CHALLENGES AND SOLUTIONS FOR SPUTUM BIOMARKERS TO SUPPORT CYSTIC FIBROSIS CLINICAL STUDIES

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INTRODUCTION

Biomarker analysis in sputum is increasingly used to assess airway inflammation and to evaluate response to treatment in individuals with respiratory diseases including cystic fibrosis (CF). While bronchoalveolar lavage (BAL) and bronchial washing are routinely used for biomarker analysis, it is highly invasive and costly.

On the other hand, measurement of biomarkers in sputum is challenging. Here we present some of the challenges and solutions in biomarker analysis in sputum samples using case studies measuring arginase and nitrite and nitrate, two biomarkers implicated in CF.

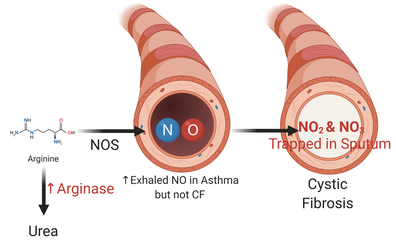

Figure 1: Elevations in arginase and nitrite/nitrate are implicated in the pathophysiology of cystic fibrosis and are potential biomarkers of disease progression.

SPUTUM AS A MATRIX FOR BIOMARKER PROFILING

Sputum is the discharge that is expectorated from the respiratory system. It is a heterogeneous substance with a mixture of mucus produced by the airways and saliva. It can be both watery and viscous. The sputum is rich in proteins, inhaled matter from the environment, and cellular debris which can provide a wealth of information on lung conditions. Biomarker profiling of sputum has the potential for use as indicators of disease status, and act as the efficacy biomarkers in response to a treatment. However, for the sputum biomarkers to be considered as accurate measures of biologic activity, it needs to be clinically relevant, reproducible, and sensitive/specific to disease pathology.

Heterogeneity of sputum samples is a major obstacle in achieving reproducible clinically relevant results. There are two processes used to achieve a homogenous mixture and to ensure that each aliquot of sample is representative of the original sputum specimen. One process is chemical digestion using dithiothreitol (DTT) or N-acetyl-L-cysteine (NALC). The active component DTT is a sulphydryl group, which cleaves disulfide bonds in the mucus. While DTT is known to be more effective than NALC, it can affect the measurement of cytokines and other proteins.

Another challenge in working with sputum is the stability of proteins/enzymes in sputum. The proteolytic activity in sputum is known to be high and, in some cases, it has been reported to be higher in the sputum of CF patients. This requires careful investigation of stability of the biomarker under investigation to ensure that the analyte is stable from collection to analysis. Different protease inhibitors (and a cocktail of them) have been used to stabilize analytes in sputum. Unfortunately, there is no one size fits all protease inhibitor cocktail that works for all biomarkers. This should be investigated on a case by case basis.

Challenge	Solution
Viscosity and heterogeneity of sputum	Add mucolytic reagents such as DTT
Proteases and ureases in sputum degrade biomarkers	Find optimum protease inhibitors and dilute sputum
Interference from DTT	Apply correction factor if appropriate to context of use. Treat standards with DTT. Report results as fold change or relative units.

Table 1. Fit-for-Purpose Solutions for Challenges Measuring Biomarkers in Sputum

CASE STUDY 1: MEASUREMENT OF ARGINASE ACTIVITY IN SPUTUM

Arginase, which converts L-arginine into L-ornithine and urea, is a key enzyme of the urea cycle. While it is mainly expressed in hepatic tissues, it is also found in bronchial epithelial cells, endothelial cells, fibroblasts, and alveolar macrophages. Studies have shown that arginase may play an important role in the pathogenesis of various pulmonary disorders including cystic fibrosis. Interest in arginase activity in cystic fibrosis patients has grown significantly due to its potential role in limiting the bioavailability of L-arginine for nitric oxide synthases (NOSs).

We have developed a robust and reproducible method for the measurement of arginase activity in sputum. Briefly, the method involves incubation of L-arginine solution with heat-activated sputum lysates. The amount of urea generated from this reaction is then determined by a spectrophotometric assay based on a reaction with α-isonitrosopropiophenon. The arginase activity is calculated from a standard curve for urea (1–30 µg urea). The results are expressed in absolute terms as units/mg protein. One unit is defined as the enzyme activity that catalyzes the formation of 1 µmol urea/min.

The first challenge faced during method development was the sample stability. We observed loss of arginase activity in collected samples over approximately seven days. The samples used for method development were from commercial sources. Th sample collection procedure involves incubation of samples for 30 minutes on a shaker in the presence of a protease inhibitor cocktail and 6.5 mM of DTT. Samples are then centrifuged at 1200xg for 20 minutes. The sample collection procedure was suspected to be the cause of the loss of arginase activity.

We have tested three different commercial protease inhibitors along with three different concentrations of DTT. The results showed that a protease inhibitor cocktail plus DTT (6.5 mM) performed best in stabilizing the samples. Lower arginase activity was observed if samples were collected using only 0.65 mM DTT or less.

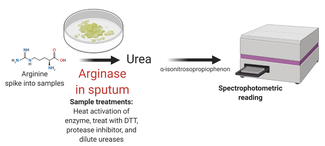

Figure 2: Arginase in Sputum Method

During the assessment of dilutional linearity it was observed that for some samples the activity of arginase increases with increasing dilution. It was suspected that the urease present in samples may be interfering with the measurement of the arginase activity. The suspected samples were spiked with urea and were assayed without the substrate to measure urease activity. To overcome interference from urease activity, the minimum dilution of samples was increased from 2-fold to 8-fold. Finally, the specificity of the method was confirmed by spiking N-hydroxy-nor-L-arginine (nor-NOHA). This specific arginase inhibitor inhibited arginase activity and the formation of urea in a dose-dependent manner.

		Conc. mU/mg		
Preanalytical condition	**sample ID**	**24 hours (T0)**	**72 hours**	**7 days**
- DTT + protease inhibitor	1P	397	388	234
- DTT + protease inhibitor	2P	354	328	276
- DTT + protease inhibitor	3P	376	209	188
+ DTT + protease inhibitor	1DP	510	488	519
+ DTT + protease inhibitor	2DP	432	409	455
+ DTT + protease inhibitor	3DP	396	401	421
+ DTT - protease inhibitor	1D	488	470	432
+ DTT - protease inhibitor	2D	398	311	235
+ DTT - protease inhibitor	3D	401	302	198

Table 2. Stability samples with DTT (6.5 mM) and protease inhibitor before storage

Sample	24hrs	72 hrs	7days	1 month	2 months	% bias 2 months
1DP	510	488	519	444	472	7.45
2DP	432	409	455	371	384	11.1
3DP	396	401	421	328	331	16.4

Table 3. Long-term stability maintained to 2 months with DTT and protease inhibitor

CASE STUDY 2: TOTAL NITRITE AND NITRATE IN SPUTUM

There is evidence that NOS activity is increased in acute pulmonary exacerbation of CF. However, while elevated exhaled NO is reported in patients with asthma and upper respiratory tract infection, it is not observed in CF patients. It is suggested that in CF patients, NOS is activated yet the NO produced by NOS does not diffuse into the airways but accumulates in sputum in oxidized form (NO2 and NO3). This suggest that the measurement of exhaled NO in CF patients is not a relevant indicator of airway inflammation and NOS activation in CF patients. Sputum levels of NO2 and NO3 are a more relevant measure of NOS activation.

Hence it is important to have a highly robust and reproducible method for the measurement of NO2 and NO3 in CF patients. We have developed a two-step method for the measurement of total nitrate/nitrite in sputum. During the first step the nitrate is converted to nitrite using nitrate reductase. In the second step, nitrite is converted to deep purple azo compound by adding. The intensity of color is propositional to the amount of total NO2 and NO3 in samples and is measured at 540 nm using a colorimetric plate reader.

It is known that the antioxidant such as DTT will interfere with the color development. In an attempt to minimize the impact of DTT, we evaluated various DTT concentrations, from 50 μM to 1000 μM. The data shows that DTT at 100 μM or greater interferes with the assay. One way the interference could be minimized is by performing standard correction. In that case the same amount of DTT added to the samples were also added in the standard curve preparation. A correction factor was developed by comparing standard curve in buffer with DTT and without DTT. The correction factor was utilized to calculate NO2 and NO3 levels in unknown samples. If the intended purpose of the assay is to measure fold changes, the correction factor may not be necessary.

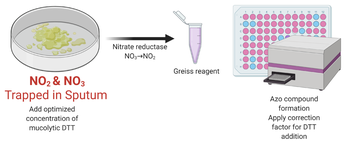

Figure 3: Nitrite in sputum method

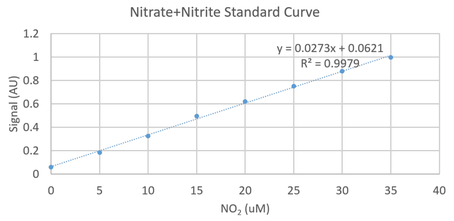

Figure 4: Linear standard curve of nitrite after standard correction for DTT interference

CONCLUSION

For sputum biomarkers to be considered as accurate measures of biologic activity and of potential relevance to disease intervention, it is critical to develop sample collection procedures that will maintain sample integrity and will be free from artifacts from sample processing. We have shown that the bioanalysis of biomarkers in sputum poses unique challenges but also demonstrated that most of these challenges can be overcome by implementing a “fit-for-purpose” method development approach and methodical investigation of assay specificity, reproducibility, and interferences.

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