SYMPOSIUM SUMMARIES

S1.1
MOLECULAR CHAPERONES AND MECHANISMS OF POLYPEPTIDE DISLOCATION DURING ER ASSOCIATED PROTEIN DEGRADATION (ERAD)

Jeffrey L. Brodsky, Ph.D.

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA

All proteins that are secreted from cells, and most that ultimately reside within the cell, must traverse the secretory pathway, a network of membranes and intracellular organelles which house the "machinery" that helps proteins mature. Critical components of these machines are molecular chaperones associated with the endoplasmic reticulum (ER). Molecular chaperones are ubiquitous proteins that play critical roles during protein folding and degradation. However, if protein folding is inefficient or kinetically retarded, a nascent secreted protein may be targeted for degradation via a process we termed as ER associated protein degradation (ERAD). Not surprisingly, molecular chaperones are required for the ERAD of mis-folded proteins and may "decide" whether a protein is sufficiently mature to transit through the secretory pathway, or whether it should be targeted for ERAD if mis-folded.

Approximately 80% of all clinical cases of CF arise from mutations that affect the folding and thus prevent the exit of the cystic fibrosis transmembrane conductance regulator (CFTR) from the ER, thereby converting CFTR into an ERAD substrate. As anticipated, molecular chaperones are associated with immature forms of CFTR in the ER. But, it has been difficult to decipher whether the association between chaperones and CFTR is because the chaperones are "trapped" with immature CFTR while trying to help the protein fold, or whether they are recruited to actively target the protein to the ERAD machinery. Moreover, the importance of developing a deeper understanding of chaperone action on CFTR is warranted as chemical modifiers of chaperone levels/activity have been shown to augment the transport and maturation of CFTR mutants to the plasma membrane (reviewed in 1).

To better understand how chaperones influence CFTR biogenesis, we developed a CFTR expression system in yeast in which the effects of rapidly inactivating specific molecular chaperones on CFTR stability could be examined. We found previously that the cytoplasmic Hsp70 chaperone, Ssa1p, is required for the degradation of CFTR (2). We have also examined the impact of inactivating the Hsp90 chaperone on CFTR stability and present data that the protein is degraded more rapidly than in wild type cells, consistent with a role for this chaperone in catalyzing protein folding. In contrast, we recently determined that Hsp90 promotes the degradation of another ERAD substrate, apolipoprotein B (3). These results suggest that Hsp90 acts at unique steps during the maturation of different polypeptides, and may be either "pro-folding" or "pro-degradative". This hypothesis reconciles the controversial roles that Hsp90 has been proposed to play during protein maturation.

Both soluble proteins in the ER lumen and integral membrane ERAD substrates, such as CFTR, are degraded by the proteasome, a multi-catalytic cytoplasmic protease. There is currently intense research aimed at deciphering the mechanisms and molecules that link CFTR mis-folding to proteasome-mediated degradation, and several models have been presented to account for how this ERAD substrate is degraded. In one view, the proteasome initiates the degradation of CFTR by "shaving" the exposed cytoplasmic loops of CFTR. In another view, CFTR is extracted from the ER membrane and "retro-translocated" or "dislocated" to the cytoplasm, and transport and degradation may be either coupled or uncoupled. The presence of CFTR "aggresomes" in the cytoplasm of CFTR over-expressing cells (or in cells in which degradation is compromised) suggests that CFTR can be extracted and that dislocation and degradation may be uncoupled under some conditions (4).

To determine definitively whether retro-translocation and degradation can be uncoupled during ERAD, we examined the fate of a soluble model protein that is retro-translocated from the ER prior to its degradation by modifying an *in vitro* ERAD assay we developed several years ago (5); other soluble ERAD substrates include proteins implicated in liver disease and diseases of the immune system. We now find that the 19S "cap" of the proteasome is sufficient to retro-translocate the soluble ERAD substrate and that subsequent addition of the 20S proteolytic core of the proteasome degrades the retro-translocated polypeptide. These results suggest that dislocation and degradation can be uncoupled for aberrant, soluble polypeptides in the ER lumen, and attempts to demonstrate this phenomenon for simple integral membrane ERAD substrates are underway.

Acknowledgments

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S1.2
PHARMACOLOGICAL UNCOUPLING OF PROTEASOME ATPASE AND PEPTIDASE ACTIVITIES RELEASES CFTR DEGRADATION INTERMEDIATES FROM THE ER MEMBRANE

William R. Skach

Division of Molecular Medicine. Oregon Health Sciences University. Portland, OR 97201

When newly synthesized CFTR fails to achieve its mature conformation it is degraded in the endoplasmic reticulum via the ubiquitin-proteasome pathway (1, 2). This process involves covalent attachment of multiple ubiquitin molecules to one or more lysine residues. Polyubiquitin chains in turn, serve as a degradation signal for the 26S proteasome, a multi-catalytic protease comprised of a 20S core and two 19S regulatory subunits (3). The 20S proteasome contains four stacked heptameric rings arranged as a toroidal cylinder; two outer rings contain alpha subunits, while two inner rings contain beta subunits. In eukaryotes, three beta subunits in each ring, beta 1, beta 2, and beta 5, function as active proteases in the interior of the cylinder. In contrast, the 19S regulatory subunit is responsible for recognizing (and removing) polyubiquitin chains. It also contains a ring of AAA-ATPases that are proposed to unfold and thread substrate in to the axial conduit of the 20S subunit (4, 5).

A key question in CFTR biology is how the 26S proteasome, which resides solely in cytosolically contiguous compartments, degrades proteins such as CFTR that contain multiple transmembrane segments and ER-lumenal peptide loops. Previous studies have demonstrated that proteasome-mediated CFTR degradation involves polypeptide extraction from the ER membrane. However, the precise relationship between extraction and degradation has been poorly defined. Under conditions of proteasome inhibition, cytosolic forms of CFTR have been identified and shown to form inclusions called aggresomes (6, 7). Other studies have demonstrated that membrane extraction and degradation of CFTR and related ABC transporters are tightly coupled (8, 9).

To address the molecular mechanism by which CFTR is removed and degraded from the ER membrane, we developed a cell-free system that reconstitutes CFTR synthesis, membrane integration and degradation via the ubiquitin-proteasome pathway. Under physiological conditions, immature CFTR is polyubiquitinated, degraded and released from the ER membrane solely in the form of trichloroacetic acid (TCA)-soluble peptide fragments. Degradation is entirely ATP-dependent and sensitive to a variety of proteasome active-site inhibitors but not inhibitors of serine, cystine, metalloproteinases or amino-peptidases. Using proteasome inhibitors, we demonstrated that all three active beta subunits contribute to CFTR degradation and that complete inhibition of beta subunit activity is required to block conversion of CFTR into TCA soluble fragments (10). In contrast, when proteasome ATPase activity is inhibited by hemin, ubiquitinated CFTR remains tightly bound to the ER membrane. Upon addition of fresh cytosol, pre-ubiquitinated CFTR can be degraded and released, again as TCA soluble fragments. These results strongly suggest that CFTR degradation occurs at the ER membrane and involves recruitment of cytosolic factors.

To explain the formation of CFTR cytosolic intermediates, we took advantage of the ability to selectively block proteasome protease and ATPase activities. We reasoned that in the absence of ATPase activity, ubiquitinated substrate should not be unfolded and hence not extracted from the ER membrane. In the absence of peptidase activity, however, protein unfolding could potentially exceed the capacity for degradation and thus give rise to unfolded degradation intermediates. This, indeed, is the case. In the presence of active site inhibitors, MG132, ALLN, and *clasto*lactacystin-beta-lactone, CFTR is released from the membrane as large heterogeneous TCA insoluble fragments (20 to ~60 kDa in size as well as higher MW species that resemble polyubiquitinated protein). These cytosolic fragments contain N- C- and R-domain epitopes and regardless of their size, remain associated with a large protein complex similar in mass to that of the proteasome. Generation of TCA insoluble cytosolic fragments correlates directly with the extent of beta subunit inhibition, consistent with the hypothesis that their generation results from progressive failure to cleave unfolded substrate. Release of these fragments is completely blocked when proteasome AAA-ATPase activity is inhibited by hemin. These results provide evidence that hemin-sensitive ATPases facilitate dislocation of CFTR from the ER membrane and transfer unfolded protein into the catalytic core of the 20S subunit.

An important implication of these studies, is that under physiologic conditions, proteolytic activity of the 20S subunit must equal or exceed unfolding activity of the 19S subunit (and/or other contributing unfoldases). This is critical to prevent the accumulation of unfolded, partially degraded intermediates that could have significant detrimental effects in cells (11). Active site proteasome inhibitors uncouple ATPase activity from peptide cleavage and thus allow unfolding and membrane extraction to exceed the rate of degradation. In the case of CFTR, we propose that this results in aberrant release of cytosolic degradation intermediates and that these intermediates are prime substrates for aggresome formation. (supported by NIH and CFF).

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S1.3
HOW THE HSC70/CHIP UBIQUITIN LIGASE COMPLEX PARTITIONS NASCENT CFTR BETWEEN FOLDING AND DEGRADATION PATHWAYS

Douglas M. Cyr

Department of Cell and Developmental Biology and the UNC-CF Center, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599-7060, USA

Eukaryotic protein quality control systems monitor the folded state of cellular proteins and target aggregation prone forms of non-native CFTR and CFTRΔF508 for degradation via the ubiquitn-proteasome system. However, how the cell selects nascent ER forms of CFTR and CFTRΔF508 for ubiquitination and proteasomal degradation is not clear. We have demonstrated that Hsc70 functions in a complex with the ER localized Hsp40 co-chaperone Hdj-2 to facilitate co-translational steps in CFTR folding/assembly (1). In addition, we recently identified CHIP as a co-chaperone of Hsc70 that functions at the ER to promote the proteasomal degradation of nascent forms of CFTR and CFTRΔF508 (2). CHIP interacts with Hsc70 via a set of N-terminal TPR domains and contains a C-terminal U-box domain that is related to the RING domain that is found in a subset of E3 ubiquitin ligases (3). E3 enzymes act as substrate selectors for E2 ubiquitin conjugating enzymes, which function to facilitate the proteasomal degradation of proteins by building polyubiquitin chain onto target polypeptides. Based on this information, we surmised that CHIP functioned with Hsc70 as a quality control factor that mediates the selective ubiquitination of non-native CFTR and CFTRΔF508. To test this model, purified components and cell extracts were utilized to reconstitute the polyubiquitination and degradation of the cytosolic subdomains of CFTR. Results from these studies demonstrated that Hdj-2 and Hsc70 cooperate to facilitate the CHIP dependent polyubiquitination of CFTR. Therefore, CHIP functions as a chaperone dependent E3 ubiquitin ligase that utilizes the polypeptide binding activity of the Hdj-2/Hsc70 chaperone pair to select non-native CFTR for degradation. In overexpression studies with cultured cells, we demonstrated that the elevation of the activity of the CHIP/Hsc70 ubiquitin ligase complex diverted nascent CFTR from its folding pathway and increased the rate of its proteasomal degradation. In addition, the inactivation of the CHIP/E3 ubiquitin ligase complex decreased the rate of nascent CFTR turnover and increased the level of folded CFTR that accumulated in the cell. Thus, Hsc70 interacts with different sets of co-chaperones to facilitate CFTR folding or degradation. In addition, these data suggest that both folding kinetics and the activity of the cellular quality control systems play an important role in controlling the partitioning of non-native CFTR between folding and degradation pathways. Therefore, the in activation of the ERQC system has the potential to extend the half-life of nascent CFTRΔF508 increase and may thereby increase its folding efficiency. Results from ongoing tests of this hypothesis will be presented and discussed.

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S1.4
CYSTEINE STRING PROTEIN: A CO-CHAPERONE IN CFTR BIOGENESIS

H. Zhang, F. Sun, K.W. Peters and R.A. Frizzell

Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261 USA

The apical membrane density of CFTR is a key determinant of CF disease severity. Protein kinase A (PKA) phosphorylation of CFTR regulates both channel gating and its distribution between the apical membrane and intracellular compartments (1). Removal of CFTR's R domain virtually abolishes its regulated membrane redistribution; (R-CFTR traffics constitutively to the plasma membrane where it forms a spontaneously active channel. This result suggests that the non-phosphorylated R domain permits CFTR to enter a regulated trafficking compartment, whereas R domain phosphorylation permits CFTR to redistribute to the plasma membrane. Accordingly, CFTR interactions with proteins involved in regulated trafficking (e.g. synaptic vesicle components) may assist in the redistribution of CFTR in response to cAMP/PKA agonists.

Cysteine string proteins (Csps) are membrane-associated, synaptic and secretory vesicle proteins that contain a J-domain (comprising a Hsc70 binding motif); Csp is required for regulated exocytosis in neurons and insulin secreting cells (2). We found that two Csp isoforms were expressed in mammalian epithelial cell lines (e.g. T84, Calu-3) that express CFTR. In Calu-3 cells, Csp co-sedimented with CFTR and with markers of the endoplasmic reticulum (ER) and the plasma membrane. Immunofluorescence co-localized Csp with calnexin in ER and with CFTR at the apical membrane domain. In accord with expectations regarding its role in regulated exocytosis, Csp at the apical domain may be involved in regulated CFTR trafficking; however, ER localized Csp may serve other CFTR associated functions.

We found that CFTR could be co-precipitated with Csp from Calu-3 cell lysates. The core-glycosylated, immature form of CFTR (band B) predominated in the Csp IP, particularly in relation to the relative levels of immature and mature (band C) CFTR expressed in Calu-3 cells at steady-state. Intracellular CFTR domains, constructed as GST fusion proteins, were added to Calu-3 cell lysates to identify Csp-CFTR domain interactions. The CFTR N-terminus and R domain pulled down Csp in these assays. Binding experiments using *in vitro* translated Csp isoforms also showed physical interactions of Csp with the CFTR R domain and with the N-terminus having sub-micromolar affinities.

In *Xenopus* oocytes expressing CFTR, Csp co-expression decreased the Cl current increases evoked by cAMP stimulation. This decreased functional response could be attributed to decreased CFTR protein expression, detected by immunoblot. In mammalian cells co-expressing CFTR and Csp, mature CFTR was not detectable, but the expression of immature CFTR was enhanced. In pulse-chase experiments, Csp co-expression blocked the conversion of immature to mature CFTR and stabilized band B. The effect of Csp co-expression was specific for CFTR, since Csp did not alter the biogenesis of VSV-G protein or perturb endogenous Hsc70 levels.

The structural requirements for the inhibition of CFTR maturation by over-expressed Csp were examined in HEK 293 cells. Co-expression of a J-domain mutant of Csp (H43Q) that disrupts its interaction with Hsc70 did not impair the biogenesis of mature CFTR. CFTR co-precipitated with myc-tagged H43Q Csp, so that the Hsc70 binding site (the HPD motif), or a complex of Csp with CFTR that relies on Hsc70 interactions, is not responsible for the physical interaction of Csp with CFTR. Nevertheless, protein binding studies showed that the J-domain was required for the interaction of Csp with the R domain of CFTR *in vitro,* indicating that the J-domain's binding sites for Hsc70 and the R domain are different. In co-immunoprecipitation assays, Csp formed complexes with CFTR, Hsc70 and Hsp90, suggesting that Csp is part of a chaperone complex involved in CFTR processing.

These results indicate that Csp interacts physically and functionally with CFTR. The co-expression studies suggest that Csp, and its interaction with Hsc70, may influence CFTR processing in one of two ways. First, as a co-chaperone Csp could stabilize intermediate forms of CFTR to promote their folding and maturation; that is, Csp would contribute positively to CFTR maturation, similar to the Hsc70 co-chaperone, Hdj-2 (3). Second, Csp interactions with non-folded CFTR intermediates may contribute to the degradation of nascent CFTR, similar to the Hsc70 interacting protein, CHIP (4). Csp over-expression could reduce the steady-state expression levels of mature CFTR in either scheme. As a co-chaperone, Csp over-expression could cause a prolonged association of CFTR with Hsc70, which can target CFTR for degradation (5).

Several findings favor the co-chaperone model for Csp's physiological interaction with CFTR. First, previous data suggest a positive role for Csp in protein folding processes, since as with other DnaJ proteins, Csp binds to unfolded model proteins and prevents their aggregation in an Hsc70-dependent manner (6). Second, the predominant interaction of Csp with CFTR band B in Calu-3 cells suggests a physiological role for Csp in the correct maturation of CFTR since the majority of the CFTR expressed in these cells is the fully glycosylated mature form. Third, the direct interaction of Csp with

CFTR subdomains in the absence of Hsc70 suggests that Csp may localize Hsc70 at specific sites to faciliate CFTR folding. Fourth, the binding sites on Csp for Hsc70 and the R domain differ supporting the concept the Csp is not targeting Hsc70-associated CFTR. Fifth, Csp over-expression stabilized CFTR band B, which is contrary to the result expected for a protein that promotes CFTR degradation. The biogenesis of CFTR is a complex process, involving multiple chaperone protein interactions that stabilize folding intermediates to facilitate CFTR maturation. Our findings suggest that Csp is a CFTR binding co-chaperone that may mediate CFTR interactions with Hsc70, and perhaps Hsp90, to promote CFTR maturation. [Supported by the NIH (DK56490) and the Cystic Fibrosis Foundation]

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S2.2
POPULATION AND CASCADE TESTING FOR CYSTIC FIBROSIS IN THE UNITED KINGDOM

Maurice Super

Department of Clinical Genetics, University of Manchester Medical School, Royal Manchester Children's Hospital M27 4HA U.K maurice.super@man.ac.uk

There is no uniform policy on cystic fibrosis carrier screening in the United Kingdom. Medical services in the UK are either free to the patient at the point of service on the NHS, the National Health Service (the vast majority) or supplied on a private, payment basis to a far lesser extent.

The four countries which comprise the UK, England Northern Ireland Scotland and Wales, administer their own health services in the regions which comprise them. All four countries are represented on and contribute to the National Screening Committee. NHS health regions are under strong pressure not to introduce any kind of health screening before it has been approved by the National Screening Committee. Some existing screening services, which predate the formation of this committee, have continued on a national or regional basis.

Only in Lothian, a Scottish health authority centred on Edinburgh, is there an antenatal *couple* screening programme for cystic fibrosis and this has run since 1991, originally as a major research project and thereafter as a locally funded NHS service, on an ad hoc basis[1]. A reduced incidence of CF in the health region has been ascribed in part to the antenatal programme.[2]

The Leeds Antenatal Screening Service has advertised carrier testing for CF on a commercial basis on its website since 1997. Similarly, the Regional Genetics Service in Manchester circulated all general practitioners and obstetricians in the region in 1999 offering carrier screening in the absence of a family story, on a pay per service basis. The uptake in both these services has been very low. University Diagnostics, a commercial laboratory, advertised CF carrier screening in the lay press from 1995 but ceased its operation in 2000, mainly because of poor uptake. Nevertheless 70% of couples continue to accept the offer of screening in the Edinburgh antenatal programme.

In 1999 "Screening for Cystic Fibrosis" a Health Technology Assessment report, commissioned by the NHS, was published.[3] This dealt with antenatal and newborn screening for CF, since neither is currently offered nationally in the U.K. The conclusions of the report were as follows.

- Antenatal genetic screening should be offered routinely
- Preconceptual screening should be offered to couples who request it
- Genetic screening should be available to all infertile men and sperm donors
- Testing should be undertaken in laboratories with an annual throughput of at least 500 cases
- Health authorities could consider introducing neonatal screening.

The current situation in the UK is that the recommendations of the report have not been implemented. Instead, following intense pressure from the Cystic Fibrosis Trust, with almost unanimous support of CF centre directors, a decision has been made to introduce national newborn *disease* screening. A number of aspects have contributed to the decision to delay implementing antenatal carrier screening. At meetings of the Antenatal Subcommittee of the National Screening Committee, concerns have been expressed about which type of screening protocol might

be used. In couple screening, as it is currently practised in Edinburgh, the results when only one partner is a carrier and the other negative have not been divulged. Concerns have been raised that the rights of the individual might be being infringed, although the patient information leaflets make clear that results will only be communicated when both partners are carriers. Conscious of these concerns, Edinburgh is changing its policy to one of informing all discovered carriers of their status together with the offer of counselling to the couple A further concern raised by the Antenatal Subcommittee is potential difficulty seen in having an antenatal and newborn screening programme running together with the aim of the former being the offer of reproductive choice (implying severity of the condition) while the latter stresses the improved outlook for affected children and hence the value of having an early diagnosis. The committee will be looking in greater depth at these issues before deciding on which advice it will give concerning a nationwide screening programme.

Cascade screening

In 1992 a health technology assessment of the implications of carrier screening was published in the U.S. It concluded that carrier testing should be confined to those with a family history of CF. When this was revisited in 1997[4], while introduction of preconceptual and early pregnancy screening was recommended, the same statement concerning the need to offer testing to relatives with a family history and their partners was once again included. An *active* cascade service has existed with regional NHS funding in the North West of England since 1993[5]. This regional service is based in the genetics department at Royal Manchester Children's Hospital and has dedicated laboratory and specialist field worker staff and is supported by the CF DNA laboratory and the specialist counselling service. Working with the support of CF clinic staff throughout the region, parents of affected children or affected adults themselves are encouraged to inform their relatives of the availability as an NHS service of counselling and carrier testing. The main target group, as realised by the families and the programme is couples or individuals of child-bearing age. An audit of those who have used the programme has shown general satisfaction with it, with very few feeling that they had been pressured into be tested. Elsewhere in the UK regional genetics departments conduct counselling and testing of those with a family story on the NHS but on referral of the couple or individual, rather than proactively as in north west England. The view in Manchester is that there is widespread failure to refer such families, nationally. There have been two successful legal actions by couples for failure to refer when a family story of CF was given and subsequently an affected child was born[6]. The Cystic Fibrosis Trust produced a fact sheet for families entitled "The family cascade screening programme for cystic fibrosis" (www.cftrust.org) and has distributed this to all CF centres. While cascade testing will perhaps not reach the majority at risk in the population, there can be no argument that it is good practice.

It is widely accepted in the UK that were a breakthrough in treatment, allowing a great reduction in the burden of CF, then the case for antenatal screening would be weakened and that for newborn further strengthened. At the same time were a cure only effective in the fetus to be discovered, then preconceptual carrier testing would become more attractive.

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S2.3
CYSTIC FIBROSIS PRENATAL SCREENING OF 41,000 WOMEN IN A LARGE HMO

David R. Witt

Genetics Department, Kaiser Permanente Northern California, San Jose, CA

Population screening for cystic fibrosis has been debated since the discovery of the CFTR gene in 1989. Concerns had included uncertainty about the effectiveness of pre-test education and post-test counseling, unprecedented scope and logistical issues due to large numbers of patients and specimens, the availability of sufficient numbers of trained health care providers to implement screening, laboratory issues (methodology, determination of mutation panel size, etc.), insensitivity of CFTR gene mutation analysis, cost, potential psychological effects in patients being informed of their carrier status, and genetic discrimination.

In the early 1990's, a national moratorium took effect due to these concerns pending the results of pilot studies designed to assess a variety of aspects of CF screening. These studies provided a consistent set of evidence that strongly supports the notion that CF population carrier screening, particularly in the prenatal setting, is logistically feasible, has value for individuals, is analogous to many other forms of genetic testing, and is not associated with significant negative consequences. These findings were assessed by a series of conferences and professional organizations including The NIH Consensus Development Conference (1997), The Sixth Scarborough Conference on Prenatal Screening for Cystic Fibrosis (1998), The American College of Medical Genetics (2001) and The American College of Obstetricians and Gynecologists (2001). All of these groups recommended the implementation of population-based screening for CF and recognized that this would largely occur in the prenatal period. Today, 13 years after the discovery of the CFTR gene, CF screening has been implemented in a variety of settings and a substantial increase in the volume of testing in the U.S. can be anticipated over the next several years.

The original qualms about CF screening were legitimate and must be adequately addressed by any screening program prior to initiating testing:

- Appropriate pre-test education must be provided to enable informed decision-making by a patient. This can be accomplished by several methods such as oral presentation, written materials, and video.
- Post-test counseling must provide adequate information to identified carriers and at-risk couples about complex issues surrounding their newly defined risk and options for pregnancy management.
- Screening may involve large numbers of patients and specimens and, therefore, systems must be in place to manage the flow of information including tracking, reporting, and follow-up. Experience shows that utilization of testing is increased when screening occurs prenatally and decreased when barriers to testing such as additional appointments for counseling or phlebotomy are present.
- DNA test insensitivity is an inherent part of CF screening and must be recognized and appropriately communicated to patients and providers. Standard mutation analysis cannot identify all deleterious mutations, but testing a small subset of mutations in Caucasians, the ethnic group at highest risk, will identify the majority of carriers. Consequently, the residual carrier and couple risks are reduced approximately ten fold to an extremely low level and, despite test insensitivity, the goal of greatly reducing a couple's risk for having an affected baby is clearly achieved.
- The dollar cost of CF screening is an important consideration and appropriate resources need to be allocated prior to implementation. However, economic considerations are increasingly being superceded by the redefinition of standard-of-care that mandates the offer of screening. Like many other forms of genetic testing and counseling, CF screening will not save money. But the cost of the benefits (the value of information to patients and dollar savings from CF births averted by pregnancy termination) is not unreasonable and is certainly in line with many other medical interventions.
- Adverse psychological effects have not occurred in the vast majority of screened individuals and should not require a great deal of attention.
- The fear of widespread genetic discrimination as a result of population-based prenatal CF screening is unwarranted. Patients' concerns, if any, can be addressed relatively simply.

A few issues surrounding prenatal CF screening remain controversial and potentially problematic. Among these, the most important ones are 1) selection of appropriate target patients based on ethnic-specific carrier frequencies and mutation analysis sensitivities and 2) limitations in genotype-phenotype correlation with potential uncertainty in clinical prognostication:

- Target Population: Both the carrier frequency and the sensitivity of CFTR mutation analysis are significantly lower in non-Caucasians. This decreases the utility, and increases the cost, of CF screening in non-Caucasians. Policy decisions regarding whom to test must be based on this fact and will be discussed in light of current recommendations.

- Genotype-Phenotype Correlation: Whereas pancreatic dysfunction can be reasonably accurately predicted, pulmonary function is less well correlated. Consequently, genetic counseling based on fetal CFTR mutations may be broad and less precise than desirable, particularly when one or both of the mutations are of the mild type. Nevertheless, reasonable prognostication can usually be achieved that adequately equips potential parents with a level of information needed to make an appropriate informed decision. The clinician must impart the appropriate ranges of possible outcomes and paint a fair picture of the possible impact on the child and family.

Approximately 200,000 CF carrier screening tests will be performed in the U.S. in 2002 in the absence of a family history of CF. A brief summary of the largest organized CF screening program in the U.S., based at Kaiser Permanente Northern California (KPNC), illustrates the kind of experience with CF prenatal screening that is attainable and expected. A woman is offered screening after receiving pre-test education through a combination of videotape, written material, and/or oral presentation in either a group or individual setting. Eligibility is based on self-reported ethnicity for herself and her partner, and sequential testing is offered if either she or her partner has at least some Caucasian ancestry. If a CFTR mutation is not identified the patient receives a letter describing her new, reduced carrier risk. There is no contact with a genetics provider. If a mutation is identified, the woman is offered genetic counseling by telephone or in-person including testing of her partner. If the male partner is also identified as a carrier then this high-risk couple receives in-depth genetic counseling and is offered prenatal diagnosis. CFTR mutation analysis is done using a 37 mutation panel. Approximately 70% of eligible women participate and ¼ of these belong to couples in which one partner is not Caucasian. During 3 years of operation approximately 260 specimens/week on average have been processed and 41,000 individuals have been screened. Interesting findings include the identification of carriers and compound heterozygotes in approximately 1/28 and 1/7,000 screened individuals, respectively, and a relatively high frequency (25%) of several mild mutations.

High-risk couples have chosen prenatal diagnosis 80% of the time, and 100% of affected fetuses have been aborted. Despite the logistical difficulties of processing and tracking large numbers of patients and specimens, the flow between the prenatal clinics, genetics laboratory, clinical genetics providers, OBGYN providers, and patients moves extremely well and without significant problems. Patients have been pleased and reported concerns are minimal.

S3.1
LYMPHOCYTES IN THE PATHOGENESIS OF ALLERGIC BRONCHOPULMONARY ASPERGILLOSIS IN CYSTIC FIBROSIS

Alan P. Knutsen, MD

Division of Allergy and Immunology, Department of Pediatrics, Saint Louis University Health Sciences Center, St. Louis, Missouri

Allergic bronchopulmonary aspergillosis is a late-phase allergic inflammatory response to *Aspergillus fumigatus* allergens that occurs in genetically susceptible patients with asthma and cystic fibrosis. In addition, *A. fumigatus* allergens, especially proteases, have direct effects on the respiratory epithelium that result in pro-inflammatory responses and increased allergen exposure to the bronchoalveolar lymphoid tissue (BALT). The allergic inflammation to *A. fumigatus* leads to pulmonary central bronchiectasis and fibrosis.

The diagnosis of ABPA is based on a set of clinical and immunologic reactivity to *A. fumigatus*. ABPA occurs only in atopic asthmatic and CF patients. Secondily, ABPA patients have an elevated total serum IgE concentration >1000 IU/ml. Thirdly, patients with ABPA develop IgE and IgG antibodies to *A. fumigatus*. Recently with the development of studying serologic reactivity to recombinant purified *A. fumigatus* allergens difference between ABPA and atopic individual has been observed. Atopic individuals develop IgE antibodies to fewer purified allergens, namely Asp f1 and f3; whereas ABPA patients develop IgE antibodies to more *Aspergillus* allergens, namely Asp f2, f3, f4, f6, f12 and f16 and at greater level. The differences between ABPA and atopic individuals' responses to *A. fumigatus* appear to be quantitative and qualitative.

There are several lines of evidence that the BALT initiates and maintains an immune response to *Aspergillus* with subsequent trafficking to the peripheral lymphoid system. Greenberger and colleagues demonstrated that IgE and IgA anti-*Aspergillus* antibodies were primarily made in the BALT; whereas IgG anti-*Aspergillus* antibodies were produced in the peripheral lymphoid tissue. Analysis of cells obtained from bronchoalveolar lavage fluid (BALF) in ABPA reveal that they are an admixture of alveolar macrophages, eosinophils and lymphocytes, similar to that found in asthma. Eosinophil infiltration predominates both in BALF and lung tissue as is evident in lung biopsy. In addition, eosinophils are activated and have released their mediators, such as major basic pro-

tein. In addition to eosinophils, T-, B- and NK-cells are also found in BALF. The T-cells are an admixture of CD4+ and CD8+ T-cells in an approximate 2:1 ratio. Both CD23+ NK cells and CD23+ CD4+ T-cells obtained from BALF of ABPA patients have been observed, indication of *in vivo* IL-4 stimulation. In previous studies of *Aspergillus fumigatus* stimulated T-cell lines of ABPA, low percentages of CD23+ CD4+ T-cells were observed. Recently, we have observed that *in vitro* IL-4 stimulated MNC from CF patients were induced to express 5-10% CD23+ CD4+ T-cells with high density CD23 expression. This was prompted by the observation that in CF ABPA patients there was increased *in vivo* CD23+ CD4+ T-cells. The role of these cells may be T-cell CD23 and B-cell CD21 ligand-counterligand interaction, which augments IgE synthesis. Similarly, CD23+ NK cells may be an important source of sCD23 and/or NK-cell CD23:B-cell CD21 interactions increasing immunoblast IgE synthesis.

From human and murine models, Th2 CD4+ T-cells and their cytokines are central to the development of ABPA. The Th2 cytokine IL-4 plays a central role in the allergic inflammatory responses observed in ABPA. IL-4 upregulates cellular activity via binding to the IL-4 alpha receptor (IL-4Rα). The IL-4 receptor is a heterodimer comprised of IL-4Rα and the common gamma chain receptor (Cγ), IL-4Rα/Cγ. In addition IL-4Rα forms a heterodimer with the IL-13 alpha (IL-13Rα), IL-4Rα/IL-13Rα. Both IL-4 and IL-13 binds the IL-13 receptor. The IL-4 receptor is present on a variety of cells including B-cells, NK-cells, mast cells, endothelial cells and subpopulation of T-cells. Both IL-4 and IL-13 induce IgE isotype switch of B-cells to IgE synthesis. IL-4 also induces CD23+ expression, the low affinity IgE receptor, and soluble CD23 (sCD23) which augments B-cell IgE synthesis. In Asp f1 T-cell lines, the phenotypes were CD4+CD25+ T-cells and had the cytokine profiles IL-4+ and IFNγ-, indicating Th2 CD4+ T-cells. Furthermore, lymphoproliferative responses for Asp f1 T-cell lines were inhibited by anti-IL-4 but not by anti-IL-2, suggesting an IL-4 autocrine response. Interestingly, atopic CF patients without ABPA also developed Asp f1 generated Th2 CD4+ T-cell lines. Importantly, tetanus toxoid generated T-cell clones showed a Th1 phenotype, indicating that the Th2 response in ABPA is specific to *Aspergillus* allergens and not a generalized Th2 skewing to all antigens.

In ABPA CF patients, the cytokine pattern to Asp f2, f3 and f4 stimulated 7 days MNC cultures were examined. ABPA CF patients had significantly increased IL-4 and IL-5 and decreased IFN-γ synthesis compared to non-ABPA and non-atopic controls. Interestingly, IL-10 synthesis was increased in all patient groups to Asp f2, f3 and f4 stimulation, but not to tetanus toxoid stimulation. IL-10 is an anti-inflammatory cytokine synthesized by a variety of cell types, including monocytes, Th1 and Th2 cells, and bronchial epithelial cells. IL-10 regulates both Th1 and Th2 lymphocytes though there is a preferential inhibition of Th1 responses. *Aspergillus* allergens, by stimulating IL-10 synthesis and preferentially inhibiting Th1 response, may promote a Th2 T-cell response toward *Aspergillus* allergens. The frequency of Th1 and Th2 T-cells were also examined in those patients. In ABPA and non-ABPA CF patients, the frequency of PMA and ionomycin stimulated IFN-γ+ CD3+ T-cells was reduced compared to normal controls. However, the frequency of IL-4+ CD3+ T-cells was increased in *Aspergillus*-stimulated cultures in ABPA. When tetanus toxoid and Asp f2, f3, and f4 stimulated T-cells were examined, IFN-γ+ CD3+ T-cells were decreased in CF patients. Thus in ABPA CF patients, there is increased frequency of *Aspergillus* Th2 T-cells and decreased IFN-γ+ Th1 T-cells. Thus, in CF patients, there is a skewing of Th2 T-cells which probably plays a role in the increased incidence of ABPA in CF patients.

In addition, IL-4 appears to up-regulate CD86 expression in B-cells in atopic patients. This is important since CD86 in B-cells is an important costimulatory molecule for augmentation of IgE synthesis. The ligand for CD86 is CD28 on T-cells. CD86 and CD28 colligation also stimulates T-cells, promoting Th2 CD4+ T-cell responses and cytokine synthesis, eosinophil airway inflammation and airway hyperresponsiveness after allergen challenge. Recently, we have examined the effects of IL-4 stimulation of CD23 and CD86 expression on B-cells of patients with ABPA. In previous studies, purified B-cells from ABPA were observed to spontaneously synthesize elevated amounts of IgE compared to *Aspergillus* sensitive non-ABPA patients, indicating *in vivo* stimulation into IgE secreting immunoblasts. In recent studies, CD23 and CD86 expression on freshly isolated B-cells of ABPA patients were increased compared to non-ABPA *Aspergillus* sensitive patients suggesting *in vivo* IL-4 or IL-13 stimulation. Following IL-4 stimulation, ABPA patients had significantly increased rates of CD23 expression per B-cells compared to atopic and non-atopic. IL-4 stimulation did not increase the percentage of CD86 in ABPA patients, but did significantly increase the number of CD23 molecules per CD86+ B-cell. Though IL-13 also increased CD23 expression, there was no significant increase compared to other groups. Thus, ABPA patients had increased sensitivity to IL-4 stimulation with upregulation of CD23 and CD86 expression compared to other atopic individuals, such that ABPA >atopic >>non-atopic patients.

As a potential mechanism for this observation, mutations of IL-4Rα polymorphisms are being evaluated. Polymorphisms of IL-4 have been identified in atopic individuals with IgE levels. Some of these polymorphisms have been associated with a gain-in-function of IL-4 and IL-4 Rα interactions promoting increased CD23+ expression and IgE synthesis. Subsequently,

seven polymorphisms have been identified that result in increased IL-4 activity. In preliminary studies, homozygous mutations of the Q576R polymorphisms were observed in 2 ABPA patients. However, increased sensitivity to IL-4 stimulation was observed in ABPA and atopic patients without the Q576R polymorphism. Thus, current studies were being conducted to identify all the known polymorphisms in these patients.

Chauhan et al investigated whether there is unique TCR recognition (T-cell epitopes), TCR-Vβ restriction, or HLA-class II restriction that would promote enhanced Th2 responses. Analysis of T-cell epitope mapping has revealed that there were three immunodominant regions of the Asp f 1 protein in ABPA patients that is recognized by TCR. Furthermore, TCR epitope mapping studies revealed limited number of epitopes reacting with TCR, TCR Vβ restriction or usage, and HLA class II restriction. Four major Vβ chains, Vβ 3,6,13 and 14, react to Asp f 1. Recently, Chauhan et al identified that there is HLA-DR2 and DR5 restriction in patients with ABPA. Furthermore, within HLA-DR2 and HLA-DR5, there are restricted genotypes. In particular, HLA-DRB1*1501 and 1503 was reported to provide high relative risk. On the other hand, 40 to 44% of non-ABPA atopic *Aspergillus*-sensitive individuals have the HLA-DR2 and/or DR5 type. Further studies indicated that the presence of HLA-DQ2 (especially DQB1*0201) provided protection from the development of ABPA. Thus, certain genotypes of HLA-DR2 and DR5 may be necessary but not sufficient to cause ABPA. Furthermore, Chauhan et al demonstrated that Asp f l allergen has a low-affinity of binding to HLA-DR. This is consistent with Th2 T cell response previously reported by others in that strong antigen HLA-DR-Ag-TCR affinity binding favors a Th1 cellular response whereas low affinity binding favors a Th2 humoral response.

In summary, a quantitative increased Th2 CD4+ T-cell response to *Aspergillus* in both the BALT and systemic immune systems characterize ABPA. Perhaps key in the immunopathogenesis is that the BALT is exposed to high levels of *Aspergillus* allergens that have a disrupting effect on the respiratory epithelium allowing for *Aspergillus* allergens causing mononcyte pro-inflammatory response. In addition, *Aspergillus* allergens may stimulate high IL-10 synthesis skewing a Th2 T-cell response. Then abnormal mucus properties due to CFTR mutations may promote growth of *Aspergillus fumigatus*. Other genetic factors are characterized by restricted TCR-Vβ usage and by HLA-DR 2 and DR5 restriction to *Aspergillus* allergens with low affinity antigen binding. This also promotes a Th2 T-cell response. Thus, there are host immunogenetic susceptibility to develop ABPA which resides within the HLA-DR-Ag-TCR signaling of the T-cells and biologic properities of *A. fumigatus* that skew a Th2 CD4+ T-cell response.

S3.2
CFF CONSENSUS CONFERENCE ON DIAGNOSIS AND TREATMENT OF ABPA IN CF

Richard B. Moss

Department of Pediatrics, Stanford University, Palo Alto CA

Allergic bronchopulmonary aspergillosis [ABPA] develops from sensitization with allergens from Af [*Aspergillus fumigatus*] present in the environment. ABPA is a disease primarily occurring in patients with asthma (1-2%) or CF (2-15%). It is manifested by wheezing, pulmonary infiltrates and bronchiectasis and fibrosis. Some immunological manifestations are peripheral blood eosinophilia, immediate cutaneous reactivity to Af antigen, elevated total serum IgE, precipitating antibody to Af, elevated specific serum IgE and IgG antibodies to Af, and increased serum IL-2 receptor concentrations. The hyphae of Af that grow saprophytically in the bronchial lumen result in persistent bronchial inflammation leading to proximal bronchiectasis.

The *diagnosis* of ABPA in CF is difficult and may often be delayed because many of the diagnostic criteria overlap with common manifestations in CF. Unlike in asthma, pulmonary infiltrates, bronchiectasis and obstructive lung disease are common manifestations of CF lung disease with or without ABPA resulting from recurrent and chronic bacterial infection. Atopy as well as an onset of a variety of immune responses to Af antigens early in life in patients with CF complicates the interpretation of various serological parameters for the diagnosis of ABPA. Early diagnosis and treatment aiming to suppress the inflammation is, however, important to prevent irreversible lung tissue damage.

Consensus Conference recommendations for diagnosis of ABPA in CF are:

Consensus Conference Full Diagnostic Criteria for ABPA in CF

1. Acute or subacute clinical deterioration (cough, wheeze, exercise intolerance, exercise- induced asthma, decline in pulmonary function, increased sputum) not attributable to another etiology.
2. Serum total IgE concentration over 1000 IU/mL (2,400 ng/mL), unless patient receiving systemic corticosteroids (if so, retest when off steroids).

3. Immediate cutaneous reactivity to *Aspergillus* (prick skin test wheal >3 mm with surrounding erythema, off systemic antihistamines) or *in vitro* presence of serum IgE antibody to Af.
4. Precipitating antibodies to Af or serum IgG antibody to Af by an *in vitro* test.

New or recent abnormalities on chest radiography (infiltrates, mucus plugging) or chest CT (bronchiectasis) that have not cleared with antibiotics and standard physiotherapy.

Minimal Diagnostic Criteria

1. Acute or subacute clinical deterioration (cough, wheeze, exercise intolerance, exercise-induced asthma, change in pulmonary function, increased sputum) not attributable to another etiology.
2. Total serum IgE >500 IU/mL (1200 ng/mL). Note: if ABPA is suspected and total IgE is 200-500 IU/mL, repeat testing in 1-3 months recommended. If on steroids, repeat when off steroids.
3. Immediate cutaneous reactivity to *Aspergillus* (prick skin test wheal >3 mm with surrounding erythema, off systemic antihistamines) or *in vitro* demonstration of IgE antibody to Af.
4. One of the following:
   a. Precipitins to Af or *in vitro* demonstration of IgG antibody against Af.
   b. New or recent abnormalities on the chest radiograph (infiltrates, mucus plugging) or chest CT (bronchiectasis) that have not cleared with antibiotics and standard physiotherapy.

Because of the relatively high risk of ABPA in CF patients and widespread differences in reported prevalence, the Consensus Conference recommended routine *screening* for ABPA in CF:

Consensus Conference Suggestions for Screening for ABPA in CF

1. Maintain a high level of suspicion for ABPA after age 6 years.
2. Obtain total serum IgE concentration annually. If the total serum IgE is >500 IU/mL, determine immediate cutaneous reactivity to Af or use an *in vitro* test for IgE antibody against Af. If positive, proceed to Minimal Criteria diagnosis consideration.
3. If the total serum IgE concentration is 200-500 IU/mL, repeat if there is increased suspicion for ABPA such as by a disease exacerbation, and perform further diagnostic tests (immediate sin test reactivity to Af or *in vitro* test for IgE antibody to Af, precipitins or serum IgG antibody to Af and chest radiography).

There are two aspects of *treatment* of ABPA. The first is the attenuation of the inflammation and immunologic activity, for which systemic corticosteroids are the mainstay of therapy. The second is the attenuation of the antigen burden arising from fungal colonization of the bronchial tree. Reducing the fungal burden in the respiratory tract might decrease antigenic stimulation, reduce inflammatory response, ameliorate symptoms, and possibly reduce the long-term risk of disease progression. There is limited evidence that itraconazole is useful in CF patients with exacerbations ABPA and it may facilitate a decrease in corticosteroid use. There is insufficient evidence to recommend other oral or inhaled antifungal agents. Inhaled corticosteroids cannot be recommended for initial therapy or for prevention of pulmonary fibrosis and chronic pulmonary dysfunction from ABPA in CF patients. Inhaled corticosteroids and possibly leukotriene modifiers may however be useful for the asthma component of ABPA as recommended by NIH asthma guidelines.

General treatment recommendations for ABPA in CF are as follows:

IgE (IU/mL)	Pulmonary symptoms and/or worsening PFT	New infiltrates CXR/CT	Positive serology*	Treatment (rx) recommendations
>1000 or >2x rise	Yes	Yes	Yes	Rx for ABPA
>1000 or >2x rise	No	No	Yes	No Rx. Monitor IgE, CXR, PFT
>1000 or >2x rise	No	Yes	Yes	Rx for CF infection. Consider Rx for ABPA if no response
>1000 or >2x rise	Yes	No	Yes	Consider Rx for ABPA, CF infection, and/or asthma
>500 in the past. No change from baseline	Yes	Yes	Yes	Rx for CF infection. Consider Rx for ABPA or asthma if no response
500-1000	Yes	Yes	Yes	Rx for ABPA

IgE, total serum IgE level; CXR, chest radiograph; CT, chest computerized tomography; PFT, pulmonary function test
*Af-specific IgG, IgE or positive precipitins to Af. Since these test results may not be available quickly, they are not required to initiate any therapy, but should be obtained.

Recommendations for pharmacotherapy for an exacerbation of ABPA in CF are as follows:

Corticosteroids	
Indications:	All patients except those with steroid toxicity
Initial:	0.5 - 2.0 mg/kg/day *po* prednisone equivalent, maximum 60 mg/day, for 1-2 weeks
Begin taper:	Then 0.5 - 2 mg/kg/day every other day for 1-2 weeks
Taper off:	Attempt to taper off within 2-3 months
Relapse:	Increase corticosteroids, add itraconazole, taper corticosteroids when clinical parameters improve
Itraconazole	
Indications:	Slow/poor response to corticosteroids, relapse, corticosteroid-dependent, or corticosteroid toxicity
Dosing:	5 mg/kg/day, maximum dose 400 mg/day *po* unless itraconazole levels obtained. BID dosing required when daily dose exceeds 200 mg
Duration:	3-6 months
Monitor:	LFT: all cases. Itraconazole levels: concern of adequate absorption, lack of response, possible drug-drug interaction. Monitor levels of concomitant drugs with potential for drug-drug interaction
Adjunctive Therapy	

The Consensus Conference was held on June 12-13, 2001. A resulting document has been submitted for publication.

S3.3
ASTHMA IN CF: ROLE OF BRONCHODILATORS

Peter König, MD, PhD

Department of Child Health, University of Missouri-Columbia, Columbia, Missouri/USA

Bronchodilators are the second most widely used group of drugs (93.3%) in CF patients, after pancreatic enzymes (94.8%).[1] Part of the justification for such use stems from the fact that both bronchial hyperreactivity[2,3] and atopy,[4] two of the main components of asthma have been shown to occur in CF patients to a greater degree than in normal subjects. Cross-sectional studies of bronchodilator responsiveness in CF yielded widely differing numbers of positive responders, between 0-50%.[5,6] Our longitudinal study,[7] for one year, with spirometry testing every 1-3 months as outpatients and twice weekly during hospitalizations, showed that virtually all patients (95%) had a significant bronchodilator response to albuterol at least once. In another 1 year open study of albuterol MDI 2 puffs b.i.d., with twice daily PEFR measurements in addition to office spirometry, it was concluded that significant (>15%) bronchodilation occurs in 25% of days and all patients respond at least sometimes.[8] Frequent responders could not be predicted by age, history of wheezing, family history of asthma or atopy or severity of baseline airway obstruction. In addition to the immediate benefit, this study also showed that maintenance therapy with albuterol twice daily caused a long-term improvement of 18% in FEV_1, while the same group had a significant deterioration in the previous year, as did a control group not treated with albuterol during the study year.[8] A later, double-blind, controlled study of albuterol MDI 2 puffs b.i.d. for 6 months showed a significant improvement of pulmonary function (both office spirometry and home PEFR) with no significant change with placebo.[9] However, the difference between albuterol and placebo did not reach statistical significance, possibly because of the relatively small number of patients (n = 21). The long term improvements in FEV_1 did not correlate with the mean daily bronchodilation on PEFR.[9] In the previous study,[8] long term improvement on FEV_1 correlated with the mean daily bronchodilation (PEFR), but improvement on FVC and FEF_{25-75} did not. Thus it seems that bronchodilation is not the only cause of the long-term benefi-

cial effect of adrenergic agents and it may not even be the important one. There was no difference in the number of hospitalizations and only a non-significant trend for less days of hospitalization on albuterol. Eggleston and associates[10] performed a controlled trial of albuterol MDI 2 puffs q.i.d. for 2 months and found significant improvement in daily PEFR measurements in those patients who had positive methacholine tests, but not in those with a negative test. There were no significant changes in symptoms or spirometry. More recently, several trials were performed with salmeterol. Hordvik,et al[11] showed a longer lasting beneficial effect with salmeterol than albuterol MDI in hospitalized patients and a better effect of large doses of salmeterol by MDI (4 puffs, 84μg) b.i.d. compared to nebulized albuterol t.i.d. Bargon and associates,[12] in a two week open trial of salmeterol, found a significant improvement in PEFR, symptom score and rescue use of short-acting B_2 agonists. In a more recent study by Hordvik, et al,[13] salmeterol high dose (100μg b.i.d.) by Diskus, was compared to albuterol 2.5 (g b.i.d. by nebulizer in a long-term (6 months) study. Salmeterol was significantly better in FEV_1 measurements, rescue use of albuterol, and symptom score.[13] Both treatments were equally safe. Thus, it seems that B_2 agonists have both a short-term and a long-term beneficial effect in cystic fibrosis and these effects are probably more than just due to their bronchodilator effect. The short-term effects may be mostly due to bronchodilation, as several studies have shown bronchodilator effects with other types of bronchodilators, such as anti-cholinergics and theophylline.[14] For the long-term beneficial effects, many possible mechanisms exist, such as increased mucociliary clearance,[15] inhibition of superoxide production by human neutrophils,[16] neutrophil accumulation (salmeterol, but not albuterol or formoterol),[17] pseudomonas adhering and mucosal damage[18] and possibly even chloride channel function.[19] In summary, short-term benefits have been shown with all three groups of bronchodilators, while adrenergic agents have also long-term benefits in CF.

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S3.4
ASTHMA IN CF: CORTICOSTEROIDS

Ian M Balfour-Lynn MD FRCP FRCPCH

Dept. Paediatric Respiratory Medicine, Royal Brompton Hospital, London, UK [i.balfourlynn@ic.ac.uk]

It is difficult, if not impossible, to determine who has both CF and asthma, and who has asthma-like symptoms due to CF lung inflammation. Currently, the diagnosis of asthma in a patient with CF is a clinical one [1]. The presence of cough is irrelevant, but recurrent wheezing is a cardinal symptom. There may be bronchial hyperreactivity and there may be bronchodilator responsiveness but both are common in patients with CF.The diagnosis is strengthened by a strong family and personal history of atopy. Ultimately, the response to anti-asthma medication may also help with the diagnosis.

Inhaled corticosteroids (ICS)

Regular ICS are the mainstay of asthma prophylaxis and they should be prescribed when a patient has recurrent wheezing that requires bronchodilators at least a few times a week. There has been an increase in their use in CF over the last decade but it is likely that they are often started and continued in patients in whom benefit has not been shown. In addition, they are often started in wheezy CF infants, and continued for many years, even when unnecessary. In fact many parents stop giving them to their children if they do not think they are working, as so many are wary of using steroids. Regular use of ICS is common nonetheless but quite variable; the European database recorded that 10% in France, 12% in Germany and 36% in the UK were prescribed them, with little correlation to age or disease severity [2]. A survey of UK paediatric centres in 1998 showed that about 40% of CF children were prescribed them, but there was a wide range between centres with a median use that ranged from 10% to 93% [3]. In the North American database, 45% with asthma were on ICS and 17% who had no asthma [4]. The latter figure is interesting, as there has been a move towards using ICS as a form of therapy for lung inflammation, regardless of asthma-like symptoms. Whether this is justified is unclear, as a Cochrane systematic review concluded that published trials have failed to prove benefit in CF [5]. A small study published since the Cochrane review has, however, shown that beclomethasone dipropionate given for 2 months led to a reduction in bronchoalveolar lavage markers of inflammation, with no adverse effect on adrenal function or infection rate [6]. It is suggested that ICS should be tried in CF patients with wheezing, and the response should guide continuation of therapy. However it is not appropriate to give higher and higher doses if there is no clinical improvement. There are concerns over potential side effects, particularly on growth, and at high doses the dose-response curve flattens out whilst the side effect profile continues to increases in a linear manner [7].

	Asthma (%) n = 3976	No asthma (%) n = 8646
Oral bronchodilators	27	12
Inhaled bronchodilators	95	76
Oral corticosteroids	31	21
Inhaled corticosteroids	45	17
Cromlyn / nedocromil	48	11

Use of pulmonary therapies reported to North American Epidemiologic Study of Cystic Fibrosis in 1995 (12,622 CF children & adults) related to the presence of concomitant asthma (reported in 31.5% of the patients). Table from Balfour-Lynn & Elborn [1], data adapted from Konstan et al [4].

Oral corticosteroids

Short courses (<7 days) of oral corticosteroids, as used in acute asthma, may provide symptomatic relief in CF patients with bronchospasm. Providing the patient does not receive these courses too often, steroid side effects are rarely seen. The issue of whether short courses are useful for CF chest exacerbations per se is not known. Long term administration of oral corticosteroids is a different matter however. Although a degree of benefit has been demonstrated (in those chronically infected with *Pseudomonas aeruginosa*), benefit was outweighed by the multitude of adverse effects seen, particularly on growth and glucose metabolism [8]. There is no data on long term use of very low doses such as are sometimes used in severe asthma (i.e. 5-10 mg/day). Despite the evidence [9], use of long term oral corticosteroids is surprisingly high. The North American CF database revealed that 31% with asthma were prescribed them and even 21% of those without asthma [4]. In Europe, their use is less common but increased with age and disease severity [2]. The figures are certainly higher than the reported prevalence of ABPA (for which they are still the main treatment), so presumably they are being given as long term anti-inflammatory therapy. Routine long term use would seem ill advised, although there will always be a few patients with intractable wheezing or severe small airways disease in whom their continued use is necessary. In some of these difficult patients, alternatives to steroids are sometime worth trying [10]. Most CF

patients with milder bronchospasm and wheeze however should not be taking oral corticosteroids regularly.

Conclusion

The diagnosis of CF asthma relies on the clinical judgement of the physician or paediatrician who should then try relevant therapy, but only continue with it if benefit is objectively proven. Inhaled corticosteroids should be the 1st line prophylaxis but doses should not escalate. Oral corticosteroids may be used for occasional short term symptomatic relief but are not advised for long term therapy.

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S4.1
SEARCHING AND EVALUATING RESEARCH EVIDENCE

Tanya Kinney LaPier, PT, PhD, CCS

State University of New York at Buffalo, Buffalo, New York, USA

Incorporating evidence based decision making into clinical practice involves several steps. First you need to formulate a refined clinical question that identifies a specific patient population, technique, and outcome measurement. Clinical questions typically address descriptive phenomenon, assessment techniques, or intervention efficacy. The next step is performing a literature search using appropriate databases and key terms based on the clinical question. The final step in evidence based decision making is evaluating the existing research literature.

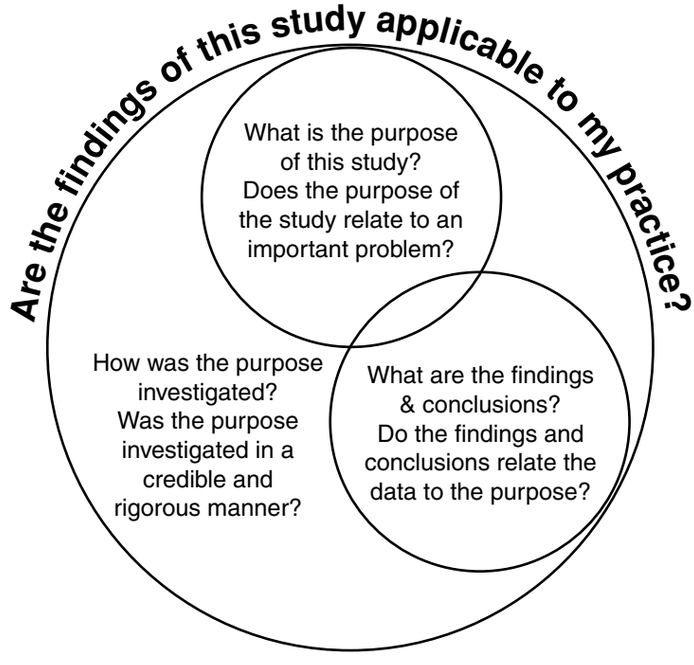
A good clinical question to initiate evidence based practice should specify four components: patient population, intervention, comparison intervention, and outcome. The following is an example of a clinical question that would be difficult to search for evidence, "Is exercise effective for patients with cystic fibrosis?" A refinement of the question might look like this, "In adolescents with cystic fibrosis *(patient population*) is daily aerobic exercise *(intervention)* better than medical management alone *(comparison intervention*) at reducing number of respiratory infections *(outcome)*? This question can be modified to further broaden or narrow the search, for example patient population can be broadened to children with cystic fibrosis or narrowed to adolescent girls with cystic fibrosis.

After you have formulated a clinical question the next step in evidence based decision making is searching the research literature. The past few decades have provided an explosion of information in the biomedical sciences. The proliferation of research data has facilitated the process of evidence based practice but has made the process of finding the evidence more challenging. A database is a compilation of information that has been grouped together based on some commonality in topic or type of information. Sometimes information databases are clustered together in a search tool or retrieval system. Ideally a search tool allows more than one data base to be searched simultaneously. The National Library of Medicine (NLM) Gateway interface searches multiple, Web-based NLM information systems. This search tool will perform simultaneous searching in multiple databases. Some of the databases in NLM Gateway

include Bioethics line, Directory of Information Resources on line, Health Services Research Projects in Progress, Health Star, Med line, Population Information on line, and Toxicology net. The NLM Gateway search tool and databases included within it are available for unrestricted use at gateway.nlm.nih.gov/gw/cmd. Some databases require a subscription or fee for use, such as the Cochrane Library.

After you have identified the information databases that you want to search, then you need to decide on what key words to use in your search. A combination of key words that is to narrow may produce a search with very few or no matches. A broad key word or words used in a search may produce an overwhelming number of matches. One method to limit a database search is by using what is called a Boolean operator. AND, OR, and NOT (must all be capitalized) are Boolean operators used by PubMed as a strategy to limit searches. AND can be used if it is known that two key terms are essential in the article being sought, by using AND you are saying all articles must contain both search terms. When using the OR operator you are saying that articles must contain at least one of the search terms. Lastly, the NOT operator can be used. By using NOT, articles will be eliminated if they contain a specific search term. Another way to streamline a database search is to set limits on specific fields, age groups, gender, type of study, publication date, specific language, and/or types of articles. Using these search strategies will help you more efficiently obtain the information you need to answer your clinical question.

The final step in evidence based practice is evaluating the existing research literature. Evaluating the evidence includes three steps: selecting articles from the search to analyze, assessing the merits of each individual study, and summarizing all the research studies. Screening research articles obtained during a literature search is usually necessary. The figure illustrates a three tiered process for screening a research article. First identify the purpose of the study and determine if it relates closely enough to the clinical question to continue reviewing the article. If the purpose relates to the clinical question, then decide if the methods used in the study were appropriate to provide reliable and valid information. If the methods were appropriate, next determine what the study results were and if they can be used to answer the clinical question of interest. Screening a research article is used to determine if you should proceed with a thorough analysis and application of the information to the evidence based decision making process. A detailed analysis of a research study requires a strong foundation of knowledge in research methodology and statistics. This further analysis of a research study is necessary to answer the overarching question, "Are the findings of this study applicable to my practice?" This evaluation paradigm can be applied to diverse types of research studies by the novice or expert researcher. Lastly, a consensus of all available evidence must be made by weighting research studies based on type. In evidence based practice it is generally considered that systematic reviews provide the highest level of evidence followed by randomized controlled trials, cohort studies, case-



control studies, then other studies (animal studies, single subject design, case reports...).

In summary, searching and evaluating the research literature is an integral part of evidence based practice. Systematic reviews, although not always readily available, are invaluable in the process of evidence based decision making.

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S4.2
ADVANTAGES AND LIMITATIONS OF SYSTEMATIC REVIEWS

Dr. Eleanor Main

Institute of Child Health, London, United Kingdom

Keeping track of the overwhelming and relentlessly growing bulk of clinical literature remains an unattainable goal for practitioners. The risk of not keeping up is significant in terms of inadequate delivery of evidence based health care. While some variation in practice may be inevitable, the delivery of poor quality or dangerous health care is unacceptable. Systematic reviews have become the pragmatic and potentially powerful response to the information explosion because of their potential to summarise information in a robust and accessible fashion for practitioners.

Early traditional or narrative literature reviews were often subjective in nature with no systematic method of comprehensively locating original articles. They often listed the range of viewpoints in the literature rather than provided practical clinical solutions to specific questions, and assessment of quality and methodology of manuscripts was haphazard or absent. Inclusion and exclusion criteria were rarely explicit and studies were permitted equivalent authoritative stature, despite enormous variability in sample size or robustness of methodology. Unpublished material was not considered and authors frequently only included studies that supported their own point of view.

By contrast, the modern systematic review claims to address some of the shortcomings of the traditional reviews by describing the review methodology accurately enough to enable replication by other authors. Systematic reviews deal with clearly focused questions, rather than collating all publications within the general clinical area. They reduce bias by considering the quality and methodology of all studies and have explicit inclusion and exclusion criteria for published and unpublished material. Standardised validity assessments for potential studies are often included and meta-analysis of data provides the potential for achieving sufficient statistical power and generalisability from pooling data in smaller trials. Results of different studies can be numerically compared using confidence intervals to identify consistency or inconsistency in results between studies. Bigger, more methodologically sound studies can be weighted to have more influence than smaller poorer studies. In so doing, systematic reviews and meta-analyses can inform both practice and further research and can bridge the gap when larger multi-centre randomised controlled trials which could produce the answers are still pending or absent.

Disadvantages of systematic reviews are commonly perceived to include the possibility that synthesis may disguise or oversimplify important distinctions between primary studies with regard to inclusion or exclusion criteria or the nature of an intervention. Particularly focused reviews may be inapplicable to the specific problems facing particular patients. Worse still, the findings from systematic reviews are not always consistent with comparable reviews (4) or with the findings of large-scale high quality independent clinical trials (1;6). Concordance between the findings of meta-analysis of several small trials and a single large trial range are claimed to range between 33 - 80% (1;6;10).

There are many examples within respiratory and physical therapy practice that provide confirmation of both the advantages and limitations of systematic reviews. Despite the existence of several reviews of physiotherapy management in cystic fibrosis, there are few that provide the kind of valuable resource, which practitioners require to inform their practice.

Systematic reviews are limited both by the quality and volume of the data available from primary studies and by the methods of synthesis, either by exclusion of relevant studies or inclusion of inadequate studies. Unless conducted under rigorous peer examination, systematic reviews will always be vulnerable to accusations of subjectivity. Other forms of bias can influence any stage of the systematic review process and include publication

bias, selection bias and language bias. In these situations meta-analysis of published trials could identify an erroneous treatment effect.

The systematic review can provide the best available evidence in the temporary or permanent absence of large well-conducted clinical trials. In future better indexing and registration of publicly funded research projects could improve the quality of systematic reviews, to make sure that findings are effectively circulated even if unpublished. Reduction in the inconsistencies of terminology used both in indexing publications and by researchers would ease selection of relevant manuscripts. Procedures to access original patient data and update systematic reviews, such as those utilised within the Cochrane review process will help to maintain current and relevant resources for clinicians.

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S4.3.1
COCHRANE SYSTEMATIC REVIEWS: "CHEST PHYSIOTHERAPY COMPARED TO NO CHEST PHYSIOTHERAPY FOR CYSTIC FIBROSIS" AND "CONVENTIONAL CHEST PHYSIOTHERAPY COMPARED TO ANY FORM OF CHEST PHYSIOTHERAPY FOR CYSTIC FIBROSIS"

S. Ammani Prasad[1], E. Main[1], CP van der Schans[2]

[1]Great Ormond Street Hospital, London, UK; [2]University Hospital Groningen, Netherlands

Persistent infection and inflammation within the lungs are the major contributory factors to airway damage and respiratory insufficiency in cystic fibrosis (CF).[1,2] Excess production of thick mucus leads to airway obstruction. Chest physiotherapy (CPT) plays an integral role in assisting the clearance of airway secretions and is a daily part of the treatment regimen. It is however burdensome and time consuming. To date there remains little clear evidence to support the efficacy of CPT in CF,[3,4] although a previous meta-analysis[5] reported standard CPT to be more effective in terms of mucus clearance than no treatment.

The following results are from two Cochrane reviews. The aim of first was to evaluate the effectiveness and acceptability of CPT compared to no treatment or spontaneous cough alone in cystic fibrosis. The second aims to determine whether the traditional method of chest physiotherapy (conventional CPT) is more effective than any other airway clearance modalities.

Search strategy

Relevant trials were identified in the Cochrane Cystic Fibrosis and Genetic Disorders Group Specialised Register of Controlled Trials. In addition unpublished work was identified by searching through abstract books of the three major CF conferences. Other sources included searches using electronic databases e.g. CINAHL.

Methods of the review

Studies were reviewed by two independent reviewers and a quality assessment of the included studies was

made.[6] Where data could not be extracted in the format required, authors were contacted to obtain the original data. For continuous outcomes either the mean change from baseline for each group or mean post intervention values and the standard deviation for each group were recorded.

Types of interventions

Interventions included conventional CPT; positive expiratory pressure (PEP); high pressure PEP; active cycle of breathing techniques; autogenic drainage; exercise and oscillating devices. Considerable variation occurs in the application of these techniques. For the purpose of these reviews these interventions were grouped within their broader definitions.

Review 1: Chest Physiotherapy Compared to No Treatment or Spontaneous Cough

19 studies which included a "no treatment" or "cough alone" control group were identified for potential inclusion. Of these two were not clinical trials, four included diagnoses other than CF, five did not evaluate chest PT and two contained no data.

The six remaining studies were included, all were short term (<7 days), cross over design but no meta-analysis was possible. This has highlighted a gap in the body of knowledge surrounding the value of CPT in CF and may be helpful in the design of future trials. While these studies suggest that airway clearance regimens may be beneficial in patients with CF in terms of mucus transport, their short term nature should be taken into context in what is a long term disease.[7]

Conclusions

Short-term crossover trials suggest that airway clearance regimens may have a beneficial effect in patients with CF. However based on this review there is currently no robust scientific evidence to support the hypothesis that chest physiotherapy for the purpose of clearing airway secretions has a long term beneficial effect in patients with CF, nor to suggest that it is in any way harmful.

Review 2: Conventional CPT Compared to Any Form of CPT

Short term duration studies (<7 days) were excluded from this review. Some studies were excluded because original data could not be located or the authors could not be contacted. Some authors agreed to provide data, which are still forthcoming. Data from 14 studies has been analysed, four of which were of two week duration undertaken during acute respiratory exacerbation and the remaining 10 were longer term (>4 weeks).

In both the overall analysis of the 14 studies and on separate analysis of the two week and longer term studies there was no overall difference between the "newer" techniques and conventional CPT in terms of FEV_1, FVC or FEF_{25-45}. Sub-analysis for specific techniques could only be performed for PEP and AD and there was no significant preference for either technique over conventional CPT.

While there did not appear to be any overall advantages to specific airway clearance techniques, it was clear from examination of the raw data that there was considerable individual variation in response to the different therapies. This suggests the need for individualised therapeutic regimens rather than blanket prescription of single airway clearance techniques.

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S4.3.2
POSITIVE EXPIRATORY PRESSURE (PEP) PHYSIOTHERAPY FOR AIRWAY CLEARANCE IN PEOPLE WITH CF

Mark Elkins[1], Alice Jones[2], Cees van der Schans[3]

[1]Dept of Respiratory Medicine, Royal Prince Alfred Hospital, Sydney, Australia; [2]Dept of Rehabilitation Sciences, The Hong Kong Polytechnic University, Hong Kong; [3]Dept of Rehabilitation, University Hospital, Groningen, The Netherlands.

Introduction

Chest physiotherapy is widely prescribed to assist the clearance of airway secretions in people with CF. Positive expiratory pressure (PEP) devices, which provide a constant back pressure to the airways during expiration, may improve clearance by increasing gas pressure behind secretions via collateral ventilation[1]. Given the widespread use of PEP devices, there is a need to determine the evidence for their effect[2].

Methods

Relevant trials were identified in the Cochrane Cystic Fibrosis and Genetic Disorders Group Specialised Register of Controlled Trials, and abstract books of three major CF conferences. Randomised clinical trials in which PEP was compared with any other form of chest physiotherapy in people with CF were included. All studies identified by the search were reviewed by two independent reviewers from different centres. The quality of the studies was assessed as described by Jadad[3]. Disagreement on inclusion or quality rating was settled by consensus.

Description of studies

Thirty-four studies were identified. Nineteen studies involving 386 participants met review inclusion criteria. Six were published only in abstract form. Three studies were excluded. In the remaining twelve studies, the study design or outcome data have been reported in insufficient detail to determine whether the inclusion criteria have been met, and may be able to be included pending further unpublished data from authors.

Among the nineteen included studies, sample sizes ranged from eight to 66. Eight were acute intervention studies. The duration of each treatment arm in the remaining studies ranged from one week to two years. Two studies were conducted in subjects experiencing a respiratory exacerbation. One study was conducted exclusively with infants. The remainder combined paediatric, adolescent and/or adult subjects. Only one of these provided results with the data from adolescent and adult subjects independently. Generally, subjects had a wide range of lung function impairment.

Fourteen of these studies involving 228 participants were cross-over trials from which data from the end of the first randomisation arm could not be extracted. Only two cross-over studies examined for carry-over or period effects. In one of these studies, strong carry-over effects were seen.

Methodological quality of included studies

None of the 19 trials were double blinded. Whilst all were described as randomised, only two described an appropriate method. Twelve trials described withdrawals and dropouts. Overall the methodological quality was low. There is therefore a risk of bias in the results of these studies.

Primary outcome measures

1. Forced Expiratory Volume in 1 second (FEV_1)

Twelve studies involving 262 subjects measured FEV_1. Eight short term studies (acute intervention up to one month of twice daily treatments) were unable to demonstrate any significant change in FEV_1 with either the PEP or the comparator intervention (forced expiratory technique, postural drainage, percussion, and vibration (PDPV), non-invasive bi-level ventilatory support, oscillating PEP (Flutter)). In a year long comparison with PEP, a non-significant greater trend downward in FEV_1 was reported with Flutter compared to PEP, although the data are unpublished[4]. In a year long comparison with PDPV in paediatric/adolescent subjects, FEV_1 improved by a mean of 5.98%pred for the PEP group, while in the PDPV group it deteriorated by 2.28%[5]. A two year trial incorporating adult subjects as well did not reproduce this result, with no significant difference in the rates of decline in FEV_1 over two years between the PEP group and the PDPV group, with annual declines of 2.76% and 2.11%, respectively. When the results for subjects aged under 19 years were examined, the PEP group declined 1.58% per year and the PDPV group 1.65% per year[6].

2. Number of Respiratory Exacerbations per Year

No studies listed respiratory exacerbation rates among their formal outcome measures. Four cross-over trials reported patients being withdrawn due to exacerbations, although these are not well defined. It is also unclear which treatments the subjects were randomised to at the time of departure from any of these studies. In a year long, parallel trial, however, two patients in the Flutter group, but none from the PEP group, were withdrawn by their physicians due to "clinically significant deterioration in pulmonary function" [4].

3. Number of Days of Intravenous Antibiotics per Year

Two studies measured antibiotic use. Fewer hospital admissions with antibiotics due to respiratory exacerbations occurred with PEP than with Flutter: 5 vs 22, respectively[4]. More days on antibiotics (29.5

days/patient/ year) were reported with PEP than PDPV (18.2 days/ patient/year), although in this study it is not stated whether the antibiotics were intravenous, nor whether they were prescribed in response to a respiratory exacerbation[7].

4. Well Being

In a two year, parallel trial of PEP versus PDPV, neither group demonstrated a significant change in Quality of Well Being (QWB) Scores, which had been similar at baseline[6].

Other primary outcomes to be discussed in the session include adverse effects, survival, exercise tolerance, and patient preference. Secondary outcome measures to be discussed include direct measures of mucus clearance, weight/volume of expectorate, FVC, FEF25-75%, TLC, RV, FRC, oxygenation, radiological/ventilation imaging, nutritional status, cost, and adherence.

Implications for Practice & Research

In the absence of evidence for the benefit of any chest physiotherapy modality over no therapy in subjects with cystic fibrosis[8], it remains impossible to recommend PEP as maintenance therapy based on the results of this review. If such a benefit from either PEP or another therapy can be demonstrated, however, this review will provide some support for the relative benefit of PEP over Flutter or PDPV in optimising FEV_1 in the long term. Cross-over trials are likely to be affected by carry-over effects. The large number of these trials highlights the need for long term, parallel, randomised clinical trials comparing airway clearance modalities.

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S4.3.3
PHYSICAL THERAPY FOR PATIENTS WITH CYSTIC FIBROSIS: A SYSTEMATIC REVIEW

Judy Bradley[1,2] Fidelma Moran[2] Peter Wark[3]

[1]Regional Adult Cystic Fibrosis Centre Belfast Northern Ireland, [2]School of Rehabilitation Sciences, University of Ulster Northern Ireland [3]Research Division, Southampton General Hospital, Southampton UK.

Background

In cystic fibrosis (CF) progressive respiratory disease results in an abnormal ventilatory response to exercise. This contributes to dyspnoea and is a major limiting factor to exercise tolerance in this population. Physical training has been incorporated into the treatment regime at most CF centers with the perception that adherence reduces dyspnoea, reduces the decline in lung function and improves exercise tolerance. It is not clear how many weeks training are required to achieve benefits, how long after cessation of training these benefits are maintained or how adherence affects the outcome of physical training. It is also not clear if there are any side effects of physical training. Therefore this systematic review was conducted to determine the effect of including a prescribed regime of physical training in the care package for patients with cystic fibrosis (CF).

Methods

The design was a systematic Cochrane review of randomised controlled trials of physical training in patients with CF (1). Trials were identified from the Cochrane Cystic Fibrosis and Genetic Disorders Group specialist trials register (date of the most recent search: August 2001). Two reviewers independently selected the trials to be included in the review using a proforma to capture main inclusion criteria. The methodological quality of each included trial was assessed using the Jadad scale and the criteria proposed by Cochrane to rank allocation concealment. Data was extracted from the original studies and between group differences in continuous measures were compared by calculating a weighted mean difference (WMD). When the appropriate data for calculation of WMD was not available, but where significant between group differences in the mean change in the variable from baseline were reported then data relating to the mean and standard deviation (SD) change in each variable from baseline for each group was recorded.

Results

Six trials met the inclusion criteria (2-7) (n = 184 patients). In all six trials, the method of allocation concealment was ranked unclear (Grade B). Scores on the Jadad scale ranged from 0 to 2 however this must be considered in the context of difficulty in double blinding physical training studies and the fact that four of the six studies were reported in abstract form only. Physical

training, in the short term, improved exercise tolerance. In a three year trial there was no improvement in exercise tolerance, but the treatment group had a significantly reduced rate of decline in FVC % predicted compared to the control group WMD 2.17 (95% CI 0.47 to 3.87). Changes in other lung function parameters showed a similar trend, but these were not statistically significant.

Conclusions

Conclusions about the efficacy of physical training in CF are limited by the small size, short duration and incomplete reporting of most of the trials included in this review. Physical training is already part of the care package offered to most patients with CF and there is no evidence to actively discourage this. Further research is needed to assess comprehensively the net benefit of the addition of physical training to the care package of patients with CF.

Acknowledgements

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S4.4
TRANSLATING EVIDENCE INTO CLINICAL PRACTICE

Professor Rosalind L Smyth

Department of Child Health, University of Liverpool, Alder Hey Children's Hospital, Eaton Road, Liverpool L12 2AP, United Kingdom

The dramatic improvements in survival in cystic fibrosis over the last four decades have largely been attributed to the use of earlier and more intense pulmonary, nutritional and other interventions. However, this has meant that treatment regimens have inevitably become more complex, expensive and time consuming. It is therefore an imperative to carefully evaluate the effectiveness of each of these interventions in improving survival, quality of life and other important outcomes for cystic fibrosis patients. In no area of treatment is this more important than chest physiotherapy. Families invest a considerable amount of time in daily physiotherapy regimens, which require the supervision and advice from a highly trained and skilled practitioner. We need to be able to advise patients about what treatment strategies to initiate and when and whether one particular regimen offers advantages over alternatives.

Rigorous evaluation of treatments by randomised controlled trials (RCTs) has occurred relatively recently in cystic fibrosis. A recent review of all RCTs, in patients with cystic fibrosis, published in the years' 1966-1997 and identified a total of 506[1]. There was a twenty fold increase in the number of RCTs performed in the years 1965-1969 compared with 1990-1994. At the time of that review, 94 of these trials were of physiotherapy interventions and included a total of 1787 participants. There were some concerns identified in the review about the quality of RCTs in cystic fibrosis. For example, in 72.7% the sample size was 30 or less and less than 10% were multicentre trials.

Currently the Cystic Fibrosis Trials Register of the Cochrane Cystic Fibrosis and Genetic Disorders Group includes a total of 187 references to 138 clinical trials (including 2838 participants) of physiotherapy interventions. The majority of these were randomised controlled trials. In 116 out of 136 physiotherapy trials, where the sample size was reported, it was 30 or less. The majority of the references (138/187 references) have been published since 1990.

In the Cochrane Cystic Fibrosis and Genetics Disorders Group, there has been considerable interest in under-

taking systematic reviews of physiotherapy interventions. Currently there are two reviews and three protocols of physiotherapy interventions published on the *Cochrane Library*. Reviewers have at times been frustrated by the lack of evidence for interventions which are used widely in clinical practice. By evaluating, and where appropriate aggregating, data from all clinical trials of particular interventions, Cochrane reviews can provide new data about whether treatments are effective and estimate the size of that effect. In the field of cystic fibrosis as a whole, although in many areas the evidence from RCTs is much less robust than we would like, important new information has arisen from Cochrane systematic reviews. This has informed clinical practice and research. Systematic reviews of randomised controlled trials have also identified important gaps in the evidence to direct cystic fibrosis care and in the UK this has led to a number of important large multicentre trials (for example, The TOPIC, CALICO and WISE Trials). Trials are also being considered for physiotherapy interventions in cystic fibrosis.

Physiotherapy, which is well-established as part of routine care in cystic fibrosis is a relatively straightforward intervention to study in large pragmatic randomised controlled trials. Much important clinical data is regularly being collected for inclusion in large national cystic fibrosis disease registries or databases. It would be very possible to utilise these routine data collection systems to capture relevant data for patients who are participating in randomised controlled trials. This would considerably ease the burden of data collection and reduce the costs of running pragmatic clinical trials in cystic fibrosis. A vision for the future is that high quality clinical trials will become accepted as an integral part of the care offered to patients with cystic fibrosis. There are many aspects of treatment of cystic fibrosis where there is true doubt about the benefits of one form of treatment compared with another. This uncertainty should be explained to patients, who with full information and informed consent, may be invited to participate in appropriate clinical trials. The ultimate aim is that patients and clinicians will have access to all relevant information about the most effective treatments available.

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S5.1
MANAGEMENT OF THE COMPLICATIONS OF PORTAL HYPERTENSION

Michael R. Narkewicz MD

Section of Pediatric Gastroenterology, Hepatology and Nutrition, University of Colorado School of Medicine, Denver, Colorado

Portal hypertension complications are the most common manifestations of advanced CF associated liver disease [1]. These include variceal or nonvariceal gastrointestinal (GI) hemorrhage, hypersplenism, ascites and porto-systemic encephalopathy.

GI hemorrhage

GI hemorrhage is the most common complication of CF associated portal hypertension. Initial management is focused on establishing vascular access and hemodynamic stability. Initial assessment should include a CBC, PT and PTT with correction of coagulopathy with FFP and vitamin K and transfusion as necessary. In the patient who is having hematemesis or melena, placement of a nasogastric tube and lavage with room temperature saline can determine if there is active upper intestinal bleeding and can improve hemostasis by removal of blood from the stomach.

The primary medical management options are vasopressin which causes a generalized vasoconstriction or somatostatin and its analogs that has a more selective effect on splanchnic blood pressure and reduces portal venous flow. This can be given subcutaneously (50-500 micrograms 2-3 times per day) or as a continuous infusion (1 microgram/kg/hour). Generally treatment is continued for 24-48 hours. Vasopressin or somatostatin have been shown to reduce or stop bleeding in 50-70% of subjects. However, without definitive treatment, rebleeding is common. Nonspecific beta blockers such as propranolol have been shown to reduce rebleeding and may be useful in prophylaxis. However, they have no role in acute GI hemorrhage.

In most patients with a significant upper GI hemorrhage, endoscopy is indicated to determine the source of the bleeding and if appropriate, institute endoscopic therapy. Findings at endoscopy can include gastritis, ulcers and esophageal or gastric varices. Varices with active bleeding or stigmata of bleeding (fresh clot, red wale sign or cherry red spot) should be treated. Variceal band ligation and variceal sclerosis are the available treatments. They have fairly similar success rates (85-95%) with perhaps a slightly lower incidence of complications with band ligation. Once variceal treatment is undertaken, repetitive endoscopy with variceal eradication should be the goal. Gastric varices are more difficult, but newer techniques of sclerosis and glue injec-

tion have met with success. Complications from endoscopic treatment include rebleeding, infection and esophageal stricture.

In patients with intractable upper gastrointestinal bleeding who fail endoscopic treatment, portosystemic shunting is appropriate. Transjugular intrahepatic portosystemic shunting (TIPS) has had good success in CF associated liver disease and should be the first option if feasible . Very small children (10-15 kg), very small livers and significant encephalopathy may make a TIPS or a surgical shunt more difficult. More aggressive such as liver transplantation as treatment for isolated GI hemorrhage is usually not necessary for CF associated liver disease.

Ascites

Ascites is common in CF associated liver disease. Initial management includes gentle sodium restriction, diuretic treatment with furosemide and aldactone. With massive ascites, single or repetitive paracentesis can be helpful [2]. Ascites is less responsive to TIPS than GI hemorrhage.

Hypersplenism

Hypersplenism is characterized by leukopenia, thrombocytopenia and anemia due to intrasplenic trapping. In general no treatment is necessary. In cases of severe thrombocytopenia with recurrent episodes of bleeding or splenic enlargement that results in significant pain or nutritional compromise, partial splenic embolization has been used successfully in cystic fibrosis [3, 4].

Encephalopathy

Encephalopathy can be either acute or chronic. Its effects can include disturbed consciousness, personality changes, decline in intellectual functioning, and disturbances of speech and motor function. Treatment is aimed at identification of precipitating factors such as excessive protein intake, GI hemorrhage or infection. In general, treatment is supportive and directed at the cause if identified. Protein restriction is useful if adequate caloric intake is maintained. Lactulose and/or oral antibiotics such as neomycin can be effective. Encephalopathy is a relative contraindication of portosystemic shunt procedures as it is very likely to worsen following a radiologic or surgical shunting procedure.

Therapies for Variceal Hemorrhage (derived from [5])

Supportive	Monitor and transfuse as needed
Pharmacological	Vasopressin Somatostatin, octreotide
Mechanical	Sengstaken-Blakemore tube
Endoscopic	Sclerotherapy Variceal band ligation Glue injection
Radiological	Transjugular intrahepatic portosystemic shunt
Surgical	Portosystemic shunt Nonshunting procedures Liver transplantation

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S5.2
DISTAL INTESTINAL OBSTRUCTION SYNDROME

Warren P. Bishop, M.D.

Division of Gastroenterology, University of Iowa, Iowa City, Iowa

Distal intestinal obstruction syndrome (DIOS), previously known as meconium ileus equivalent, is a common complication of cystic fibrosis, occurring in 20-40% of CF patients[1, 2]. In this syndrome, obstruction of the distal small bowel or colon result from impaction of high-viscosity stool. Surprisingly, there are still many unanswered questions about the pathogenesis of DIOS and its proper treatment. The clinician treating intestinal obstructive symptoms in cystic fibrosis patients must also be aware of pitfalls in the diagnosis and management of this condition, which may be mimicked by other disorders and complicated by associated defects.

What causes DIOS? The major possibilities include: 1) excessive intake of fat-enriched foods in the presence of inadequate pancreatic enzyme supplements, giving rise to fatty stools that somehow clog the intestine, 2) Thick mucus resulting from defective electrolyte and water secretion, and 3) motility defects. Because DIOS has been described in patients with pancreatic sufficient forms of cystic fibrosis[3, 4], is not associated with fat content of diet [5], and does not respond to higher-dose enzyme replacement therapy[1], the maldigestion hypothesis is probably not correct. However, thick, viscous mucous is produced by the intestine with abnormal CFTR, and can cause rubbery, putty-like stools which can easily cause obstruction.. Retained mucus is seen within fecal material in these patients, and at the microscopic level within goblet cells of surgical specimens[6]. Intestinal motility problems do not clearly exist in cystic fibrosis, although prolonged small bowel transit time has been reported in DIOS patients[7]. Treatment of DIOS patients with cisapride, a drug which enhances small bowel motility, has been reported to reduce symptoms, but does not abolish the need for other intervention in a well-designed double-blind study[8]. Thus, the primary mechanism of DIOS appears to be obstuction of the intestine by viscous intestinal mucus, with a possible contribution of increased transit time.

The usual symptoms of DIOS are crampy abdominal pain, distention of the belly, and bilious vomiting. These symptoms are non-specific indicators of intestinal obstruction, and can also be caused by many other obstructive lesions. It is therefore critical that cystic fibrosis patients presenting with such symptoms be adequately evaluated for other problems. Several causes of intestinal obstruction are common in cystic fibrosis. Intususception can occur as a result of DIOS, with inspissated stool serving as the lead point[9, 10]. Conversely, DIOS may be aggravated by other partial obstruction, including adhesions from prior surgery[11], diverticulitis[12], and colonic fibrosis. Appendicitis can occur as the result of inspissated mucus[13, 14], and an appendiceal abscess can mimic the typical right lower quadrant fecal mass seen in "uncomplicated" DIOS[15]. Thus, extreme caution must be exercised in the diagnosis and subsequent treatment of DIOS to avoid missing or exacerbating other conditions.

Diagnostic studies should include plain abdominal radiographs and the usual laboratory studies obtained for acute abdominal pain. These include a complete blood count, urinalysis, serum chemistries, and amylase. A contrast enema should be performed early if there is any significant suspicion of an obstructive mechanical lesion, and may be therapeutic. A computerized tomography scan and/or abdominal ultrasound may be warranted to rule out abscess or intussusception. Finally, a therapeutic trial of intestinal lavage (see below), in the absence of evidence for infection or anatomic obstruction, may be begun.

At the present time, intestinal lavage using a polyethylene glycol-containing electrolyte solution (PEG-ELS) is the most effective therapy available for DIOS. Prior to the availability of these solutions, treatment was limited to the use of acetylcysteine and sodium diatrozoate (water-soluble X-ray contrast) enemas[16-18]. Intestinal lavage offers an effective purging effect. PEG-ELS is generally administered as a continuous infusion via a nasogastric tube to promote the hydration and expulsion of the inspissated stool[3, 18, 19]. The use of lavage solutions presents a hazard, however, in the event of high-grade obstruction downstream. There is risk of intestinal perforation and compromise of bowel perfusion if lavage is continued in the absence of fecal output. Lavage should be slowed or discontinued if stool output does not occur or if it causes a significant increase in abdominal girth, and further studies obtained as described above.

Prophylactic therapy for DIOS should be considered in susceptible. Years ago, daily oral adminstration of acetylcysteine was suggested[20], but is no longer reported in the literature and is not in general use. PEG-ELS have been shown to be of benefit in prophylactic therapy or at the earliest sign of symptoms[18]. Unfortunately, these solutions are unpleasant-tasting and difficult to adminster orally to children and some adults. Although no published data is yet available for DIOS, functional constipation in children responds extremely well to small daily doses of electrolyte-free polyethylene glycol[21]. This is a highly palatable product that can be dissolved in the patient's favorite beverage. Electrolyte-free PEG is a simple osmotic agent that results in increased fecal water content. Although it is

now commonly being used in DIOS patients, no study has yet been published to confirm its efficacy in this condition. Because of the risk of electrolyte disturbance, this product should probably not be used in the higher volumes or the prolonged period of time generally required to clear an obstruction in active DIOS.

In summary, there have been some advances in the therapy of DIOS, particularly the use of PEG-ELS solutions to clear the intestine of thick stool. Because of the high incidence of other obstructive lesions in DIOS-like symptoms, caution must be exercised in diagnosis and the therapeutic use of intestinal lavage. Much work remains to be done to investigate new therapies, possibly including agents which promote intestinal secretion, prokinetic agents, and electolyte-free PEG.

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S5.3
RECENT ADVANCES IN ENDOSCOPIC AND SURGICAL MANAGEMENT OF GERD IN CYSTIC FIBROSIS.

Virendra Joshi MD

Section of Gastroenterology, Department of Medicine, Tulane University Medical School, New Orleans, Louisiana USA

Gastroesophageal reflux in patients with cystic fibrosis occurs at a higher frequency than general population ranging between 25% in patients less than 5 yrs to 81% in patients older than 5yrs[1,2,3].Early detection and aggressive management of complications of cystic fibrosis in these patients has lead to longevity in this population, since these patients are living longer the management of these patients will change and approach will be more like how we deal with average patients with reflux.

Most children respond to antireflux medical management. However, children with GERD unresponsive to conservative measures may need antireflux surgery. The most popular surgical technique is laproscopic Nissen fundoplication, with reduction of operative times to half compared to open technique and complication rates as low as 2.6% and wrap failure rates only 3.4%[4]. Unfortunately data on long- term follow up of these patients is not yet available. Finally, Barrett's esophagus is being increasingly recognised in older children attributed to longstanding unrecognized reflux[5]. An antireflux treatment may halt the advance of transformation of Barrett's to dysplasia and then adenocarcinoma, and may reduce the need for frequent need for surveillance endoscopy. There is also some belief that antireflux surgery coupled with ablation of abnormal epithelium may reduce the malignant potential in patients with metaplastic epithelium[6].

Adults with reflux disease are more symptomatic and have higher incidence of erosive esophagitis[3]. The first line of treatment is pharmacological using new generation of proton pump inhibitors (*Isomeric technology*). Endoscopy should be reserved for failure of symptoms to resolve on PPI or for long standing GERD in order to evaluate for presence Barrett's esophagus (*ACG guidelines*). There is no data among this group of patients as to the long term results with surgical therapy. Assuming that the natural history of GERD in these patients are similar to average population: Anti-reflux surgery and medical therapy were found to be equal in all outcome measures in a recent study in carefully selected patients[7]. In another landmark study by Spechler and colleagues with a 13 yr follow up 62% patients were back on PPI[8,9]. This suggests that surgical therapy not superior to medical therapy as previously suggested. Another issue in adult patients is Barrett's and

role of surgery in reducing risk of malignant transformation. This issue is addressed in a study by Zaninoto and colleagues[10] , which indirectly shows that Barrett's does not regress after fundoplication. Another well-designed case – control study eliminates the role surgery in reducing esophageal cancer related to reflux[11].

Though both medical and surgical therapy for GERD are successful and effective, many requiring long term therapy would delight in a non surgical, non- pharmacologic option for treatment of symptoms. This has lead to extensive research and development of endoscopic procedures designed to treat GERD . Two endoscopic procedures have gained FDA approval, radiofrequency energy[12] delivery to gastroesophageal junction and transoral flexible endoscopic suturing. These treatments are only designed for medically responsive nonerosive GERD. These techniques are not appropriate as first line therapy or definitive therapy until we have more data. Finally, endoscopic therapy seems an excellent and exciting optional treatment for GERD for carefully selected patients , it is to be seen how it performs long term.

Other endoscopic treatments in development are Bipolymer injection into LES and modifications of sewing devices . These are not yet FDA approved.

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S5.4
FIBROSING COLONOPATHY/ENTEROPATHY - IS IT A DINOSAUR OR IS IT STILL ACTIVE?

John Lloyd-Still, David M Beno, Robert Kimura.
Dept. Ped., Rush Medical College, Chicago, Il

Objectives

1, Review the epidemic of fibrosing colonopathy. 2. Experimental models of fibrosing colonopathy. 3. Management options and potential therapies. 4. Discuss the associations between fibrosing colonopathy, Crohn's disease, ischemia and CF; so-called "fibrosing enteropathy".

The iatrogenic disease "Fibrosing colonopathy" described by Smyth et al in 1994, was initially thought to be related to the use of high dose pancreatic enzymes (1).Case control studies showed that the total dose of enzymes given for a prolonged period was responsible. Significant associations occurred with previous surgery, intestinal obstruction, H2 blockers, corticosteroids, and Dnase (2). Over 100 cases were reported with 65% in certain centers. Although the epidemic "ceased" another 37 cases were seen from 1995-1999 (CF Foundation data). In some centers up to 61% of patients were exposed to high doses of enzymes and are supposedly symptom free.

The original pathology (only surgical resections) described the end result with a foreshortened colon, strictures, marked submucosal fibrosis and a lack of inflammation. The most severely affected developed chylous ascites and focal nodular hyperplasia of the liver. Subsequently, the spectrum of disease included the pre-stricture lesion with marked eosinophilia and sometimes involvement of the ileum (3).The pathogenesis remains unknown (4). Controversy has involved the role of the enteric coating Eudragit in UK case control studies and animal studies. However, after the Redfern report (5) and the disappearance of the original specimens, there has been a request for recall of these publications (6). Conspicuously, the US case control study could find no association with any particular brand of enzyme.

We hypothesized that the increased intestinal permeability of CF might be involved and used the chronically catheterized rat model for a variety of experiments which have been published (7-10) and show :1.Pancreatic enzymes require increased intestinal permeability to produce the enteropathy which involves both the small and large intestine. 2. Enteropathy is produced by both Eudragit containing and non-enteric enzymes. 3. Eudragit alone does not produce significant damage. 4. NSAID (Indomethacin & Ibuprofen) and pancreatic enzymes are synergistic for enteropathy. 5. This enteropathy can be ameliorated with ursodeoxycholic acid.

The treatment of fibrosing colonopathy is not definitive since the etiology remains unknown. The major objective should be to reduce the excessive dosage of enzymes and in early disease this is usually effective. Associated C.difficele toxin should be treated. Some patients improve with bowel rest and parenteral hyperalimention or enteral feeding with elemental diet. There is no proof that treatment with steroids and 5-ASA drugs are effective. Surgery is usually required for intestinal obstruction. Procedures include limited resection of the obstructed segment, to partial or total colectomy with ileostomy. Risk of recurrence post resection is significant with 8 of 12 patients requiring further surgery post resection.

Possible contributors to colonopathy include 1. constituents of the enzymes (protease, lipase, elastase) or enteric coating (Eudragit etc.). 2. activators of the inflammatory cascade (eosinophils, neutrophils, TGF beta, cytoskeletal effects on actin, collagens. myofibroblasts, bile acids, enteric nervous system dysfunction, bacteria and toxins with translocation, angiogenesis and endothelial activation). 3. impairment of defensive factors such as alpha 1 antitrypsin (not present in the colon) or alpha 2 macroglobulin, trefoil peptides, defensins, immunoglobulins, essential fatty acid deficiency, and anti-oxidants. 4. specific abnormalities of the CF intestine - increased permeability, bile acid malabsorption,

CFTR dysfunction, defective tight junctions, hyperacidity, DIOS, and an increased risk of Crohn's disease (17x more common) with phagolysosomes (11). 5. Dnase (significant association in case control study).

There have been an increasing number of reports of an inflammatory bowel disease with features of ischemic bowel disease (secondary to meconium ileus surgery), Crohn's disease and fibrosing colonopathy. Many of these have a past history of high dose enzyme intake. Symptoms include a colitis like presentation with hematochezia and diarrhea or abdominal pain and obstruction. Sometimes major pathology may be present without gastrointestinal symptoms. This disorder resembles the findings in our rat model and is a "fibrosing enteropathy." We believe that all three disorders share a final common pathway that can become a chronic inflammatory bowel disease. Theoretically CF patients treated with high dose ibuprofen could also be at increased risk if the findings in our rat model are applicable to the human (12)

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S6.1
SEX AND GENDER ISSUES IN CYSTIC FIBROSIS: AN OVERVIEW

Cy Worby

Department of Psychiatry and Behavioral Sciences, University of Nevada School of Medicine, Reno, NV

Sex and gender inequality have characterized many societies over the millennia. The 21st. century is no exception. In some societies today, women's unequal status is egregiously blatant while in others it is more subtle. In such social contexts the health status of women is adversely affected. Sex refers to biologically based differences such as genetic, hormonal, physiological and anatomical aspects and gender refers to culturally and socially shaped variations between men and women, including the person's self-representation as male or female, and society's construction of sex roles. In the last half of the 20th. Century social justice movements eventually examined the assumptions and practices in Western medicine affecting women's health. Important disparities between men and women are evident when viewed through both lenses of sex and gender (1,2) Often it is difficult to disentangle the contribution of other variables such as race, ethnicity and poverty and their effects on sex and gender in health and disease. An Institute of Medicine study found that "Racial and ethnic minorities tend to receive a lower quality of healthcare than non-minorities, even when access-related factors…are controlled. The sources of these disparities are complex, are rooted in historic and contemporary inequities, and involve many participants at several levels…" (3). This question has been examined in the CF population (4).

The purpose of this symposium is to probe the relevance of these contemporary questions to the world of cystic fibrosis. Some speakers focus on the biomedical aspects of the bio-psycho-social totality, others on the psychosocial dimension. Perhaps at the end of this symposium we will have a better understanding of a central puzzle in cystic fibrosis: the fact that men with CF live longer than women by about two years.

A Developmental Perspective of Sex and Gender in Cystic Fibrosis

A developmental perspective offers a useful framework within which to examine these issues. The journey from infancy to adulthood for the person with CF, for her or his family and siblings, and for the CF team is ardu-

ous and complex. Dividing this journey into five life-stages allows us to ask what bio-psycho-social issues are central to each stage. We are also able to ask what it means to be female and male in each of these stages. A bio-psycho-social matrix within which to view the developing person across time cautions us to avoid reductionism. George Engel's seminal admonition in *Science*, is still relevant after 25 years: "The dominant model of disease today is biomedical with molecular biology its basic scientific discipline. It assumes disease to be fully accounted for by deviations from the norm of measurable biological (somatic) variables. It leaves no room within its framework for the social, psychological and behavioral dimensions of illness."(5).

It helps to view the sex and gender question as they really are: for the most part inseparable. Menarche for the girl is simultaneously a normative physiological phenomenon, a psychological milestone and a social event: biopsychosocial. It is also important to emphasize that the disease itself has its stages, characterized by progression and unpredictability, in the individual case. The disease intrudes, sometimes forcefully and dramatically, into the person's and family's life stages (6).

The first stage covers birth & infancy, the second the preschool years. In these stages the primary environment is the family, with some peer socialization in preschool experiences. The third stage covers the grade school years. The boy and girl travel daily between the family world and the school world. Illness characteristics such as cough and foul smelling stools, and illness episodes necessitating absences take on social significance with peers. The fourth stage is adolescence characterized by issues of identity, autonomy, sexual maturation and experimentation. Adulthood is the fifth stage with a focus on increasing autonomy from the family of origin, achieving emotional and sexual intimacy with a partner, and securing a satisfying role in the work world. Keeping the illness at bay is a central feature for the majority of persons with cystic fibrosis in each stage. Several salient issues dealing with sex and gender will be discussed within each stage. For example, in stage I whether it's a boy or a girl at birth matters greatly in some cultures. What if it's a sickly boy or girl (not yet diagnosed). Does that matter? And when the diagnosis of CF is given does it matter if it's a boy or a girl? In some countries it is possible to make an informed guess: it matters greatly. But in any one of the 113 CF Centers in the U.S. the best answers are, "We don't really know" and "It all depends on the specific family." Depending on geographic location, ethnic mix and numbers of patients CF Centers differ, one from another, in important ways. For example, the range of center medians for FEV_1 for patients aged 6-13 ranges from 70.1 to 104.4% and for patients age 18-30 the median is 40.0 to 85.8%. Median length of stay per hospitalization for <18 years ranges from 2.7 days to 18.7 days. For adults it varies from 2.5 to 16.5 days. Even the use of Pulmozyme varies greatly among centers: the national rate of use is 51.1% with a range of 8.3 to 100% among centers (7). Conclusion: it is always important to individualize, whether it is the patient, the family or the CF Center team.

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S6.2
SEX AND SURVIVAL IN CYSTIC FIBROSIS

Pamela B. Davis, M.D., Ph.D.

Department of Pediatrics, Case Western Reserve University School of Medicine, Cleveland OH

In nearly every year since accurate records have been kept, female patients with cystic fibrosis (CF) have reduced survival compared to male patients in the United States. Though the difference between males and females varies from year to year from, it averages about 3 years, or about 10% of the median survival age predicted for patients with CF in the United States today. Such a difference is substantial. For example, if a new drug for CF prolonged survival by 10%, we would be very pleased!

The death rate for female patients exceeds that for males for patients age one to twenty years. Above the age of 20 the female CF population is probably already censored by death. However, the early age at which the sex difference in mortality is evident argues that factors other than the pubertal hormone surge must account for the difference in survival. This sex differences in mortality are surprising for several reasons. The main cause of death in CF is progressive airways disease. Early in life, boys usually suffer greater morbidity from airways diseases because their airways are smaller than those of girls relative to body size. In addition, many studies show that CF girls show better canalization of growth than CF boys, and we usually associated poorer growth rate and poorer weight-for-height with poorer clinical status and greater likelihood of death. Despite these apparent advantages, the girls have greater mortality. (1)

There have been several studies addressing sex differences in key parameters for survival in CF. Demko et al (2) studied age at colonization with *Pseudomonas aeruginosa* and the age at which the mucoid phenotype is acquired. Acquisition of the mucoid phenotype of pseudomonas is associated with acceleration of the rate of decline of pulmonary function in patients with CF, and patients who acquire the mucoid phenotype of pseudomonas before the age of six years have significantly increased mortality compared to those who acquire the organism later in life. Girls acquire pseudomonas infection about a year earlier than boys, and the mucoid phenotype appears about 18 months earlier in girls. Thus, the accelerated decline in pulmonary function begins earlier in CF girls, and this may contribute to poorer survival. Complementary studies have been performed in animal models, in which chronic infection with mucoid pseudomonas is simulated by embedding the organisms in agar beads and delivering them to the lower airways, where the beads ensure retention of the live organisms despite the host's attempt to clear the infection. In this model, female mice have greater mortality than male littermates, and their BAL fluid contains higher levels of inflammatory cytokines (TNF-α, IL-1$, IL-6, KC, and MIP-2) than BAL fluid from males. Whether these differences relate to sex differences in ion transporter expression in the airways of mice, or to other factors, has not been determined. In mice, ion transport recorded in the nose differs between male and female mice. Moreover, the sex hormones have been shown to regulate levels of important ion transporters, including CFTR, although small changes in CFTR transcription are probably irrelevant if the protein itself is dysfunctional, as it is in CF. Regulation of the epithelial sodium channel, which is regarded by some investigators as the main cause of airway desiccation in CF, may be more important. Airway desiccation leads to bacterial trapping and poor mucociliary clearance. Production of the α and (subunits of the epithelial sodium channel is enhanced by estrogen and progesterone (3). Even a subtle increase in sodium reabsorption, persistent over many years, might increase retention of bacteria in the airways, promote colonization, and accelerate the decline in pulmonary function.

Certain infections have especially poor outcome for females. Infection with *Burkholderia cepacia*, for example, can result in "cepacia syndrome", a fatal illness with rapid decline, more often in women than in men. Following infection, women have greater acceleration of the rate of decline of pulmonary function than men, and in the first year following infection, one third of females, but 21% of males, succumb (4). However, acquisition of *B. cepacia* is rare and occurs in patients during adolescence and adulthood, so differential response to this organism cannot account for all of the excess mortality of females with CF.

Although pregnancy has been considered a risk factor for female CF patients, few women actually become pregnant, and most who do suffer no ill effects. Only those with very poor pulmonary function at the outset, or those who are infected with *B. cepacia*, sustain serious declines or death with pregnancy. Other, more subtle, differences must be sought.

Fitness is strongly related to mortality in CF - the more fit the subject, the lower the mortality rate (5). In that study, girls achieved lower peak work load and lower oxygen consumption than did boys with comparable pulmonary function, and so were less fit. Thus, fitness favored survival of boys. However, very early differences in mortality, evident from 1 year of age, may not be easily attributed to differences in fitness. Since fitness can be altered by training, however, it merits attention.

To some extent, biology is destiny for patients with CF, for the specific mutation has profound influence on the course of the disease, modifier genes may also contribute to survival, and females with CF have significantly poorer survival for the first twenty years of life. However, modifiable risk factors may be embedded in the sex difference in survival. For example, improved fitness may be attained by a careful program of exercise training, and infection control policies can dramatically reduce the probability of acquiring particularly noxious organisms.

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S6.3
BIOMEDICAL ISSUES FOR THE ADOLESCENT AND ADULT MALE & FEMALE, PARTICULARLY REPRODUCTIVE CONSEQUENCES

M.Götz

CF Center, Dept of Pediatric & Adolescent Medicine, Wilhelminen Hospital, Vienna, Austria

Prolonged life spans for persons with CF increase the likelihood of altered clinical presentations and additional burdens both medically and emotionally. Clinically significant differences in survival persist between countries and women are significantly more likely to die at a younger age (1).In both sexes, cardiorespiratory complications are the predominant concern and generally are the major determinant of length and quality of life. In addition, pancreatic function, nutrient uptake, and liver disease may also contribute to physical fitness and everyday coping abilities. Detailed studies assessing potential medical risk factors for poorer survival of females have failed to explain gender differences (2). However, lung development ceases earlier in women and , as they age, women retain smaller airways in relation to lung parenchyma. These gender differences may spell earlier and more profound effects of CF lung disease.

Sexual maturation is frequently delayed with late onset of puberty and menarche. Reproductive consequences in general largely depend on physical health. This is of particular importance concerning pregnancy. Thus, pregnancy related risks must be evaluated carefully (3).

In males, infertility occurs in about 98% as a consequence of obstructive azoospermia caused by absent or atretic vas deferens, body and tail of the epididymis, and seminal vesicles. Obstructed sperm transport may be overcome in some males by reconstructive techniques of the reproductive tract or, more successfully, by microsurgical sperm aspiration from the epididymis or the vasa efferentia for subsequent insemination. Reasons for reproductive pathology are still unclear but significant defective CFTR expression has been established in the epithelium of the vas deferens and the epididymis.

Notwithstanding infertility, males with CF in rare cases have been shown to be able to procreate normally and spermiograms should be done in any adolescent or adult male contemplating fathering children. Clinicians and clinical psychologists have to recognize that procreation and fertility may be an important issue for adolescent males at a relatively early stage. Reassurance that CF is not a barrier to sexual desire and activity should be offered at the onset of puberty. Fertilty and potency must be clearly differentiated and reassurance as to sexual fulfillment is an important aspect of maintained self-esteem and prevention of emotional stress (4). Only when offered in an empathic caring way worries may be alleviated and infertility strain reactions such as tension, worry, interpersonal alienation and depressive symptoms may be overcome .

In couples, infertility may be a major source of anguish but does not preclude marital accord once the condition has become accepted. In some cases psychotherapeutic support and marital counseling may be needed. Recent fertility research offers viable options and perspectives for males with CF.

In females the major concern is pregnancy. Suboptimal fertility has been desribed repeatedly but does not constitute a real problem. Secondary amenorrhea generally is a consequence of progressing lung disease and poor weight. In case of amenorrhea the help of a gynecologist versed in CF should be sought to exclude other causes. At present, several hundred pregnancies in women with CF have been documented. Nutritional state

and pulmonary function prior to and during pregnancy unanimously seem to be the dominant determinants of pregnancy outcomes (3). Hypoxemia and pulmonary decompensation may adversely affect the pregnant mother and, of course, the fetus. Maternal inabilty to increase cardiac output may put the fetus at risk of getting inadequate oxygen supply. Prematurity and perinatal death have been reported but recent data are lacking.

Maternal concerns beyond the actual course of pregnancy center on transmission of disease and influence of required maternal medical treatment on the fetus. Obligatory heterozygosity of the offspring should be discussed in the light of modern diagnostic techniques of asssessment of gene carrier status of future partners. Although antibiotics and physical therapy remain standard treatment during pregnancies few if any untoward side effects specific for CF have been recorded. Pregnancies should be closely monitored by gynecologist and CF team. Preconception counseling and psychological guidance during pregnancy (and afterwards) are highly desirable.

Overall, sexual and reproductive issues relating to adolescent and adult health in persons with CF are an important aspect of well being. Reproductive options and consequences must be discussed comprehensively both from a medical but also psychological viewpoint. Lack of doing so will greatly contribute to inadequate care of this age group. In order to achieve optimal results interaction between CF team, reproductive specialist and patient is mandatory.

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S6.4
CULTURALLY CONSTRUCTED GENDER ISSUES FOR ADOLESCENTS AND ADULTS WITH CF

Ilse Götz

University Children's Hospital, Vienna, Austria

Physicians are increasingly aware of the influence of culture and gender on the care of persons with chronic conditions, yet very few medical schools in the United States and Canada have separate courses specifically addressing these issues (1). Culture is a set of explicit and implicit guidelines that determine how individuals within a group view the world and behave in relation to other people, to supernatural forces or gods, and to the natural environment (2,3). It is in the context of normative cultural values that feminine and masculine behavior develops. The implications of traditional notions of femininity and masculinity are fundamental for constructing and practicing gender identity but in persons with CF partially conflict with disease management.

A growing body of literature suggests that boys and girls behave differently both in daily life activities and in the area of health. Despite differences between cultures and the many sub-groups even within a given society some of the culturally constructed gender issues (CCGI) apply to many adolescents and adults and therefore also to those with CF: Boys are expected to be tough and to control their emotions, to develop power and leadership qualities, and to produce offspring. Girls are allowed to express feelings, they may be passive and weak in certain areas, and child rearing may socially be more rewarded than persuing a career.

CCGI that greatly influence the medical regimen in CF include body image, nutrition, exercise, and career. The issue of appearance predominates in the media and dictates young women to remain slim and young men to present physically fit. In CF, females had significantly lower energy and fat intakes than males (4) and tended to overestimate their weight while young men underestimated it (5). Young women did not mind their thinness whereas young men were discontent with their body image that did not conform to the normative masculine body image (6). With regard to sport and exercise young women were less physically active than young men who seem to be more socially rewarded in cultures where sport is associated with masculine behavior (6). The issue of setting vocational goals was more important for males than for females (6), the latter being at higher risk of CF-attributed work disability (7).

Cultural factors also influence attitudes towards CF-carrier testing and prenatal diagnosis (8) and potentially diminish parents' ability to both absorb information on infertility and discuss it with their sons (9). Even the fact of having CF may need to be kept a secret due to cultural values that do not easily accept weaknesses, in particular when it comes to marriage.

CCGI also concern the health professionals (HP) and may unintentionally color their recommendations and decision-making. Numerous studies investigated the effect of physician gender, gender condordance between physicians and patients or cultural disease models on the patient-physician relationship. As an example, patients of female physicians had more visits in which a participatory decision-making style was used (10). In CF, the actual clinical ramifications of culture with regard to the HP have rarely been examined, hence a new area of research is unfolding.

Even in CF centers that serve a rather homogeneous group of patients HP should take the cultural background into account. In Viennna, for instance, approximately 15% of the patients represent 12 nations/cultures. The challenge rests in both the recognition of a patient's normative cultural values and their implementation in clinical practice. Culturally competent HP integrate health-related patient/parental beliefs and practices in the delivery of health care, pay adequate attention to language problems, and are aware that poor adherence could be a result of the incompatibility of requirements. Such an approach may be rewarded by increased patient satisfaction and greater clinical effectiveness.

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S7.1
CFTR INTERACTIONS WITH THE ACTIN CYTOSKELETON

Horacio F. Cantiello.

Renal Unit, Massachusetts General Hospital East, Charlestown, Massachusetts, USA

The cystic fibrosis transmembrane conductance regulator (CFTR) is an anion-selective channel whose dysfunction leads to the onset of cystic fibrosis. CFTR activation is paradigmatically elicited by stimulation of the cAMP pathway, which effects the activation of the cAMP-dependent protein kinase A (PKA), and consequent phosphorylation events. Several PKA phosphorylation sites have been identified in CFTR. Cyclic AMP-PKA independent CFTR activation has also been observed, in particular by maneuvers that modify cytoskeletal dynamics (1). Early changes in actin filament networks directly activate CFTR, while cytoskeletal collapse inhibits CFTR activity, which becomes insensitive to PKA activation, but is readily regained by addition of exogenous actin from the cytoplasmic side of the channel (1). A similar pattern of CFTR activation to that elicited by the actin filament disrupter, cytochalasin D (1) can be observed by intracellular dialysis with the actin-severing protein gelsolin. Further information also indicates that normal actin networks are required for a proper cAMP activation of CFTR (2). CFTR activation by actin structures is independent of the changes in CFTR-containing apical membrane delivery, also elicited by the cytoskeleton-mediated vesicular fusion after cell activation. The encompassed data are most consistent with the possibility that actin may directly interact with CFTR to elicit its activation, further suggesting that this channel protein may bind actin as well. The precise molecular nature of the interaction between actin and CFTR, however, remains largely unknown. In this report, new evidence is summarized where direct interactions between actin and purified human epithelial CFTR were demonstrated by imaging the channel protein by atomic force microscopy (AFM) (3). CFTR-containing liposomes in solution were deposited on freshly cleaved mica and imaging was performed in tapping-mode AFM. Liposomes flattened either spontaneously or after addition of $MgCl_2$, providing a flat lipid surface from which CFTR molecules protruded. Images of single CFTR molecules were obtained, which were identified by changes in height associated with anti-CFTR antibody binding (either external or internal epitopes). Addition of monomeric actin below its critical concentration showed the formation of actin filaments associated with CFTR, suggesting that CFTR may behave as a novel actin-nucleation protein. CFTR function was also determined in identical liposome preparations to determine its functional conformation in the lipid membranes. CFTR function was modulated by addition of actin in the presence or absence of the actin-binding protein DNAse I, suggesting that the actin interaction with CFTR is sufficient to elicit CFTR activation in the complete absence of PKA and ATP. Further indication that CFTR acts as an actin-binding protein was also determined by fluorescence changes of pyrenyl-actin

polymerization, which was modulated by the CFTR containing-liposomes in solution. The data indicate that a direct interaction between actin and CFTR exists, which may explain the regulatory role of the cytoskeleton in its ion channel function.

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S7.2
INTERMOLECULAR AND INTRAMOLECULAR INTERACTIONS THAT MEDIATE CFTR CHANNEL FUNCTION

M. Ramjeesingh, C. Li, I. Kogan, J.Kidd, Y. Wang, L-J Huan and C.E. Bear

Research Institute of the Hospital for Sick Children and Departments of Biochemistry and Physiology, University of Toronto, Toronto, CANADA

CFTR is a member of the ABC superfamily of transport ATPases. Recent high resolution structures of intact procaryotic transporters from this family permit predictions regarding the functional quaternary structure and interdomain interactions in CFTR. Defining the quaternary structure of CFTR is fundamentally important for understanding the mechanism of action of the normal protein and possibly in defining the molecular basis for disease caused by certain mutations. Further it is clear by analogy with related ABC proteins, that by mapping the interfaces between domains we will likely uncover regions which are pivotal to the regulated channel function of CFTR.

Quaternary Structure of CFTR

The solved structures of the lipid flippase: MsbA (1) and the vitamin B12 transporter: BtuC/D proteins (2) show that they dimeric. As the CFTR molecule possesses an internal duplication of the domains found in MsbA and BtuCD, we would predict the functional unit of CFTR is a monomer. Our recent biochemical studies support this prediction and show that purified, reconstituted CFTR monomers are fully functional as chloride channels (3). Furthermore, the chloride channel activity conferred by reconstitution of purified CFTR into planar lipid bilayers is similar to that of the native channel studied in biological membranes with regard to it's unitary conductance, selectivity for anions and regulation by ATP (3).

However, we found that CFTR can self-associate as dimers in our reconstitution system, in stably transfected CHO cells (3) and in human epithelial cells (unpublished observations) which endogenously express the protein. These data are consistent with electrophysiological data reported by other labs suggesting there may be physical and/or functional interaction between CFTR molecules in biological membranes (4) (5) (6). At present however, it is unclear whether CFTR molecules interact directly or indirectly. We reported that we could purify and functionally reconstitute both CFTR monomers and dimers from Sf9 cells over-expressing the protein, arguing that CFTR molecules can interact directly (3). However, purified dimers function as two independent channels indicating that the two CFTR molecules are likely to interact via regions that are peripheral to the channel pore and gate. This interpretation is consistent with the finding that CFTR molecules with different tags have not been co-immunoprecipiated in studies published by Marshall et al. (7). On the other hand, CFTR molecules may interact indirectly via other molecules. Several labs have reported that CFTR interacts via its carboxy terminus with scaffolding molecules linked to the cytoskeleton (8) (5).

Our recent studies of the quaternary structure of CFTR expressed in CHO cells revealed a possible physiological significance for CFTR dimerization. It is well known that following translation, CFTR protein is complex glycosylated in the golgi and then trafficked to the cell surface where it functions to mediate its physiological role in chloride flux. We found that dimeric CFTR is comprised of fully mature protein (Band C) protein whereas monomeric CFTR is comprised predominantly of immature protein (unpublished findings). These findings suggest that dimerization may facillitate correct protein processing. A similar mechanism has been shown underlie efficient maturation of the KATP channel (9). Our current work focusses on determining whether dimerization of CFTR serves a similar purpose. As the major mutation; CFTR delta F508 and many other mutations cause inefficient protein processing, it is

important to determine if defective dimerization may also contribute to this molecular phenotype.

Interdomain interactions in CFTR

Functional and biochemical studies of various intact ABC transporters, reveal that activation requires long range conformational changes to transmit signals between domains or subunits. Ligand interaction with the one protein subunit signals to distinct subunits to induce a change in their structure and function. Specifically our work with purified, reconstituted CFTR, we have shown that ligands which interact specifically with the membrane domains of CFTR, large anions such as glutathione and DPC, induce changes in ATP catalysis by the nucleotide binding folds (NBDs) (10). Conversely, our group (unpublished findings) and others (11) have shown that hydrolytic and nonhydrolytic interactions of nucleotides with the NBDs of CFTR can cause distinct changes in the properties of the pore in the membrane. The recent crystal structures of intact procaryotic ABC transporters highlight distinct interfacial regions between the membrane domains and the NBDs through which communication occurs. The nature of these interfacial regions in procaryotic transporters and our studies of the corresponding regions in CFTR will be discussed.

Numerous electrophysiological studies of CFTR reveal that chloride channel function requires phosphorylation at several key serine/ threonine residues as well as nucleotide binding and hydrolysis. From our synthesis of the current literature and our own work, we suggest that intrinsic ATP hydrolysis promotes gating of the CFTR chloride channel (12), (13), (14), (15), (16). Further, optimal hydrolysis requires functional interaction between the two NBDs (16). These findings are consistent with other biochemical studies of CFTR and other ABC proteins which suggest that the physical interaction between the two NBDs is an important part of the mechanism underlying ATP binding and hydrolysis (17) (18). Recent crystal structures of a number of NBDs from different ABC transporters have revealed a variety of possible dimerization interfaces. Our approach to studying the physical basis for interaction between the NBDs of CFTR in the intact protein will be discussed in this symposium.

There are major hurdles to be overcome before we can understand the molecular basis for the function of CFTR. However, published structures for ABC transporters provide clues that are currently guiding our work.

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S7.3
STRUCTURE AND MECHANISM IN NUCLEOTIDE INTERACTIONS WITH WT AND MUTANT CFTR CL CHANNELS

D.C. Gadsby, P. Vergani, C. Basso, A.G. Dousmanis, and *A.C. Nairn

*Laboratories of Cardiac/Membrane Physiology, and of *Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY 10021-6399, USA*

CFTR is unique among ABC proteins in that it forms an ion channel but, like other ABC proteins, CFTR is known to bind MgATP at both of its nucleotide binding domains (NBDs), and it hydrolyzes MgATP at a rate comparable to that of the conformational changes that open and close the ion pore (changes collectively referred to as gating). However, the link between nucleotide binding and hydrolysis at the two NBDs on the one hand, and opening and closing of the channel gate on the other, remains unclear and controversial. Much work has shown that CFTR channels must be phosphorylated by protein kinase A, and probably also by protein kinase C, before MgATP can act at the NBDs to cause the channels to open and close. From detailed measurements of the rates of CFTR channel opening and closing, before and after modifying the chemical structure of either the nucleotide, or the nucleotide binding site, or both, we have been able to assemble the following picture of gating of phosphorylated CFTR channels. It seems that ATP binds with higher affinity to NBD1, and with lower affinity to NBD2. Only when both NBDs are occupied, a slow step occurs that requires Mg^{2+} ions and is very sensitive to the structure of the β-γ phosphate moiety of the nucleoside triphosphate, and that rate limits opening of the channel. The open state of the channel is stabilized by the MgATP bound at the NBD2 catalytic site, and the open state eventually becomes destabilized following hydrolysis of that nucleotide. Channel closure, and loss of the hydrolysis products from the NBD2 active site, likely occurs without recycling of the nucleotide tightly bound at the NBD1 active site, which appears to remain occupied for many gating cycles. The channel then reopens when MgATP next binds at NBD2 to initiate another gating cycle.

This picture of CFTR channel gating derives from extensive analyses of gating kinetics of wild type (WT) and mutant CFTR, bearing mutations of key catalytic site residues in the NBDs, during exposure to ATP and/or its poorly hydrolyzable analogs AMPPNP, ATPγS, or AMP-PCP. WT CFTR channels do not open in the absence of MgATP, but as [MgATP] is raised from zero to mM the average rate of channel opening increases in a saturable manner along a Michaelis-Menten curve, and is half maximal at ~60 μM and maximal at ~1 mM. Mutation of the invariant Walker A Lys in NBD1, K464, or in NBD2, K1250, or of the invariant Walker B Asp in NBD2, D1370, in all three cases substantially reduced the apparent affinity for MgATP to open the channels. Because, for mutant K464A and D1370N CFTR channels, the deficit in opening at low [MgATP] could be largely overcome by raising [MgATP], and as all structural models of CFTR's NBDs to date place K464 and D1370 in different catalytic sites, these findings imply that both NBDs must be occupied by MgATP before a CFTR channel will open.

Two other results support this conclusion. First, the closing rate (reciprocal of the mean open burst duration) of all, WT and mutant, CFTR channels was independent of [MgATP], which indicates that no further binding of nucleotide to the open channel is required to close it. Second, the closing rate of WT CFTR channels exposed to MgAMPPNP alone was lower than that during exposure of the same channels to MgATP alone, whereas the closing rate of the same channels exposed to a mixture of MgATP and MgAMPPNP was far slower still. This latter result indicates that (at least) two nucleotide molecules interact with a single CFTR channel during one gating cycle, and since the former result ruled out nucleotide binding to the open channel, this also means that both nucleotide molecules must bind to a CFTR channel before it opens.

This conclusion is also in accord with our detailed kinetic measurements of the gating of D1370N CFTR channels, which at low (15 μM) [MgATP] both open and close more slowly than WT, thus demonstrating that the influence of this point mutation in NBD2 is felt during every gating cycle. Because D1370N channels possess a fully intact (i.e. WT) NBD1, this result argues against earlier proposals that gating cycles may involve solely nucleotide interactions with NBD1 when [MgATP] is very low. Together, these findings suggest that both NBDs are normally occupied by nucleotide during each CFTR channel gating cycle.

Measurements of the rate of channel closing show that mutation of key catalytic site residues influences (greatly slows) closing only when those mutations are introduced into the NBD2 catalytic site, not the NBD1 site. A qualitatively similar, strong stabilization of the open channel state also occurs when MgAMPPNP or orthovanadate (VO_4) is added during exposure of CFTR channels to MgATP. These findings imply that occupancy of CFTR's NBD2 catalytic site by nucleotide that cannot be hydrolyzed (MgAMPPNP or ADP-VO_4 complex in WT CFTR, or MgATP in D1370N, K1250A, or E1371S CFTR) causes the observed marked stabilization of open state, and this in turn suggests that hydrolysis of the MgATP at the NBD2 site normally precedes the usual rapid channel closure.

The fact that the NBD1 mutation K464A does not affect the channel closing rate (i.e. the stability of the open state) under normal hydrolytic conditions, but it speeds closing under non-hydrolytic conditions (i.e., in the presence of MgAMPPNP plus MgATP, or of VO_4 plus MgATP, or of MgATP alone in the K1250A background), illustrates that gating reflects interactions between CFTR's two NBDs. The different stability of the open state obtained with MgAMPPNP, depending on the simultaneous presence or absence of MgATP, provides another indication of such interactions between the two NBDs.

Measurements of nucleotide occupancy of the NBDs, using photolabeling with α^{32}P-8-azido-nucleotide or γ^{32}P-8-azido-nucleotide and split CFTR molecules to unequivocally allocate labeling to NBD1 or NBD2 catalytic sites, indicate that ATP binds with highest affinity to NBD1, from which it dissociates slowly, with a time constant on the order of 10 min. Much of this nucleotide is still in the form of nucleoside triphosphate, i.e. it can remain bound at NBD1 for minutes without being hydrolyzed. Nucleotide dissociates much more rapidly from NBD2, because labeling at NBD2 is essentially abolished by washing and resuspending before applying the UV flash to crosslink bound 8-azido-nucleotide, even after incubation with nucleotide and VO_4.

Thus, the overall picture that emerges is of NBD1 remaining occupied by nucleotide (much of which survives unhydrolyzed) for many gating cycles, while nucleotide binding and hydrolysis at NBD2 time the opening and closing of the CFTR channel.

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S7.4
REGULATION OF THE CFTR CL$^-$ CHANNEL

Michael J. Welsh, Lynda S. Ostedgaard, Olafur Baldursson and Christoph Randak

Howard Hughes Medical Institute, Departments of Internal Medicine and Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242

The activity of the CFTR Cl^- channel is controlled by its two nucleotide-binding domains (NBD) and the R domain. The R domain serves as the major physiologic regulator of the CFTR Cl^- channel. Upon elevation of cAMP levels, cAMP-dependent protein kinase phosphorylates the R domain allowing the NBDs to bind and hydrolyze ATP to open and close the channel. How phosphorylation activates the channel is not well understood. Some models propose that the R domain prevents the channel from opening and that phosphorylation relieves this inhibition. Other models suggest that phosphorylation of the R domain stimulates activity. Recent studies of CFTR with portions of the R domain deleted, and studies in which various portions of the R domain are added back to the channel provide clues as to how the R domain contributes to channel regulation. In addition, recent structural studies suggest that the R domain is predominately random coil in solution, a property not changed by phosphorylation. These observations suggest that a defined amino acid sequence is not critical, but PKA consensus motifs, a certain degree of flexibility, and possibly an optimal length are key in determining channel activity. These results are consistent with the increasing recognition that many protein domains and full-length proteins are intrinsically unstructured, and that this has advantages for signaling and regulation. In CFTR, an R domain composed predominantly of random coil would retain the flexibility that permits facile interaction with multiple regions within the rest of CFTR and allow prompt, discrete, and variable reactions to phosphorylation of different serines.

S8.1
GENES THAT MODIFY THE HEMOCHROMATOSIS PHENOTYPE

Nancy C. Andrews M.D., Ph.D.

Howard Hughes Medical Institute, Children's Hospital, Harvard Medical School, Boston, Massachusetts USA

Hereditary hemochromatosis is a multisystem disease that is caused by chronically increased intestinal iron absorption, resulting in tissue iron deposition. It typically presents in mid-life with a characteristic constellation of signs and symptoms including arthropathy of digital joints in the hands, increased skin pigmentation, depression, impotence, liver dysfunction and diabetes. Patients can expect normal duration and quality of life when hemochromatosis is detected early and phlebotomy is instituted to remove iron. However, when the diagnosis is not made, organ damage progresses, leading to potentially lethal complications including cirrhosis, cardiomyopathy, diabetes and increased susceptibility to hepatocellular carcinoma.

Most patients with hemochromatosis are homozygous for a missense mutation (C282Y) in *HFE*, a gene encoding an HLA class I-like molecule of unknown function, which is involved in the regulation of intestinal iron absorption. However, there is a wide range in phenotypic expression among C282Y homozygotes; some individuals never develop clinical disease, while others have lethal complications by the third decade of life. This variability appears to be due to both environmental and genetic factors.

We have made two models of HFE hemochromatosis in mice. We have disrupted the murine *Hfe* gene to produce a null allele, and we have introduced the C282Y mutation into an otherwise intact *Hfe* gene to model the human disease. We bred each of these mutations to homozygosity on an inbred, 129SvEvTac background, and compared the phenotypes. We found that both strains accumulated storage iron in the liver and had increased intestinal iron absorption but that iron loading was more severe in the *Hfe-/-* mice than in the *Hfe C282Y* knock-in mice, indicating that the C282Y mutation results in a partial, but not total, deficiency in Hfe function.

We showed that these mice model most aspects of human hemochromatosis. Iron overload begins after birth, when intestinal iron absorption starts. It increases rapidly over the first three months of life, but then plateaus, much as iron loading has been shown to plateau in human patients. Although hepatic parenchymal cells develop marked iron overload, liver and spleen macrophages are iron-depleted, as are the functionally equivalent bone marrow macrophages in human hemochromatosis. Like human patients, the *Hfe* mutant mice also have a marked predisposition to the development of hepatocellular adenomas and carcinomas and are more prone to develop porphyria cutanea tarda, an acquired abnormality of uroporphyrinogen decarboxylase that results in accumulation of massive amounts of liver porphyrins. Thus, *Hfe-/-* and *Hfe C282Y* mutant mice are useful for studying many phenotypic features of hemochromatosis. The only significant deficiency in their suitability for use as a model is in end organ damage – they do not develop end stage cirrhosis, cardiomyopathy or pancreatic dysfunction seen in humans. However, these limitations do not impair the use of *Hfe-/-* mice as a model for understanding the alterations in iron homeostasis in hemochromatosis.

To study the pathogenesis of hemochromatosis, we bred *Hfe* mutant mice to mice carrying mutations in other genes involved in iron transport to produce compound mutant animals. These experiments proved that mouse hemochromatosis results from increased flux of iron through the normal intestinal absorptive pathway, rather than activation of a new pathway. Our results suggested that polymorphisms and mutations affecting transport proteins might modulate the human phenotype.

There is no phenotypic variability observed in murine hemochromatosis when animals of uniform genetic background are compared. However, we do see differences in the extent of iron loading when the *Hfe* mutations are bred onto different inbred backgrounds, consistent with the notion that there are genetic modifiers of the hemochromatosis phenotype. We undertook an unbiased approach to search for genes that modify iron loading in wild type mice, hypothesizing that some or all of them will correspond to modifiers in hemochromatosis. Initially, we examined liver and spleen iron content in six well-characterized strains and found marked variability. The greatest differences were between C57Bl/10, which had low liver and spleen iron, and SWR, which had high liver and spleen iron content. The differences, approximately 6-fold for liver iron and 10-fold for spleen iron, were sufficient to support a genetic approach to identify modifying genes. We crossed the two strains and then backcrossed the F1 offspring with their inbred parents to generate N2 animals for analysis. We found evidence for one major modifier of spleen iron loading (LOD > 7) on mouse chromosome 9, and evidence for three modifiers of liver iron loading (LOD > 3) on mouse chromosomes 1, 4 and 18. Further analysis of these modifier loci is currently underway.

In summary, targeted gene mutation in mice has provided a faithful model of human hemochromatosis, allowing us to investigate the pathogenesis of this common disorder. It has also provided a means to search for modifying genes that may help to explain phenotypic variability in human patients.