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Observations of, and Insights into, Cystic Fibrosis Mucus Heterogeneity in the Pre-Modulator Era: Sputum Characteristics, DNA and Glycoprotein Content, and Solubilization Time

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Abstract: Airway obstruction with chronic inflammation and infection are major contributors to the lung damage and mortality of cystic fibrosis (CF). A better understanding of the congested milieu of CF airways will aid in improving therapeutic strategies. This article retrospectively reports our observations, and discusses insights gained in the handling and analysis of CF sputa. CF and non-CF mucus samples were surveyed for morphological features by electron microscopy and analyzed for the macromolecular dry weight (MDW), total protein, lipid, carbohydrate, and DNA. Mucus character was investigated with chemical solubilization time as a comparative tool. CF mucus appeared distinctly thick, viscous, and heterogeneous, with neutrophils as the dominant immune cell. CF sputum DNA content varied markedly for and between individuals (~1–10% MDW), as did solubilization times (~1–20 h). CF Sputum DNA up to 7.1% MDW correlated positively with solubilization time, whereas DNA >7.1% MDW correlated negatively. 3D analysis of CF sputa DNA, GP, and solubilization times revealed a dynamic and predictive relationship. Reflecting on the heterogeneous content and character of CF mucus, and the possible interplay in space and time in the respiratory tract of polymeric DNA and mucous glycoproteins, we highlight it's potential to affect infection-related airway pathologies and the success of therapeutic interventions.

Keywords: cystic fibrosis; mucus; sputum; sputa; DNA; glycoprotein; analysis; character; bronchiolar plugs; solubilization

1. Introduction

Cystic fibrosis (CF) is an exocrine and genetic disorder hallmarked by thick mucus in the respiratory tract, bronchitis, and frequent and persistent infections [1–6]. Progressively chronic infection, inflammation, and airway obstruction contribute to bronchiectasis, diminished lung function, and shortened life spans [1–6]. Loss of function mutations in the DNA for the cystic fibrosis transmembrane conductance regulator (CFTR) lead to imbalances in salts (including bicarbonate) and water transport across the secretory systems throughout the body, and contribute to altered mucus rheology (viscosity and elasticity), diminished mucociliary clearance (MCC), and declining pulmonary function [4,7–11]. Differences in CF and non-CF airways and how they manage infection have been recognized for decades and investigated intensively with functional assays and chemical and microscopic analyses [1,12–18]. Structural elucidation studies of CF and non-CF tracheobronchial mucins have added insight into the chemical changes associated with CF resulting from the CFTR mutations, the inflammation,

the recurrent infections, or the constellation of these and other factors [7,15,19–26]. Countless animal, cell, and organelle culture models have been, and continue to be, employed to give valuable guidance on how to improve the health of patients with CF [1,8,27–33].

Therapeutics development for CF has provided many tools for better managing this disease, and individuals with CF are now living much longer, higher-quality lives [5,6,34–36]. Much respiratory research has been toward combating opportunistic pathogen colonization and improving MCC. Strategies continue to evolve aimed at rehydrating the airway surface, (including the collapsed periciliary and mucus layers); dissociating and mobilizing the abundant entangled dehydrated mucous glycoproteins; and fragmenting the significant quantity of polymeric neutrophilic DNA arising from persistent inflammation and reduced MCC [3,4,6,31,37–46]. Rigorous individually-tailored daily treatment regimens now often include oral, intravenous, and/or aerosolized antibiotics and intense chest physiotherapy with or without inhaled recombinant human DNase (rhDNase), mucolytics, and/or osmotic agents [3,5,6,31,34,35,43–45]. Novel oral therapeutics targeting the basic defects are ushering in a new era for CF [4,6,34]. Addressing the synthesis, trafficking, and function of the mutant CFTR, "modulators" are proving successful at increasing functional CFTR activity, and in doing so, are improving airway hydration, MCC, and pulmonary function [4–6,11,34]. Even so, chronic obstruction, infection, inflammation, and resulting lung damage remain significant CF life challenges [4,5,33,47]. This is particularly so for individuals for whom either the lung pathology is already extensive [6] or the new agents are not available, accessible, or effective [4–6,48].

With chronic airway obstruction as a central issue in CF, the character of the so-called "CF mucus" is the focus of this research report. Normal airway mucus is innately protective and cleansing. CF mucus is more adherent, viscous, dehydrated, and impairs normal mucociliary escalator clearance [1,4,9,10,16,17,28,30,32,40,42,49–51]. Alterations in, and macromolecular complexity of, CF secretions create physical and chemical barriers to the immune system, to regional ventilation, to antibiotic and mucolytic agents, and to potential gene therapy vehicles [4,37,48,50,52–56]. CF mucus is a rich mixture of plasma proteins, inflammatory cells, DNA, bacteria, and bacterial products, with alveolar and tracheobronchial epithelial and submucosal gland secretions. CF mucus has both organic and inorganic constituents in the 'sol' and 'gel' phases at the airway epithelial interface, and shows abnormal acidification, reflecting the defective CFTR expression and chloride, sodium, and bicarbonate transport activities [9,10,47,53]. CF airway mucus is typically purulent, i.e., DNA content >0.025% of mucus dry weight, with the elevated DNA content affecting mucus viscosity, elasticity, and transport [37–39,45,47,52]. CF airway mucous glycoproteins may also be hypersecreted, defectively expanded, and/or hyperconcentrated, also impacting mucus character and MCC [4,17,18,28,31,36,40, 42,51,56].

Our laboratories endeavor to bring basic research findings to the clinical CF community to contribute to increasing the understanding, and reducing the pathologies, of this disease. Emphasizing CF respiratory tract health, infection, and related damage, we have focused on mucins, pathogens, and drug interactions at epithelial cell interfaces. Investigations have included structural elucidation of sulfated oligosaccharides liberated from tracheobronchial mucous glycoproteins [2,19–22,26], characterizations of the heterogeneity of host-adapted *Pseudomonas aeruginosa* (a major opportunistic pathogen in CF) [23,57,58], and in vitro studies of the effects of bacterial virulence factors on the human host [59–61]. Recent reporter cell-based assays revealed cytotoxic synergies toward bronchial epithelial cells of Pseudomonas phenazines with a polymyxin antibiotic used in CF, colistimethate [60]. This was especially concerning considering the chronically obstructed, often Pseudomonas colonized, status of adult CF airways and prompted us to prepare the current retrospective review of our data and insights on CF mucus character.

Here we report and discuss observations about CF mucus from our extensive experience handling CF and non-CF sputa. While these specimens were gathered for structural elucidation studies, for which the structural data have been reported, the insights gleaned from the handling and analyses of these samples have not previously been detailed, summarized, and formally discussed. Here we

offer microscopic, compositional, and biochemical observations about the character and heterogeneity of CF mucus, relative to non-CF mucus and with respect to individuals with CF over time. The data highlights the variable amounts of DNA between and within individual patients, and the combined effects of DNA with mucous glycoproteins on sputum chemical solubility, with solubility used as a measurable and comparative research tool. The discussion includes insights into potential impacts of this variable dynamic between DNA and mucous glycoproteins on CF airway infection and damage, and suggests potential opportunities to more effectively therapeutically intervene.

2. Materials and Methods

2.1. Study Declarations

This research was performed as an exempt human subject research study, in accordance with the National Health and Human Services (HSS) Regulations for the Protection of Human Subjects, 45 CFR 46, Exemption 4, following the rules of the Declaration of Helsinki of 1975. Protocols for obtaining and handling the de-identified human specimens, and the data obtained from them, were reviewed and approved by the University of Missouri Health Sciences Institutional Review Board (IRB) and the University of Missouri Institutional Biosafety Committee. As per national regulations, this laboratory research was an IRB-exempt human research investigation, no consent for participation was required, and no patient identifier or other patient private health information was associated with these data.

This research was initially designed for the study and structural analyses of tracheobronchial mucous glycoproteins from de-identified sputa as "unwanted materials" in IRB classification. As such, patient demographic information, and related microbiological culture data of these sputa, were not collected at the time.

Data generated or analyzed during this study are included in this published article and its Supplementary Materials. No patient-derived specimens are available for acquisition from the authors; patient-derived specimens reported on in these studies were either consumed in the research or forwarded on to the parallel structural elucidation studies for which they were originally obtained, and consumed in those analyses.

2.2. Collection and Chemical Analysis of Sputum Samples

Sputum samples from patients with cystic fibrosis were obtained by expectoration or via postural drainage, and from chronic bronchitic patients by endotracheal aspiration during bronchoscopy or by expectoration. With great care not to mix the samples, aliquots were taken for chemical analyses, solubilization studies, and microscopy.

In a unique time-collection study of sputa DNA content within a hospital stay of duration, four individual patient volunteers, coordinated with a research nurse, were able to donate sputum three times a day for five consecutive days. Specimens, acquired in sufficient amounts at each time point, were collected at approximately 08:00, 12:00, and 16:00. While no patient identifier, demographic or microbiological culture data are available related to these de-identified specimens, the notes provided indicate that during their clinical stay, these patients varied from each other in their antibiotic treatments with tobramycin, carbenicillin, gentamicin, ticarcillin, and others, in addition to the use of Pancrease, Bronchosol and Mucomyst.

Sputum aliquots for determining water content and dry weight were weighed, immediately frozen, lyophilized, and dry masses recorded. The resulting dry mass was considered the macromolecular dry weight, (MDW), and here referred to as synonymous with "dry weight".

Sputum aliquots designated for total protein, lipid, carbohydrate, and DNA analysis were immediately frozen after the addition of sodium azide to a final concentration of 0.02% and the samples were stored at −25 °C until analyzed. For total lipid, frozen weighed samples were delipidated by extraction three times each with ether:acetone (1:1, vol/vol) and ether:chloroform:methanol (1:2:1, *v*/*v*), 24 h each at 4 °C. The organic solvents were pooled for each sample, dried under nitrogen, and the

lipids weighed. Sputum DNA was quantitated by both the colorimetric and fluorometric procedures of Cerriotti [62] and Kissane and Robins [63], respectively. Protein was assayed by the procedure of Lowry et al. [64], and phospholipids analyzed, after silica gel [65] and two-dimensional thin-layer chromatography [66], by the procedure outlined by Lowry and Tinsley [67]. For carbohydrate analysis, acid hydrolysis and gas-liquid chromatographic determination of neutral and amino sugars were performed as described by Mawhinney et al. [68]. Sialic acid, liberated by hydrolysis in 0.05 M H_2SO_4 at 80 °C for 1.5 h and separated by ion exchange chromatography, was determined by the thiobarbituric acid assay [69] and by gas-liquid chromatography [70].

For specimens designated for collection of mucous glycoproteins, delipidated sputum residue was then dialyzed exhaustively against distilled water (Spectrophor III dialysis tubing, 3500 MW exclusion), lyophilized, weighed, and resuspended in five volumes of 0.15 M NaCl. Large molecular weight mucous glycoproteins (>1.5 MDa) were then isolated from reduced, carbamidomethylated, DNase treated sputum by Bio-Gel A-5m, as previously described [13,19]. Aliquots were lyophilized, weighed, and analyzed for protein and for carbohydrate. Carbohydrate profiles of crude sputa and isolated high molecular weight glycoproteins were compared. Isolated high molecular weight glycoproteins with carbohydrate profiles typical of tracheobronchial mucous glycoproteins, and notably free of mannose (characteristic of membrane glycoproteins), were considered mucous glycoproteins (GP). Resulting dry weights were used for calculation of GP % of sputum MDW. Such values were determined for the 87 CF sputum used in the solubilization studies, described below.

Sputum compositional survey data were initially expressed as mg/mL sputum basis for comparison. Sputum components were then additionally compared by class as a percent of macromolecular dry weight, i.e., protein % MDW, lipid % MDW, carbohydrate % MDW, and DNA % MDW. The differences in the combined values of the protein, lipid, carbohydrate, and DNA contents and the total lyophilized mass MDW represent the components not chemically analyzed, such as salts and semi-volatile components, and are referred to as "undetermined".

As no comprehensive sputum culture reports were available associated with these CF specimens, quick in-house laboratory screens at the time (i.e., growth on MacConkey and cetrimide agars) were used to check for the presence of *Pseudomonas aeruginosa.*

2.3. Assessment of Variation in DNA Content within Individual Sputum Samples

To study the variability of DNA content within individual sputum samples, sputum aliquots of sufficient volume (>5.0 mL) were immediately frozen and kept at −70 °C to minimize any mixing. Five such purulent non-CF specimens and twelve such CF specimens were assigned to this analysis. Without thawing, the samples were then individually lyophilized to a dry firm residue. From each lyophilized sample, twelve 1.0 mg aliquots were randomly taken, and each individually assayed for DNA content. The remaining residue was rehydrated with 3–4 mL distilled water and assayed for total DNA, protein, lipid, and carbohydrate, as described above.

2.4. Sputum Solubilization Studies

Sputum solubilization investigations arose from in-laboratory observations that noted variations between, and appearances of, sputum gels as they were processed for mucous glycoprotein isolation. In an attempt to better understand these differences further, and in addition to ongoing chemical studies, a dissociation approach was developed that employed a consistent, gentle, and low-temperature chemical solubilization protocol. Sputum samples designated for solubilization studies were weighed immediately after collection. Aliquots were then taken for DNA and carbohydrate determination. Samples were then placed in 25 volumes (wt/vol) of 5 mM phosphate buffer, pH 8.0, containing 5 M guanidinium hydrochloride, 1% β-mercaptoethanol, 0.01% disodium ethylenediaminetetraacetate (EDTA), and stirred at a constant rate at 4 °C. After 30 min, solution aliquots were then constantly monitored by passage through a Waters Associates Differential Refractometer. Solubilization was considered complete when a stable baseline with less than a 3% deviation was achieved, a gelatinous

pellet was not noted following centrifugation at 20,000× *g* for 30 min at 5 °C, and greater than 98% of the sputum amino sugars could be found in the supernatant following centrifugation. In several experiments, β-mercaptoethanol and/or EDTA were eliminated from the solubilization solution. Typically, data were plotted as hours for complete solubilization vs. DNA % of macromolecular dry weight (MDW), also referred to as "% dry weight". Similarly, data were plotted for solubilization time vs. glycoprotein % MDW. Sputum GP content was calculated from the masses of aliquots of chromatographically isolated pure mucous glycoprotein preparations, as described above. 2D plots and trend lines were generated with Microsoft Excel and SigmaPlot (Systat) software, while 3D graphical analysis was performed using SigmaPlot. Where original plotted historical data existed only as hard copies, digital data points for replotting were assigned with the digitization program PlotDigitizer (published by Free Software Foundation, http://plotdigitizer.sourceforge.net).

2.5. Collection, Chemical Analysis, and Solubilization of Bronchiolar Plugs

Bronchiolar plugs were collected at autopsy within 1 to 2 h post-mortem from CF patients. Aliquots of 77 random plugs were then assayed for DNA, mucous glycoproteins, and the time required to solubilize in chaotropic solution, as described above.

2.6. Electron Microscopy

CF and non-CF samples for electron microscopy (EM) were collected in sputum tubes and covered with an equal volume of 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, at 4 °C. Within 24–48 h samples were removed from the tubes, minced into 1–2 mm cubes, placed into fresh 2.5% glutaraldehyde for an additional 2 h, and rinsed in 0.1 M cacodylate buffer containing 7.5% sucrose. Post-fixation was carried out at room temperature for 1 h in 1.0% osmium tetroxide in Millonig's phosphate buffer, pH 7.2 [71]. Dehydration was accomplished through a graded series of ethanol, which was then exchanged for propylene oxide, and finally, specimens were embedded in Epon 812 [72]. One-micron sections were cut on a Sorvall MT-2 ultramicrotome. For light microscopy, sections were stained with 1.0% toluidine blue in 1.0% sodium borate. For EM, silver-gold sections were stained with uranyl acetate (saturated in 50% ethanol) and Reynold's lead citrate [73]. Grids were examined on a Hitachi HS-8 Electron Microscope. Micrographs were adjusted for appropriate contrast with Adobe Photoshop.

3. Results

3.1. Morphological Observations of CF and Non-CF Sputa, and CF Bronchial Plugs

Morphological studies of CF sputa, as shown in Figure 1a, demonstrated many consistent features. In general, there were extensive numbers of neutrophils clumped together, often in a linear fashion within a mucous matrix, or in amorphous clumps consisting of cell fragments and cytoplasm organelles. The neutrophil population tended to be synchronous in its development, stage of activity and degeneration, and be increased with increased sputum DNA content. The presence of disintegrating nuclei was so characteristic of the CF sputa that specimens could be identified as CF based on this finding.

Microscopic analysis of non-purulent and purulent bronchitic sputa in this study included patients with bacterial pneumonias, chronic asthma, chronic lung disease with bronchiectasis, and infection secondary to lung cancer. In general, mucoid non-purulent sputa showed predominant areas of mucous glycoprotein-like sheets with small areas of cell debris, very few inflammatory cells, an occasional macrophage, and some particulate matter. As illustrated with Figure 1b, purulent non-CF sputa typically displayed an abundance of mucous material with scattered inflammatory cells, very little cellular debris, with the neutrophil as the predominant inflammatory cell associated with pulmonary bacterial infections.

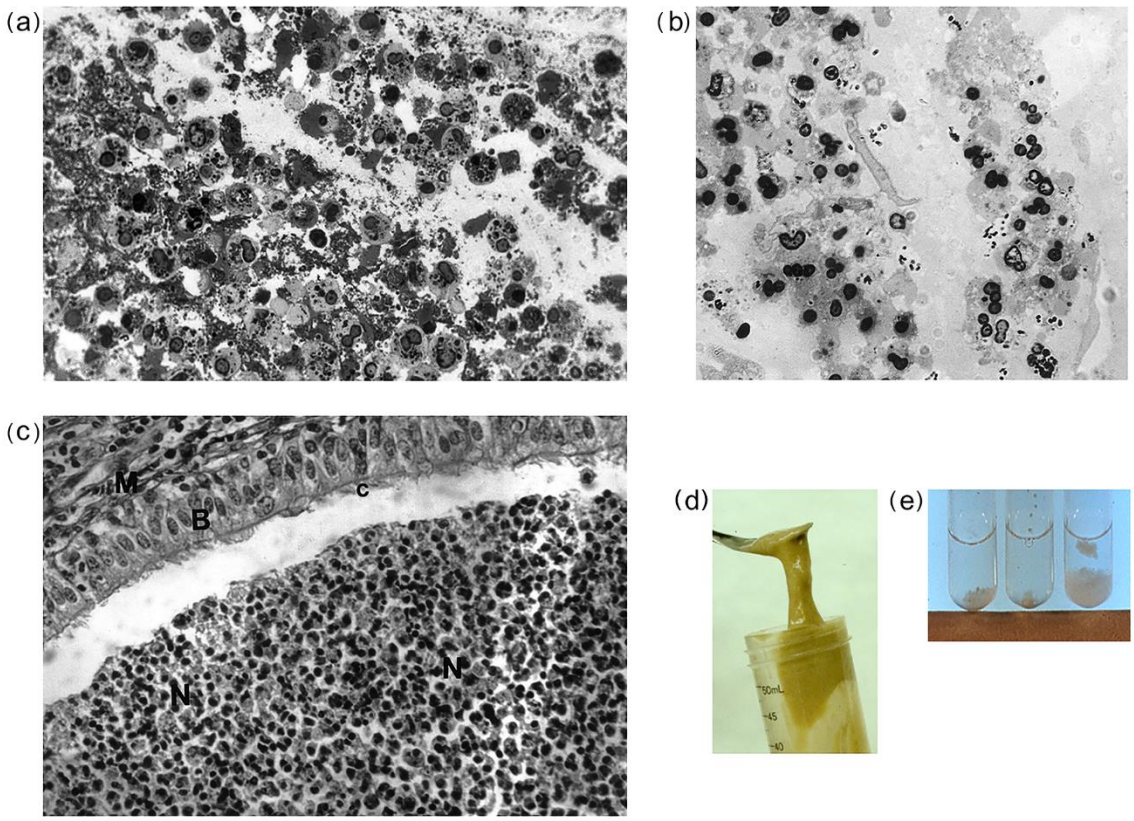


Figure 1. Visualizing cystic fibrosis (CF) and non-CF sputum and the bronchial epithelium-mucus interface. (**a**) Electron micrograph of an epoxy embedded section of sputum from a CF patient showing a synchronous population of degenerating neutrophils with swollen nuclei. The background consists of cytoplasmic components from neutrophils and amorphous dead cells. Stain: uranyl acetate and lead citrate. Original magnification 950×; (**b**) Electron micrograph of an epoxy embedded section of purulent control sputum from a non-CF patient showing predominantly intact mature neutrophils and bacteria in a mucous stroma. The non-cellular background appears primarily as an amorphous material forming a bridging mucoid lattice, with only small amounts of cellular components, debris, or membranous material. Stain: uranyl acetate and lead citrate. Original magnification 950×; (**c**) Light micrograph of a bronchus in a patient with CF who died of respiratory failure showing the lumen filled with degenerating neutrophils (N) adjacent to bronchial epithelial cells (B) with intact cilia (C). The bronchial wall is surrounded by a mononuclear cell infiltrate (M). Stain: 1.0% toluidine blue in 1.0% sodium borate. Original magnification 200×; (**d**) Photographic example of material harvested from the bronchial tree of a CF patient within 2 h post-mortem. This very thick "sludge-like", with almost tar-like, feel and appearance was not uncommon to see for late-stage CF sputa and post-mortem specimens; (**e**) Visual depiction of the heterogeneity of mucus character of three sputum specimens undergoing solubilization in guanidinium hydrochloride. The left two more dense specimens are examples from two CF patients, and the right more expanded looking material is sputum from a non-CF volunteer.

Examination of histological sections from the lungs of two patients with CF who died of respiratory failure showed ~80% of the small bronchi containing primary plugs of degenerating neutrophils and free DNA. Notably, DNA was determined to make up 9.6% and 9.1% of the plug specimens' dry weights. Figure 1c is characteristic of the histology observed for CF bronchial plugs, with the lumen filled with degenerating neutrophils and the bronchial wall remarkably intact, with preservation of epithelial cilia and very little detectable mucous glycoprotein being observed.

Mucus materials collected from the CF bronchial tree post-mortem often presented as very thick, tar-like material, as depicted in Figure 1d, quite in contrast to what is observed for normal airway mucus. From healthier CF patients, sputa could, at times, resemble normal or non-CF purulent sputa visually. However, when these samples are subjected to chaotropic agent solubilization analysis, CF sputa most often tended to be denser and expand less readily than normal airway secretions, as seen in Figure 1e.

3.2. Chemical Results

The chemical compositions of non-purulent, purulent non-CF, and CF sputa (n = 1003 total specimens) are presented in Table 1, and with more detail in Supplementary Table S1. A selection of this data is included in a recently published abstract [74]. Purulence was based on the percent DNA composition of the total sputum macromolecular dry weight (MDW), with the non-purulent classification assigned to sputa with DNA of less than 0.025% MDW, and purulent for all specimens with DNA >0.25% MDW. The water content of surveyed sputa from CF patients was significantly lower than that observed for both purulent and non-purulent non-CF sputa. Concomitantly, the MDW, protein, and lipid content of CF sputa were markedly elevated when compared to values from non-CF sputa. Notably, of the lipids, phosphatidylcholine (primarily as dipalmitoylphosphatidylcholine) represented ~50% of the total lipids in CF sputa, a significantly higher percentage than for other sputa. The total carbohydrate content of CF sputum, though increased on a mg/mL of sputum basis, actually comprised a smaller percentage of the dry weight of sputum when compared with non-CF sputum samples. The compositional analysis, as shown in Supplementary Table S2, of chromatographically isolated tracheobronchial mucous glycoproteins (TBGs) revealed carbohydrate constituents typical of TBGs for both non-CF and CF sputa [2,7,13,15,16,19,24]. Elevated sulfate and sialic acid content were found for CF specimen glycoproteins, consistent with previous reports on sputa of severely infected individuals with chronic bronchitis or CF [1,2,7,13,15,24,25]. Unfortunately, for the specimens of this retrospective biochemical study, no correlative data on patient disease severity, pulmonary function, or infection type and load are available. While the CF sputa used in the solubilization assay (n = 87) did screen positive in the laboratory for *Pseudomonas aeruginosa*, no quantitation data is available.

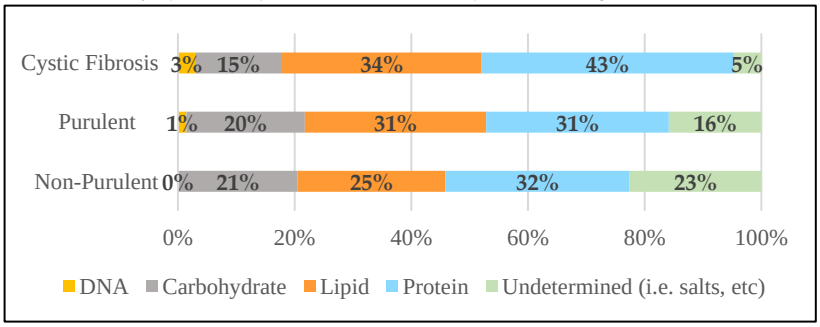
Total DNA content in CF sputum was significantly increased when compared to all non-CF sputum values, whether based on mg/mL of sputum or % MDW. As reported in Table 1, sputa mean DNA contents were: 2.94% MDW for CF sputa vs. estimated ~0.014% MDW for non-purulent sputa and 1.44 DNA % MDW for purulent non-CF sputa.

Of note, upon examination of compiled chemical data of the wide variety of specimens, it was statistically apparent, via both content and related standard deviations, that there was considerable heterogeneity in specimens. This was more apparent for purulent non-CF and CF sputa. Based on combined average determinations of protein, lipid, carbohydrate, and DNA for specimens, as indicated in the figure within Table 1, there was similarly a sizable % of the dry weight (% MDW), which is not chemically defined. In non-purulent sputa, for example, with the expectation that measured components would approximate 100% of MDW, and that the unmeasured NaCl of these sputa would average about 20% of the MDW (i.e., saline at 9 mg/mL, physiologically), this would account for much of the remainder of the significant "undetermined" category displayed (23%). For the compiled data determined for CF sputa, a much lower "undetermined" % was seen (~5%, representing ~5 mg/mL sputa). This lower value likely reflects the marked heterogeneity of the collected CF sputa in terms of both its content and density. Furthermore, mass per volume differences are likely due to the character of the lipid and protein content, as well as the expansion and gelling of DNA and mucous glycoproteins within the sputa. As data on the demographics and disease states of the individuals from whom the sputa were attained are unfortunately not available, we cannot retrospectively test the hypothesis of specific compositional differences based on age, disease severity, or CFTR genotypes.

Table 1. Chemical composition of non-purulent, purulent, and cystic fibrosis sputum [a,b,c].

Constituent	Non-Purulent [a] (*n* = 213)	Purulent (*n* = 322)	Cystic Fibrosis (*n* = 468)
Water (mg/mL)	959 ± 15	939 ± 17	862 ± 35 [d]
Macromolecular dry weight (MDW, mg/mL)	43 ± 2.4	61 ± 7.6 [d]	98 ± 13.4 [d]
Protein, mg/mL	13.6 ± 1.2	19.1 ± 4.9 [d]	42.2 ± 19.4 [d]
Lipid, mg/mL	10.9 ± 0.6	19.0 ± 1.5 [d]	33.5 ± 4.1 [d]
Carbohydrate, mg/mL	8.8 ± 1.1	12.4 ± 1.6 [d]	14.6 ± 2.5
DNA, mg/mL	0.006 ± 0.003	0.88 ± 0.26 [d]	2.88 ± 1.36 [d]
Protein % MDW	31.6%	31.3%	43.1%
Lipid % MDW	25.3%	31.1%	34.2%
CHO % MDW	20.5%	20.3%	14.9%
DNA % MDW	<0.025% *(0.014% estimate)*	1.44%	2.94%

Survey Sputa Compositional Data as Component Average % MDW [e]



DNA % MDW of Sputa Evaluated for Intra-Specimen Heterogeneity [f]

	DNA % MDW +/−S.D.	DNA % MDW range
Purulent non-CF (*n* = 5)	1.4 +/−0.6	0.54–2.15
Cystic Fibrosis (*n* = 12	3.9 +/−2.5	0.95–8.7

DNA and Mucous Glycoprotein % MDW of CF Sputa Evaluated for Effects on Sputum Solubility [g]

CF Sputa (*n* = 87)	% MDW +/−S.D.	% MDW range
DNA % MDW	6.7 +/−2.2	2.9–9.8
GP % MDW	4.6 +/−1.4	2.2–8.3
DNA + GP % MDW	11.3 +/−1.7	6.1–14.2

[a] Sputa are classified as non-purulent if DNA content is <0.025% MDW; purulent, if DNA is >0.25% MDW. [b] Data expressed as mg/mL sputum ± S.D., unless otherwise indicated. (See additional detail in Supplementary Material Tables S1 and S2). [c] Number of samples assayed (*n*), four separate samples assayed per CF patient, 1–2 samples for all others. [d] Significance of $p < 0.005$, Student's t-test, compared with non-purulent and purulent values. [e] Undetermined % MDW, the difference between lyophilized sputum mass MDW and total chemically determined components. Ex. non-purulent sputa 0.9% NaCl, for MDW of 43 mg for 1 mL sputum, 9 mg or 21% undetermined could be NaCl. [f] Total DNA content for random individual specimens of >5 g. These specimens were used to determine variability among multiple samplings of the same specimen. See Section 3.3 for within-specimen DNA distribution study data. [g] GP % MDW based on the lyophilized dry weight of chromatographically isolated mucous glycoproteins. These specimens were used to test DNA and GP content effects on the chemical solubility of CF sputa. See Section 3.5 for solubility study data.

For the study of intra-specimen heterogeneity of distribution of DNA of purulent non-CF sputa and CF sputa, larger specimens were selected to ensure enough material to do these analyses. The total DNA content for these larger volume samples, highlighted in Table 1, demonstrates a much broader DNA content range than is evident in the survey summary data.

Similarly, for CF sputum solubility studies, larger specimens were also selected. These sputa were analyzed for both DNA and mucous glycoprotein % MDW. As indicated in the methods, the glycoprotein (GP) values represent the % of the sputum dry weight that was calculated based on the mass of aliquots of chromatographically purified high molecular weight glycoproteins derived

from these sputa with the carbohydrate profile typical of tracheobronchial mucous glycoproteins. These data are also included in Table 1. For comparison with the literature, the combined content as % MDW for DNA and GP is also calculated, and illustrates that the percent of solids (% dry weight), here portrayed as % MDW, ranges ~6–14%, averaging ~11% MDW. The contribution of DNA and glycoproteins to the character of CF mucus are investigated and discussed with the solubility data.

3.3. Within-Specimen DNA Content Variability for CF and Purulent Non-CF Sputa

A wide range of values of total DNA for CF specimens was observed when evaluating particularly large volume CF sputa (>5 g) dedicated to "within-specimen" uniformity/heterogeneity studies. As noted in Table 1, and detailed in Table 2, CF sputa DNA in these representative large specimens ranged from ~1–9% of the dry weight, whereas total DNA in large purulent non-CF specimens spanned a narrower range (0.5–2.2% MDW).

Table 2. Variability of DNA content within individual purulent non-CF and CF sputum samples [a,b].

Purulent Non-CF			Cystic Fibrosis		
Total DNA	**Range**	**% Variation**	**Total DNA**	**Range**	**% Variation**
0.54	0.46–0.59	14.8	0.95	0.09–1.93	103.2
1.13	1.02–1.31	9.7	1.18	0.56–3.07	160.2
1.3	1.10–1.47	13.1	1.27	0.48–3.19	151.2
1.82	1.63–2.09	14.8	1.97	0.35–5.13	160.4
2.15	1.84–2.23	3.7	2.54	0.74–6.22	144.9
			2.88	0.63–5.65	96.2
			3.46	0.87–6.33	82.9
			4.72	1.17–7.49	58.7
			5.35	3.45–6.32	18.1
			6.51	4.72–7.83	20.3
			7.13	6.33–7.95	11.5
			8.7	7.81–9.05	4.0
		range of % variation			*range of % variation*
		3.7–14.8%			4.0–160%

[a] Total DNA expressed as % of macromolecular dry weight (% MDW). [b] Range of DNA % MDW among multiple sampling aliquots (12) of the same individual patient specimen (5 non-CF patients; 12 CF patients); % variation from the mean DNA % MDW, reflecting the heterogeneity of DNA distribution within the individual's sputum specimen.

Questioning the uniformity of distribution of DNA within individual purulent non-CF and CF sputum specimens, random samplings from each designated unmixed lyophilized sputum residue were assessed for total DNA content as % MDW, and data are reported in Table 2. For representative purulent non-CF sputa with total DNA of the range 0.54 to 2.15% MDW, replicate samplings from within each collected sample demonstrated that within-specimen DNA distribution for purulent non-CF sputa varied 15% or less from the mean total sputum DNA % MDW (3.7–14.8% variation). Representative CF sputa, with total DNA contents of the range 0.95 to 8.70%, showed extreme within-specimen heterogeneity, with the range of variability for individual CF sputum replicate aliquot samplings of 4–160%. Comparison of within-specimen DNA variation of only CF sputa of comparable total DNA content to non-CF purulent (i.e., CF sputa with DNA values 0.95 to 2.54% MDW), still yielded greater variability in DNA distribution among CF samples (>100%) than non-CF sputa. As total CF sputum DNA content increased from 2.88% to 6.51% DNA, variation in distribution across the specimen decreased from 96% to ~20% of the mean. CF sputa with the highest total DNA content, i.e., >7%, exhibited the least heterogeneity at <12% variation in their DNA content across the respective samples.

3.4. Individuals' CF Sputum DNA Content over Several Days of Treatment

Sputum DNA content was also assessed over time for four individuals with CF. Total DNA % MDW was determined for sputa collected, at three time points per day, for five successive days during

hospitalization and treatment. Initial DNA content varied between patients, ranging between 3.2–5.5% MDW. As plotted in Figure 2a and noted in a recently published abstract [74], by the end of five days sputum DNA content was reduced by 2% MDW for each patient, reflecting improvements of 56.3%, 38.2%, 50.0%, and 41.2%, respectively, for patients 1–4. Additionally, a consistent cyclic phenomenon was perceived, with sputa acquired at 12:00 having higher DNA content than those obtained earlier or later in the day.

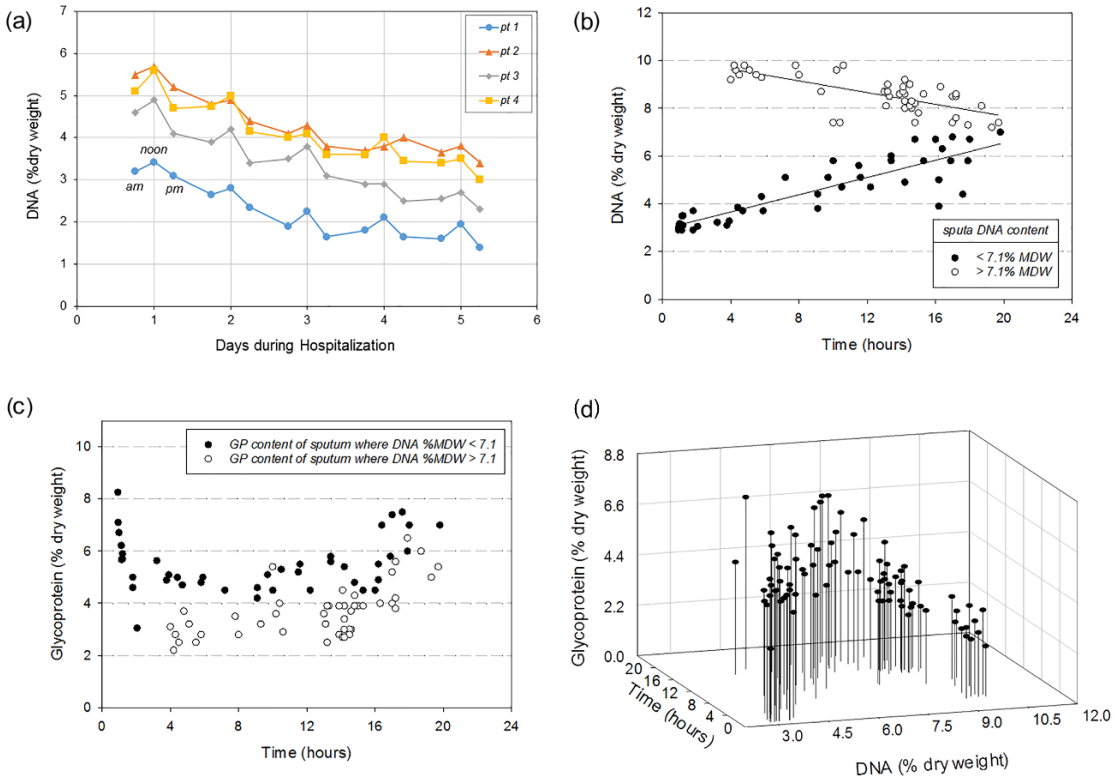


Figure 2. Graphical depiction of CF sputum DNA variability from four individuals over several days (**a**), and CF sputa (87) DNA and glycoprotein content heterogeneity and relationships affecting chemical solubilization time (**b**–**d**). (**a**) Variations in DNA content of sputa were obtained from four hospitalized CF patients during antibiotic and airway clearance treatment of clinical exacerbations. Sputa were collected at approximately 0800 h, 1200 h, and 1600 h. Patients 1–4 demonstrated a 56%, 38%, 50%, and 41% decrease, respectively, in their sputum DNA (% of macromolecular dry weight, MDW) content during five days of their clinical stay. In addition, a consistent rise in DNA content for the 12:00 sampling was noted, suggesting, at least in these patients, a possible cyclic phenomenon. (**b**) Chemical solubilization time in hours, and DNA for these 87 CF sputa varied widely. The amount of DNA appeared to affect chemical solubility in a complex manner, showing both positive and negative correlations with solubility as the level of DNA increased. The shortest solubilization times (i.e., <4 h) were observed for specimens with DNA content of 4% or less. Time to complete solubilization increased with sputa DNA content up to 7.1% MDW, and decreased as levels increased to ~10%, indicating additional factors at play; (**c**) Glycoprotein (GP) content from these same 87 CF sputa also varied markedly between individuals yet yielded no direct graphical correlation with solubility. The GP % MDW did appear to be less when the DNA content was greater than 7.1%, suggesting a shift in macromolecular dominance; (**d**) 3D graphical analysis of these CF sputa features of DNA and glycoprotein content with solubilization times revealed a horseshoe-shaped curve. This data pattern suggests a predictable solubility based on the relationship of DNA to glycoprotein within an individual specimen, and implies that the total macromolecular composition may be dynamic and important in airway clearance.

3.5. Chemical Solubilization Studies

Investigating the complexity of CF sputa, solubilization time (in hours), and its relationship to DNA and mucous glycoprotein content, was employed as a gauge to compare sputa of numerous CF patients and discern potential distinctions between CF and non-CF samples. Eighty-seven CF specimens were analyzed for time to complete solubilization in a chaotropic agent and plotted vs. specimen DNA and mucous glycoprotein content in Figure 2b,c, respectively. CF sputa DNA content ranged 2.9–9.8 DNA % MDW (with mean of 6.71%), and solubilization times ranged from ~1–20 h (0.92–19.8 h). A positive linear trend for correlation of DNA content and increasing solubilization time (up to 20 h) was observed for CF specimens which possessed <7.1 DNA % MDW, ($y = 0.1806x + 2.9352$, $R^2 = 0.7572$, $p < 0.05$), and an inverse relationship, i.e., negative trend down to 4 h, was observed for sputa with DNA concentrations >7.1%($y = -0.1228x + 10.132$, $R^2 = 0.5093$, $p < 0.05$).

Comparing purulent CF sputa to purulent non-CF sputa with DNA and solubilization time as the measurable features, in contrast to CF sputa, the non-CF sputa in Supplementary Figure S1, failed to show a consistent correlation between increased DNA content and increased solubilization time ($y = 0.0684x + 2.2588$, $R^2 = 0.1638$, $p < 0.1$). While 89% of CF specimens had DNA of >4% MDW, the majority of non-CF sputa (85%) had total DNA % MDW of <4%. Of note, this level of DNA (<4%) was also achieved by the four hospitalized CF patients in Figure 2a by the end of the 5-day sampling period. In this narrower range of DNA ~2–5% MDW, the solubilization times of purulent non-CF sputa still ranged from ~1–20 h.

Addressing the mucin component of CF mucus as a potential effector of sputum solubilization, Figure 2c illustrates the heterogeneity observed in mucous glycoprotein (GP) content for the 87 CF sputa for which DNA content and solubilization were depicted in Figure 2b (and referred to in Table 1). GP content ranged between 2.2–8.3% MDW, with a mean of 4.55%. Solubilization times relative to GP content showed only a slight correlation ($y = 0.0113x + 4.4259$, $R^2 = 0.0022$, $p < 0.05$). GP vs. solubilization values are plotted in Figure 2c as specimens with DNA breakpoint values of <7.1% or >7.1% MDW. As shown in this figure, the sputa with the lower DNA content tended to have higher % GP (mean 5.5%), and those with greater amounts of DNA had lower GP % MDW (mean 3.7%).

The ratio of GP:DNA content per CF sputum specimen and its effect on solubilization time was assessed, and data are depicted in Supplementary Figure S2. CF sputum GP:DNA values ranged from 2.84 to 0.22. Specimens with ratios of >1.5 were generally soluble in <4 h; and sputa requiring >10 h for solubilization typically showed GP:DNA ratios <1.5 (with 76% being < 1). Combining, per sputum specimen, the content of DNA and of GP for % of dry weight, the combined mean was 11.3% MDW (range 6–14%). Seventy-six percent of the specimens had combined totals of greater than 10% MDW with solubilization times ranging ~1–20 h.

CF bronchial mucus plugs obtained post-mortem ($n = 77$) were also examined for DNA and GP content and their relationships with plug solubilization. These data are presented in Supplementary Table S3. These specimens as a group shared many of the features of CF sputa, though bronchial plugs often possessed more DNA than expectorated CF sputa. For example, the hospitalized CF patients' sputa DNA in Figure 2a were all <6% MDW, whereas 47 of 77 bronchial plugs had DNA of >7% MDW. The majority of the plugs also tended to show less glycoprotein than DNA on % MDW basis (GP:DNA ratios <1). The heterogeneity and ranges of DNA and GP content, and effects on solubility, as a group, were similar to CF sputa. Bronchiolar plugs' DNA % MDW ranged 2.9–9.8%, GP % MDW ranged 2.2–7.5%, and solubilization times ranged from 1.2–20 h. For ease of comparison, the 77 plugs were grouped by data into fractions of the collection with similar characteristics. Fraction I had specimens with DNA 2.9–5.9% MDW, and a slightly narrower range of GP % MDW, and complete solubilization was achieved by 11 h. Fraction II of samples with moderate and comparable amounts of both DNA and GP (~6% MDW each), and Fraction III with plugs of DNA > 7% and a moderate amount of GP (mean of 4.1% GP), required longer to completely solubilize, ranging 10–20 h. Fraction IV, plugs with on average the highest % DNA (mean 9.5% DNA), possessed the lowest % GP (mean 2.9%) and the lowest GP:DNA ratio (0.31), and these plugs were solubilized by 11 h with a mean solubilization time

of 6.5 h. These trends paralleled the CF sputum positive and negative solubilization trends based on DNA content, seen in Figure 2b, and the relative GP to DNA content relationships in Figure 2c and Supplementary Figure S2. These data suggest that the absolute and relative amounts of both glycoprotein and DNA affect the behavior of the CF mucus specimen.

To further assess the combined impact of these two CF mucus characteristics (DNA and GP content) on solubilization time, 3D graphical analysis was utilized and presented in Figure 2d. The data revealed a horseshoe-shaped curve (resembling a tilted n-shaped parabola), suggesting a predictive pattern of relationships of GP and DNA affecting sputum solubilization.

4. Discussion

These observations, from the pre-CFTR modulator era, about CF and non-CF sputa have focused attention on the multitude of factors at play in the chronically obstructed and infected CF airway. The heterogeneity illustrated in CF sputa characteristics in this study, across a collection of specimens, and even for individuals over several days of time, indicate that the "state" of CF mucus is a dynamic conglomerate. The findings reported here direct us toward an understanding of the macromolecular players for which perturbations in the normal relationships with one another may affect the character of the mucus being elaborated by individuals with CF and its likelihood of being obstructive or being cleared from the airway.

4.1. CF Sputa Visual and Compositional Characteristics

CF sputum is known to have unique physical properties, including increased adhesivity, tenacity, and altered viscoelastic rheology, marked dehydration, and diminished transportability [1,4,12,13,17,18,32,37,38,40–42,46,50,52,55,56,75]. It has also been said that CF sputum gets it CF character from its purulency [14]. EM surveys here revealed consistent tendencies when comparing sputa from CF patients with sputa from patients suffering from other forms of chronic bronchitis. Visually, relative to non-CF sputa, a significant proportion of CF sputa was composed of acute inflammatory cells, cellular debris, and few bacteria, whether phagocytized or within a mucoid matrix. Marked neutrophil degranulation was common in CF specimens and appeared synchronous in its occurrence. Earlier studies suggest that components of CF sera may direct or affect neutrophil lysosomal degranulation activity [76]; if so, then this synchronicity could be expected.

As CF pathology is reported to include hyperproduction, hypersecretion, and/or hyperconcentration of gel-forming mucins [16–18,40,42,51,75], one might anticipate a significant amount of tracheobronchial mucous glycoproteins in CF patient sputum. In this investigation, microscopic examinations of CF sputa failed to demonstrate such an increase of mucous-like material relative to non-CF samples. CF bronchiolar mucus plugs illustrated that often in late-stage disease, homogenous dense populations of neutrophils were present with very little mucous glycoprotein matrix visibly apparent. At the time when these specimens were screened, no mucin-specific antibody staining was available to further detail this finding. Others, though, have also noted that with chronic infection, increasing age, and progression of lung disease, there appears a shift in dominance in the CF mucus from mucin, as the major macromolecular species, to DNA [18,39,52,77].

CF sputa chemical analyses reported here were consistent with previously published compositional data [1,7,12,13,15,24,25]. CF sputum, compared to non-CF sputum, was significantly increased in lipid, DNA, protein, sulfated mucus, and dehydration. Compositionally, expressed on a percentage of MDW, CF sputa showed a decreased amount of mucin-bound carbohydrate. From numerous studies, this increase in amounts of solids in the CF sputum would negatively affect the in vivo mucus rheology and cause increased mucus layer osmolarity, reduced periciliary layer fluid, and impaired ciliary movement and MCC [17,28,42,51,56], and result in the accumulation of lower lung constituents. This is clearly seen in the significant alveolar-derived surfactant contents in CF sputa, i.e., dipalmitoylphosphatidylcholine (DPPC) [56,78,79]. Altogether, the altered mucus rheology, ciliary movement, and adhesivity [1,56,79]

no longer supports normal airway mucus clearance. Plugging of smaller airways is expected to occur, and thus, contribute to gas trapping and obstructed airflow [1,10,54,56].

Together, both the microscopic and compositional data presented strongly suggest that the elevated DNA level determined in these CF sputa (up to 9.6% MDW) originate from neutrophils, rather than from epithelial cells or bacteria. The viscous, charged, and hydrophilic properties of DNA are expected to greatly alter the physical properties of CF sputa [14,37–39,52]. Elevated sputum DNA is anticipated with both acute and chronic inflammation in CF, and may be particularly obvious during pulmonary exacerbations (PEx) [18,36,56,77]. Interestingly, in other reported studies of CF mucus alongside mucoid, mucopurulent, and purulent non-CF sputa, CF mucus was less viscous than mucoid, mucopurulent, or purulent materials from patients with chronic bronchitis [52]. Also, sputa of patients with established CF lung disease possessed markedly reduced levels of the secreted MUC5AC and MUC5B mucins (as detected with specific antibodies) [39,52]. These observations, and those of others more recently, suggest a shift from mucin to DNA as the major macromolecular constituent of CF mucus with disease progression [39,52,77]. In our study, no such patient disease status was available to address this hypothesis.

4.2. Variable DNA and Glycoprotein Content Affect Sputum Solubilization

The opportunity to measure sputum DNA content during the clinical stay of four patients with CF was included in this present study. Throughout their 5-day stay, a >35% reduction in total sputum DNA % of MDW was determined. While we do not possess pulmonary function data to compare with these sputa DNA levels, declining DNA content would be predicted during successful treatment of PExs [36]. Of note, a transient rise in DNA content was observed for 12:00 specimens, possibly reflecting morning activities (therapeutic or otherwise), and/or a transition of DNA from the less mobile gel phase of mucus to the more transportable sol phase. Additionally, this may relate to the synchronicity of neutrophil populations observed microscopically. Upon seeing these results, and in conjunction with the range of DNA content observed in our larger sputa sampling, it became of interest to determine if increased DNA concentration had an effect upon sputum chemical solubilization, a step commonly employed in the isolation of mucous glycoproteins from secretions.

Sputum solubilization, under control conditions, was chosen as a method in which to provide additional insight about their character. The chaotropic conditions are not intended to mimic physiology, but rather to provide a constant condition with which to compare physiologically diverse specimens. The process of solubilization has the potential to disrupt or displace physiologically significant interactions or associations, including salt bridges, and hydrophobic and hydrophilic interactions [46,80]. While in this retrospective data analysis, we do not have mucus rheological data to compare with, others have recently provided physiologically relevant CF sputum rheology measurements showing correlations with clinical status [81,82]. In one study of CF sputa during PEx, increased elastic and viscous moduli, as well as decreased mucus transportability were observed, and these measures were seen as tightly associated with lung function [82]. This is consistent with an earlier clinical study in which altered CF sputum rheology correlated with FEV_1 status [81]. In that report, not only were higher elastic moduli noted with more severe disease as predicted by lower FEV_1, elastic moduli also showed a differentiation pattern between types of bacterial colonization [81]. Higher elastic moduli were reported for sputa possessing *P. aeruginosa* than for those with *S. aureus* [81].

In the current study, while monitoring the time to completely solubilize sputa with guanidinium hydrochloride at 4 °C, a complex heterogeneity in this mucus characteristic was observed. For CF specimens with DNA concentrations between 2.5–7.1% MDW, solubilization time did correlate with DNA content (i.e., increasing DNA content corresponded to increased solubilization times from <2 h to 20 h). These results suggested that DNA in CF sputum may play a role in the structure of the gel, possibly via hydrogen bonding or as complexes with itself, protein, cellular debris, or with the highly anionic glycoproteins present in these secretions. In contrast, for purulent non-CF sputa, for which DNA content was typically <4% MDW, the solubilization time ranged from <2 h to 20 h with no direct

linear correlation with DNA % MDW. Why the DNA content correlation with solubilization time was evident with CF sputa and not purulent non-CF sputa is not clear, though it may relate to other CF mucus conditions not explored.

As CF sputa DNA content increased above 7.1% MDW, there was a significant negative correlation with solubilization time. Based on the morphological observations, these sputa were likely dominated by neutrophils and cellular debris. This parallel ex vivo solubility data indicates, as other studies have shown, as well [1,37,39,47,52], that the neutrophil presence, death, and released DNA does alter CF mucus character, and implies that in the airway, it will also affect overall mucus rheology. Observations of shorter solubilization times with higher DNA content would be consistent with reduced interactions of DNA with other macromolecules when sputum DNA content is high.

Investigating other macromolecules with the potential to affect solubilization, we assessed both glycoprotein (GP) and DNA content of 87 CF sputa. GP levels alone did not correlate with increased, or diminished, solubilization times. GP content was generally higher when DNA content was lower (<7.1% MDW), and lower when DNA was >7.1% MDW. No direct correlation of GP:DNA ratio to complete solubilization time was observed, though CF sputa with GP:DNA ratios >1.5 typically had shorter solubilization times. The majority of CF specimens (76%) had GP:DNA <1. No patient data were available for these specimens to assess for clinical correlations with chemical content, solubilization time, and/or glycoprotein to DNA ratio. Finding greater DNA than GP content for so many of the specimens was consistent with earlier mucus specimens from established CF lung disease showing viscous, hydrophilic polymeric neutrophil DNA and very little mucin [37,39,52].

3D analysis of the solubilization data in this study offered insight into this mucus characteristic. Specific combinations of DNA and glycoprotein constituents in CF sputa appeared to predict the solubility, rather than the amount of one component alone or the component ratios.

These studies suggest that, included in the design of future laboratory investigations of CF mucus character should be multiple macromolecular variables, such DNA, mucous glycoproteins, and lipids, and the clinical status details of the patients from which the specimens are attained. As possible, correlative studies of sputum solubilization values with viscoelasticity measurements would also be informative. Combining such biochemical, biophysical, and clinical observations, may contribute to developing new screening procedures for potential therapeutics and provide valuable guidance on which to refine potential interventional strategies.

4.3. Potential Contributors to CF Mucus DNA Heterogeneity and Related Solubility

Relative to the normal airway scenario, numerous factors may lead to greater macromolecular interactions in CF mucus, and therefore, potentially contribute to observed slower sputa solubilization times. Possibly, significant increases in DNA and lipid content, dehydrated state, and lower clearance rate, may provide for an unusual 'condensed' situation in which tracheobronchial gland and epithelial cell mucin secretions may further contribute to associations and/or entanglements within CF mucus [9,10,17,18,28,30,46,50,77].

The dynamic nature of the DNA and glycoproteins relationships in CF mucus is predicted by the variability in the amount of DNA among CF specimens from different, and same, patients, over time. The particularly wide heterogeneity of distribution of DNA within the individual non-mixed CF specimens may also affect sputum solubilization. Such DNA distribution in vivo would be anticipated to impact local lipid and mucin densities, and thereby also influence mucin intermolecular interactions, gelling, adhesivity, and viscoelastic properties [1,17,51,52,56]. In the CF airway, these and numerous other physiological heterogeneities [4,9,10,28,32,46,50,56], are likewise expected to affect the complex interactions and vary the viscosity, elasticity, adhesivity, and mobility of CF mucus. Going forward in laboratory investigations toward improving airway clearance therapeutics, it will be important to consider fluctuations and alterations in the macromolecules, as well as changes in lipids, ionic strength, and other chemistries which impact the interactions and partitions of these polymeric species [46].

Interventions, for example, which redistribute the macromolecules within CF mucus from the gel to the sol phases, may also beneficially affect mucus rheology and assist MCC [79,83].

More recent studies draw attention to the larger numbers of naked and lysed neutrophil nuclei observed in CF sputa, and suggest a high likelihood that a portion of the DNA in CF mucus exists in neutrophil extracellular traps known as NETs [47,84,85]. The dense and heterogeneous nature of the collected CF mucus, and the neutrophil-laden obstruction observed in the smaller CF airways, may be indicative of pockets of the more structured NETs DNA which did not combine with other secretory components during cough, MCC, and expectoration. NETs may be expected to reduce the dynamics of mucus macromolecular interactions, and in the laboratory to extend the solubilization times. NET formation may be a neutrophil stress response to the altered CF airway chemistries, congestion, and/or to the presence of microorganisms [47,84,85]. It is, however, likely to be detrimental in CF, as the structured architecture further localizes, in time and space, the neutrophils' destructive activities [47,84,85]. DNA NET formation in CF may be triggered by mucus stasis, hypoxia, *P. aeruginosa*, and/or by the enzyme phospholipase C (PLC) produced by colonizing *P. aeruginosa* [1,18,47,58,86]. Of the 87 sputa of solubilization study reported here, no data is available to confirm DNA NET presence, though all screened positive for *P. aeruginosa*, suggesting the possibility of impacting responding neutrophils. Therapeutically, combining medical intervention to down-regulate NET formation [85], with current rhDNase treatments to dissociate DNA [45] would be expected to reduce the amount of inflammation and tissue damage caused by the concentrated cytotoxic components, as well as to beneficially impact mucus viscosity, MCC, and lung function.

4.4. Potential of CF Mucus Character to Promote Bacterial Adaptation and Survival

CF chronic infection with opportunistic pathogens, and its treatment, continue to be actively investigated [3,34,87–91]. Considering the congested CF airway and the heterogeneity of CF mucus composition and solubility profiles, also brings to the discussion the spatial heterogeneity of the CF lung and the potential impact of its stagnant contents on microbial colonization.

In CF, with dense mucus and ineffective cough, MCC, and immune cell activities, microorganisms are provided the time and space for diversification. Opportunistic pathogens, such as *P. aeruginosa*, are known to adapt to their environment, acquiring and expressing traits needed for survival, and down-regulating characteristics which would damage their host or incite their removal [87,89–92]. Bacteria within protective matrices (i.e., mucoid *P. aeruginosa*), are difficult to completely eradicate by physio-mechanical and/or therapeutic means [3,89–91], and as aggregates associated with host polymeric mucins or DNA, are more antibiotic-tolerant [7,90]. In the confined spaces of mucus obstructed CF airways, and those of other chronic obstructive pulmonary diseases (COPDs), microbial communities may also share the "common goods" and "social cheaters" may advantage of the proximity of their neighbors and neighbors' products for survival [93]. Bacterial-CF host adaptation was apparent in recent metabolic screens of sputum isolates, where we saw a higher incidence of lipase activity among CF mucoid *P. aeruginosa* isolates than for non-mucoid CF or non-CF *P. aeruginosa* isolates [58]. In the current report, the DPPC elevation in CF sputa, relative to non-CF sputa, suggests that this lipid alteration in the CF airway may promote the elaboration of PLC by chronically colonizing bacteria. In a stagnant airway, PLC and other bacterially-derived hydrolytic enzymes may accumulate to sufficient concentration to alter the host surfactant functions and airway resistance, and affect mucus rheology and pathogen clearance [1,79,94].

4.5. Obstructive CF Mucus Potential to Increase Risk and Time of Exposure to Cytotoxic Agents

With chronic obstruction, there are longer durations for opportunistic pathogens, neutrophils, and the airways to be exposed to one another. There are also increased risks for the individual associated with the duration of exposure and accumulation of drugs, bacterial products, and debris. An example of this increased risk is suggested by in vitro studies showing time and iron-dependent cytotoxicity of the *P. aeruginosa* phenazine pyocyanin on epithelial cells in culture [59]. The polymyxin antibiotic

colistimethate, which is commonly used in CF, can be cytotoxic to airway cells at physiologically relevant concentrations, and may be even more cytotoxic in conjunction with *P. aeruginosa* virulence factor pyocyanin [60]. Preliminary in vitro investigation of the metallic anti-microbial agent gallium, which is currently in clinical trials for CF Pseudomonas infection [34,89], has also revealed synergistic cytotoxicity between gallium, which itself shows very low cytotoxicity toward airway epithelial cells, and pyocyanin [61]. As such, in the congested CF airway, the time of airway clearance of the combination of therapeutic agents and bacterial virulence factors may be a major contributor to the overall health or damage of the airway.

4.6. Obstructive CF Mucus Potential to Limit Access and Effectiveness of Beneficial Agents

Respiratory mucus, as a protective barrier, may also limit the delivery and effectiveness of helpful agents [43,50,56,91]. This may be especially important for the use of aerosolized antibiotics, as pneumonias with multi-drug resistant organisms continue to increase, in non-CF conditions and CF [43,44,91,95]. The current study suggests that in gauging how to maximize benefits and minimize adverse effects in the airway, one needs to be cognizant of the elements of mucus obstruction.

Many factors may impact overall mechanical airway clearance, drug dispersal, distribution, activity, efficacy, and potential for cytotoxicity. Suggested by our study and others addressed above, these factors may reasonably include whether respiratory secretions are purulent or not; whether they contain polymeric mucins existing as entangled strands or as mucoid sheets or flakes; whether DNA is free, polymeric or in static NETs; whether bacteria are in aerobic or anaerobic pockets of host extracellular matrices or exist in self-made biofilms; and numerous other permutations. Antibiotic eradication therapy of *P. aeruginosa* in CF, for example, has not always been successful within the specific therapeutic opportunity windows [3]. While the cause of therapeutic failure is not clear, it likely includes host factors related to airway obstructions, which limit drug delivery and dispersion [3]. Considering the dynamic nature of the DNA and/or mucin content of CF airways over the long term, as well as throughout the day, continued therapeutic refinements may come from focusing attention on the individual patient's airway mucus composition at the time therapy is being applied.

4.7. CF Mucus Content as Potential Guide to Enhanced Airway Therapeutics Interventions

With a wide variety of therapeutic interventions, developed over many decades, quality of life and longevity of individuals with CF have dramatically improved [5,6,34–36,43,96,97]. It remains paramount, however, to address the issues of chronic obstruction, inflammation, and infection in CF, with the development of additional novel mucolytic, anti-inflammatory, and anti-infective strategies and agents, especially for those individuals with advanced lung disease and those for whom current pharmacological agents are not effective or accessible [3–5,18,28,31–34,46,47,55,77,91]. Our ex vivo hands-on observations predict that continued improvements in therapeutic options for the chronic obstruction, infection, and inflammation in CF and COPDs will address in vivo CF mucus as a dynamic conglomerate.

Our work, as well as that of others, points to the promising potential for intervening in the CF respiratory infection and muco-inflammatory cycle at the interplay of the DNA and mucous glycoproteins [4,18,31,77,79,83]. Our study revealed that the relationship of these macromolecular species, more so than the amount of one component or the other, affects mucus character and solubilization times. These data suggest that for airway specimens where one component dominates, addressing that component would be predicted to be beneficial to reduce aberrant mucus characteristics and improve MCC. Such strategies would include, for sputa with high DNA content, targeting DNA degradation [31,37,38,45,77], and for sputa with high mucous glycoprotein content, addressing the mucin polymer interactions and providing more hydration [4,17,18,31,32,46,49,55,77,83]. CF sputa solubilization in the lab was more readily achieved when the combined mucous glycoprotein and DNA content was less than 10% MDW, suggesting that therapeutics to reduce infection and inflammation may also assist in lowering GP and DNA levels, potentially lowering total solids, and thereby improving

osmotic conditions and MCC. Our work also suggests that airway clearance will be most difficult when the CF respiratory mucus possesses moderate to high levels of both DNA and mucous glycoprotein. For these individuals, multi-modal therapies are anticipated to be most effective [28,46,77]. This laboratory study suggests that muco-active agents [4,18,36,55,77] which possess elements which address both the DNA and the mucous glycoproteins, in all their complex forms and interactions, would potentially improve airway clearance for the widest population of individuals with CF, and considering the heterogeneity of CF mucus, in time and space, may potentially prove useful at all stages of exacerbation, infection, inflammation, and obstruction.

5. Conclusions

As described in these research observations, CF sputa, over the population, and over time for individuals, reflect a spectrum of relative concentrations of DNA and mucous glycoprotein, and these relationships affect sputum solubility. As CF airway clearance strategies continue to evolve to hydrate, solubilize, mobilize, and remove the viscous obstructive materials, these data suggest that therapeutics which address both the DNA and mucous glycoprotein components, in the high lipid concentration environment, may prove beneficial in treating this ever-changing airway dynamic and be effective and versatile across patient populations, and over time for individual patients. Progress toward new anti-microbials for CF pathogens will undoubtedly also benefit from an improved understanding of bacterial behaviors in the in vivo environment in which the bacteria exist and adapt, i.e., the dense and heterogeneous matrix known as "CF mucus". In this light, the knowledge gained in the study of the complex "worst case" CF airway, of its altered mucus composition and function, is anticipated to continue to aid and guide new strategies for airway clearance and control of infection, virulence, and inflammation, for CF and for a wide spectrum of pneumonias and chronic obstructive pulmonary diseases.

Supplementary Materials: The following are available online at http://www.mdpi.com/2673-527X/1/1/2/s1, Table S1. Additional Details of Chemical Composition of Non-purulent, Purulent and Cystic Fibrosis Sputum; Table S2. Chemical Composition of Tracheobronchial Glycoproteins from Patients with and without Cystic Fibrosis; Figure S1. Examples of Solubilization Times of Purulent CF and Purulent Non-CF Sputa; Figure S2. Examination of Effects of Glycoprotein:DNA Content Ratios on CF Sputa Solubilization Times; Table S3. CF Bronchiolar Plugs Solubility Times with Corresponding DNA and Glycoprotein Content.

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