⁻) secretion

The respiratory transepithelial potential difference (PD) is by far the most extensively validated biomarker. This functional examination allows to measure in vivo voltage potential resulting from transepithelial ion fluxes at the mucosal surface, and has mostly focussed on the nasal mucosa [4]. Nasal PD (NPD) has been successfully used to measure CFTR modulator therapy in G551D patients with the potentiator ivacaftor [5]. In this phase 2 study NPD responded to ivacaftor in a dose-dependent fashion to treatment, similarly as was observed for airway cultures. Most importantly, improvements in CFTR ion-channel function in the nasal mucosa were also related to improvements in lung function [6]. These data demonstrate that NPD clearly reflects physiology of the native respiratory tissue and correlates to clinical status, consistent with the fact that this biomarker might predict clinical outcome and can be used as a valuable outcome parameter for future clinical trials.

1.1.2. Sweat gland and sweat Cl⁻ concentration

Sweat Cl⁻ concentration is responsive to CFTR functional rescue, as shown in the phase 2 ivacaftor dose-escalation studies [5]. This responsiveness seems however larger than change in nasal transepithelial Cl⁻ transport suggesting higher sensitivity of the sweat Cl⁻ biomarker to CFTR altering treatment. Indeed, ivacaftor induced a mean change in sweat Cl⁻ of about 40–50 mEq/L, which establishes a “current” in vivo benchmark for CFTR modifier responses [5,6], and reduced sweat Cl⁻ levels to levels lower than the diagnostic threshold of the typical CF (60 mEq/L). New sweat gland biomarkers (see below) that have discriminative power at higher levels of CFTR function (e.g. between pancreatic sufficient CF and healthy controls) may be useful for a clear diagnosis in these treated individuals and as an adjunctive outcome measure of treatment.

The current sweat Cl⁻ measurement is not a perfect surrogate endpoint for CF lung disease, as shown by the limited correlation of its change with improvement in FEV1 in phase 3 studies both with single-drug ivacaftor and combination ivacaftor/lumacaftor in F508del homozygote patients [6,7]. This may be explained by the fact that FEV1 reflects a heterogeneous expression of CFTR in the lung, which is modified by many factors including the environment, whereas sweat Cl⁻ is reflective only of the absorptive activity of CFTR activity in the sweat duct.

1.1.3. Intestinal epithelium and trans-rectal epithelium Cl⁻ secretion

CFTR is the dominant, if not sole, apical Cl⁻ channel in the intestine. Intestinal current measurements on ex vivo

biopsies isolated from the mouse show that expression of 20% of wild type-CFTR protein is already sufficient to normalize transepithelial anion secretion indicating the high sensitivity of this biomarker. Small gain in CFTR expression or function induced by CFTR modulator compounds (e.g., 1–5% of wild-type values) will thus result in a large gain in intestinal Cl^- transport, e.g., from 5 to 25% of wild-type controls (Hugo de Jonge, personal data). Importantly, the aggregate response to multiple secretagogues (carbachol and forskolin) of rectal transepithelial Cl^- transport correlates with Cl^- in sweat. Moreover, it discriminates between pancreatic insufficient CF patients and healthy subjects and is related to pancreatic function and FEV1 [8–10].

This test is very useful when NPD measurements may be impossible, for example because of acute upper respiratory tract infection, extensive nasal polyps or after prior sinus surgery or when infants are too small to allow performing NPD. Moreover, it is done on native epithelium, in a tissue with exceptionally high cell turnover, suggesting that the effect of correction might be seen very early and importantly, it is the only biomarker that can directly assess the beneficial effects of pure CFTR correctors or potentiators *ex vivo*. However, the difficulty of this test is its current limited availability and the impossibility of centralized laboratory because of the short viability of rectal tissue, thus still precluding its widespread use.

1.1.4. CFTR biomarkers: current challenges and future prospects

Before using CFTR biomarkers as a surrogate outcome measure in clinical trials, several issues have to be resolved. Data are needed on repeatability (intra- and inter-assay variation in the same individual). Although a limited number of studies have been performed on this topic, all three bioassays described above seem to have large variability within and between centers. These issues should be improved by test standardization, which is a work in progress led by the European ECFS Clinical Trials Network (CTN) and the Therapeutics Development Network (TDN) of the US Cystic Fibrosis Foundation (CFF) [4]. Reference parameters for nasal and rectal transepithelial Cl^- secretion and, more generally, clinically relevant thresholds of response to CFTR bioactive compounds need to be further investigated. Studies are lacking in infants and preschool children because those tests are difficult to perform (e.g. NPD or diagnostic thresholds have not been established). Studies combining these three biomarkers are required to gain more accuracy in CFTR expression pattern according to the tissues and to implement the most sensitive panel of biomarkers as clinical trial endpoints.

1.2. Innovative methods to assess CFTR function in sweat glands by Tanja Gonska

Gonska pointed out that the current sweat Cl^- test, based on quantitative pilocarpine iontophoresis (QPIT) [11], still serves as the gold standard test to confirm a clinical diagnosis of CF [12]. Further, it is a very responsive biomarker to assess the efficacy of novel CFTR targeting drugs [5]. Despite the good

performance of this test, there are large overlaps of sweat Cl^- test results among healthy controls, obligate heterozygotes (i.e., parent of CF patients who carry just one CFTR mutation), patients with a diagnosis of a CFTR-related disorder carrying 1 or 2 CFTR mutations associated with residual function, and even some patients with a diagnosis of CF [13,14]. Further, based solely on QPIT, patients carrying certain CFTR mutations such as 3849+10kbC>T or D1152H may not be identified as CF [15,16].

The lab of Paul Quinton and the Toronto group have recently jointly developed two new methods to assess CFTR function at different levels of the sweat gland: 1) the sweat gland potential difference (SPD) assay which targets CFTR function at the level of sweat duct capturing its absorptive function; [17] and the β -adrenergic sweat secretion assay which allows assessment of CFTR as secretory channel in the secretory coil of the sweat gland [18].

Gonska explained these two methods in further detail. The SPD can be measured between a topical and an intra-cutaneous placed electrode following sweat stimulation using iontophoresis of a cholinergic, or cholinergic plus β -adrenergic agent(s). Following iontophoresis application of water-saturated mineral oil on the stimulated skin area is important to prevent current shunts [17]. As published previously, the SPD was found to be more negative in patients with CF compared to healthy controls [19], due to loss of the Cl^- diffusion potential across the CF sweat duct [20]. Refinement of the measuring electrode as well as development of an easier-to use software program has now allowed the transfer of SPD into clinical studies. Direct comparison of the SPD and QPIT in subjects with assumed different CFTR functional levels such as healthy controls, obligate heterozygotes, pancreatic sufficient (CF-PS) and pancreatic insufficient CF (CF-PI) patients demonstrated similar discriminatory abilities of both tests. Thus, while the SPD offers an immediate read-out of the result, it does not add functional information for a diagnosis of CF.

The second method Gonska presented was the β -adrenergic sweat secretion test. Again, this test was based on an earlier observation that sweat stimulation with β -adrenergic agents induces small, but significant sweat secretion by the sweat glands which was half maximal in heterozygotes and completely absent in CF patients [21,22]. The introduction of an evaporimeter enabled capturing these low volume sweat secretions and led to the development of a clinically applicable sweat test protocol [18]. This protocol includes intracutaneous injections of 1) a cholinergic agonist, 2) atropine to inhibit cholinergic sweat secretion and 3) a cocktail of β -adrenergic stimuli including isoproterenol, aminophylline and atropine and measurement of the resulting sweat secretion. Under these conditions, the resulting sweat secretion can be recorded by placing the evaporimeter probe on the stimulated and treated (water-saturated mineral oil) skin area. Direct comparison of the β -adrenergic sweat secretory assay and the QPIT in subjects presenting with assumed different CFTR functional levels such as healthy controls, obligate heterozygotes, patients with a CFTR-related disorder, CF-PS and CF-PI demonstrated different discriminatory ability of both tests. As shown earlier, the β -adrenergic sweat secretion was half-maximal in

heterozygotes when compared to healthy controls and completely absent in all CF patients including those with 3849+10kbC>T or D1152H and normal sweat Cl^- results. Clear discrimination among healthy controls, heterozygotes and CF patients, supports this test as being the first functional *in vivo* assay to identify carriers of one CFTR mutation. However, the β -adrenergic sweat secretion assay was not able to discriminate between CF-PS and CF-PI patients, whereas the sweat Cl^- test discriminated between the CF-PS and CF-PI patients in the validation trial. Thus, the β -adrenergic sweat secretion test, which offers immediate read-out results, adds important functional information for a CF diagnosis. Responsiveness of both novel sweat tests to CFTR-targeted treatment is currently investigated in ongoing studies.

2. Gut models

Three presentations focussed on the usage of intestinal tissue from CF patients to assess CFTR function: Nico Derichs and Marcus Mall who focussed on measurements of CFTR-mediated Cl^- secretion in native tissues (rectal biopsies) and Jeffrey Beekman on intestinal organoids.

Originally, two distinct micro-Ussing chamber setups and pharmacological activation protocols to analyze CFTR function directly on native rectal tissues were developed. One setup originally developed at the University Hospital Rotterdam uses traditional re-circulating micro-Ussing chambers adapted to accommodate small human rectal biopsies and a pharmacological protocol that focussed primarily on Ca^{2+} -mediated (i.e. carbachol-induced) Cl^- secretion as the primary outcome of CFTR function in the rectal epithelium [23–27]. The other setup was originally developed at the University of Freiburg and uses continuously perfused micro-Ussing chambers to facilitate studies of the effects of secretagogues and/or pharmacological compounds on CFTR function under different experimental conditions (e.g. in the absence and presence of endogenous CFTR activation) in the same tissue in a strictly paired fashion. The pharmacological protocol developed with this setup relies primarily on cAMP-mediated Cl^- secretion as an outcome of CFTR function [8,28–32].

From these, several independent protocols have been developed more recently providing different measurements of CFTR-mediated Cl^- secretion with different sensitivities (Table 2).

Comparison of these distinct approaches based on published data, including a series of recent studies (see Table 2) indicates that the perfused Ussing chamber setup with testing of cAMP-mediated Cl^- secretory responses as primary outcome measure is more sensitive for quantitative assessment of wild-type and mutant CFTR function. This leads to higher discriminant power of CF patients, especially for those CF patients with residual CFTR function when compared with those without CFTR function. The evaluation of this pharmacological stimulation (with some variations) as a multicenter standardized operating procedure (SOP) both within ECFS-CTN and CFF-TDN using commercially available recirculating Ussing chambers (to enhance standardization) is currently ongoing. Hence, evidence from a series of independent studies suggests that measurements of CFTR-mediated Cl^- secretion in rectal biopsies as a robust and reliable CFTR biomarker that may be useful to assess response of mutant CFTR to modulator therapy [8–10,23,24,29,33,34]. However, it remains unknown whether the usage of commercial equipment will be sensitive to detect residual function in CF-PS (not assessed so far [35]), valuable for CF prognosis, and modulation of mutant CFTR function.

A novel approach of intestinal organoids (also obtained from the rectal biopsies) has been developed which not only allows measurement of CFTR activity in biological samples of unlimited growth potential but also seems to allow additional unprecedented applications of the tissue, including its usage in high-throughput screens for drug discovery (see below).

2.1. Rectal biopsies: intestinal current measurement (ICM) by Nico Derichs

Nico Derichs introduced the topic of intestinal current measurements (ICM) in rectal biopsies, by stating that it has proven to be a sensitive CFTR biomarker for difficult CF diagnoses and is a useful outcome measure to evaluate CFTR

Table 2
Summary of cAMP-induced bioelectric responses in rectal biopsies from non-CF subjects and pancreatic sufficient (PS) and pancreatic insufficient (PI) patients with CF determined by perfused and recirculating Ussing chamber protocols. Assessment of CFTR function in perfused Ussing chambers was performed under open circuit conditions and measurements in recirculating Ussing chambers under short circuit conditions.

	Perfused Ussing chamber (I_{sc-eq} -cAMP)		Recirculating Ussing chamber (I_{sc} -cAMP)			
	Multicenter trial SOP		Original protocol		CFF TDN SOP	ECFS CTN SOP
	Mean \pm SEM	Mean \pm STD	Mean \pm SD			
Non-CF	94.3 \pm 8.6 (n = 19)	55.5 \pm 11.1 (n = 26)	16.6 \pm 14 (n = 28)	4.8 \pm 4.0 (n = 16)	99.5 \pm 71 (n = 17)	59.0 \pm 24.9 (n = 37)
CF-PS	28.1 \pm 2.9 (n = 18)	16.3 \pm 2.8 (n = 12)	9.8 \pm 6 (n = 7)	n.d.	n.d.	25.9 \pm 15.3 (n = 21)
CF-PI	-9.3 \pm 1.7 (n = 27)	-11.3 \pm 0.8 (n = 55)	1.9 \pm 2 (n = 34)	0.2 \pm 0.8 (n = 21)	-5.0 \pm 12 (n = 44)	5.4 \pm 16.7 (n = 40)
Reference	[9]	[8]	[10]	[41]	[35]	Preliminary data [37]

(n) indicates the number of individuals analyzed.

modulating therapies. Historically, differences across Europe in equipment (e.g. perfused vs. recirculating Ussing chambers), protocols (e.g. the order of CFTR stimulating agents) and registration mode have complicated the comparison of results and the establishment of center-independent diagnostic reference data [36]. First diagnostic reference data to sensitively discriminate between CF-PI, CF-PS and non-CF had been published for the ‘original’ recirculating protocol [10]. Consequently, the ECFS Diagnostic Network Working Group and ECFS Clinical Trials Network worked to standardize ICM across European sites (www.ecfs.eu/ecfs_dnwg). Based on previous experience with both protocols, an SOP for ICM is being developed and validated under the auspices of ECFS. This includes a multicentre comparison for the definition of diagnostic cut-offs for discrimination between residual CFTR function in CF-PS and normal CFTR function in non-CF. Preliminary data of this analysis have been presented at the 2013 North-American CF Conference [37]. Importantly, this European SOP for ICM (under implementation in 10 European sites) is being harmonized with the one by the TDN of the Cystic Fibrosis Foundation in the US [35], to allow for transatlantic multicentre trials on CFTR modulation in CF patients with either severe and/or mild *CFTR* mutations.

Based on preliminary data, the ICM performed according to the current European SOP for ICM seems to be highly reproducible [38], sensitive to detect residual CFTR function [37,38], and has excellent responsiveness to both *ex vivo* incubation with CFTR correctors and *in vivo* oral ivacaftor treatment [38,39], suggesting that this multicentre SOP may have great value as CFTR biomarker in preclinical and clinical studies.

Short- and long-term comparisons to other CFTR biomarkers (sweat test, nasal potential difference) and clinical surrogate parameters are underway and will help to define the best strategy for designing next clinical trials of CFTR modulators.

2.2. Assessment of CFTR function in rectal biopsies (perfused chamber protocol) by Marcus Mall

Marcus Mall summarized the development and studies that have been performed with the perfused micro-Ussing chamber setups and discussed potential advantages as well as limitations of this technique. The perfused Ussing chambers were originally developed to study normal ion transport and CF pathophysiology in the human colon. The use of continuous perfusion approach has made it possible to study effects of secretagogues under different experimental conditions (e.g. in the absence and presence of cAMP-mediated stimulation) within the same tissue. These studies demonstrated that CFTR is essential for Cl^- secretion in human colon and that CFTR-mediated secretion relies on co-activation of cAMP- and Ca^{2+} -dependent K^+ channels in the basolateral membrane of the colonocyte that provide the driving force for luminal Cl^- exit through CFTR [28–30].

In patients with CF, assessment of CFTR function by this protocol allows discrimination between two functional

phenotypes, a group with little or no detectable CFTR function and a smaller group (~15–20%) with residual CFTR function evidenced by an attenuated Cl^- secretory response to cAMP-dependent activation [8,9]. Further, two international multicentre studies including patients with rare CFTR genotypes and atypical CF phenotypes at study sites in Europe and South America demonstrated that CFTR function determined by this technique correlates with the CFTR genotype and CF phenotype. CF patients lacking detectable CFTR function typically carry two severe CFTR mutations (classes I to III) and present with a classical CF phenotype with exocrine pancreatic insufficiency (PI), whereas CF patients with residual function carry at least one mild CFTR mutation (classes IV and V) and present with a milder or atypical form of CF including pancreatic sufficiency [8,9,40].

A recent proof-of-concept study using the first generation potentiator 1-EBIO (1-ethyl-2-benzimidazolone), a K^+ channel opener demonstrated to potentiate CFTR-mediated Cl^- secretion, demonstrated that this ICM protocol is sensitive to detect pharmacological potentiation of mutant CFTR function in native rectal tissues from patients with a large spectrum of CFTR genotypes [34]. These results suggest that ICM may be a sensitive quantitative endpoint of CFTR function in studies investigating the response of F508del-CFTR, but also rare CFTR mutations to emerging CFTR modulators in individual patients, and may thus guide the implementation of individualized mutation-specific therapy for patients with CF.

Taken together, these studies suggest that this ICM protocol provides a sensitive biomarker of CFTR function that can be applied for diagnostic workup of patients with equivocal diagnosis and characterization of CFTR mutations with unknown functional consequences, and provide valuable information. Compared to other ICM techniques (see Table 2), the perfused Ussing chamber protocol detects substantially higher cAMP-induced currents in non-CF rectal tissues suggesting that it may also be more sensitive to quantify residual CFTR function in CF (Table 2). This quality may be critical when ICM is considered as a novel quantitative endpoint in preclinical and early clinical testing of emerging mutation-specific therapies for CF [8–10,41].

2.3. Intestinal organoids by Jeffrey Beekman

Recent advances in adult stem cell culture technology have enabled the long-term expansion of human intestinal epithelial tissue *in vitro* (reviewed in [42]). This technology was applied to expand intestinal CF tissue using biopsies from ICM measurements. Crypts are isolated from biopsies and are placed in a 3D-culture environment containing essential growth factors that maintain the “stemness” of the epithelial stem cell compartment. The crypts rapidly close and grow into self-organizing spheroid structures consisting of a single epithelial cell layer and containing multiple crypts and villus-like domains surrounding a central lumen. Forskolin-induced luminal fluid secretion is completely CFTR dependent and can be accurately quantified by measurement of organoid swelling using live cell imaging [43].

A clear advantage of this approach is the relative ease of culturing intestinal organoids when compared with other existing primary cell models for CF such as primary airway epithelial cell cultures that require multiple culture conditions and transfer to filters for terminal differentiation at the air–liquid interface. Within weeks after culture, a large dataset can be generated in which CFTR residual function and response to (combinations of) CFTR modulators is able to be accurately measured for an individual patient sample. Importantly, CFTR residual function measurements in organoids can quantitatively reflect ICM measurements in the biopsies from which the cultures were derived [43]. After measurements of currently available CFTR restoring drugs, cells can be biobanked for future analyses of new therapeutic agents without requiring further sampling and patient discomfort. This assay can thus play an important role in prospectively defining optimal (combination of) treatments for patients in a cost-effective and patient-specific manner.

Intestinal organoids can also play an important role at the preclinical phase of CFTR modulator development [44]. The ease of culture, and straightforward and robust CFTR function measurement would allow rapid development of high-throughput drug discovery approaches for CF in a primary cell model for the first time. In collaborations with Dr. Lukacs and Dr. Bear, the intestinal organoid model has been shown to be suitable for identification of compounds that synergize with VX-809 in correcting CFTR-F508del, and showed similar results for interactions between CFTR modulators and isolated CFTR in proteoliposomes [45,46].

Finally, novel advances in gene editing of adult stem cells may offer the development of novel disease models and regenerative medicine approaches for CF. Schwank et al. recently published the functional repair of CFTR by CRISPR/Cas9 technology in intestinal organoids [47]. This proof-of-concept study has indicated that CFTR gene corrected clonal intestinal stem cells can be selected and characterized in vitro. This remains a highly experimental approach, yet may lead to future therapies in which the patient's own stem cells are genetically repaired in vitro and used to replace resident tissue stem cells for the regeneration of disease-free tissue in affected organs. For now, this approach is ideal to generate novel disease models and reporter cells for drug discovery and scientific studies.

3. Airway models

Four presentations focussed on the use of airway cells from CF patients to assess CFTR function, drug discovery and potentially other applications: Anil Mehta, Margarida Amaral and Mitch Drumm who focussed on primary cultures of lower and upper respiratory tract airway cells and Ulrich Martin on stem cells.

Indeed, despite the acceptance of rectal tissue in diagnosis and prognosis [39], it still involves some degree of invasiveness and requires an experienced gastroenterologist/ endoscopist who is not always available at all CF centers. Moreover, it is often claimed that CFTR function would be most appropriately determined in the airways, as the major focus of CF morbidity and mortality.

Monolayers of primary human bronchial epithelial (HBE) cells have thus been used in many studies as the gold standard for validation of both mechanisms of CF disease and for pre-clinical validation of CFTR modulator therapies.

Despite their outstanding value, primary HBE cells are scarce, difficult to obtain and not easily grown at every point-of-CF care. These reasons prevent its more widespread use in CF diagnosis, in determining prognosis and in assessing response to therapy in a prospective manner. Growth limitations also limit the application of HBE cells in drug discovery programs.

However, innovative approaches are emerging which seem to allow the usage of nasal cells for more widespread use. Similarly, new possibilities are arising from increasing knowledge on how to generate induced pluripotent stem cells (iPSCs), which grow indefinitely and thus have potential for use in high-throughput drug screening projects. These recent advances help moving the CF field forward at a much faster pace.

3.1. Nasal cells by Anil Mehta

The airway epithelium lining the inferior turbinate bones of the nose, when cultured ex vivo at an air liquid interface (ALI) culture recapitulates many of the ion transport characteristics of the airways of the lung [48]. Because of the relative ease of access and the minimal discomfort during nasal scrapings or brushings, this multicellular epithelial source has become the de facto “standard” for many groups. However, many different (and often unclear) protocols are used to culture cells that may impact ion transport characteristics and response to modulators which limits uniformity in data generated between different centers [49].

For example, two recently published methods provide a compendium of the applied protocols that underscore two different approaches: the use of serum-free culture media with the addition of synthetic hormones and growth factors [50] or the use of media supplemented with Ultrosor G serum substitute [51]. In addition, a debate is still ongoing regarding the levels of transepithelial resistance (TER) that airway (and in particular nasal) cells have to achieve in culture and the meaning of the observed values of current when compared to data obtained from analysis of isolated biopsies of airway mucosa [52]. Despite the presence in the literature of reports attesting the effects of different concentrations of hormones and growth factors for the culture of airway cells, at present an agreement on cell culture conditions as one or more SOPs has not been established yet [49].

One of the objectives of the recently created ECFS Working Group on Basic Science of CF is to standardize all of the above. An equally important goal is the interpretation of the resultant ion transport characteristics that is currently hampered by the paucity of normal ranges from healthy subjects. The absence of such apparently simple baseline data hinders the work of newly entrant research groups and biotechnology companies to the field. The overall goal of all the protocols published is to demonstrate the efficacy of the different methods to expand and differentiate isolated cells into fully polarized ciliated

monolayers of pseudostratified airway epithelium, comprising (albeit variable numbers of) basal, goblet and ciliated cells, with a measurable TER and demonstrable vectorial ion transport. Ultimately, these *in vitro* cultures should recapitulate the clinical *in vivo* situation as accurately as possible, and hence, protocols that most robustly correlate with clinical observations are required.

3.2. Primary cultures of lung and nasal cells by Margarida Amaral

Until recently, although useful for many studies [53–59], nasal cells freshly obtained from CF patients could not be used in Ussing chamber for bioelectric measurements because of the relatively low number of cells obtained by this approach (typically $1\text{--}2 \times 10^6$ cells) and limited ability to promote expansion through culturing as primary cultures because of the high cell mortality after a few passages. Notwithstanding, usage of these cells in electrophysiological measurements has been demonstrated previously, albeit through patch-clamping which is a difficult to standardize technique [60].

However, in a recent real breakthrough in epithelial biology, Schlegel et al. reported that normal adult epithelial cells can be induced to a “stem-like” state in which they proliferate indefinitely *in vitro*, through a Rho kinase inhibitor (Y-27632) in combination with fibroblast feeder cells [61,62].

Data were presented confirming these findings applied to nasal epithelial cells obtained by brushing as previously described [57]. Very promisingly, preliminary data indicate that monolayers of these cells can be assessed in perfused Ussing chambers for CFTR-mediated Cl^- secretion, similarly to rectal biopsies (see above) or primary cultures of lung cells.

Further work is ongoing to validate this *in vitro* approach, which integrates the individual’s combination of CFTR mutations plus other favorable or unfavorable variants of modifier genes, for its use in CF diagnosis, disease prognosis and for predicting *in vivo* drug efficacy.

3.3. Induced pluripotent stem cells by Ulrich Martin

Induced pluripotent stem cells (iPSCs) represent another patient / disease-specific cell source for disease modeling and drug screening [63]. Similar to embryonic stem cells, these cells show an unlimited potential for proliferation and differentiation and they can now be readily generated from various somatic cell types including easily accessible sources such as blood [64]. Recent critical advances in the field include scalable mass production [65] and targeted differentiation of such cells [66], indicating that technologies exist for large-scale production of patient and mutation-specific cell lineages. Importantly, with patient-specific iPSCs a “universal cell type” will be available and thus provide the basis for different disease-relevant organotypic *in vitro* models including lung, bile duct and intestine.

Moreover, the development of novel highly efficient genome engineering approaches, including the CRISPR/Cas9 system and TALE Nucleases (TALENs) [67], allows the

generation of CFTR gene-corrected human cells as crucial isogenic controls for studying the effects of a specific CFTR mutation in a given genetic background. Likewise, CFTR mutations of choice can be introduced and studied, e.g. in case of rare mutations for which no homozygous patient is available. Furthermore, iPSC reporter lines with constitutive expression of halide- or voltage-sensitive fluorescent proteins for functional drug screens or molecularly tagged endogenous CFTR allowing for direct visualization of trafficking can be generated for drug screening purposes.

Although iPSCs can already be differentiated into CF relevant cell types such as respiratory derivatives [68] and bile duct epithelium [69], existing complex and cost-intensive differentiation protocols definitely represent the major bottleneck for utilization of iPSCs in CF disease modeling and drug screening. With the likely development of improved culture techniques during the next few years, genetically engineered patient-specific iPSC derivatives will provide a basis not only for a better understanding of the different CF disease phenotypes but also for identification of further clinically applicable drugs that are able to functionally correct the organ-specific consequences of the different classes of CFTR mutations.

3.4. Modification of human respiratory cells by Mitch Drumm

The ability to expand primary airway epithelial cells provides immense potential for both mechanistic studies as well as a resource for drug screening. However, the limitations of cells expanded through this method are currently not fully understood. One use of the cells is the creation of matched, isogenic cells in which the only genetic difference is the CFTR genotype. To create such cells, gene editing procedures are being explored, such as zinc-finger nucleases and the CRISPR/Cas9 system. Although these procedures are efficient, there is significant variation among cells exposed to the procedure as some cells will be heterozygous for an intended mutation, some homozygous and some not mutated at all. Thus, selection and growth of clonal cells with identical gene-editing events are necessary to achieve pure populations for optimal analysis. Data from Mitchell Drumm (and also generated by Calvin Cotton at Case Western Reserve University) demonstrated that single-cell clones in which the CFTR gene had been inactivated by CRISPR/Cas9-mediated mutagenesis could be created and expanded. To select potential gene-edited cells, cells were transfected by CRISPR guide RNA recognizing CFTR and a Cas9-expressing plasmid also expressing GFP. This allowed efficient sorting of GFP expressing cells using fluorescence-activated cell sorting. Cells were subsequently seeded on irradiated 3T3 fibroblast feeder layers and expanded. Clones were subsequently moved to transwell filters for polarization and those cells are currently being analyzed for their ability to differentiate at air–liquid interface (ALI) culture conditions and their electrophysiological and gene expression properties. These preliminary studies indicate the primary cells can be genetically manipulated and that clonal selection is possible.

4. Conclusion

Clear progress has been made over the recent years in developing standardized objective readouts for individual CFTR residual function and the use of such measurements for CF diagnosis, defining disease severity (prognosis) and the *in vivo* efficacy of CFTR modulating therapy. Many of the different techniques show distinct dynamic ranges for the measurement of CFTR function, indicating that these multiple approaches are required to differentiate between distinct levels of CFTR function that are associated with severe and milder forms of CFTR-related diseases and the various CFTR-targeting therapies.

In addition, novel advances in stem cell culture technology indicate that *in vitro* cultures of patients can play a role in measuring CFTR residual function and prospectively defining the optimal (combination of) therapy for each individual with CF. These models will also allow for the development of more effective drug screening platforms. Emphasis is required on using tissues and culture protocols that combine accurate prediction of clinical disease characteristics with as limited patient discomfort as possible.

For both the *in vivo* and *in vitro* biomarkers, reference values and thresholds for CF diagnosis, correlation to disease severity and significant responses to therapy need to be further established. European research efforts should be coordinated to speed up the promising developments presented at this conference and to jointly potentiate the efforts of the National Patient Associations.

A Working Group on Basic Science of CF has recently been created at ECFS in order to complement the already established European ECFS Clinical Trials Network and the ECFS Diagnostic Network Working Group. The aim is to i) further standardize human *in vitro* models across various European CF research centers to help defining correlations between CFTR genotypes, CFTR function, and CF phenotypes along with response to CFTR-modulating therapies for the individual patient, and to ii) further standardize the platforms for research and preclinical drug development across various locations so that they can be used as *bona fide* CF disease biomarkers.

Conflicts of interest

MDA has served as a consultant to Vertex and Galapagos, has been supported to attend and to speak at Symposia (Novartis, Gilead and Vertex) and to participate in an educational grant program by Facilitate Ltd. JMB is an inventor on a patent application describing the CFTR function measurement in intestinal organoids. ND has served as consultant to Vertex and Bayer, and participated in an educational grant program by Facilitate Ltd. No conflicts of interest are indicated for other authors.

Acknowledgments

Work in the authors labs has been supported by: strategic grants PEst-OE/BIA/UI4046/2011 (BioFIG) and FCT/MCTES PTDC/SAU-GMG/122299/2010 from FCT (Portugal) and

CFF-Cystic Fibrosis Foundation, USA (MDA); Myrovitis Trust (AM); the NCFS-Dutch CF Foundation and the Wilhelmina Research funds (JMB); the German CF Association — Mukoviszidose e.V., the Christiane Herzog Foundation, Germany, Cystic Fibrosis Research Inc., USA, and ECFS CTN and ECFS Diagnostic Network Working Group (ND); the German CF Association — Mukoviszidose e.V. (MAM); CF Canada and CFFT (TG); French CF Association — Vaincre La Mucoviscidose (IS-G) and CFF — Cystic Fibrosis Foundation, USA (MD).

The authors are grateful to Sylvia Hafkemeyer (Mukoviszidose e.V.), Veronique Marguerite (VLM), Vincent Gulmans (NCFS), David Debisschop (ECFS), and Christine Dubois (ECFS) for the initiative and organization of the meeting.

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Jeffrey M. Beekman

Department of Pediatric Pulmonology, and Laboratory of Translational Immunology, Wilhelmina Children's Hospital, University Medical Centre, Utrecht, The Netherlands

Corresponding author.

E-mail address: J.Beekman@umcutrecht.nl.

Isabelle Sermet-Gaudelus

Unité de Pneumo-Pédiatrie Allergologie, Hôpital Necker, INSERM U 1551, Université Paris Sorbonne, Paris, France

Kris de Boeck

Pediatric Pulmonology, Department of Pediatrics, University Hospital Gasthuisberg, Leuven, Belgium

Tanja Gonska

Department of Pediatrics, University of Toronto and Research Institute, The Hospital for Sick Children, Toronto, Canada

Nico Derichs

Christiane Herzog Cystic Fibrosis Centre, Pediatric Pulmonology and Immunology, Charité-Universitätsmedizin, Berlin, Germany

Marcus A. Mall

Department of Translational Pulmonology, Division of Pediatric Pulmonology & Allergy and Cystic Fibrosis Centre, Translational Lung Research Centre Heidelberg (TLRC), University of Heidelberg, Germany

Anil Mehta

Division of Medical Science, University of Dundee, United Kingdom

Ulrich Martin

Leibniz Research Laboratories for Biotechnology and Artificial Organs (LEBAO), Department of Cardiothoracic, Transplantation and Vascular Surgery, Hannover Medical School, Germany

Mitch Drumm

Department of Genetics, School of Medicine, Case Western Reserve University, Cleveland, OH, USA

Margarida D. Amaral

University of Lisboa, Faculty of Sciences, Centre for Biodiversity, Functional and Integrative Genomics, Lisboa, Portugal

Corresponding author.

E-mail address: mdamaral@fc.ul.pt.