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Biomarkers for Cystic Fibrosis Drug Development

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Abstract

Purpose—To provide a review of the status of biomarkers in cystic fibrosis drug development, including regulatory definitions and considerations, a summary of biomarkers in current use with supportive data, current gaps, and future needs.

Methods—Biomarkers are considered across several areas of CF drug development, including cystic fibrosis transmembrane conductance regulator modulation, infection, and inflammation.

Results—Sweat chloride, nasal potential difference, and intestinal current measurements have been standardized and examined in the context of multicenter trials to quantify CFTR function. Detection and quantification of pathogenic bacteria in CF respiratory cultures (e.g.: *Pseudomonas aeruginosa*) is commonly used in early phase antimicrobial clinical trials, and to monitor safety of therapeutic interventions. Sputum (e.g.: neutrophil elastase, myeloperoxidase, calprotectin) and

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blood biomarkers (e.g.: C reactive protein, calprotectin, serum amyloid A) have had variable success in detecting response to inflammatory treatments.

Conclusions—Biomarkers are used throughout the drug development process in CF, and many have been used in early phase clinical trials to provide proof of concept, detect drug bioactivity, and inform dosing for later-phase studies. Advances in the precision of current biomarkers, and the identification of new biomarkers with 'omics-based technologies, are needed to accelerate CF drug development.

Introduction

Cystic fibrosis (CF) is caused by mutations in a gene coding the cystic fibrosis transmembrane conductance regulator (CFTR) protein, an ion channel regulating chloride, bicarbonate, sodium and fluid fluxes at epithelial surfaces (1). While there have been steady improvements in outcome, median predicted survival for a newborn with CF in the United States in 2014 was 41 years, and median age of death in 2014 was 28 years (Cystic Fibrosis Foundation 2014 Patient Registry Report) underscoring the need for better treatments. CF lung disease is characterized by defects in ion transport, mucociliary clearance, inflammation, bacterial infection, and airway remodeling that culminates in bronchiectasis and respiratory failure. CFTR may also cause intrinsic abnormalities in host defense cells including epithelia, neutrophils and macrophages.

Given its complex pathophysiology, the U.S. CF Foundation (CFF), academic investigators, industry partners and other sponsoring agencies have taken a multipronged approach targeting the different elements of CF pathophysiology. In particular, efforts aimed at treating *Pseudomonas aeruginosa* infection, thinning CF mucus and restoring airway surface liquid have shown clinical benefit but have not halted decline in lung function. Recently, a new class of agents termed CFTR modulators has had clinical impact (dramatic lung function improvement with ivacaftor for patients with CFTR gating mutations; modest with lumacaftor-ivacaftor for F508del homozygous patients) (2-5). However, other measures more sensitive than lung function may accelerate drug testing and biomarkers are becoming recognized as a critical tool for CF drug development.

A biomarker, by US Food and Drug Administration (FDA) definition, is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or biological responses to a therapeutic intervention" and offers great promise to speed drug development (6). The potential uses of biomarkers in drug development include enabling assessment of safety, efficacy, and patient selection for the purpose of enrichment. The FDA recognizes the importance of biomarkers and drafted a qualification process that addresses aspects of biomarker development and clinical utility (7); here are great challenges to meeting this rigorous qualification process for a rare disease such as CF (8).

To advance biomarkers in CF drug development, the CFF has convened a CF Biomarker Consortium consisting of investigators with particular expertise in biomarker research. This document focuses on biomarker validation and qualification for assessing CFTR function and detection, infection, and inflammation, examines relationships of existing biomarkers to

established clinical outcomes in CF, highlights new technologies for biomarker development, and summarizes areas requiring further development.

Biomarker Validation and Qualification Within The Context of Utilization

As biomarkers for CF drug development are advanced, their application or utilization will dictate the necessary validation and qualification process. All biomarkers to be used in the context of drug development should reliably and sensitively capture the biologic activity of a therapy. Hence, any novel assay, procedure, or test must be validated for its ability to accurately and reproducibly measure a biologic process. Beyond biomarker validation, additional qualification of a biomarker would establish a linkage with clinical outcomes (clinical validity); validation of such a linkage strengthens the utilization of the biomarker throughout later phases of clinical development and in rare cases can promote a biomarker to the level of a surrogate endpoint that substitutes for a clinical endpoint in pivotal registration trials (8, 9).

It is important to recall that the role of biomarkers throughout drug development, and particularly in early phase studies, is to demonstrate biological efficacy of new therapies, confirm mechanism of action, and inform dose selection. Biomarkers used in early phases of drug development do not require qualification, or even evidence of strong correlation with measurable clinical outcomes. In early phases they can be used to explore and inform go/no go decisions regarding later-phases, but notably the level of risk to the overall development program is ultimately dependent on confidence that promising biomarker results will predict clinical efficacy.

Biomarkers typically play a supportive role to confirm mechanism of action in later-phase studies, however in some settings it may be desirable for them to replace traditional clinical efficacy measures in order to streamline drug development by enabling more rapid assessment of efficacy with potentially fewer numbers of patients. In most cases this would require that the biomarker meets qualification standards as a surrogate endpoint for clinical efficacy (7). Surrogate endpoints serve as substitutes for accepted measures of clinical efficacy and can accelerate drug approval by responding over a shorter duration (e.g. compared to survival), or by requiring fewer study participants because of increased measurement precision. The process of qualifying a biomarker as a validated surrogate endpoint for phase 3 trials requires that the marker correlate with a meaningful clinical endpoint and capture the net effect of the intervention or drug on the efficacy endpoint (10). The latter is more difficult to achieve because it demands that late-phase studies simultaneously capture the biomarker and the clinical endpoint to evaluate predictive net effect (11). To date, no biomarker has formally gone through the qualification standards of a surrogate endpoint for clinical efficacy in CF. Importantly, however, there are instances in other rare diseases, such as Fabry's disease, for which a non-validated surrogate endpoint of a physiologic biomarker of disease has been used as a pivotal endpoint in a registration trial (12).

While more traditional drug development focuses on prognostic biomarkers that are correlates of disease outcome and are intervention-independent, there is also a role for

predictive biomarkers in drug development which offer information that is more specific to a particular intervention. Predictive biomarkers have been an area of focus for many years in oncology research (13). In a drug development program, a predictive biomarker may be an analyte, gene, or protein that identifies candidate study participants who are more likely to benefit from treatment and enable enrichment trials that are meant to optimize trial outcomes. As with the more general classification of biomarkers, development and validation of predictive biomarkers generally demands rigorous, multi-staged assessment of their clinical utility through retrospective and prospective controlled trials (14).

As in any rare disease setting, opportunities for flexibility in this rigorous process balanced against level of risk will be necessary given the limited patient population. Strategic utilization of biospecimens and well characterized phenotypic data in conjunction with rigorous study design and close collaboration with regulatory agencies are essential for advancing biomarkers in CF.

Cf Relevant Pulmonary Biomarkers: Current Status and Opportunities

Progressive lung disease is the primary cause of CF morbidity and mortality, and a critical target for therapeutic development. Several therapies have been developed to address CF lung pathologies, and their clinical use has been associated with improved outcomes. The most common primary outcome measures supporting regulatory approval of CF pulmonary therapies are discussed below. Physiologic outcome measures have recently been published (15).

Accepted Clinical Efficacy Measures in CF

Change in FEV_1 has become one of the most established endpoints to demonstrate clinical efficacy in CF clinical trials (2, 4, 16-18). Pulmonary exacerbations (risk, frequency, time to) have also served as primary clinical efficacy endpoints, but typically require larger and longer studies to demonstrate treatment impact. Important secondary measures that have supported approval and clinical use of pulmonary therapies include patient reported outcomes (e.g.CF Quality of Life – Revised instrument), weight gain and bacterial density (4, 18-22). To date, no CF-specific anti-inflammatory drug has gone through regulatory approval and thus there has been little guidance as to what the accepted clinical efficacy measures will be for these agents, which have not produced the type of immediate and sustained FEV_1 improvement observed with mucolytics, antibiotics, or CFTR modulators (23, 24).

Need for More Sensitive Clinical Efficacy Endpoints

Important gaps that have emerged include relevant outcome measures for patients with either mild or advanced lung disease, and young CF patients who typically have both mild disease and poorly standardized outcome measures. Multiple breath washout testing and the Lung Clearance Index as recently published (15) has advanced as a putative endpoint with greater sensitivity than FEV_1 to detect biologic effect in younger patients with mild lung disease, and are currently included in several CF clinical trials. Imaging may serve as a correlate of structural lung injury (e.g. bronchiectasis). The remainder of this review will examine the

existing data supporting the validity and qualifications of several biomarkers of CFTR activity, infection and inflammation, and the investigations needed to optimize these biomarkers for use in CF drug development.

Cftr Activity and Ion Transport in Vivo

There are three *in vivo* biomarkers of ion transport which have primarily been used to monitor restoration of CFTR function. These include sweat-chloride concentration, nasal potential difference (NPD) measurement, and intestinal current measurement (ICM). Sweat-chloride is a simple, portable, and reliable test of CFTR function that clearly discriminates between patients with minimal, partial and full CFTR activity (25, 26). It has been carefully standardized for both clinical use and for incorporation into clinical trials, and remains the mainstay of CF diagnosis. Different levels of CFTR function quantified by the sweat-chloride test also correlate with important markers of disease severity, including age of diagnosis (pre-newborn screening), pancreatic sufficiency, isolated male infertility, microbiology, and lung disease severity (25-30). Profound reductions in sweat-chloride have been observed in all studies of ivacaftor monotherapy in CF patients with gating mutations (3, 5, 31-34). Intermediate effects were observed with ivacaftor in CF patients with the R117H mutation, and smaller effects in CF patients with two copies of the F508del CFTR mutation treated with ivacaftor or ivacaftor/lumicaftor (35-37). The effects of CFTR modulators on sweat chloride generally parallel the clinical benefits observed for these three populations (FEV_1, risk of pulmonary exacerbations – see Table 1). While individual changes in sweat-chloride have not correlated directly with FEV_1 improvements (38, 39), aggregate data have demonstrated excellent assay performance and detection of biologic activity. Future studies utilizing sweat-chloride will need to monitor long term relationships between sweat-chloride and key clinical outcomes in CF to support this biomarker as a key measure to accelerate drug development.

NPD is a direct measure of CFTR function in respiratory epithelium, and isolates CFTR activity across the nasal mucosa independent of sodium transport and the activity of other chloride transporters (40, 41). It is more difficult to perform than sweat- chloride, and requires specialized equipment and extensive training. Recent efforts have standardized NPD performance and analysis across the US and Europe, including SOPs and centralized coordination and interpretation of trial data (42-46). It has been incorporated into small investigator-initiated trials of CFTR and other ion transport modulators (47-50) and also into early phase trials of CFTR modulators that proceeded to seek regulatory approval (3, 46). In multi-center trials, NPD measurements had sufficient sensitivity to detect dose-dependent bioactivity of ivacaftor in patients with the G551D CFTR mutation, but failed to detect bioactivity of systemic ataluren or lower-dose lumacaftor monotherapy in phase 2 and 3 studies performed in patients with PTC and F508del mutations, respectively (3, 44). Neither of these interventions had measurable clinical benefits, suggesting that the NPD assay may be specific for clinically relevant modulator bioactivity. Studies focused on improving NPD reliability and examining relationships between CFTR (or other ion transporter) restoration and clinical response are gaps in CFTR biomarker development and would clarify the future role of this assay in CF drug development.

ICM is another assay that can isolate CFTR function, and has the advantage of a large dynamic range between CF and non-CF (28, 51-54). Rectal biopsies are dissected and then studied in Ussing chambers to monitor CFTR-dependent ion transport *ex vivo*. Like NPD, efforts have standardized ICM performance, and a universal SOP has been adopted by both US and European centers with centralized data interpretation for use in multicenter clinical trials (53, 55). Like sweat- chloride and NPD testing, ICM can clearly discriminate different levels of CFTR function based on CFTR genotype (nonfunctional, partial and full function) with clinical correlates dependent on level of CFTR activity (28, 51). Demonstrating reliability of ICM is difficult due to the need for repeated biopsies, and performance of the assay is limited to centers with expertise in specialized electrophysiologic measurements. For these reasons, ICM will likely remain an early phase CFTR biomarker performed in a limited number of standardized centers.

Biomarkers of Infection

Viral and fungal infections are critical aspects of CF lung disease, but infection with defined pathogenic bacteria such as *Pseudomonas aeruginosa, Burkholderia cepacia complex* and methicillin resistant *Staphylococcus aureus* are linked to CF morbidity and mortality (56-60). Thus, developing anti-microbial interventions is a key goal of CF therapeutics. Infection can be considered as a progression from early transient infections to chronic, often biofilm-dominated conditions that are poorly reflected *in vitro* (61, 62).

The most direct biomarkers of infection are from the lower respiratory tract (bronchoalveolar lavage (BAL) fluid, sputum) but other sources such as cough swabs, oropharyngeal swabs and nasopharyngeal (NP) samples are used in non-expectorating patients, albeit with unclear sensitivity and specificity for lower airway tract infection that limits their applicability to drug development.

Commonly used biomarkers for antimicrobial studies include bacterial density (CFU/g for sputum or CFU/mL for BAL fluid) and/or detection of CF pathogens as primary or secondary endpoints. Only drugs treating chronic *Pseudomonas* infection have sought regulatory approval, and relied on reduction in microbial density as a key supportive endpoint (17, 63-65). However in many trials change in FEV_1 is the primary endpoint and this may not always overlap with reductions in *Pseudomonas* density (66).

Targeted PCR-based detection, which is routine for viruses, is being introduced to detect CF bacteria, yet these methods have not been used extensively (67, 68). Panels to detect frequently encountered bacteria are in development, and are often based on detection of bacterial enzymes or virulence factors that may enable early detection of resistance.

The CF Lung Microbiome

Evaluation of the CF lung microbiome (through unselected detection of 16S ribosomal bacterial RNA using deep sequencing methods) is an emerging technology. Microbiome studies to date have generally shown that decreased measures of microbial diversity are associated with more advanced disease and may change prior to exacerbations (69-71). Methodological differences in extraction and sequencing, poor distinction of live vs. non-

viable bacterial DNA, and the lack of quantitation are current limitations to their clinical and biomarker use. Detection of bacteria using molecular methods has enhanced sensitivity and potentially may reduce processing times, but monitoring of disease status and drug effect via molecular detection and microbiome analysis remains exploratory.

Future directions include functional evaluation of the microbiome either through interactions between taxa and/or changes in metabolic activity correlating to clinical outcomes. To examine metabolic activity, DNA and RNA based metagenomics or metatranscriptomics (i.e. the study of the function/activity of the transcriptome -RNA-seq) have been used. Technical challenges include contamination with human genetic material and mapping of sequence data (72). Early reports indicate that DNA based (total) and RNA based (metabolically active) community structure show overlap for the predominant taxa, but less prevalent organisms may be metabolically very active (73). Further, metabolic activities in CF samples show less patient-to-patient variation than the total microbiome and differ from results in other pulmonary conditions (74), and metabolic profiles of bacteria are dynamic with adaptation to the lung disease (75). Although limited to small study numbers, early findings suggest changes in disease status based on exhaled volatile bacterial metabolites (76). Measuring bacterial volatile organic compounds and inflammation may allow rapid monitoring of lung disease progression. Methods may include ‘electronic noses’ that distinguish patterns of volatile organic compounds rather than specific compounds, and have the benefit of portability. Technical challenges, standardization, contribution of non-respiratory vs. respiratory factors, and defining CF vs. non-CF patterns remain barriers to overcome prior to their use as biomarkers for antimicrobial therapies (77).

Biomarkers of Inflammation

Inflammatory biomarkers could play a critical role in the development of anti-inflammatory drugs, and reflect downstream improvements in CF lung disease for disease-modifying treatments. They could be used in early phase studies to confirm the proposed mechanism of action of drug candidates. Alternatively, as correlates of clinical endpoints, they could help select agents for further Phase 3 investigations. Results from previous CF clinical trials indicate that anti-inflammatory therapies may not result in immediate improvements in pulmonary function, but could slow the rate of lung function decline (23, 24). This requires many patients being studied over a prolonged period to demonstrate efficacy, highlighting the urgency of identifying biomarkers that can more rapidly screen candidate drugs.

Lung-derived Biomarkers of Inflammation

The most direct method to assess CF lung inflammation is via bronchoscopy with BAL. BAL inflammatory markers have been used as clinical endpoints in pathophysiological studies (e.g. Australian Respiratory Early Surveillance Team for CF) (78, 79), and in clinical trials of inhaled tobramycin (80) and recombinant human DNase (81). A recently published document from the European CF Society Clinical Trial Network concluded that the use of BAL in clinical research should be limited due to its invasive nature, but that it may be applicable to early-phase clinical trials conducted in specialized centers and in trials involving young children with early/mild lung disease (82).

Due to drawbacks of bronchoscopy, the two most commonly collected biospecimens to measure inflammation are sputum and blood. Spontaneous sputum expectoration is generally limited to those with more advanced lung disease (adolescents and adults). Sputum induction (inhalation of hypertonic saline) improves sample acquisition in individuals who do not routinely expectorate sputum, and biomarker measurements are reasonably comparable between induced and spontaneously expectorated sputum (83-85). For studies in which the primary outcome is a sputum biomarker in children and/or those with preserved lung function, sputum induction likely should be performed throughout the trial. When sputum biomarkers are secondary outcomes, collecting spontaneously expectorated sputum (with induction as a back-up) is a reasonable approach.

The validity of sputum biomarkers as endpoints in CF clinical trials has been extensively reviewed (85). Neutrophil elastase (NE) is currently the most informative sputum biomarker to monitor CF lung disease. Sputum NE activity correlates with bronchiectasis in CF (86), tracks with and is predictive of future lung function decline (87, 88), relates to treatment response in pulmonary exacerbations, and predicts time to next exacerbation (89, 90). Increased BAL NE is also a predictive biomarker of impaired lung function and bronchiectasis in young children with CF (79). Other sputum biomarkers that are associated with and predictive of key clinical events in CF include calprotectin (91), myeloperoxidase (92), high-mobility group box 1 (HMGB-1) (93, 94), and YKL-40 (95).

A proof of concept study assessing the responsiveness of sputum biomarkers to intravenous antibiotic treatment during pulmonary exacerbations demonstrated that decreases in sputum inflammatory markers, correlated with pulmonary treatment response (89). These findings though were not replicated in a more recent study of exacerbation treatment (96). Differences in sputum NE were observed over a six month study of azithromycin compared with placebo (19), but more recent interventional trials using sputum inflammatory biomarkers have generally failed to show significant changes in NE activity and other sputum biomarkers (except for modest reductions in sputum IL-6 (97-99)). Also, ivacaftor, which has been shown to improve many clinical outcomes, did not change sputum inflammatory biomarkers in a G551D CF cohort (33). The lack of treatment effect in these studies may be due in part to the intrinsic variability, particularly between-subject variance, of sputum biomarkers (99). This variance makes it challenging to rely upon sputum biomarkers during drug evaluation over short treatment periods, and sputum biomarkers may only be sufficiently sensitive to demonstrate anti-inflammatory effects in longer trials (≥ six months). To minimize the potential effects of sputum collection and processing on biomarker variability, recommendations include using SOPs (sputum induction, processing), centralized laboratories for sputum processing and analysis (CFF National Resource Centers: https://www.cff.org/Our-Research/Therapeutics-Development-Network/Working-with-the-TDN/National-Resource-Centers/), training of research personnel, and ensuring quality control (85).

Blood-based Biomarkers of Inflammation

Systemic inflammatory markers would be ideal since blood measurements are easily standardized, repeatable, and can be obtained from subjects of any age and disease severity.

Systemic inflammation may also link pulmonary and non-pulmonary comorbidities of CF. While there are less data correlating systemic inflammation with clinical outcomes in CF, systemic inflammatory biomarkers correlate with key clinical events including pulmonary exacerbations and lung function decline (100-104). Circulating biomarkers have consistently changed more than sputum markers of inflammation with exacerbation treatment (91, 96, 105), suggesting that systemic inflammatory measures may be more sensitive in short term interventional studies focused on mitigating exacerbations. Based on results from a multicenter exacerbation study (106), serum C reactive protein (CRP), serum amyloid A (SAA) and calprotectin declined during azithromycin treatment in a CF interventional trial (107). These reductions correlated with improvements in lung function and weight gain, providing indirect evidence that the changes were associated with clinically meaningful outcomes. Other candidate systemic biomarkers relating clinical status to CF outcomes include neutrophil elastase antiprotease complexes (NEAPC) (101), various cytokines including interleukin-6 (many studies), IgG (100), and circulating mononuclear cell RNA transcripts (108).

Additional studies are required to develop sputum and systemic inflammatory biomarkers for CF drug development. We must determine associations between inflammatory biomarkers and key clinical outcomes (FEV_1 decline, pulmonary exacerbations) in broader CF populations, which will be facilitated through biospecimen collection in longitudinal studies. We need to know whether short term changes in inflammatory biomarkers predict longer term clinical outcomes, and how airway and systemic inflammation may change with CFTR modulators. Additional data examining the effects of freezing and delayed sputum processing on analyte measurements are required, and would inform multicenter trials that collect sputum samples and perform centralized processing and analysis.

New Technologies and Tools

‘Omics-based Biomarker Development for New CF Therapeutics

The use of ‘omics based tools to identify and validate biomarkers relevant to CF pathologies have only recently begun to be applied, and elevation to regular biomarker use is not established. These tools allow for an unbiased analysis of complex cellular processes in a variety of substrates. Discovery of novel molecular biomarkers in CF has to date been limited, and the majority of discovered biomarkers represent acute response markers. Furthermore, no molecular biomarkers have been shown to reliably predict acute or chronic CFTR restoration. The advent of novel approaches in high throughput technologies coupled with advancements in analysis of “big data” may allow investigators to ask important questions with high sensitivity and precision.

Gene-array studies have suggested the dysregulation of numerous pathways in CF. In an elegant study Wright and colleagues examined gene expression in the nasal epithelia of CF patients with either mild or severe lung disease (109) and identified abnormalities in gene expression regulating lipid metabolism, ubiquination, and mitochondrial/whole-cell redox regulation that segregated cohorts by disease severity. More recently, Nick and colleagues identified a panel of RNA transcripts that were predictive of pulmonary exacerbations (108). All of these observations require validation prior to clinical extension.

Mass spectrometry (MS)-based proteomics and/or metabolomics have been used for either non-biased or targeted/selected biomarker discovery, with lipidomics gaining much recent interest. MS can precisely and rapidly examine markers of disease or response to therapy broadly, allowing for global analyses of individual samples in a non-biased, data-driven approach. Metabolomic analyses of non-fasted CF patients revealed a decrease in β-oxidation of fatty acids, which is a marker of mitochondrial dysfunction (110), and CF plasma lipidomic studies have detected significant decreases in anti-inflammatory lipids (111). In CF sputum, a metabolomic/lipidomic analysis by Yang and colleagues identified a number of proinflammatory lipids (oxylipins) previously not identified in the CF lung (112). These studies also found a significant decrease in lipoxin A4 in CF lungs, which had previously been identified and validated by other approaches (113). The fact that these broad analysis studies also accurately detected the known lipoxin A4 deficiency in CF increases confidence in the application of MS-based lipidomic analyses in CF.

In the case of proteomics, no broad analysis of markers of CF disease progression in serum has been reported. Studies have focused on CF versus non-CF comparisons, which require less extensive analyses to identify differences versus studies where all cohorts have CF with varying degrees of disease. Proteomic analysis of CF sputum discovered a relationship between myeloperoxidase, protein oxidation and pulmonary inflammation (114), which was validated in BAL (115). When the proteome of CF-patient nasal epithelia was examined, a significant decrease in the expression of a number of anti-inflammatory proteins was detected (116). Proteomic screens have discovered a previously unknown mechanism for antioxidant downregulation in CF, namely Nrf2 dysfunction (117, 118). Follow-up biochemical studies in CF primary tissues linked this dysfunction to increased inflammatory signaling, and demonstrated that activation of Nrf2 in CF mice produced anti-inflammatory benefits (119). These results highlight the potential of non-biased proteomic analyses in discovering novel biomarkers and previously unknown mechanisms of disease.

Although ‘omics approaches hold much promise for biomarker discovery in CF, it is important to note that MS-based analyses are semi-quantitative, and therefore it is essential that observations made in proteomic, metabolomic, and lipidomic studies are validated by other approaches. Furthermore, while instrumentation has improved, the techniques used to conduct proteomic analyses also vary and can influence results significantly. Standardization of methodology, data analysis and incorporation of these tools into therapeutic clinical trials offers the opportunity to fully realize the promise of these technologies to advance CF care and drug development.

Summary, Needs and Conclusions

The status of biomarkers to advance CF therapies varies considerably across the different pathogenic targets. Many CF biomarkers have demonstrated their ability to inform early drug development by assessing drug bioactivity and potentially enabling dose selection for trials. Sweat chloride appears to be a biomarker with great potential to guide early phase CFTR modulator development and enable regulatory decision making, and further studies examining relationships between improvements in sweat- chloride and long term outcomes may broaden the utilization of this biomarker. NPD and ICM can also provide supportive

data of bioactivity, but technical challenges limit these biomarkers to early phase trials. Biomarkers of infection including sputum bacterial density and pathogen detection remain valuable tools for early phase trials. Molecular platforms to assess the microbiome (and host response) also hold promise to advance biomarkers of airway infection and drug activity, and standardization of techniques coupled with their incorporation into studies of new therapies are required to understand their role in drug development. Inflammatory biomarkers in sputum (NE) and blood (CRP, calprotectin, SAA) correlate with clinical status and interventions, but gaps in our understanding of CF inflammation are a limitation to the development of novel CF anti-inflammatories. The revolution in 'omic technologies over the past decade is beginning to identify novel biomarkers of various disease manifestations, but advances in data analysis and the need for candidate validation are critical before these technologies become mainstream players in CF drug development. We hope that this review serves as a valuable summary of biomarkers relevant to CF therapeutics, and guides future research to advance this field and to accelerate the development of new treatments for CF patients.

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Table 1
Impact of CFTR Modulators on Sweat-Chloride in Clinical Trials

* Ivacaftor monotherapy	Enrollment (number on modulator)	Change in sweat [chloride] (treatment effect)
Accurso (4) (G551D, >18 yrs)	N = 8	-42.3 mM (p<0.01)
Ramsey (5) (G551D, >12 yrs)	N = 83	-48.1 mM (p<0.001)
Davies (34) (G551D, 6-11 yrs)	N = 26	-54.3 mM (p<0.001)
Davies (36) (gating, 2-5 yrs)	N =19	-46.9 mM (p<0.001)
De Boeck (6) (gating, age >6 yrs)	N = 39	-49.2 mM ((p<0.001)
Moss (37) (R117H, >6 yrs)	N =34	-21.9 mM ((p<0.001)
Rowe (35) (G551D, >6 yrs)	N = 151	-53.8 mM (p<0.001)
Flume (39) (F508del/F508del, >12 yrs)	N = 112	-2.9 mM (p=0.04)
Lumacaftor monotherapy Lumacaftor/ivacaftor co-therapy	Enrollment (number on modulator)	Change in sweat [chloride] (treatment effect)
Clancy (45) (lumacaftor, 200 mg every 24 hrs, F508del/F508del, >18 yrs)	N = 19	-8.21 mM (p<0.01)
Boyle (38) Lumacaftor 400 mg every 12 hrs and ivacaftor 250 mg every 12 hrs, F508del/F508del, >18 yrs)	N = 11	-10.3 mM (p=0.002)

*Studies listed by lead author, with genotype and age of enrolled subjects as noted. Patients >6 yrs of age were dosed with ivacaftor 150 my every 12 hrs. Patients age 2-5 years were dosed with ivacaftor based on weight.

†Dose of lumacaftor and ivacaftor as noted.

Table 2
Microbial Biomarkers in Acute and Chronic Infection

Infection Biomarker	Early infection	Chronic infection
Culture: Presence or CFU/mg (mL)	Common biomarker for antibiotic development May be of interest in CFTR specific therapies	Small changes in density with most therapies. May show changes with antimicrobials and CFTR modulators.
*Molecular detection and diversity measures	No differences prior to onset of a primary pathogen, followed by decreasing diversity with advance in disease.	Long term decrease with advanced disease. Short term changes less pronounced. Internal consistency within a given patient.
Bacterial metabolites	Few data available and low bacterial biomass compared to host derived signal, thus many signals represent host inflammation.	Changes in bacterial phenotypes and metabolites with disease progression. Some could serve as markers of disease severity.
Measures of bacterial cooccurrence	Few data are currently available, but indicate competition and communication.	Need to develop network analyses – not yet informative for pathogenesis or disease severity.

* Diversity can be measured using extensive culture conditions, but typically considered a measure in microbiome studies measuring relative abundance.