S13.4
3-D PROTEOMICS: A NOVEL, QUANTITATIVE PARADIGM FOR DISCOVERY OF CANDIDATE BIOMARKERS FOR CYSTIC FIBROSIS

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Proteomic techniques are being widely pursued in the search for meaningful biomarkers for cystic fibrosis, and for novel targets for CF drug discovery. The driving force for this emphasis on proteomics in CF research has come at some cost to the genomics approach. One reason has been the concern that while the messages, defined by genomic studies, might be transcribed from only 30,000-50,000 genes, the proteome might be represented by up to several million possible proteins. A second reason is the possibility that while genomic data might yield information on the immediate intention of the cell in terms of mRNA synthesis, it is possible that only the resultant proteins could be expected to yield information on what is truly relevant to the cell over time. A final reason is the concern over the apparent disconnect between genomic and proteomic data, since changes in levels of message and protein seldom seem to coincide. These concerns might reasonably lead one to the compelling conclusion that CF research would be well served by concentrating on "CF Proteomics", and worrying less about "CF Genomics".

However, there are some fundamental reasons to pause before accepting this formulation of the problem at face value. One troubling concern is the fact that the sensitivities of conventional genomic and proteomic analyses actually differ by approximately 10,000. The number can be estimated from the fact that genomic analysis easily measures *ca*. 10^4actin mRNA's, while conventional silver staining methods need ca. 10^8actin protein molecules for ready detection. To detect global protein expression ("proteomics"), the conventional approach is to separate proteins by 2D-gel electrophoresis, and to locate the proteins by silver staining. The challenge with conventional silver staining technology is that it is relatively insensitive. Furthermore, the narrow dynamic range means that only qualitative data can be obtained in terms of relative amounts of different proteins. Fluorescent types of mass labels have been developed which have wider dynamic ranges. However, no profound advantages are actually derived in practice in terms of sensitivity or real quantitation. Finally, and possibly more significant, is the fact that the protein located at any one place on a 2-D gel can come from many cellular compartments, and could have been synthesized at any time in the life of the particular cell or tissue. Messenger RNA, by contrast, is frequently nascent, and degradation is frequently fairly rapid. In addition, actually quantitating different proteins under different conditions has proven to be a Herculean task. At the moment, this is accomplished by differential mass labeling of individual peptides, one-by-one, and analysis by mass spectrometry. It is therefore perhaps expecting too much to correlate significant changes in small numbers of messenger RNA's over short time periods, with equally changes in large numbers of cognate proteins over much longer time periods.

We have approached this 21st Century problem with what could be termed a 20th Century solution: pulsing the cell or tissue sample with ^{35}[S] methionine, and then measuring incorporation into each protein spot by autoradiography and phosphorImaging. The application of a radiolabel to the proteomics problem brings the sensitivity, quantitation, and the dynamic window, into the realm hitherto reserved for genomics. The specific advantage of this approach is that it actually yields a map, in the third dimension, of all proteins on the 2-D gel in terms of their individual rates of biosynthesis. We have therefore termed this biosynthetic map "3-D Proteomics". Importantly, if the identity of the protein is known, and the specific activity of the ^{35}[S] is also known, then the exact amount of a given protein can be calculated. If the calculation is performed globally, then every protein on the 2-D gel can be quantitated at once in the third dimension.

As a useful example of the application of 3-D Proteomics to CF, we have studied the CF lung epithelial IB3-1 cell and the derivative AAV-[wildtype]CFTR-repaired IB3-1/S9 cell. We find that nearly every silver stained protein spot on a 2-D gel is also labeled with ^{35}[S]. However, some proteins occurring in large amounts by silver stain may sometimes be marked by only small amounts of label. These data mean that in some cases certain "housekeeping" proteins with slow turnover may dominate the conventional 2-D gel, thereby obscuring the dynamic activity of possibly more interesting proteins. Conversely, some proteins that are barely detectable by silver stain are profoundly radio-labeled. Not surprisingly, we find that many more proteins are detectable by this method than by conventional silver stain. For example, we have estimated that different populated regions of 2-D gels, as defined by conventional silver stain, may contain

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up to 3-5 fold more proteins when viewed from the perspective of the ^{35}[S] label. We have now used this approach to identify which proteins are significantly over- or under-synthesized in CF cells relative to repaired cells. By way of illustrating how powerful this approach can be, we have found that approximately 30 proteins fall into these categories if the cut-off is 4-fold-or-more, or 4-fold-or-less. We are presently using mass spectrometry and other methods to identify the illuminated proteins, and molecular techniques to quantitate cognate messenger RNA's in CF cells and tissues. Preliminary data indicate that at least some members of the class of differentially synthesized proteins in CF cells, identified by 3-D Proteomics, may be correlated with equivalently differentially synthesized cognate mRNA's. We suggest that such correlated proteo-genomic activities may prove to be important in resolving the apparent problematic impass hitherto thought to exist between genomic and proteomic analysis of CF.

In conclusion, while we have been using incorporation of ^{35}[S] methionine into proteins in CF and repaired cells to develop 3-D Proteomics, it is clear that other substrates can also be employed. These might include other radio-labels or mass labels; other amino acids; other biochemical building blocks or precursors such as nucleotides, sugars or lipids; and inorganic species such as $^{35}[S]O_4$ or $^{32,33}[P]O_4$. Although this discussion has focussed on biosynthetic rates, we might emphasize that the method also lends itself easily to identify post-translational modifications or pharmacoproteomics on a global scale. In fact, the novelty of 3-D Proteomics does not lie so much in the method *per se*, but in the context with which the method is applied. Fortunately, the technique of using these pulse-chase methods to study proteins of interest has been well tested in the 20th Century. However, application of the technology to global proteomics would be impossible without 21st Century inventions in computer science, mass spectrometry and knowledge of the human genome. We suggest that the application of 3-D proteomics to CF, and possibly other related problems, promises to create a simple, dynamic, sensitive and quantitative pathway to the development of robust CF biomarkers and relevant targets for CF drug discovery.

S14.1
ANION AND FLUID TRANSPORT IN THE MAMMALIAN AIRWAY

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CF lung disease is typically characterized by the presence of inspissated airway mucus and impaired mucociliary transport. Currently, it is unclear how this characteristic pathology results from genetic defects in the CFTR that cause CF. Because the CFTR is known to function in normal airway epithelia as a chloride and bicarbonate channel, we hypothesized that alterations in airway mucus and mucociliary transport in CF were largely a consequence of the loss of anion secretion capability.

Because of their morphological similarities to human airways, porcine airways were used in these studies. Initial experiments from our laboratory found evidence that secretion of both chloride and bicarbonate occurred in bronchi (6), which are richly populated with submucosal glands, but not in bronchioles, which are aglandular (1). This finding, along with reports that CFTR was highly expressed in serous cells of the submucosal glands (4), suggested that secretion of chloride, bicarbonate, and liquid might be localized to glands. Indeed, when porcine bronchi were treated with bumetanide and dimethylamiloride (DMA), respective inhibitors of transepithelial chloride and bicarbonate secretion, the liquid secretion response to ACh was reduced by about 90%, a response that was preserved even when the surface epithelium was removed (3). Similar inhibition of ACh-induced liquid secretion was seen when airways were pretreated with the anion channel blockers NPPB and DPC, but not DIDS (3), a response profile that is consistent with a CFTR-dependent process. Pretreatment of pig bronchi with bumetanide and DMA also induces obstruction of gland ducts with mucin (5), which is also seen as the earliest pathological development in CF lung disease (6). Further, pretreatment of bronchi with chloride and bicarbonate secretion inhibitors leads to the production of a thick, relatively dehydrated mucus resembling that seen in CF airways (7). These observations suggest that the CFTR is an important mediator of glandular liquid secretion and that when this process is impaired, changes occur which mimic early CF lung disease.

We reasoned that inhibition of chloride- and bicarbonate-dependent liquid secretion in glandular airways might also affect mucociliary transport. When porcine tracheas were treated with NPPB or the combination of bumetanide and DMA, the rate of mucociliary transport in the presence of ACh was profoundly inhibited (2). Exposing the airway lumen to benzamil, to reduce ENaC-dependent absorption of airway fluid, preserved mucociliary transport even in the presence of these anion secretion inhibitors (2). To examine longer term consequences of anion secretion inhibition, isolated perfused

pig lungs were treated via the vasculature with bumetanide and DMA for 4.5 hours. In osmium-fixed tissue sections, the surface mucus layer appeared to be plastered to the epithelial surface, and the cilia were flattened. This phenomenon was most prevalent when the glandular secretogogue ACh was combined with the anion secretion inhibitors with more than 98% of the epithelial surface exhibiting this plastered mucus appearance. In the presence of only ACh, no plastered mucus was observed in any tissue sections, and the cilia morphology appeared normal.

We conclude from these studies that submucosal glands of the bronchial airways secrete fluid by a chloride- and bicarbonate-dependent mechanism. Because of the pattern of responses to potential anion channel blockers and the relatively high expression of CFTR in glands, we speculate that this transporter participates in this process. Because inhibition of anion and liquid secretion in this model causes mucus to become plastered to the airway surface and profoundly impairs mucociliary transport, we propose that CFTR-dependent anion and liquid secretion is critical to normal mucus clearance processes in the lung.

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S14.2
MOLI1901: NOVEL ACTIVATOR OF ALTERNATIVE CHLORIDE CHANNELS

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Background

Cystic Fibrosis (CF) is an autosomal recessive, inherited disease caused by mutations in the Cystic Fibrosis Transmembrane Regulator (CFTR) gene. CFTR is a cAMP-mediated chloride channel that regulates ion and water balance across epithelia. Absence of the CFTR-regulated chloride permeability in airways of CF patients is associated with increased viscosity of respiratory secretions (1). Impaired clearance, chronic infection, and inflammation lead to progressive, obstructive lung disease and decline in pulmonary function. One strategy to re-hydrate the CF mucous is to inhale an alternative chloride channel agonist to bypass CFTR, thereby increasing airway mucociliary clearance and lessening the rate of decline in pulmonary function.

Introduction

Moli1901, a stable, 19 amino acid polycyclic peptide produced from *Streptoverticillium cinnamoneus*. Moli1901 stimulates a Ca^{++}-dependent alternative chloride ion channel in human, respiratory epithelial cells (2), thereby increasing chloride secretion and concomitant airway hydration, as demonstrated in dogs (3,4).

Mechanism of Action

In vitro studies of Moli1901 demonstrate a relatively sustained increase in chloride secretory response in primary human cultures of normal and CF respiratory epithelium (3,4). These effects were measured under short circuit current (Isc) conditions employing cells or tissues mounted in Ussing chambers where the sodium current was removed with amiloride. The chloride secre-

tory response in CF tissue was approximately twice that seen in normal tissue. The analysis established that the maximal dose response to Moli1901 was achieved at 1μM, with an ED_{50} of 0.3 μM, when applied to the mucosal surface of the cell.

Preclinical Pharmacology

A dog model was used to demonstrate water secretion into the airway. When dogs were exposed to 50 μM aerosolized Moli1901, achieving 0.3 μM at the bronchial surface, fluid measurements demonstrated a doubling in the amount of fluid recovered, as well as a sustained response (>160 minutes) (3,4).

Absorption, Disposition, Metabolism, and Excretion (ADME)

Moli1901 undergoes little, if any, systemic absorption following aerosol exposure in the lungs of rats and dogs. Intravenous administration of Moli1901 to the mouse or rat indicates that Moli1901 undergoes biliary excretion of the parent compound. With this disposition profile, it is unlikely that aerosolized Moli1901 would show toxicity associated with systemic absorption.

Toxicology

The toxicity of Moli1901 by multiple dose inhalation has been studied in rats and dogs. In rats, total depositions of up to 1,000 μg/kg were maintained for one month without signs of toxicity. At doses of 2,000 μg/kg, minimal to slight squamous metaplasia of the epithelium covering the laryngeal surface of the epiglottis was noted. No other areas or organs were affected histologically at depositions of up to 5,000 μg/kg. In dogs, a similar range of total deposition was studied and the results will be presented.

Human Pharmacology and Pharmacokinetics

The bioelectrical properties of the nasal epithelium resemble those of the airways, making the nasal mucosa an appropriate surrogate model of the lung mucosa (4). The phase I trial, Moli1901-001, where intranasal application of Moli1901, superfused as solutions of up to 10 μM (0.02 mg/ml), confirmed that Moli1901 produced an acute and sustained response by inducing chloride transport across nasal respiratory epithelial cells in healthy volunteers (n = 4), as well as patients with CF (n = 4) with no adverse events, and provided the initial *in vivo* proof of concept (5).

Protocols Moli1901-002 (6) and Moli1901-003 evaluated the safety and pharmacokinetics of aerosolized Moli1901 administered as single doses of up to 5 ml at four concentrations of 0.01 mg/ml (5 μM), 0.1 mg/ml (50 μM), 0.3 mg/ml (150 μM), and 0.5 mg/ml (250 μM) to healthy volunteers (n = 16) and CF patients (n = 16). No serious adverse effects were considered to be associated with Moli1901. Therefore, Moli1901 was judged to be safe for aerosolized use in humans, both healthy and with CF disease, at a dose of up to 5 ml of 0.5 mg/ml.

Analysis of human plasma samples from subjects enrolled in Moli1901-002 and –003, and receiving the highest dose level (0.5 mg/ml), showed no detectable levels of Moli1901 at all time points evaluated, using a validated assay that detects 10 ng/ml of Moli1901 in plasma. Based on data from animal studies, it is anticipated that Moli1901 will not be systemically absorbed to any appreciable extent in humans.

Conclusions

Moli1901 activates an alternative chloride channel in CF and non-CF nasal epithelium and may restore epithelial chloride ion permeability and water balance in pulmonary epithelial cells. Aerosolized Moli1901 is safe and well-tolerated up to a dose of 5 ml of 0.5 mg/ml. Long-term therapy with Moli1901 may improve the efficiency of mucociliary clearance, thereby preventing bronchial infections and inflammation in CF patients, and could alter the natural disease by prolonging survival. Exploitation of the pharmacological effect of increasing chloride secretion when Moli1901 is administered directly to the pulmonary epithelium via inhalation is a rational and novel approach to CF therapy.

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S14.3
SUPPRESSION OF PREMATURE STOP MUTATIONS IN CFTR: EFFICACY IN CF CELL LINES, MICE, AND PATIENTS

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Cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein. While (F508 is the most common CF mutation, approximately 10% of CF patients carry a premature stop mutation in one or both alleles of the *CFTR* gene. Certain ethnic groups such as the Ashkenazi Jewish population carry a much higher percentage of CF stop mutations. Thus, a therapeutic strategy aimed at suppressing this class of mutation would be highly desirable for the treatment of this common genetic disease. Recent reports of *in vitro* studies showing that aminoglycoside antibiotics can suppress premature stop mutations in the *CFTR* gene using a vaccinia-T7 expression system in HeLa cells (1); in a CF bronchial epithelial cell line (2); and in CF bile duct cells (3) have generated considerable interest.

It has been suggested that translation termination signals consist of a tetranucleotide sequence comprised of the stop codon and the first nucleotide 3(of the stop codon (4). Consistent with this model, the tetranucleotide termination signal has been shown to be the primary determinant for aminoglycoside-mediated suppression (6-8). This suppression occurs via the misincorporation of an amino acid through the pairing of a near-cognate aminoacyl tRNA with the stop codon (5). Most studies on the suppression of stop mutations have been carried out using gentamicin. However, a recent study found that two other clinically relevant aminoglycosides, tobramycin and amikacin, can also suppress premature stop mutations to varying extents (9). Using readthrough reporter constructs as well as mammalian cDNAs containing naturally occurring premature stop mutations, it was shown that gentamicin and amikacin can suppress premature stop mutations containing all possible tetranucleotide signals (although to differing extents). The levels of termination suppression achieved by tobramycin were generally much lower than gentamicin or amikacin, and tobramycin was unable to suppress termination signals containing the UGAA or UGAC tetranucleotide. Based on these results, both gentamicin and amikacin are predicted to suppress the G542X, R553X, R1162X stop mutations (which all have the UGAG tetranucleotide) and the W1282X mutation (which contains the UGAA tetranucleotide). In contrast, tobramicin is predicted to more weakly suppress the G542X, R553X, and R1162X mutations, but is not expected to suppress the W1282X mutation.

To address whether aminoglycosides can suppress a *CFTR* premature stop mutation in an animal model, a transgenic mouse was constructed that expressed a human *CFTR*-G542X cDNA under control of the intestinal fatty acid binding protein (FABP) promoter in the context of a *Cftr* null (*Cftr-/-*) background (10). It was then asked whether the daily administration of the aminoglycoside antibiotics gentamicin or tobramycin could restore the expression of a detectable level of CFTR protein. Immunofluorescence staining of intestinal tissues from *Cftr-/- hCFTR*-G542X mice revealed that gentamicin treatment resulted in the appearance of hCFTR protein at the apical surface of the glands of treated mice. Weaker staining was also observed in the intestinal glands following tobramycin treatment. Short-circuit current measurements using intestinal tissues from these mice demonstrated that a significant increase in cAMP-stimulated trans-epithelial chloride currents could be observed following gentamicin treatment and a near significant increase following tobramycin treatment. When taken together, these results indicate that gentamicin, and to a lesser extent tobramycin, can partially restore the synthesis of functional hCFTR protein by suppressing the *hCFTR*-G542X premature stop mutation *in vivo*.

The ability of gentamicin treatment to restore CFTR production and function in CF patients with premature stop mutations has also been addressed in two pilot studies. In the first study, nine CF patients with stop mutations received gentamicin drops intranasally three times daily for 14 days (11). Following this treatment regimen, nasal potential difference (PD) measurements indicated that a significant repolarization of the nasal epithelium had occurred, suggesting that the gentamicin treatment partially corrected the chloride transport defect in CF patients with premature stop mutations. In the second study, five CF patients with stop mutations and five CF controls were treated with parenteral gentamicin for one week, and underwent repeated nasal PD measurements (12). During the treatment period, the number of nasal PD readings in the direction of chloride secretion increased significantly in the stop mutation patient group compared with controls. Four of five subjects with CF stop mutations had at least one reading during gentamicin treatment with a chloride secretory response of greater than -5mV (more hyper-polarizing), a response that was not seen in any of the CF controls. The results of these two studies suggest that gentamicin treatment

can suppress premature stop mutations in airway cells of CF patients and produce a partial restoration of CFTR function *in vivo*.

The results discussed above suggest that aminoglycoside therapy aimed at suppressing *CFTR* stop mutations can induce a partial restoration of CFTR function *in vivo.* However, significant barriers to the therapeutic use of these compounds remain, such as the significant side-effects associated with aminoglycosides. Current work is aimed at identifying strategies that limit the side-effects of these compounds. In addition, high-throughput screens carried out by PTC Therapeutics have identified new drugs that are unrelated to the aminoglycosides that have the ability to suppress stop mutations at much lower concentrations than aminoglycosides in cell-based assay systems. There is promise that the aminoglycosides or one of these next-generation compounds can be developed into clinically-useful drugs to suppress premature stop mutations that cause CF, and possibly many other genetic diseases caused by premature stop mutations.

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S14.4
HIGH THROUGHPUT SCREENING FOR ACTIVATORS OF ΔF508-CFTR

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Drugs that enhance the activity of the defective ΔF508-Cystic Fibrosis Transmembrane Conductance Regulator (ΔF508-CFTR) is a therapeutic-based approach to improving lung and pancreatic function of CF patients. We initiated a program to look for agents that correct two known defects in ΔF508-CFTR; low number of CFTR molecules in the cell membrane and decreased channel activity. This approach relies on the ability to identify compounds from a collection of more than 100,000 drug-like molecules that can correct either, or both, defective properties. Two high-throughput, cell-based screens were developed based on the ability to detect compounds that increase ΔF508-CFTR-dependent chloride transport. The potentiator screen identified compounds that potentiate, or increase, the conductance of preexisting ΔF508-CFTR, whereas the correction screen was designed to find compounds that increase the number of active ΔF508-CFTR channels in the membrane.

Both potentiator and correction screens exploit the ability to monitor cellular membrane potential changes through changes in Fluorescence Resonant Energy Transfer (FRET) between a membrane-soluble, voltage-sensitive dye, $DiSBAC_2(3)$, and a fluorescent phospholipid, CC2-DMPE, which selectively binds to the outer leaflet of the plasma membrane and acts as a FRET donor (1,2). Changes in membrane potential (V_m) cause the negatively charged FRET acceptor, $DiSBAC_2(3)$, to redistribute across the plasma membrane resulting in a change in the amount of energy transfer from the FRET donor, CC2-DMPE. The changes in fluorescence emission are monitored using an instrument called the Voltage Ion Probe Reader (VIPR™), an integrated liquid handler and fluorescent detector designed to conduct cell-based screens in microtiter plates (2). The screens were carried out in NIH-3T3 cells stably expressing human ΔF508-CFTR (3). The basic format consisted of establishing a Cl^- - gradient and then activating the channel by forskolin addition and measuring depolarization due to Cl^- - efflux.

Since the potentiator assay was designed to identify compounds that act rapidly on channels already at the membrane, cells were grown under conditions that maximized the number of channels, i.e., 27°C (4). Using this assay, 122,000 compounds were screened and the resulting active compounds were prioritized by their potency, percent potentiation, chemical attractiveness, ability to increase the chloride current (*I*sc) in monolayers of polarized epithelial cells expressing ΔF508-CFTR and ability to potentiate ΔF508-CFTR gating in NIH3T3 cells as measured by patch clamping. The results of this prioritization process resulted in the identification of several chemical scaffolds that are being considered as starting points for further optimization.

The correction screen differs from the potentiation screen in two key parameters. First, the cells are grown at 37°C. This decreases the number of channels at the surface (compared to growth at 27°C) and more closely mimics the *in vivo* condition. Second, since a possible mechanism of action of correction compounds is to alter the trafficking of *de novo* synthesized CFTR, cells are incubated with compounds for 16 hr. 160,000 compounds were screened in this assay and the active compounds prioritized by a process similar to that described for the potentiator compounds, with the addition of biochemical assays to directly quantify changes in channel density on the surface.

This approach raises several important issues as compounds progress through medicinal chemistry, preclinical development and clinical study design. These include: i) the possible requirement for treatment with both a correction and potentiator compound in order to demonstrate clinical efficacy, ii) addressing the relationship between the level of ΔF508-CFTR activity that can be restored *in vitro* to the level needed to confer a clinical benefit, iii) developing compounds in the absence of a highly predictive animal model, and, iv) determining the profile of the patient who would benefit most from this therapeutic approach. Though these issues present us with significant challenges, we believe that improving ΔF508-CFTR function with the kind of safe, potent and selective drugs that we envision will be an important step in expanding the therapeutic options open to CF patients and their physicians.

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S14.5
ACTIVATORS OF CFTR AND CHLORIDE TRANSPORT: BUILDING A BRIDGE FROM THE BENCH TO THE BEDSIDE

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More than 1,300 mutations in the CFTR cause CF, a disease characterized in part by a lack of Cl secretion in epithelial tissue. CF presents as exocrine pancreatic insufficiency, an increase in sweat NaCl concentration, male infertility and airway disease. The lungs of CF patients become infected with *Pseudomonas aeruginosa*, setting in motion a cycle of inflammation, tissue damage, impaired lung function and eventually death. The lack of Cl secretion is though to play a major role in the development of lung disease in CF. Thus, numerous laboratories and biotechnology companies have launched drug discovery programs to identify novel therapeutic agents to correct defective Cl secretion in CF. Although it has not been demonstrated that correction of Cl secretion normalizes epithelial function or reverses the CF disease process, recent data are promising.

Mutations in the CFTR have been classified into five major categories (13). Because each class of mutations reduces Cl secretion by different mechanisms, it is likely that a minimum of five different therapeutic strategies will be needed to correct defective Cl secretion in all patients with CF. In addition, because some mutations cause several defects in CFTR function, it is also evident that some mutations, such as ΔF508, which affects ~70% of CF patients, may require several therapeutic agents to correct defective Cl secretion.

Class I mutations cause defects in the synthesis of stable CFTR mRNA resulting in an absence of CFTR protein. Approximately one half of all mutations in CFTR fall into this class. Mutations leading to premature stop codons produce truncated mRNA transcripts that are unstable and fail to produce functional CFTR protein. Aminoglycosides, including G418 and gentamicin, cause readthrough of unstable mRNA, including those caused by the W1282X and R553X mutations, to product functional CFTR protein (1).

Class II mutations include ΔF508, the most common mutation in CF. This class of mutations results in an abnormal protein that fails to escape the endoplasmic reticulum (ER), therefore, little, if any, CFTR is expressed in the plasma membrane. Although early studies showed that a reduction in temperature, so-called chemical chaperones such as glycerol, TMAO and DMSO could induce ΔF508 to exit the ER, these studies uncovered additional defects in ΔF508, including a greatly reduced half-life in the plasma membrane compared to wt-CFTR, reduced activity as a Cl channel and reduced half-life in the plasma membrane (2;7). Accordingly, treatment of individuals with ΔF508 is likely to require combined therapy that will include: (1) promoting ΔF508-CFTR exit from the ER; (2) activating ΔF508-CFTR in the plasma membrane

and (3) retaining ΔF508-CFTR in the plasma membrane. Several drugs have been identified that promote ΔF508-CFTR exit from the ER (e.g., phenylbutyrate, CPX, thapsigargin, desoxyspergualin, doxorubicin (4;9;10;12),(6) and activate ΔF508-CFTR in the plasma membrane (e.g., genistein, MPB-07, 2-(4-pyridinium)benzo[h]4H-chromen-4-one bisulfate, and 3-(3-butynyl)-5-methoxy-1-phenylpyrazole-4-carbaldehyde (5;8). However, to date, no drugs have been developed to retain ΔF508 in the plasma membrane.

Class III mutations disrupt activation and regulation of CFTR at the plasma membrane. Mutations in this category, such as G551D, cause a severe phenotype. However, several compounds, including those noted above that activate Δ508CTR, also activate G551D (e.g., genestein).

Class IV mutations reduce chloride conductance, and, thus, result in a reduced Cl current. Mutations in this class, such as R117H and P574H, cause a mild phenotype. Milrinone, a class III phosphodiesterase inhibitor, in combination with forskolin, which increases cAMP levels, activates these mutations. Although milrinone is unlikely to be of practical use in CF, because of its effects on cardiovascular function, a derivative of milrinone or a related compound may be useful.

Class V mutations reduce the level of CFTR protein due to alterations in the promoter or by affecting alternative splicing. Examples of Class V mutations include A455E, and 5T.

Class V mutations are associated with a pancreatic sufficient phenotype, or even the absence of CF symptoms. Drugs that activate CFTR (e.g., genestein) or increase the stability of CFTR in the plasma membrane may provide some clinical benefit to individuals with Class V mutations.

Additional drug discovery programs have focused on activating non-CFTR Cl channels in the airways. The rationale behind this approach is that activation of alternative Cl channels may correct the CF phenotype by replacing absent or defective CFTR Cl channels with Cl channels that are normally inactive. Activation of P2Y2 receptors stimulates non CFTR mediated Cl secretion in CF airway epithelial cells (11). Similarly, studies of peptide drugs (*e.g.,* duramycin) that increase intracellular calcium and stimulate Ca-activated Cl channels are also being evaluated for CF (3).

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S15.1
BACTERIAL BIOFILMS AND OXYGEN TENSION:AN INTRODUCTION TO BACTERIAL BIOFILMS

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Biofilms are complex bacterial communities attached to a surface. The transition from the planktonic to the biofilm mode of growth and the subsequent return to a planktonic mode of existence is a regulated developmental process. Examples of bacterial biofilms are chronic P. aeruginosa infections in the lungs of CF patients, oral microbes on teeth, the "slime" layer on the surface of submerged objects in aquatic environments, bacterial contaminants on medical devices, clogged pipes, and bacterial colonization of plant surfaces. Surfaces that support biofilm formation range from abiotic surfaces to biotic surfaces such as the eukaryotic cells.

There are several lines of evidence that support the contention that biofilm cells develop properties that are markedly different from their planktonic counterparts. Planktonic bacteria living as individual, free-living cells transition to organisms that live attached to a surface and in close spatial proximity. When living in these communities, bacteria develop a distinct architecture. Protein and gene expression patterns of planktonic and biofilm cells show multiple differences, presumably reflecting differences in their physiological states. Biofilm formation is also marked by the production of exopolysaccharides. Finally, biofilm-grown bacteria have been long known to develop increased resistance to a wide range of antimicrobial agents. In some cases, biofilm-grown bacteria can become up to 1000-fold more resistant to an antibiotic than their planktonic counterparts.

Although these recent studies point towards a role for biofilms in the CF lung, the physiological state of P. aeruginosa under these conditions is poorly understood. In vitro studies using microprobes and other techniques suggest that biofilms are heterogeneous in terms of their physical-chemical properties, including the presence of steep oxygen, nutrient and pH gradients. One emerging idea suggests that P. aeruginosa biofilms found in the mucus layer of the CF lung are oxygen limited and may even be anoxic. The availability of oxygen can have a profound impact on the physiological state of bacteria and may also influence their response to antimicrobial agents. In this presentation, I will provide an overview of bacterial biofilm development and what is known regarding the physical and chemical heterogeneity of biofilm communities especially in terms of potential oxygen availability.

S15.2
ENVIRONMENTAL SIGNALS THAT TRIGGER BIOFILM FORMATION BY *STAPHYLOCOCCUS AUREUS* AND *PSEUDOMONAS AERUGINOSA* IN CYSTIC FIBROSIS AIRWAYS.

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Hypotheses which link the basic defect in CF to bacterial lung disease concern altered glycoprotein composition on the apical membrane of airway epithelial cells [1], mutated CFTR itself [2], inactivation of defensins by abnormal high luminal salt concentrations [3] and abnormally low production of interleukin 10 by airway epithelial cells of CF patients [4]. These findings predict that bacterial lung disease develops because increased numbers of receptors for bacterial respiratory pathogens on airway epithelial cells lead to abnormally high colonization of bacteria in CF lungs [1], mutated CFTR does not internalize and kill bacteria and consequently bacteria multiply on airway surfaces [2], increased luminal salt concentrations inactivate released defensins from epithelial cells which normally kill bacteria on epithelial surfaces [3], and low IL-10 or high IL-8 will lead to abnormally high levels of inflammation in CF airways which facilitates bacterial infection [4].

In contrast to these hypotheses, it was proposed that in CF airway epithelial cells abnormal secretion of chloride through mutated CF transmembrane conductance regulator (CFTR) and abnormal absorption of sodium through the epithelial sodium channel (ENaC) cause water absorption which results in a volume-reduced but salt-isotonic airway surface liquid [5]. Water absorption also would explain the highly viscous mucus layer on CF respiratory epithelial cells which impairs the ability of the host to keep airways sterile by mucociliary clearance [5].

As a consequence of abnormal sodium absorption, CF respiratory epithelial cells may also display an abnormal high activity of the basolateral membrane-

bound sodium/potassium ATPase which may lead to a significantly increased oxygen consumption. Indeed, increased oxygen consumption of CF cells versus normal control cells was measured [6]. This in turn may deprive the ASL/mucus layer on the apical side of airway epithelial cells of oxygen. We demonstrated that CF airway epithelia produce a steeper O2 gradient than control cells and that this ability reflects a unique feature of CF airway epithelia [7].

Since bacteria sense their environment and change their genotype and phenotype according to environmental stimuli, bacterial genotypes and phenotypes may differ in vitro and in vivo. We hypothesized that the hypoxic environment in the mucus layer on epithelial cells in airways of CF patients determines the genotype and phenotype of S. aureus and P. aeruginosa in CF airways. When then showed that both S. aureus and P. aeruginosa respond to the hypoxic environments as present in CF mucus with a switch from nonmucoid to a mucoid phenotypes in vitro. Finally we demonstrated that these phenotypes are present in the airways of CF patients. Using DNA microassays for P. aeruginosa we defined up and down-regulated genes under anaerobic versus aerobic growth conditions.

In CF therefore, at a site which is normally sterile, a local anaerobic environment is present which impairs the innate host defense system in addition to defective mucociliary clearance. Our data lead us to conclude that therapeutic strategies to treat CF lung disease should include novel drugs designed to clear the lung of retained mucus plaques/plugs, which initiate and perpetuate CF lung disease, and antibiotics that effectively treat P. aeruginosa growing under hypoxic/anaerobic conditions.

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S15.3
CONSEQUENCES OF QUORUM SENSING FOR GROWTH OF P. AERUGINOSA

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Pseudomonas aeruginosa, has two complete quorum sensing systems (las and rhl) that it utilizes to regulate genes in a density dependent fashion (1,2). The las system is composed of LasR, a transcriptional regulator protein belonging to the LuxR family and LasI, an autoinducer synthetase responsible for the synthesis of the autoinducer 3O-C12-HSL (3,4). When cells reach a critical density the concentration of 3O-C12-HSL inside the cell reaches a threshold level and activates the LasR protein which in turn activates /or represses multiple genes. RhlR and RhlI act similarly but the autoinducer synthesized by RhlI is C4-HSL (5,6). Gene-Chip analysis indicates that the las and rhl systems together regulate approximately 600 genes, which is ten times the number previously identified (7,8).

P.aeruginosa chronically infects the lungs of CF patients. In this environment the bacteria grow slowly as a biofilm enmeshed in mucus plugs. Recent data suggests that the organisms are also growing anaerobically (9). The role of the quorum sensing regulon under these conditions is not completely understood and in some cases is controversial. Thus one study concludes that the las quorum sensing system is required for normal biofilm development whereas another study concludes that quorum sensing is not involved in formation or development of *P.aeruginosa* biofilms (10,11). What is becoming clear is that *P.aeruginosa* responds dramatically to different nutrients and growth conditions. Thus over half of the genes found to be activated by the las and rhl quorum sensing system when the cell were

grown aerobically in one growth medium were found not to be transcribed when the cells were grown in another media under anaerobic conditions (7). Such experiments have implications for understanding the role of quorum sensing in chronic lung infections of CF patients.

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S15.4

ANAEROBIOSIS AND *HAEMOPHILUS INFLUENZAE*

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H. influenzae, a human-restricted, upper respiratory commensal and occasional pathogen is acquired shortly after birth. Bronchoalveolar lavage studies of infants with cystic fibrosis find high densities of nontypeable *H. influenzae* ($\geq 10^7$ cfu per ml) and numerous polymorphonuclear leukocytes. However, passive lung function testing at that time does not show any detectable abnormality. *H. influenzae* is particularly well suited for life in the respiratory tract of patients with cystic fibrosis: it has several adhesins for mucin and is facultative anaerobe, possessing the ability to adjust its metabolism according to the available oxygen supply. Monosaccharides are metabolized by glycolysis aerobically and anaerobically, with pyruvate being converted to L(+) lactate, acetate and formate. Under conditions of oxygen limitation, as in a mucus plug, there is increased flow of pyruvate to L(+) lactate, oxaloacetate conversion to fumarate and the catabolism of glutamate/aspartate to fumarate. Fumarate reduction to succinate provides electrons for the first step in the generation of ATP via bacterial electron transport. When growing anaerobically *H. influenzae* produces fimbriae and other surface structures which facilitates adherence to mucin or respiratory epithelial cells. *H. influenzae* grows in mucus as macrocolonies, which do not meet the classic definition of a biofilm. However, like other Gram-negative bacteria *H. influenzae* produces a quorum-sensing molecule, called autoinducer 2 (AI-2). In *P. aeruginosa* biofilm formation is dependent upon the synthesis of autoinducer 1 (AI-1). Without AI-1 a mature biofilm is not formed and many virulence factors are not synthesized and secreted. *H. influenzae* mutants in AI-2 synthetase show a media-dependent growth defect, are avirulent in a rat-model of otitis media and fail to secrete a hemolysis. In addition the *H. influenzae* AI-2 mutants grow poorly under microaerophilic or anaerobic conditions.

Normal human respiratory epithelial cells grown at air-liquid interface (ALI) are unable to clear low inoculae (≤ 200 cfu) of *H. influenzae*, while they readily eliminate *P. aeruginosa*. Colonization of normal human respiratory epithelial cells grown at ALI aborgate the *P. aeruginosa* clearance mechanism. Thus *H. influenzae* can make the normal respiratory epithelium susceptible to infection by *P. aeruginosa.*

Conclusion

H. influenzae possesses a strategy for infection of the respiratory tract by growing in mucus. In that environment where it is shielded from antibiotics, phagocytes and antibody-complement it uses quorum sensing molecules to facilitate macrocolony formation, secrete certain virulence factors and infect the human respiratory tract.

S16.1
USE OF IBUPROFEN FOR THE TREATMENT OF AIRWAY INFLAMMATION IN CF: AN UPDATE

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Chronic inflammation of the CF airway results in lung destruction. Anti-inflammatory therapy has been advocated as a means to slow progression of CF lung disease (1). In a 4-year clinical trial in CF patients age >5 years with mild lung disease (FEV_1> 60% pred), ibuprofen, taken twice daily in doses sufficient to decrease PMN migration, slowed the decline in pulmonary function, preserved the percentage of ideal body weight, and decreased the frequency of hospitalizations compared to placebo (2). The youngest patients (age 5-12 years) benefited the most.

Despite the knowledge that inflammation in the CF lung occurs early in the course of disease and is responsible for lung destruction, and that the results of the ibuprofen trial suggest beneficial effects, the therapy has not been widely adopted. According to the U.S. CFF Patient Registry, <10% of patients per year were treated with high-dose ibuprofen during 1996-2000 (Table 1). Even among the group expected to benefit the most (5-12 year olds with FEV_1 > 60% pred), the use was only 11.1%.

Table1. Use of ibuprofen by age and disease severity (CFF Registry:1996-2000)

AGE (yrs)	N[1]	% on IBU
All	19,773	6.0
<5	2,903	1.0
≥ 5, <13	6,239	9.6
≥ 13, <18	3,601	8.5
≥18	7,030	3.5
FEV_1 (% pred)	N[1,2]	% on IBU
All	15,152	7.1
>60	10,247	8.5
40-60	2,687	4.9
<40	2,218	3.6

[1]average across the five years 1996-2000
[2]excludes subjects with no PFT data

Based on a survey of CF Care Centers in the U.S., infrequent use of ibuprofen appears to be attributable to concerns regarding safety and to the complexity of obtaining a pharmacokinetic study to initiate therapy (3). Regarding safety, the factor most cited is the concern of GI hemorrhage, a known adverse effect of NSAIDs. Based on the CFF Registry, CF patients treated with ibuprofen during 1996-2000 had a higher incidence of GI bleeds requiring hospitalization, but the overall incidence was quite low (<0.5%) (Table 2). Renal failure associated with ibuprofen therapy has been the subject of several case reports in the literature, but based on the CFF Registry data, the incidence of renal failure is not increased among CF patients treated with ibuprofen (Table 2).

Table 2. Annual Incidence (%) and Relative Risk of Adverse Effects (CFF Registry:1996-2000)

	IBU	No-IBU	RR (95% CI)
All (ave N = 19,773)			
Ulcers	.32	.22	1.44 (0.86, 2.44)
GI Bleed	.49	.23	2.12 (1.40, 3.21) p = .0004
Renal Failure	.06	.21	0.30 (0.11, 0.85) p = .02
Age 5-12; FEV_1 > 60% (ave N = 4,505)			
Ulcers	.18	.08	2.42 (0.84, 6.97)
GI Bleed	.32	.10	3.19 (1.30, 7.85) p = .01
Renal Failure	.00	.01	--

The lack of additional data assessing the effectiveness of ibuprofen therapy for CF is another reason cited for infrequent use. A 2-year placebo-controlled trial currently being conducted in Canada will provide additional data. We are currently assessing follow-up data from the 4-year trial.

For the original 84 subjects in the 4-yr ibuprofen trial, we obtained PFT data over the 8-yr period following disenrollment and combined it with data from yrs 1-4 of the trial. The entire 12 yrs of PFT data were analyzed with mixed models using change from baseline in FEV_1% predicted as the outcome, with two-slope spline models representing early (0-4 yrs) and late (4-12 yrs) slopes. The number of years of follow-up PFT data (mean, range) were 10.7 (4-12) and 11.2 (7-12) for IBU and Placebo randomized groups, respectively. During the 8-yr period following disenrollment, patients could be treated with ibuprofen according to physician preference; approximately equal proportions of the IBU and Placebo group were treated after the trial. To date, 6 IBU and 9 Placebo patients have died, all after disenrollment (median years from enrollment to death 10.5 and 10.1, respectively).

Least-squares means (± 95% CI) and estimates of early and late slopes for the 84 subjects in the original intent to treat analysis (Fig 1), indicate that the difference between groups found at the end of the 4-yr

Figure 1. Intent—to—Treat Groups

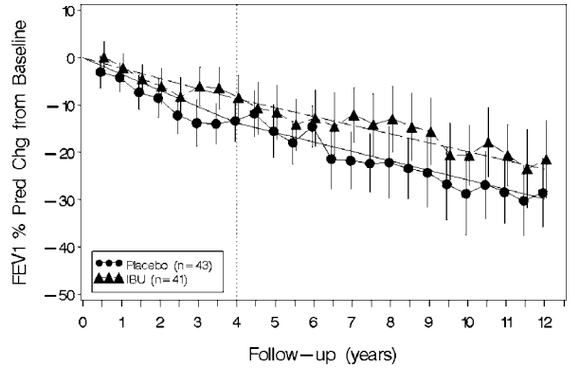
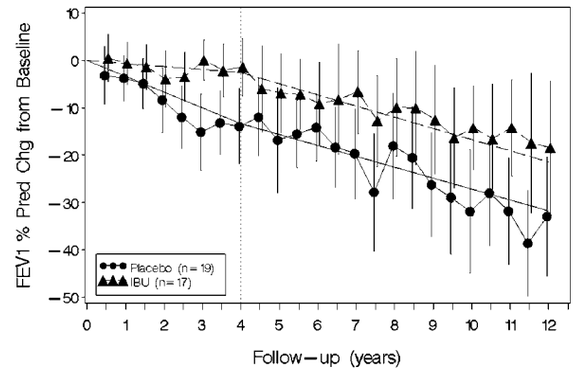


Figure 2. Compliant patients age 5—12



trial (difference = 5.1 % predicted) persists; the estimated difference in means at 12 yrs is 6.4% predicted. For those age 5-12 yrs who were compliant during the trial, among whom the largest treatment effect was seen (Fig 2), a similar pattern is seen where the difference in means at 4 yrs of 10.8% predicted is maintained over continued follow-up (difference = 10.3% predicted at 12 yrs). However, only about half the patients had follow-up data up to 12 yrs. Small sample sizes and variation in patterns of ibuprofen use precluded further analysis according to those who subsequently went on or off ibuprofen in each group.

Data thus far suggest that the overall benefits of ibuprofen therapy outweigh the risks. Strategies to prevent GI bleeding should be considered to safely increase the use of ibuprofen. Continued monitoring is required to further determine the efficacy and safety of this therapy for CF.

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S16.2
INHALED STEROIDS IN CYSTIC FIBROSIS

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Background

Lung inflammation is a predominant feature of CF lung disease and contributes to morbidity and mortality.

Hypothesis

Anti-inflammatory therapy is a good thing - providing the benefits outweigh the adverse effects. Inhaled corticosteroids may be the drug of choice.

Methods

Study design - randomised double-blind placebo controlled trials. Trials published in abstract form only have been excluded. Patients - children and adults with CF. Outcomes - lung function - spirometry in those over 6 years; lung inflammation - measured in serum, sputum and bronchoalveolar lavage; adverse effects - principally adrenal suppression or infection rates.

Adverse effect

Well tolerated, although most trials did not formally assess adrenal function. In the 2 studies that did, there was a decreased response to ACTH stimulation in 1 study [6] but no changes in another [7]. No increase in quantitative bacterial colony counts or *Pseudomonas aeruginosa* (PA) acquisition in two studies [6,7]. All studies were too short to properly assess long term growth.

Discussion

The Cochrane systematic review concluded "there is not enough evidence at present from trials to show whether inhaled steroids are of benefit in cystic fibrosis. Similarly, there is not enough evidence to show that their regular use does no harm" [8]. The conclusions in the updated review will not be any different, despite publication of the more recent trials, although it is encouraging that it has now been shown that lung inflammation, as assessed in BAL, is reduced. Despite this, the use of ICS in CF is common, and probably still increasing, in Europe and North America. Databases recorded ICS use in all CF patients as 36% in UK (reported 1997) [9] and 26% in North America (reported 1999) [10]. I believe this is because many CF clinicians feel that in theory ICS should be beneficial, are unlikely to cause harm, and that lack of evidence for benefit is not necessarily the same as evidence for lack of benefit. Unfortunately the lack of evidence is largely due to problems with the published trials, mainly related to size and duration. This is not meant as a criticism, since large clinical trials are notoriously difficult to perform on a large scale without drug company support - even if this only involves providing drug & placebo inhalers. Such support is not always available unless there is a new product at stake, in which case huge resources are made available for trials (*e.g.* Pulmozyme, TOBI). We still do not have the answer over benefit, and the issue of risk is also not so clear cut. Most trials have not properly assessed effects on adrenal function and growth. However there is increasing evidence that high doses, particularly of fluticasone can cause adrenal crises and significant hypoglycaemia in asthmatic children [11,12,13], although these

Results

	Patient no.	Age (years)	Drug	Daily dose (mcg)	Time (weeks)	Inflammation	Lung function
Schiøtz et al 1983 [1]	26	4 – 29	BDP	400	16	No change in serum	No change
Van Haren et al 1995 [2]	12	16 - 45	BUD	1600	6	—	Reduced BHR
Nikolaizik et al 1996 [3]	49	20 ± 7	BDP	1500	4	—	Reduced TGV
Balfour-Lynn et al 1997 [4]	23	6 – 17	FP	400	6	No change in sputum	No change
Bisgaard et al 1997 [5]	55	9 – 29	BUD	800	26	—	Increased FEV_1
Wojtczak et al 1999 [6]	28	<5	FP	440	16	Trend to reduction in BAL	—
Wojtczak et al 2001 [7]	12	1.5 - 13	BDP	420	8	Reduction in BAL	—

BDP - beclomethasone dipropionate, BUD – budesonide, FP - fluticasone propionate, BAL - bronchoalveolar lavage, BHR - bronchial hyperreactivity, TGV - thoracic gas volume, FEV_1 – forced expiratory volume in 1 second

few case reports must be put in the context of the hundreds of thousands of patients who have not come to harm. Either way, it would seem sensible to avoid long term use of high doses.

In the UK, we have been unable to recruit sufficient patients - not already on ICS - to a multicentre trial of starting ICS. Hence our new approach, in which we are currently undertaking a UK CF Trust-funded multicentre double-blind randomised controlled trial of the effect of stopping ICS in CF children and adults already taking them. We aim to report results at the 2003 NACFC.

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S16.3
INDUCED SPUTUM AS AN OUTCOME MEASURE IN STUDIES OF CYSTIC FIBROSIS

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Investigators of airway inflammation and infection in cystic fibrosis (CF) have primarily examined bronchoalveolar lavage (BAL) fluid or spontaneously expectorated sputum. The application of either method is limited in studies of new therapies for several reasons. Bronchoscopy is relatively invasive and its risks make the use of repeated measurements in BAL for studies less acceptable to patients. In addition, there is no data on the repeatability of inflammatory markers in BAL fluid, and repeated bronchoscopy in patients to establish reproducibility of results is difficult. Furthermore, expectorated sputum is produced chronically only by patients with moderate to advanced lung disease, which limits the number of patients that can be studied. These problems have limited clinical studies in young or reasonably healthy patients at a stage of their disease when modifying the airway inflammatory response may have the most therapeutic benefit.

Sputum induction is a noninvasive method of sampling the lower airway compartment that may be a useful

tool in the study of airway infection and inflammation in CF. It has been well validated for studying airway inflammation in asthma (1). More recently, induced sputum (IS) has been studied as a potential outcome measure for studies in CF. Several investigators have established the feasibility of sputum induction in CF patients including adults with mild to moderate lung disease (2,3), chronically expectorating children with mild to moderate lung disease (4,5) and non-expectorating children with mild lung disease (5,6). Two methods of sputum induction have been used: 1) inhaling 3% saline nebulized from an ultrasonic nebulizer over 12 minutes, and 2) inhaling increasing concentrations of hypertonic saline from 0.9 – 3% to 4.5 – 6% saline over 5 minute intervals. Both methods appear to be equally successful in obtaining adequate samples of IS from CF patients.

Safety of the procedure is of major concern in patients with CF. In asthmatics sputum induction has been determined to be safe, the major risk being bronchospasm (measured by a decrease in FEV_1), which can be prevented by pre-treatment with bronchodilators (1). Studies of IS in CF patients have also determined it to be well tolerated. In all the studies published to date, investigators pretreated subjects with bronchodilators and monitored safety using spirometry, peak flow and oxygen saturation measurements. Overall, children with CF had more frequent drops in FEV_1 or symptoms with sputum induction that led to discontinuation of the procedure than adults with CF. The percentage of subjects with a drop in FEV_1 was not related to the concentration of saline used.

Sputum induction has been shown to be more sensitive than either expectorated sputum or BAL in detecting bacterial pathogens (3). Studies comparing IS cultures to oropharyngeal cultures have shown that concordance exists but is not absolute in detection of the most common bacterial pathogens in CF (4, 5). The inflammatory profile of IS is similar to that of expectorated sputum and BAL. The cell differential count and levels of inflammatory markers, when corrected for dilution using urea concentration, in IS, expectorated sputum and BAL fluid were similar (3). Analysis of samples collected sequentially at 4 minute intervals showed similar inflammatory content in all the aliquots (7). Other investigators have also demonstrated that the levels of inflammatory markers in CF adults were repeatable at a 3-week interval (6), and in non-expectorating children bacterial cultures and inflammatory markers were repeatable at 1 wk-intervals, and within-week repeatability in adults was also high (8).

Studies to date of IS as an outcome measure in CF suggest that the technique can be employed in a range of ages and CF lung disease severity. Though no serious adverse reactions have been reported in patients with mild-moderate disease, safety needs to be monitored carefully as bronchospasm can occur despite pre-treatment with bronchodilators. In order to determine whether IS is useful in research on new CF therapies, studies are needed to determine whether a significant change can be detected following an intervention. A recent multicenter study whose preliminary findings will be presented at this meeting demonstrates that bacterial density and levels of interleukin-8 and free neutrophil elastase in IS decrease after treatment with intravenous antibiotics for a pulmonary exacerbation. In summary, induced sputum using hypertonic saline is a useful and safe method for studying airway inflammation and infection in cystic fibrosis, and has applicability in investigations of new therapies.

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S16.4
INDUCED SPUTUM & EXHALED BREATH MARKERS IN CF

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Introduction

Airway inflammation is a major characteristic of cystic fibrosis. It begins at early age and persists and progresses throughout life. Controlling and monitoring lung inflammation is the key to improving prognosis. Inflammatory markers are raised in bronchoalveolar lavage fluid (BAL), but this is an invasive procedure that is not suitable for routine or repeated use. Therefore, less invasive methods have been studied for both clinical management and research. There is a large range of options: induced sputum, exhaled gases, breath condensate and others. Because these techniques are noninvasive, they can be used repeatedly and some can be used in children and patients with severe CF.

Methodological issues

Sampling: Alveoli, airways, upper respiratory tract and environmental contaminants can contribute variably to samples. For example, induced sputum is a mixture of tracheobronchial secretions and saliva; exhaled NO come from upper and lower respiratory tract and sampling must avoid nasal gas contamination. In contrast most substances measured in breath condensate are present not only in the lower airway but throughout the respiratory tract including nasal passages.

Storing & measuring: Processing sputum samples need to be done shortly after induction, currently a maximum of 2 h for storage is recommended. The analysis of exhaled gases normally performed immediately. Exhaled condensate may be stored at $^{-}70°C$ and is subsequently analyzed.

The equipment used differs in both size and cost; ideally it should be cheap, portable and give a immediate result. These requirements are not yet met by any of the systems.

Patient variables: All the inflammatory markers measured are influenced by such factors as physiotherapy, inhaled and systemic treatment, diet and time of day. Futher characterization of these variable is needed.

Induced Sputum

This was first advocated in 1992 and has been extensively studied since (1). Inflammatory findings correlate best with bronchial washings and more variably, but reasonably, with bronchial biopsies and BAL(2,3). The methods of sputum induction have been reviewed elsewhere (4). The procedure is somewhat unpleasant to undergo and is not suitable for small children. Furthermore, the technique itself induces an inflammatory response so that it cannot be repeated in less than 24 h (5).

In CF there is a significant increase in indices of airway inflammation, including total cell counts, absolute neutrophil counts, interleukin-8, tumour necrosis factor, interleukin-1β and neutrophil elastase activity (6). Sputum total protein concentration was elevated in the CF groups, whereas urea and albumin levels were not significantly different. On the other hand, while exhaled NO does not increase in CF, sputum NO_2/NO_3 was significantly higher in acute patients compared with both stable patients and control subjects, which suggests that NOS is activated during acute pulmonary exacerbations of CF(7).

Exhaled Gases

Nitric Oxide is the most extensively studied exhaled marker and is used as an important noninvasive marker of lung inflammation in asthma and other lung diseases. Variations in the methods of collection of samples have been described, and guidelines with suggestions for the collection and analysis of exhaled NO have been published.

In contrast to asthma, exhaled and nasal NO levels are significantly lower in patients with CF than in normal subjects, despite the intense neutrophilic inflammation in the airways(8). There is no good association between exhaled NO and CF genotype, disease severity or infection with *Pseudomonas*. The reason for the low levels of NO in patients with CF is not fully understood. Firstly, there is a deficiency of NOS2 in patients with CF(9). Secondly, there is an association between the length of a repeat polymorphism in the NOS1 gene and exhaled NO in patients with CF(10). Other possible explanations include poor diffusion of nitric oxide across increased and viscous airway secretions, removal of nitric oxide by reaction with reactive oxygen species in the inflamed environment and failure of upregulation of epithelial inducible nitric oxide synthase in chronic suppurative conditions. Carbon Monoxide is probably derived from the degradation of haem to bilirubin in the lung during oxidative stress. In CF patients, exhaled CO levels are elevated and increase further during exacerbations and fall with antibacterial treatment(11). Furthermore, CO levels in CF are lower in patients receiving oral corticosteroid treatment.

Exhaled ethane is elevated in CF, reduced in steroid-treated patients and correlates with CO and RV/TLC. Hence, it may be a useful noninvasive marker of oxidative stress(12).

Breath Condensate

Subglottic gas is saturated with water and can be collected as exhaled water vapour condensate which may

contain low molecular weight (ie not protein) markers of inflammation. Salivary contamination may influence the levels of several markers detectable in exhaled breath condensate. Moreover, since the quantity of droplets in exhaled condensate is very low, the levels of inflammatory mediators may vary by a factor of 100 or more, depending on variations in the dilution of respiratory droplets by the water of vaporization (13).

Exhaled Hydrogen Peroxide levels in adult CF patients are not significantly different from those of healthy controls(14,15). Conversely, a study found that in CF children with acute infective pulmonary exacerbations, higher levels were detected than in healthy children, and a significant decrease of exhaled H_2O_2 levels in the course of treatment with antibiotics was also found(16). Thus, exhaled H_2O_2 may not be a suitable marker of airway inflammation in stable CF patients, but is of potential value to monitor the effect of anti-inflammatory treatment for exacerbations.

Elevated levels of nitrite and nitrate and nitrotyrosine have been found in exhaled condensate of patients with CF during both the stable period and exacerbations(17).

Endogenous airway acidification, as assessed by pH in expired breath condensate, has been implicated in asthma pathophysiology. The pH value were significantly lower in patients with COPD and bronchiectasis compared with patients with asthma and control subjects and correlated with both sputum neutrophilia and oxidative stress.. The pH of the expired breath condensate has not yet been adequately studied CF(18).

Concentrations of 8-isoprostane in the breath condensate of patients with stable CF are increased about threefold compared with those in normal subjects and were negatively correlated with FEV1(19). IL-8 levels in exhaled condensate are mildly elevated in stable CF but are more than doubled in patients with unstable CF compared with normal subjects. Exhaled Na^+ and Cl^- are elevated in exhaled condensates of patients with CF and correlate with the sweat test and disease severity.

Other Methods

Exhaled temperature measured under controlled conditions is low in CF and COPD when compared with normal subjects(20). Exhaled breath temperature may serve as a nonspecific, simple and inexpensive method for home monitoring of several upper and lower respiratory conditions including CF.

Conclusions

Noninvasive markers have important potential as measures of airway inflammation in patients with CF. They are not yet adequate for use in clinical management but all are being actively studied with encouraging findings. The development of convenient measurement analyzers and devices is a high priority.

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S17.1
HOW WE DO IT: CARE OF NEWLY DIAGNOSED INFANTS WITH CYSTIC FIBROSIS IDENTIFIED THROUGH NEWBORN SCREENING – THE INITIAL VISIT

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The care of infants with cystic fibrosis (CF) identified through newborn screening is structured to promote health, avoid early complications of CF, and empower families through education about CF. In this brief review we highlight some of our strategies for caring for newly diagnosed infants with CF. Some approaches are supported by evidence while many are not. In this "How We Do It" symposium we describe the approaches of our CF care center as a starting point for discussion.

The initial relationship between the CF Center and families of infants identified through newborn screening differs from the relationship that is formed with families of infants or children identified conventionally. Following conventional diagnosis, the CF care team becomes involved after the family has seen several medical providers. Families have suspected that something is wrong with their child. Although it is difficult to cope with the diagnosis of CF, many families feel some relief that a diagnosis is finally made. In contrast, most families of infants identified through newborn screening have viewed their infant as perfectly healthy. Families are contacted by either their primary care provider or by a member of the CF care team and told that their infant has CF. This happens within a few weeks after birth, an extremely vulnerable time. The initial contact and visit are therefore crucial in establishing a relationship between the family and the CF care team.

After the initial phone conversation notifying the parents that their infant has CF, they will usually contact friends or other family members who may relate painful anecdotes about CF. In addition, they frequently go to older textbooks or the Internet and read unreviewed accounts of CF. All of this leads to additional anxiety. This is why the initial diagnostic visit with the CF care team, which should occur as soon as possible, is critically important. The two major themes we try to convey at that visit are optimism and support. We try to alleviate their anxiety by introducing optimism very early in the initial visit. After introductions, the first thing we say is that their infant has CF but that we expect him/her to do very well. Families are often relieved at that point. We then outline the rest of the initial visit, which includes discussions of the diagnosis of CF, what is CF, and what resources are available.

In discussing the diagnosis of CF, we review the initial screening tests (immunoreactive trypsinogen [IRT] levels), and the diagnostic tests (sweat test and/or genotyping results). We explain that the IRT results gave us some clue that there was something wrong with the infant's pancreas. Often a brief explanation of the role of the pancreas is then needed. We then discuss the sweat test. The families ask about the actual values. They ask especially about how often there can be falsely elevated numbers.

Next, we discuss the implications of CF. We say that CF is an inherited disease that causes changes in many organs in the body, including the pancreas, lungs, sinuses, digestive system, reproductive system, and sweat glands. We usually begin with the pancreas and potential problems in digestion. Much is known about nutritional and pancreatic status in early infancy in CF. Sixty percent of infants identified through newborn screening will be pancreatic insufficient by two months of age and 85% will be insufficient by one year of age (1,2). In addition, one-third of children with CF have evidence of fat soluble vitamin deficiencies (3). This data provides justification for early enzyme replacement. The decision to begin enzymes is based on multiple factors including current symptoms of malabsorption (frequent, greasy foul-smelling stools, excessive

flatus or gas), poor weight gain, or a low serum albumin level. Fecal elastase tests are likely to be increasingly useful in deciding about early enzyme therapy. We tell the families that even if enzymes are not started initially there is a good possibility that they will be needed in the first year of life. Parents ask many questions about enzyme therapy. They particularly want to know whether infants will become "dependent" on enzymes. This has not been studied well in infants but we have followed a few infants who were started on enzyme treatment and then were found to be pancreatic sufficient. These infants have done well following discontinuation of enzymes. Also, we explain that the use of enzyme therapy early on is not likely to accelerate pancreatic injury. We start all infants on fat soluble vitamin supplementation.

We then talk about the lungs. Again, there is a substantial body of medical knowledge that provides the basis for our discussion of lung involvement in early CF (4). We explain that the mucus in the airways is thick and sticky. This thick mucus blocks the airways and traps bacteria and other particles. Parents often ask at this point about severity of lung disease and especially whether all children with CF get lung problems. Our response is that there are clearly different degrees of severity but virtually all patients have lung disease at some point. This is usually a proper time to stress that all children with CF are different. The question of survival is often raised. We address this by giving them median survival information based on the current CF Foundation registry. We emphasize that this data is based on patients born 20-30 years ago. However, we do mention that occasionally a child does not make it out of childhood though this is very uncommon. Parents often ask about sports activities and physical limitations. Here we can confidently tell them that children with CF are for the most part just as active as other children.

Other topics which are discussed at this initial visit include the genetics of CF, the relationship between the CF center and their primary care providers, and finally, the CF Foundation. It is very comforting to parents to know that the CF Foundation started by parents, advocates for both care and research in cystic fibrosis. We provide the families with a booklet and the CF Foundation web site address. We also encourage them to contact our center at any point. We are careful not to overwhelm families with information at the initial visit. We understand that in many cases they cannot absorb even a fraction of what we say. The most important part of the approach is that they see the CF care team as optimistic and supportive. In addition, we emphasize that it is important for the parents to know as much as possible about CF and that we will do our best to help them get the information they need. We usually call the family within a couple days of their initial visit. The infants are seen for a follow-up appointment in two weeks and then monthly or every other month during the first year.

We have focussed here on the initial visit because it sets the tone for what is hoped will be a long relationship. There is increasing evidence that early diagnosis through newborn screening improves growth in CF and can avoid complications in infancy. More states are adopting newborn screening. We will need to further refine our approach to care of infants with CF identified through newborn screening.

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S17.2
EVALUATION OF YOUNG CHILDREN AND INFANTS WITH AN UNCERTAIN DIAGNOSIS OF CF

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Our attempts to define the limits of the CF diagnosis have been complicated by CF-like clinical phenotypes in patients that do not fulfill the CFF diagnostic criteria, a "grey-zone" of mild CFTR dysfunction (sweat test, NPD) that straddles unequivocally normal or abnormal CFTR function, and a high prevalence of CFTR heterozygote mutations in the general population. There is increasing evidence that mild CFTR dysfunction associated with CFTR mutations can result in "non-classic" forms of CF disease isolated to specific organs (e.g. congenital absence of the vas deferens (CABVD), idiopathic pancreatitis and chronic sinusitis) (1). The introduction

of newborn screening for CF is likely to add a further pool of infants with a questionable diagnosis of CF.

Patients with "non-classic" CF who are found to have evidence of mild CFTR dysfunction (intermediate sweat Cl^- or decreased nasal Cl^- permeability) and inconclusive CFTR genetic analysis present a significant diagnostic challenge. The successful diagnostic resolution of atypical presentations of possible CF depends on a comprehensive evaluation of clinical features, CFTR function and CFTR mutations.

Clinical evaluation: An atypical phenotype in which a diagnosis of CF cannot readily be made is thought to exist in 2% of CF patients (2). Most infants and children who trigger concerns about the possible diagnosis of CF present with a variety of respiratory and gastrointestinal symptoms. The sino-pulmonary symptoms in these patients are similar to symptoms resulting from a number of common childhood disorders (e.g. nasal allergies, asthma, immunodeficiency, gastroesophageal reflux). Likewise, the gastrointestinal symptoms of atypical CF resemble those of pancreatitis (recurrent abdominal pain), other causes of pancreatic insufficiency (e.g. Schwachman-Diamond syndrome), intestinal malabsorption, and other causes of failure to thrive. A fastidious clinical evaluation is a crucial starting point for an evaluation of possible CF. Evaluation of respiratory symptoms should include radiological studies (including sinus and chest CT scans for sinus/lower respiratory symptoms) microbiological studies (expectorated sputum, gag sputum and/or BAL) lung function testing if possible, and tests that exclude other potential causes of the symptoms (e.g. immune function studies, studies of gastroesophageal reflux). A diagnosis of fat malabsorption should not be made without quantitative measurement of stool fat content or fecal elastase, other markers of pancreatic damage should be sought (e.g. serum trypsinogen), and empiric trials of pancreatic supplements should be avoided. In boys, a testicular examination or ultrasound should be performed to determine the presence or absence of a vas deferens.

CFTR function: The sweat test is the gold standard for evaluation of CFTR function. Whereas the great majority of patients with CF will have a sweat Cl^- of >60mMol/l, there are numerous reports of CF patients with mild or atypical presentations who have normal or "intermediate" ($[Cl^-]$ 35 – 60 mMol/l) sweat Cl^-. Despite efforts of the CFF to standardize sweat testing, there is considerable variability in performance of the test at CF Centers resulting in unnecessary inclusion of CF in the differential diagnosis in some cases. In addition, sweat Cl^- in CF patients may increase with age and a cut-off value of 60 mMol/l may not be appropriate for diagnosis of CF in infants and small children (3). Other tests of CFTR function can provide further supportive evidence for or against CF, but are not available at most CF Centers. Nasal potential difference (NPD) studies are particularly useful since they test CFTR function in the respiratory epithelium, the most common site of symptoms. However the test is complex and only offered in a few centers. Recently the test has been adapted for use in children and infants (4), but most infants and children less than 6 years require sedation for NPD, adding to the complexity of the test. Colonic potential difference testing and determination of sweat secretion rates are adjunctive tests that may be useful to determine CFTR function in indeterminate cases. The usefulness of these tests could be improved by increasing their availability.

CFTR mutation analysis: Mutation screening has greatly enhanced our diagnostic capability since the great majority of CF patients have mutations can be detected with the mutation panels that are available commercially or at selected CF Centers. However, only a minority of the more than 900 described CFTR mutations are tested for with these diagnostic panels, and not all the described mutations are known to cause clinical symptoms or abnormal CFTR function. In addition, CFTR intron-8 polythymidine sequence variations are thought to determine the effectiveness of CFTR transcription, and polymorphisms (5T) of this intron should be sought in any patient with an indeterminate diagnosis. The presence or absence of exonic and flanking intronic CFTR mutations can be definitively shown by gene sequencing, and this kind of exhaustive genetic testing has uncovered CFTR mutations in patients with atypical symptoms and intermediate sweat chloride values (5). Gene sequencing is available at a few CF Centers and has recently become commercially available.

Summary

Evaluation of an infant or child with atypical CF symptoms, a "borderline" sweat test and inconclusive CFTR genetic screen depends on a combination of careful clinical evaluation, detailed age-appropriate testing of CFTR function and an exhaustive CFTR genetic analysis. Before this work-up is initiated, it should be determined if the clinical symptoms that initiated the CF investigation are real, the presence of other diseases that could explain the symptoms should be identified and treated, and the accuracy of the sweat test result should be verified by multiple concurring tests. No single test of CFTR function or CFTR mutation will likely resolve the diagnostic quandary and a combined approach should be sought. While comprehensive genetic testing is now more widely available, detailed tests of CFTR function, particularly in children and infants, is limited and should be improved. Skilled interpretation of the results of CFTR genetic testing is critical. The current CFF guidelines for making a diagnosis of CF are not challenged by this group of patients. The patients who fulfill the diagnostic criteria for CF have, in the great majority of instances, a clinical phenotype and prognosis that is very different from the cohort of "non-classic" CF patients. In children or infants with atypical symptoms, the chal-

lenge is whether the patients' symptoms can be attributed to CFTR dysfunction or another disease. Investigation of children and infants with CF-like symptoms need clinical evaluation and testing that is age appropriate. In patients whose disease is attributed to CFTR dysfunction, clinical judgment is needed to determine which, if any, of the standard therapies used in patients with CF should be applied to this group. The complete clinical characterization of these young patients with non-classical CF will need to await long-term follow-up.

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S17.3
CLINICAL CHARACTERISTICS AND OUTCOMES IN OLDER ADULTS DIAGNOSED WITH CYSTIC FIBROSIS

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Through greater awareness of mild cystic fibrosis (CF) phenotypes, increasing numbers of adults with chronic pulmonary infections, infertility, or sinus disease are being evaluated for CF. Patients present to the Colorado Adult CF Clinic through referrals from local physicians, the Infectious Disease Service at National Jewish Medical and Research Center (NJC), or are self-referred. Patients diagnosed with CF in adulthood typically have histories of chronic cough, sinusitis, and infertility (in males). Patients usually have been diagnosed with "asthma" or told they have "recurrent pneumonia". These patients are nearly always pancreatic sufficient, although a history of recurrent pancreatitis is not uncommon. On exam, features of obstructive lung disease are nearly always present, but clubbing is usually absent. Radiographic studies of these patients demonstrate bronchiectasis, and spirometery is consistent with an obstructive pattern of airflow limitation. Sputum cultures demonstrate typical CF pathogens such as *P. aeruginosa,* and *S. aureus*. Many of these patients are co-infected with nontuberculous mycobacterial species, and frequently they were referred to NJC because of this finding.

When patients age 40 or older who were diagnosed with CF in adulthood are compared with patients of the same age diagnosed in infancy, remarkably few differences are observed. A retrospective chart review of clinical records of forty CF patients age 40 and older (range 40-73 yrs, mean = 46.97+/-1.03 years) seen at the Colorado Adult CF Clinic over the past decade was performed. All patients included in the analysis had either two identified CFTR mutations (n = 30) or 2 sweat chloride values > 60 (n = 10). The mean age at diagnosis of CF patients diagnosed as infants less than 2 years of age was 0.14 +/- 0.11 years; while the mean age of adult CF patients diagnosed at age 18 years of age or older was 35.3 +/- 0.14 years ($p < 0.0001$). Patients diagnosed as infants did not have a significantly different genotype distribution than those diagnosed in adulthood, with compound delta F508 heterozygotes comprising the most common genotype in both groups. As expected, patients diagnosed in childhood were more likely to be pancreatic insufficient ($p<0.03$). Infection with mycobacteria was common in both study groups. Although detection of mycobacteria in the sputum prompted the diagnosis of CF in a number of adults, the prevalence of mycobacterial disease was not significantly different between the two groups ($p = 0.16$). Lung function of CF patients who survived to age 40 or beyond was equivalent between individuals diagnosed in childhood or as adults, with severe pulmonary disease present in the majority of patients (mean FEV1 31 % predicted +/- 4.7). This substantiates the large survivor effect for patients who were diagnosed as infants, as the majority of patient with severe pulmonary disease die before age 40.

Diagnosis of CF in adults presenting with one or more clinical feature of CF was established following the recommendations the CF Foundation Consensus Statement (1). Evidence of CFTR dysfunction must be present as defined by elevated sweat chloride concentration (Cl^- > 60 mmol/L) analyzed by quantitative pilocarpine iontophoresis on two occasions, or by identification of two CF mutations. The identification of a single CF mutation and the presence of a 5T allele of the polythimidine tract in the intron 8 (IVS8) of the CFTR gene also provides sufficient evidence of CFTR dysfunction. Individuals with suggestive clinical features and an elevated but not diagnostic sweat chloride value are referred for analysis of nasal potential difference (NPD) at The Children's Hospital (Denver). Patients identified as heterozygous for a CFTR mutation and clinical features highly suggestive of CF are often followed in our clinic with the anticipation that newer methods of mutation analysis will ultimately confirm CF in a large number

of these individuals. However, these individuals are not identified as CF patients until the above referenced criteria is met.

When the diagnosis of CF is confirmed in an older adult with CF, a number of questions can be anticipated. At the time of diagnosis, genetic counseling is made available to the patients. Patients who are interested in family planning, those that have succeeded in having children, as well as those with siblings are usually concerned over the potential ramifications of carrying the gene for CF. Generally, patients with bronchiectasis or other clinical features of CF are relieved to be given the diagnosis of CF. Typically these patients have seen numerous physicians throughout the course of their illness, and often their care has been inadequate. The diagnosis of CF typically allows a more standard therapeutic approach which emphasizes airway clearance, bronchodilators, and periodic courses of antibiotics. Likewise, the approach to recurrent pancreatitis, sinusitis and infertility is modified by the diagnosis of CF. Rarely, adults in whom the diagnosis of CF seems plausible will wish to postpone mutation analysis for fear that their ability to obtain insurance will be jeopardized.

Our initial therapeutic approach is dictated by the pulmonary infections and extent of lung disease of the individual. Sputum analysis by our laboratory is compared with previous results (when available). Based on clinical status, we attempt to treat patients with a 2-3 week course of IV antibiotics soon after presentation if there is evidence of a pulmonary exacerbation. This allows us to determine the baseline pulmonary status of a newly diagnosed individual, and usually results in a significant improvement in pulmonary function and general well-being. Patients chronically infected with *P. aeruginosa* are started on inhaled Tobramycin following treatment with IV anti-pseudomonal antibiotics. Patients are introduced to a range of available maneuvers and devises to assist with sputum mobilization. Newly diagnosed CF patients with MAC colonization are usually not treated, but individuals with mycobacterium classified as "rapid-growers" and evidence of recent clinical decline are started on 3 or 4 drug therapy based on the sensitivities of the organism. Nutritional and psychosocial evaluations are conducted soon after the time of diagnosis, as these individuals have frequently been chronically ill for their entire adult life. A considerable number of non-CF related co-morbidity is seen in older CF patients, including hypertension and malignancies. Symptoms not characteristic of CF need to be evaluated aggressively, and close cooperation with primary care physicians is essential.

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S18.1
THE PREVALENCE AND CLINICAL MANIFESTATIONS OF BONE DISEASE IN CF

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Prevalence of decreased bone mineralization in adults with CF

The CF Foundation Patient Registry began collecting bone densitometry information in 1997, and the 2000 data reports that osteoporosis is present in 2.6% of adult patients and osteopenia in 3.3%[1]. These numbers clearly underestimate the true prevalence of decreased bone mineral density (BMD) since uniform screening has not been implemented. The results seem particularly low when compared to reports from several large cross-sectional studies. Elkin[2] evaluated BMD in 107 patients of varying clinical status and found that 38% had osteopenia and 13% had osteoporosis. Flohr[3] performed a cross-sectional analysis of 75 adults (mean age 25 years) using both DEXA and quantitative ultrasound. They report that osteopenia was present in 34% and osteoporosis in 27%. Haworth[4] studied 151 CF adults and found that 34% had a T score greater than –2.0. Conway[5] evaluated 114 CF adults and found that 66% had osteoporosis or osteopenia.

Several risk factors of osteoporosis have been identified in CF adults. For example, the prevalence of low bone mineral density appears to be particularly high in patients who have poor clinical status. Aris[6] evaluated 70 CF adults referred for lung transplant evaluation and found that 57% had osteoporosis and 39% had osteopenia. Shane[7] studied 70 patients awaiting lung transplant and reported 49% had T scores consistent with osteoporosis at one site or more and 35% and osteopenia. Studies[6-8] have identified that chronic use of oral or intravenous corticosteroids is another major risk factor of osteoporosis. These findings are not surprising since studies in people who do not have CF have reported the negative effects of systemic steroids on bone density[9].

Studies have reported that worsened nutritional status is a risk factor for osteoporosis[6-8]. These results are supported by reports of normal BMD in normally nourished CF adults[10], and are further supported by multiple studies documenting the significant correlation between lean tis-

sue mass and BMD[11]. Hypogonadism has been reported as another risk factor for osteoporosis in CF[6,8] ; however, these findings are likely tied to poor nutritional status. Starvation and low body weight have been associated with low levels of sex steroids in other clinical disease states[12], and studies have shown that pubertal onset and normal menstrual cycling are dependent on maintenance of a minimum body weight[13]. Therefore prevention of bone loss secondary to low sex steroid levels should first be addressed by improving nutritional and clinical status.

Prevalence of CF related bone disease in children and adolescents

The 2000 CF Foundation Annual Report states that 0.2% CF patients less than 18 years have osteoporosis and 0.2% have osteopenia[1]. Similar to the adult data, these estimates of prevalence are under-reported secondary to lack of uniform screening, and are markedly lower than several large cross-sectional reports. In 1979, Mischler[14] measured bone mineral content (BMC) in 27 CF patients ages 5-24 years and found that 33% had low BMC when data was corrected for body weight. Using quantitative CT, Gibbens[15] reported decreased BMD in a cohort of 57 patients whose ages ranged from two to 21 years. They also noted that decreased BMD was more common in CF patients with greater disease severity and poorer nutritional status. Henderson[16] reported decreased BMD in 40 CF children, again noting variability in BMD and concluding that the percent deficit in bone mineral was related to weight Z-score, lean body mass and to FEV1. Bhudhikanok[8] evaluated BMD by DEXA in 21 patients less than 18 years and found that 8 had BMD consistent with osteopenia and concluded that risk factors for osteopenia/osteoporosis included poor maintenance of body mass, use of systemic corticosteroids and delay of puberty.

Delayed pubertal maturation has been well documented in children with CF and may represent a major risk factor for future development of osteoporosis. Puberty is the time of greatest bone mineral accrual[12] and despite normal BMC during childhood, studies[3,6] have documented low BMC in adolescents with CF. Lack of normal accrual could be secondary to delayed pubertal maturation or could be secondary to the blunting of peak growth velocity once puberty is initiated. The cause of pubertal abnormalities does not appear to be secondary to hypogonadism. Bone age- and Tanner stage-appropriate sex steroid levels have been reported in both male and female children and teens with CF[10]. Both lean tissue mass and height are strong correlates of BMD[11]; therefore effort should be made to maximize them in children and adolescents.

Clinical Consequences of Osteoporosis.

The most important clinical consequence of decreased BMD is non-impact fracture, and one of the most serious types of fractures for a person with CF is a vertebral body fracture. These fractures can interfere with pulmonary function and with ability to participate in chest physiotherapy. In 1998 the CF Foundation Patient Registry began collecting information on fractures. The 2000 Annual Report states that 0.4% of adults had fractures during the previous year[1]. However, there has been no routine screening for fractures in people with CF, and several studies suggest occurrence of fracture in people with CF is much greater. In a study of 70 CF adults awaiting lung transplant, Aris[17] found that 37 had a history of fracture. In this cohort 62 non-reported vertebral fractures had also occurred. In a review of 94 chest radiographs, Elkin[2] found 23 vertebral fractures. Clearly uniform screening is needed to provide information on the prevalence of fracture in CF.

Conclusion

The actual prevalence of bone disease in people with CF has yet to be determined; however it is clear that some groups are at high risk. Uniform screening measures should be instituted inclusive of relating BMD to fracture risk. Screening protocols implemented as a result of recommendations from the consensus conference on bone disease will help provide much needed information about the prevalence and clinical consequences of decreased bone density in people with CF.

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S18.2
PATHOGENESIS OF CF BONE DISEASE

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CF bone disease is complex and multifactorial. Poor bone mineral accretion from late childhood through young adulthood results in lower than expected peak BMD (1). Increased bone loss, particularly in adults, probably contributes to low BMD. Several authors have reported increased levels of bone resorptive markers in CF patients (2-4). Histomorphometry on bone samples from CF patients have shown severe osteopenia/osteoporosis in trabecular and cortical bone with decreased osteoblast (bone forming cells) and increased osteoclast (bone resorbing cells) activity resulting in imbalances in bone turnover resulting in a net loss of bone (5). Bone biopsies rarely report osteomalacia (profound mineralization defects due to lack of vitamin D or calcium.

Vitamin D and Calcium: Over 20 studies (largely reviewed in 6) have found low 25OHD levels (the best measure of vitamin D supply) despite supplementation with oral vitamin D according to CF clinical practice guidelines, but PTH level elevation is less common (3). Low 25OHD levels can occur in CF patients due to malabsorption (7), reduced sunlight exposure or, possibly, accelerated 25 OHD catabolism. Vitamin D deficiency is clearly a factor in some cases of osteopenia in CF. Hahn et al first demonstrated a correlation between low 25OHD levels and BMD in CF (8). Some reports have not found an association between serum 25OHD levels and BMD in CF, but this is not unexpected since BMD reflects bone health over the lifetime of the patient and vitamin D levels may fluctuate day to day based on the season, sunlight exposure, diet, etc. Hanley et al.found that only 7 of 15 of their vitamin D deficient patients achieved normal serum 25OHD levels after 4-10 weeks of standard supplementation therapy (400-800 IU/d) and only 30% had normal 25OHD levels after 1 year (9). The current guidelines for vitamin D supplementation in CF are being revised to address this problem. Reduced calcium absorption may also occur in CF (10). The above results indicate that vitamin D/calcium-dependent mechanisms are very likely to contribute to low BMD in CF.

Nutrition and Activity: A variety of nutritional factors may contribute to low BMD. Several authors have correlated poor nutrition or reduced body mass index (BMI) with low BMD (3, 11). Malabsorption of fat soluble vitamins may increase the risk of bone disease in CF, but low BMD has been reported in both pancreatic sufficient and insufficient patients. Vitamin K insufficiency may increase the levels of undercarboxylated osteocalcin (an important bone formation protein), possibly contributing to reduced BMD. Inadequate caloric intake may delay puberty, a time when the greatest accrual on bone occurs. Whether poor nutrition plays a causal role in CF bone disease is not clear as it may result from active inflammatory processes that simultaneously contribute to altered bone turnover and, ultimately, bone disease. Consumption of caffeine, alcohol and tobacco has also been shown to adversely impact bone metabolism in non-CF groups. Finally, factors such as weight bearing exercise and physical therapy are generally beneficial while inactivity may hasten bone loss.

Inflammation: Chronic inflammation is a risk factor for osteoporosis in non-CF groups. Indirect evidence supports a role for inflammation associated with pulmonary infections in the disordered bone remodeling so prevalent in CF patients. The number of intravenous antibiotic courses negatively correlates to BMD (12). Mononuclear and T and B lymphocytes are the predominant cell types identified in nasal tissues of patients with CF and these cells have been implicated in augmenting osteoclastic bone resorption through the production of CFU-GM and other cytokines. Acute lung infection in cystic fibrosis associated with an increase in the osteoclast-stimulatory inflammatory cytokines, IL-6, IL-1 and TNFα that is concomitant with an increase in biochemical markers of bone resorption, NTX and Dpd, as well as a reduction in the bone formation marker, osteocalcin (13). These abnormalities improved with antibiotic therapy for infection, but did not return to normal. In a second study, BMD was related to levels of IL-6 ($r = -0.60$) and TNFα soluble receptors ($r = -0.42$ and -0.50) (14). Patients with a low fat free mass (FFM) had greater concentrations of IL-6, which suppressed less after antibiotics than those with a normal FFM.

Endocrine Abnormalities: Sex hormonal deficiency from delayed puberty and hypogonadism probably play a role in CF bone disease. Low levels of serum testosterone and abnormal menses have been reported in adults, but are mosre common in advanced disease (1, 15). Diabetes, a recognized complication of CF, has been associated with losses in bone mass in non-CF groups.

Medications: Oral and inhaled corticosteroids are the most common cause of iatrogenic osteoporosis in society and probably contribute to low BMD in CF based on studies inversely correlating BMD and cumulative prednisone usage. Medroxyprogesterone acetate (Depo-Provera) has become a popular form of contraception, but has been shown to increase bone resorption in non-CF women.

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S18.3
SCREENING FOR CF BONE DISEASE

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The life expectancy of patients with Cystic Fibrosis (CF) has increased dramatically over the past few decades. As the population of patients with CF ages, medical diseases and complications are emerging that were infrequently seen in past years. CF related bone diseases are becoming increasingly recognized as significant clinical complications of CF. A variety of clinical factors predispose to the development of CF bone diseases but a complete understanding of the pathophysiology is lacking. Further, most of the current reports of CF-related bone disease are observational studies. This paucity of data makes it difficult to formulate reliable recommendations. To assist practitioners in the understanding and management of CF bone diseases, guidelines regarding the screening, diagnosis and management of this problem have been drafted in a consensus conference sponsored by the CF Foundation.

Bone loss in cystic fibrosis is a complex, multifactorial process. Malabsorption of calcium and fat-soluble vitamins, particularly vitamins D and K clearly contribute to the problems. Chronic inflammation associated with lung disease, inactivity, malnutrition, gonadal insufficiency and delayed puberty may also contribute to the problem. A variety of drugs used to treat patients with CF, particularly steroids, have been associated with bone loss. Finally, the risk of bone loss increases after lung transplantation. Recommendations for screening for CF bone diseases begin with identification of persons at risk for bone loss and bone fragility as well as identification of prior fractures. Guidelines for screening patients at risk are then outlined further. Screening for bone mineral density should be performed by dual energy x-ray absorptiometry (DXA) of the PA lumbar spine and proximal femur. Interpretation should be

based on the Z score for patients under age 30 years, T score for patients over 30 years of age.

General Recommendations

Management of lung disease to minimize the effect of inflammation and infection.
A regular exercise program that includes weight-bearing exercise is recommended.
Use of tobacco and excessive alcohol consumption should be avoided.

Nutritional Recommendations

Maintain adequate weight (≥ 90% IBW) and body mass index (>25% predicted).
Insure adequate Calcium intake for age. (1300-1500 mg/d for ages 9 and up)
Vitamin K supplementation of 0.3-0.5 mg/d.
Mg, Zn and Cu intake should at least achieve RDA levels.
Vitamin D:
Monitor 25(OH)D levels annually in all age groups.
Target circulating 25 (OH)D levels range from 30-60 ng/ml.
Obtain serum levels of 25(OH)D preferably prior to the winter months.
For circulating 25(OH)D levels <30 ng/ml, a repletion protocol is outlined.
Maintenance supplementation of 400 IU/d (ages 0-1) and 800 IU (age 1 and up).
Higher maintenance doses of may be necessary in some individuals.

Endocrine Recommendations

Identify patients with CF related (or type I) diabetes.
Monitor bone age as outlined by the recent nutritional consensus conference.
Screen adolescents for Tanner staging (females ≥ 9 years, males ≥ 13 years).
Pubertal delay is defined, with criteria for evaluation and management.
Recommendations for monitoring free testosterone levels in older males are outlined.
Screen for age of menarche during clinical assessment.
Clinically assess menstrual regularity in females annually.
Screen for menopause in women over 40 years old.
Consider hormonal replacement therapy for pubertal delay and hypogonadism.
Monitor steroid use.

Radiographic Recommendations

Monitor chest radiographs for evidence of fractures and bone loss.
Review fracture history regularly.
Baseline bone density testing (DXA) is recommended as follows (age 8 and older):
Delayed puberty.
Nutritional failure (IBW <90%).
Diabetes.
Moderate / severe lung disease.
Organ transplant candidate / post-transplantation.
Use of drugs associated with bone loss:
Systemic glucocorticoids >90 days per year.
Depot medroxyprogesterone acetate (Depo-Provera)
Heparin
Low trauma (fragility) fracture.
Age 18 if not done previously for other indication.

Repeat study recommendations

For Z or T score -1.0 and above, repeat every 5 years, sooner if clinically indicated.
For Z or T score -1.0 to –2.0, repeat every 2-4 years, sooner if clinically indicated.
For Z or T score –2.0 and below, repeat annually or sooner if clinically indicated, until stable or improved.
For steroid use, transplantation or documented bone loss, repeat annually.
Timing of subsequent studies may vary as clinical indications change.
Follow absolute bone mineral density, not T or Z scores.

Bone growth and development begins in infancy and early childhood then accelerates during adolescence. By adulthood, bone structure is established and subsequent bone health relies on the balance of bone resorption and bone deposition. The above consensus recommendations were developed with two goals. First is to assure adequate nutrition and an appropriate endocrine environment to allow strong, healthy bone development. The second goal is early identification of patients with increased risk of fracture, such that preventative measures may be implemented prior to subsequent fractures.

S18.4
TREATMENT OF CYSTIC FIBROSIS BONE DISEASE

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Cystic fibrosis bone disease has a multifactorial aetiology[1] and the histomorphometric appearances are heterogeneous with both osteoporosis and osteomalacia being described[2,3]. Treatment strategies must therefore be individualised.

General recommendations to improve bone health in children and adults include:

- ensuring that total dietary calcium intake is appropriate for age
- ensuring that 25-hydroxyvitamin D levels are between 30 – 60 ng/ml
- minimising the use of corticosteroid therapy
- maximising lean body mass
- encouraging regular weight bearing exercise
- minimising pulmonary infection / inflammation
- hormone replacement therapy for patients with delayed puberty or hypogonadism

Bisphosphonates are potent inhibitors of osteoclastic bone resorption and effective treatments for postmenopausal osteoporosis and corticosteroid induced osteoporosis. Bisphosphonates might be efficacious in patients with cystic fibrosis as premature bone loss is prevalent in young adults[4] and bone turnover studies demonstrate an imbalance of bone resorption over formation[5]. There are however several reasons why bisphosphonates might be problematic in patients with cystic fibrosis: 1) There is a high incidence of vitamin D insufficiency/deficiency[1]. 2) The gastrointestinal absorption of bisphosphonates in the general population is low (~0.75%) and might be further reduced in patients with cystic fibrosis. 3) Oral aminobisphosphonates can cause erosive oesophagitis, the incidence of which might be increased in patients with cystic fibrosis due to the prevalence of gastro-oesophageal reflux. 4) Adherence to oral bisphosphonates might be sub-optimal in patients with cystic fibrosis as treatment regimens are often already demanding. In these circumstances, once weekly oral bisphosphonates might improve adherence. Alternatively, adherence and gastrointestinal absorption concerns could be overcome by the intermittent administration of intravenous bisphosphonates, but this is more invasive to the patient and causes an acute phase response in approximately 10% of the general population. 5) Bisphosphonates are a relatively new treatment for osteoporosis and have been largely evaluated in the postmenopausal women. In view of the relative uncertainty about the long-term effects of bisphosphonates on skeletal development and their potential to be teratogenic, careful consideration is required before prescribing them in a young population.

Two bisphosphonate trials (both using intravenous pamidronate) have been reported in adults with cystic fibrosis, one in non-transplant[6] and the other in post-lung transplant patients[7]. In the non-transplant study there was a 5.8% and 3.0% difference in lumbar spine and total hip bone density between the pamidronate and control groups after six months treatment[6]. However, there was a high incidence of bone pain (lasting up to three days) following pamidronate infusion in patients not taking oral corticosteroid therapy[8]. It has been suggested that intravenous pamidronate causes an acute phase response that is diminished by the concomitant use of oral corticosteroids[9]. In the post-transplant study, bone density in the pamidronate group increased by 8.8% and 8.2% in the lumbar spine and proximal femur after two years treatment, in comparison to control subjects who gained 2.6% and 0.3%, respectively[7]. None of the patients reported bone pain following pamidronate, which further suggests that immunosuppressive agents have a protective effect.

In the adult population, until further safety and efficacy data are available, it seems prudent to limit bisphosphonate therapy to patients at high risk of developing fragility fractures:

- patients with a previous fragility fracture
- patients requiring long-term or frequent courses of corticosteroid therapy in whom a significant reduction in BMD has been documented*
- patients awaiting solid organ transplantation in whom a significant reduction in BMD has been documented*
- patients with reduced lumbar spine or hip BMD ($Z < -2.0$ in patients < 30 years of age and $T < -2$ in patients ≥ 30 years of age), in whom a significant reduction in BMD has been documented*
- patients following solid organ transplantation

* Patients at high risk of developing a fragility fracture in whom bisphosphonate therapy is being considered should ideally have a DXA scan every six months to document change in absolute BMD. A significant reduction in absolute BMD can be defined as > 3% in the lumbar spine or > 5% in the proximal femur.

In the paediatric cystic fibrosis population, the prescription of bisphosphonates should be limited to patients with a history of fragility fracture and patients following transplantation. In these patients supervision by a paediatric bone specialist is strongly advised.

The choice of bisphosphonate for patients with cystic fibrosis is limited by the trial data currently available[6,7]. For this reason, a trial of intravenous pamidronate is rec-

ommended in conjunction with measures to reduce the likelihood of bone pain, until studies evaluating oral preparations have been reported. Measures to reduce the risk of bone pain include administering the infusion at the end of a course of intravenous antibiotic therapy, commencing a three day course of oral prednisolone (20-30mg daily) two days before pamidronate infusion, and slowing the infusion rate to three hours. In the non-transplant cystic fibrosis population, patients should be counselled about the risk of developing bone pain after pamidronate infusion and that the side effects are likely to be less marked with subsequent doses. Female patients should be counselled about the potential risk of bisphosphonates to a developing foetus and all females who choose to have bisphosphonate therapy should use adequate contraception.

In conclusion, cystic fibrosis bone disease is a heterogeneous condition and requires individualised management. General recommendations for improving bone health are listed above. Bisphosphonate therapy should be reserved for patients at high risk of fragility fracture until further safety and efficacy data are available.

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