EXPERT REVIEWS

Using bacterial biomarkers to identify early indicators of cystic fibrosis pulmonary exacerbation onset

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Acute periods of pulmonary exacerbation are the single most important cause of morbidity in cystic fibrosis patients, and may be associated with a loss of lung function. Intervening prior to the onset of a substantially increased inflammatory response may limit the associated damage to the airways. While a number of biomarker assays based on inflammatory markers have been developed, providing useful and important measures of disease during these periods, such factors are typically only elevated once the process of exacerbation has been initiated. Identifying biomarkers that can predict the onset of pulmonary exacerbation at an early stage would provide an opportunity to intervene before the establishment of a substantial immune response, with major implications for the advancement of cystic fibrosis care. The precise triggers of pulmonary exacerbation remain to be determined; however, the majority of models relate to the activity of microbes present in the patient's lower airways of cystic fibrosis. Advances in diagnostic microbiology now allow for the examination of these complex systems at a level likely to identify factors on which biomarker assays can be based. In this article, we discuss key considerations in the design and testing of assays that could predict pulmonary exacerbations.

The need for biomarkers of pulmonary exacerbation in cystic fibrosis

Cystic fibrosis (CF) lung disease is characterized by a self-perpetuating cycle of airway obstruction, chronic bacterial infection and vigorous inflammation that results in bronchiectasis. Over 90% of people with CF die as a consequence of progressive lung damage resulting from bacterial infection [1,2]. CF patients typically have periods of clinical remission interspersed with acute episodes of increased respiratory symptoms, known as cystic fibrosis pulmonary exacerbation (CFPE). CFPEs are a major cause of morbidity for CF patients [3] and are often accompanied by a decline in lung function that may not be recovered following treatment [4,5]. CFPEs and associated lung damage have a major impact on quality of life [6–9], short-term mortality [10–13] and healthcare expenditure [10]. However, the lack of a specific disease marker or a unifying, commonly-accepted indicator to initiate treatment for a CFPE hampers the improvement of patient care. There is, therefore, substantial effort directed towards finding a marker to enable prompt diagnosis and treatment of CFPEs before irreversible lung damage occurs.

A key problem in diagnosing CFPE is the lack of a consensus definition of what it constitutes. Diagnosis typically revolves around a clinician's decision to treat a constellation of symptoms [14], including cough, sputum production, dyspnea, decreased energy level and appetite, weight loss and decreases in spirometric parameters [15]. There is a continuing debate as to which combination of these measures most accurately represents a significant clinical change necessitating intervention [16]. CF patients differ widely in the way in which they manifest their condition [17] and the large number of treatment options may have side effects that mask or mimic some clinical signs [18–20]. Increasingly, patients who appear or feel 'not quite right' are being treated with antibiotics to prevent the development of more worrying symptoms [16]. The treatment of

patients with mild symptoms that do not yet meet typical CFPE definitions might, in some cases, reduce the incidence of CFPE over the long term [21,22]. However, the consequentially increased antibiotic use and the serious accompanying side effects of such an approach are a significant cause for concern.

Patient-reported symptoms might be an important complement to physician-assessed clinical signs in the diagnosis of CFPE, and newly developed diaries are currently being validated for this purpose [15,17,23,24]. However, patient-reported outcome scores are not without drawbacks. Subjective judgments about symptom severity differ significantly between patients [25], and could potentially be influenced by the desire to more firmly control therapy.

It would be ideal if biomarkers could be identified that had the capability of directly reflecting objective disease activity rather than relying entirely on clinical markers. Factors involved early in the CFPE initiation pathway could prove useful as predictive markers, allowing earlier intervention. The aim of early intervention would be to quickly reduce the development of a full inflammatory response and to shorten and reduce the severity of the CFPE, with the hope of preventing the development of irreversible lung damage. In this way, an effective and reproducible diagnostic marker for the early stages of a CFPE could offer the exciting possibility of disease-modifying therapy that could prevent permanent loss of lung function and the associated morbidity and mortality.

Bacterial infections in the CF airways are currently monitored through routine microbiology. While useful in the long-term surveillance of disease progression, these data are unlikely to provide sufficient detail to be predictive of CFPE onset. The application of molecular diagnostics to monitor biomarkers of bacterial infection continues to expand [26–28]. The increasing availability of commercial assays, in conjunction with a growing recognition of the utility of such molecular diagnostics in the characterization of clinical infections, means that routine surveillance in the management of CF respiratory disease using these methods may now be possible. However, which biomarkers are to be measured requires careful consideration.

In this article, we examine the potential of microbial factors to provide biomarkers for the early detection of CFPE onset, as well as the challenges that must be overcome for such molecular diagnostic surveillance to be implemented routinely.

Cystic fibrosis pulmonary exacerbation

The reasons for the occurrence of periods of CFPE are often not known, although a number of potential causes have been suggested (**Box 1**). These include the acquisition of new strains of bacterial species [29], the expansion of existing bacterial populations in the airways [29], blooms of planktonic bacterial cells released from biofilm populations [30], the expression of bacterial virulence factors [31], viral infections [32,33] and ambient air pollution (**Box 1**) [34]. Despite the lack of clarity regarding the triggers of CFPE, once initiated, the typical increase in severity of respiratory signs and symptoms is thought to reflect an upregulation of local inflammatory responses. As highlighted by a number of review articles, this inflammatory response is complex and multifactorial [17,23,24,35]. Broadly, CF airway disease is considered to be

dominated by persistent neutrophilic infiltration, with elevated IL-8 and neutrophil elastase in airway secretions [36–41]. There is evidence that proinflammatory cytokines and other mediators are abnormally elevated in CF patients, relative to the burden of infection [42]; it has been suggested that dysregulation directly results from the underlying CF defect [41,43,44]. However, inflammation is increased by local airway epithelium–pathogen interactions and is elevated during CFPE [41].

The elevated inflammatory response that characterizes CFPE forms a positive-feedback loop (**Figure 1**). Activated neutrophils release large amounts of elastase and other proteases that overwhelm the local host defenses [15]. Furthermore, as these neutrophils break down, they release large amounts of high-molecular-weight DNA that further increases the viscosity of the endobronchial secretions, which hinders mucociliary clearance [45]. In this way, a vicious cycle of chronic infection and inflammation occurs that encourages persistence of pathogens, promotes obstruction of the airway lumen and causes the destruction of airway wall architecture [15,46].

CFPE biomarkers

Biomarkers enable objective measurement and evaluation of "normal biologic processes, pathogenic processes, and pharmacologic responses to a therapeutic intervention" [47]. They have been shown to be useful in the diagnosis and characterization of a wide range of clinical conditions, including cardiovascular, renal and metabolic diseases, as well as sepsis and cancer [48–50]. Biomarkers should reflect biological activity and correlate with established clinical outcome measures, such as how a patient feels, functions or survives [51–53]. Their potential use in CF is particularly attractive given the diverse manifestations of CF airway disease and the response to therapy among individual CF patients.

Key biomarker characteristics that must be considered when designing assays for CFPE generally have been set out by Mayer-Hamblett *et al.* [53]. However, some of these considerations are more relevant to the use of biomarkers as early indicators of CFPE onset. Biomarkers useful in this context must be considered on the basis of two key areas; the degree to which they reflect biologically informative processes predictive of CFPE onset, and their suitability for use as a routine diagnostic tool.

Reflection of biologically informative processes predictive of CFPE onset

Biomarkers must consistently show a change in level prior to the onset of CFPE when compared with levels during clinical stability, at least among a subset of patients or exacerbations. Determining such a change relies on defining the point at which a patient is experiencing CFPE. However, in the absence of a consensus definition for CFPE, biomarker levels can only be correlated with an increase in accepted clinical markers and symptom scores. Although measurements of sputum inflammatory biomarkers obtained from the same patient on different occasions have been shown to be reproducible (although not specifically as predictors of CFPE), they can vary considerably between patients [54,55]. It is therefore important that the baseline

values of such clinical markers are recognized as specific to individual patients, and that the assay can cover the effective range of clinically relevant test results.

Useful biomarkers should have a turnover rate that allows them to reflect short-term changes within the airways, but be sufficiently stable for accurate and reproducible measurement. Biomarkers that are highly unstable may be present for a very short period of time and may therefore only be detectable through frequent sampling. Furthermore, such instability could lead to appreciable biomarker degradation during sample handling. By contrast, highly stable biomarkers may accumulate over time in the airways in such a way that they do not reflect short-term changes in disease or return to baseline levels following successful treatment.

Suitability for use as a routine diagnostic tool

It is essential that the biomarker being measured is easily available from samples that can be routinely collected from patients, such as blood, urine, exhaled breath (EB), saliva or sputum. Inflammation in the CF lung appears to be primarily driven by local stimuli, mediators and chemoattractants, and is not the local effect of a systemic inflammatory reaction [56]. As such, systemic indicators of inflammation are likely to have low sensitivity and show only modest increases during acute exacerbations. Blood-based biomarkers are therefore less appropriate for diagnosing the onset of CFPE. Sputum is readily obtainable in the vast majority of adult CF patients and has been shown to be a useful basis for characterizing CF airway infection [57–59]. However, it should be noted that owing to the uneven distribution of infection within the CF lower airways, performing frequent assays is necessary in order to minimize sampling bias [60,61]. EB and EB condensate (EBC) have been used as samples for measuring biomarkers in a range of respiratory conditions [62,63]. While some concerns have been raised about the sensitivity and reproducibility of these sample types [63], they are noninvasive and easily collectable. EB and EBC samples have been used for the measurement of a number of biomarkers in CF patients, including nitric oxide [64–66], condensate acidity, nitrate, nitrite, 8-isoprostane, hydrogen peroxide and IFN-γ [67].

Since analysis will not be performed immediately in most instances, biomarkers must be stable at room temperature or have the capacity to be stabilized with the addition of specific reagents or by freezing. This is particularly important in the case of highly unstable molecules, such as mRNA. By contrast, although data regarding protein markers are sparse, for the majority of such proteins the process of freezing of samples for batch processing does not appear to affect levels observed [68].

Assays must be sufficiently inexpensive, both to carry out and in terms of equipment, expertise or reagents, in order to allow repeated use in routine surveillance.

The process of identifying clinically informative biomarkers typically involves comparison with an existing measure that is considered to be the 'gold standard' (something that definitively identifies a condition) [27]. However, in the context of CFPE, no such gold standard exists. This situation greatly complicates the process of evaluating potential assays. Nevertheless, the central

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Box 1. Hypothesized triggers of cystic fibrosis pulmonary exacerbation.

Bacterial
- Acquisition of new bacterial strains [84,124]
- Expansion of existing populations [125]
- Planktonic blooms [30]
- Increase in virulence gene expression [126]
- Shifts in bacterial community structure [93,94]
- Acquisition of new bacterial species, such as atypical *Mycobacteria* [127]

Environmental
- Particulate matter [34]
- Ozone [34]

Viral
- Respiratory viral infection [84,128,129]
- Human herpes virus [130]

Fungal
- Allergic bronchopulmonary aspergilliosis [127]
- Colonization by *Exophiala dermatitidis* [131] or *Candida albicans* [132]

These triggers do not include structural changes in the airways, such as lobar/segmental collapse, or nonadherence to treatment for factors outlined.

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role of the local inflammatory response in CFPE has led to the investigation of a wide array of inflammatory mediators as biomarkers (as discussed by Sagel *et al.* in 2007 [54]). Particular attention has been given to IL-8 [24,38,53,69–75], neutrophil elastase [38,53,69–71,75,76] and myeloperoxidase [39,69,77–81] in sputum samples. In each study, biomarker levels were elevated during CFPE, then decreased following the initiation of antibiotic therapy. In addition to immunoenzymatic assays, techniques such as ^{18}F-fluorodeoxyglucose PET/CT imaging allow the direct determination of the physical distribution of neutrophilic inflammation [82]. However, this method remains too expensive and technically complex for routine use at this time.

As before, these studies have primarily sought to identify biomarkers that can provide accurate and reliable measurement of the biologic activity surrounding CFPE and/or as a sensitive marker of inflammation, primarily for use in assessing treatment strategies or disease progression. Here, a wide range of host-derived biomarkers have the potential to be useful. An increase in levels of these factors could be detectable prior to an elevation of clinical signs and symptoms. However, this is likely only to occur once an upregulation in inflammatory response has been established. As such, while having the potential to be informative in the diagnosis of CFPE, disease progression and the evaluation of therapy, these inflammatory biomarkers will be limited in their predictive power, because the point at which inflammation is elevated may be too late to prevent irreversible lung damage. Instead, in order to indicate the likely onset of CFPE, biomarkers that are associated with triggers of inflammatory response upregulation must be identified. Consideration must therefore be given to where such novel predictive biomarkers might be found.
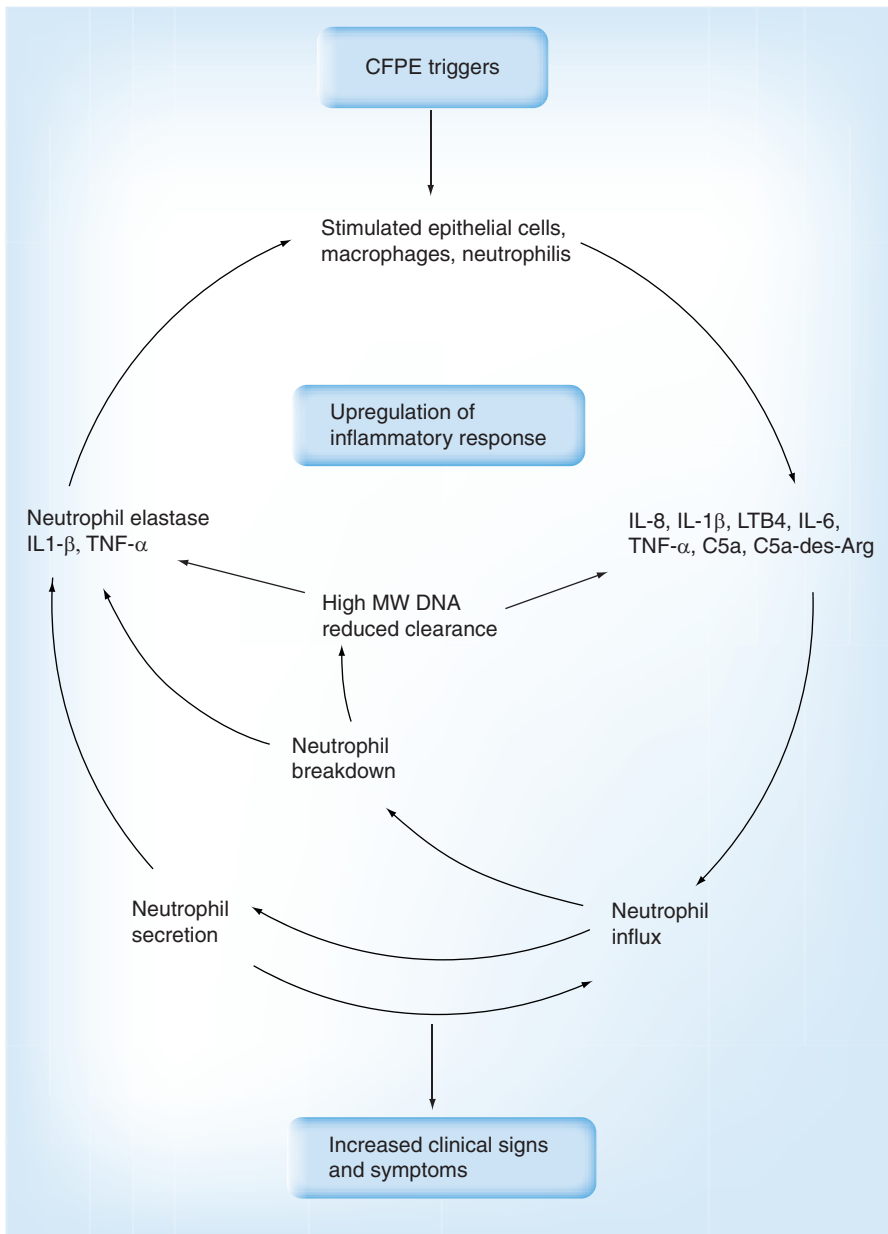
Figure 1. Proposed path of cystic fibrosis pulmonary exacerbation onset.
Conventionally, CFPE is diagnosed on the basis of an increase in the severity of clinical signs and symptoms. Changes in these markers follow an upregulation of the local inflammatory response. While molecular diagnostic assays based on components of the inflammatory response offer an earlier and more specific indication of the onset of CFPE, the ability to monitor factors associated with triggers of CFPE could allow intervention prior to the infliction of airway damage.
CFPE: Cystic fibrosis pulmonary exacerbation; MW: Molecular weight.

CFPE-predictive biomarkers

A wide range of potential triggers have been proposed for the increased neutrophilic influx that characterizes CFPE. Such a diversity of potential triggers may translate into a number of different types of CFPE, each with different characteristics. For example, it has been suggested that episodes of CFPE that result from bacterial and viral infection represent distinct phenomena [83]. As such, separate diagnostic strategies may be required.

Infections caused by common respiratory viruses are associated with disease progression in CF patients [33,83–86]. While such infections are symptomatically acute, there is evidence that respiratory viruses may persist for extended periods in the airways of patients with a range of chronic respiratory disease [87,88]. Therefore, it may be most appropriate to analyze their presence in respiratory secretions through the enumeration of viral particles, with data compared against a threshold level associated with respiratory symptoms, rather than a simple presence/absence assay. Increasingly sophisticated molecular diagnostic strategies are being developed for such analysis [89–92], making the application of these assays a realistic proposition in the surveillance of CF patients.

Bacterial infections of the CF airways are typically chronic. The interactions between these bacteria and the host, which may give rise to episodes of CFPE, therefore, are again unlikely to be characterizable by presence/absence assays, and would require more detailed analysis. For example, characterization of factors such as relative species abundance and gene-expression profiles may be necessary [93,94]. However, in each instance, the response of the host to changes in airway bacterial populations (and the resulting upregulation of the inflammatory response among leukocytes and/or epithelial cells) should be the focus of analysis (attempts to identify predictive biomarkers form part of the wider effort to determine the nature of the relationship between bacteria and their products in the CF lower airways, and the occurrence of CFPE). From a bacterial perspective, potential trigger mechanisms may occur through different routes. Such mechanisms could include the activation of neutrophils and epithelial cells when detecting the presence of bacterial cells in the airways. Other mechanisms may involve the upregulation of the immune response through the secretion of specific compounds by bacteria in the airways. In the case of the former inflammatory response triggers, assays must focus on the detection and enumeration of bacterial cells. For the other triggers, assays must be directed towards the measurement of levels of either secreted compounds in the airway or the transcription of the genes that encode them by bacterial cells **(Figure 2)**.

Traditional, culture-based diagnostic microbiology is of limited use in reporting subtle changes in airway bacterial populations because of the presence of slow-growing bacterial variants [95,96] and potentially viable but nonculturable bacterial cells [97–100]. However, the application of culture-independent quantitative PCR (Q-PCR)potentially offers a more accurate means by which changes in bacterial numbers prior to the onset of CFPE can be characterized [101], regardless of whether species are refractory to *in vitro* culture [102]. Q-PCR technology is well validated and used widely in the characterization of bacteria in clinical samples [103]. However, where clearance of material from the site of infection is poor, as in the lower CF airways [104,105], the presence of nonviable cells and extracellular DNA can have a substantial impact on the accuracy of quantification by molecular methods. In order to address this problem, new molecular protocols have been developed that exclude from analysis any DNA not derived from viable bacterial cells [106]. These protocols, based on the use of propidium monoazide photo-crosslinking chemistry to block extracellular DNA or DNA from nonviable cells from serving as a template for PCR reactions, have been adapted to the CF respiratory context [107]. Furthermore, they have been shown to be capable of identifying short-term changes in bacterial levels in sputum not detected by standard Q-PCR techniques, such as a reduction in bacterial density in CF respiratory secretions following the intravenous administration of certain antibiotics [108]. Such approaches are rapid, relatively inexpensive and can be performed using automated systems. While requiring further validation, such pretreatment of samples could provide a basis for the routine surveillance of either total bacterial density in airway secretions, or the densities of specific species or strains of particular interest using multiplex PCR reactions [109].

It may be that it is the expression of particular traits by bacteria, rather than simply their presence in the airways, that is key in triggering the onset of CFPE. In such circumstances, early indications of CFPE onset might be derived from the determination of levels of virulence behavior. Specific immunoenzymatic assays (ELISA) have previously been used to directly determine levels of bacterially secreted compounds, such as *Pseudomonas aeruginosa* elastase, exotoxin A and alkaline protease, in CF airway secretions [31]. While not yet available as commercial kits, such assays may provide a rapid and relatively inexpensive routine assay of bacterial virulence biomarkers. Real-time PCR assays could be used to determine the transcription rates of virulence genes [110,111]. However, owing to the instability of mRNA, such assays would be dependent on sample handling and, as such, would be inappropriate for routine surveillance.
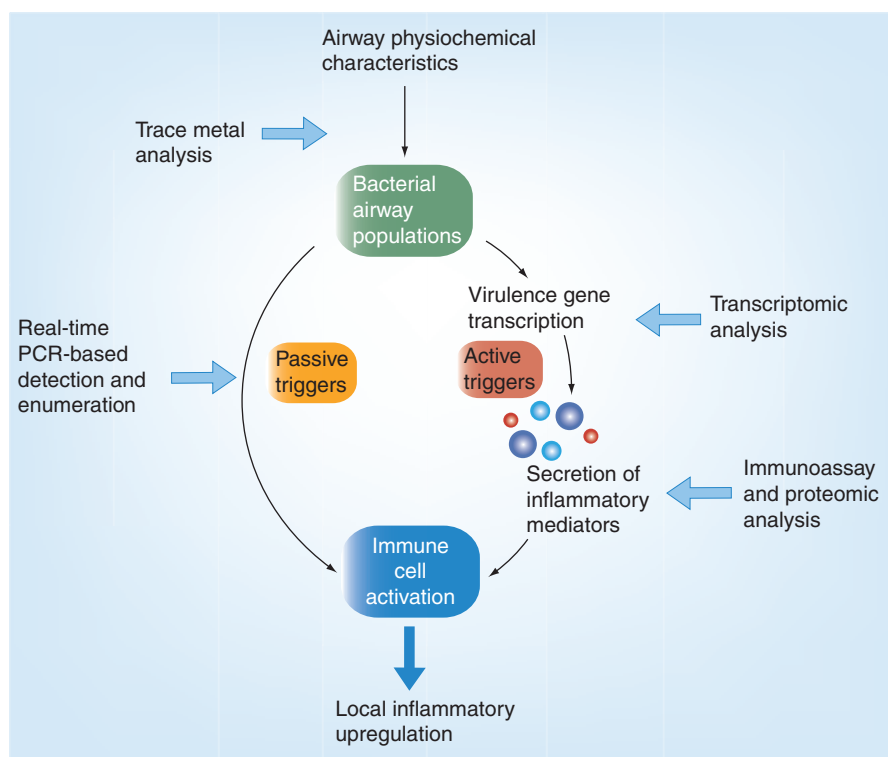

Figure 2. Passive and active potential microbial triggers for cystic fibrosis pulmonary exacerbation, with suggested analytical points (white arrows) for onset prediction.

In some cases, changes in the behavior of bacteria can be driven by alterations in the physiochemical characteristics of the airways. Trace metals required for bacterial growth may be particularly important in determining the way in which bacteria grow. For example, lactoferrin, an iron-chelating protein abundant in human external secretions, blocks the formation of biofilms by *P. aeruginosa*. Biofilms are a bacterial growth phenotype implicated in CF respiratory pathogenesis [112,113]. Scavenging of trace metals by secreted molecules such as lactoferrin may represent an important host antimicrobial strategy [72]. Direct spectrometric measurement of copper, zinc and iron has revealed elevated levels in CF sputum compared with controls; furthermore, zinc levels in sputum decrease following treatment of CFPE and derangements of this defense may impact lung disease in CF [72]. While requiring significant technological investment, elemental level analyses have the advantage of not being affected by the activity of proteases [41,114]. However, further investigation will be required in order to assess the clinical value of these measurements.

A number of secreted peptides that confer antimicrobial effects through the chelation of trace metals may provide the basis for a more practical biomarker assay than trace-metal measurement. For example, the neutrophil-secreted zinc chelator, calprotectin, is present in high levels in CF airway secretions, and correlates with disease markers, including neutrophil count [77,115,116]. The assays required to quantify calprotectin are comparatively simple. If this biomarker is predictive of CFPE, the relative ease of quantification would provide a range of practical benefits.

Expert commentary

As yet, there has been little investigation into whether inflammatory or microbial biomarkers can provide an early indication of the likely onset of CFPE. This is partly owing to the fact that many patients can remain stable for months or even years without experiencing a CFPE episode. Substantial efforts are required to assemble the comprehensive data sets needed to study and validate candidate markers by taking multiple airway secretion samples during periods of pulmonary stability leading up to CFPE.

Several assays have now been validated analyzing potentially informative inflammatory biomarkers in the assessment of CF sputum. The development of microbial biomarkers is far less advanced. The utility of both types of measurement in predicting CFPE when applied to longitudinally collected sample sets must now be investigated.

Owing to a variation in the way in which CFPEs manifest themselves between patients, and even within the same patient [70], a panel of biomarkers may be more predictive than a single measure. The use of composite biomarker panels has been shown to provide improved diagnostic power in other contexts, including in the prediction of cardiovascular disease risk [117] in relation to myocardial infarction and death [118]. Furthermore, since the mechanisms by which CFPE occurs are not understood, it is potentially problematic to select biomarkers on the basis of proposed mechanistic models. A composite CF lung biomarker panel may therefore be more appropriate for CFPE prediction than any individual biomarker [119].

Five-year view

Informing treatment strategies

The identification of biomarkers that provide an early indication of CFPE would represent a major advancement in the management of CF airway infections. However, the impact that such biomarkers could have is dependent on their ability to inform clinical decision-making, and thus improve patient outcome. Current treatment strategies center around long-term maintenance therapy to retard disease progression and short-term therapy during periods of CFPE to reduce symptoms. However, little consideration has been given to what the most appropriate treatment would be if an impending pulmonary exacerbation was detected prior to the upregulation of the local immune response. As efforts to measure

biomarkers in CF airway disease increase, such considerations will be necessary.

Microbial community

In addition to recognized CF pathogens, the CF lower airways often contain a complex polymicrobial community [92–94,120–121]. It is therefore important to consider if and how this wider community, consisting of bacteria, fungi, and eukaryotic viruses and phage species, contributes to triggering CFPE, and, as such, presents potential targets for diagnostic biomarker design. Microbial species may act as triggers, either through direct interaction with the host, or through interactions with recognized CF pathogens such as *P. aeruginosa* [92,122,123]. The potential complexity of these multispecies interactions represents a significant challenge to predictive biomarker design and further elucidation is required in order for them to inform this process.

Biomarker assay roll out

The value of performing certain mainstays of routine sputum diagnostics, such as the determination of the antibiotic susceptibility of clinical isolates, are being called into question [201]. The process of re-evaluating the components of CF sputum analysis provides an opportunity to apply resources to emerging assays that may be clinically informative. Furthermore, the expanding role being played by molecular diagnostics within healthcare laboratories provides a skills base and infrastructure for the performance of increasingly complex routine biomarker assays. Where assays can take advantage of sample types already provided on a regular basis (e.g., for routine diagnostic microbiology), surveillance through novel strategies might be possible without substantial disruption of existing patient routines. However, while an exciting prospect, the identification of appropriate predictive CFPE biomarkers requires further investigation.

Financial & competing interests disclosure

Key issues

- A biomarker that objectively reflects disease activity, rather than relying on clinical markers, would be a major advantage in the management of cystic fibrosis pulmonary exacerbations.
- The identification of biomarkers that are predictive of pulmonary exacerbation might allow early intervention prior to the establishment of an elevated inflammatory response.
- The suspected infective nature of these events suggests that microbe-derived biomarkers may be most useful.
- Potential assays can be based on the detection and enumeration of microbes, or on measures of expression of clinically significant microbial traits.
- Both the accurate enumeration of key bacterial species and their gene-expression markers are now possible through the application of molecular genetic assays.
- While still in its infancy, this work has the potential to be useful in the management of cystic fibrosis respiratory disease.

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