MECHANISMS OF POLYUNSATURATED FATTY ACID ALTERATIONS IN CYSTIC FIBROSIS

By

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To my amazing parents, Mom and Dad, infinitely loving and supportive

and

To my beloved fiancé, Adam, who is my everything

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LIST OF ABBREVIATIONS

4-phenylbutyrate 4-PBA

5-lipoxygenase 5-LOX

Acetyl-CoA carboxylase ..... ACC

Adenosine monophosphate-activated protein kinase ..... AMPK

Adenosine triphosphate ..... ATP

Arachidonic acid ..... AA

ATP binding cassette transporter ..... ABC

Bovine serum albumin ..... BSA

Bronchoalveolar lavage ..... BAL

Calcium/calmodulin-dependent protein kinase kinase beta ..... CaMKKβ

Calcium release-activated calcium channels ..... CRAC

Carnitine palmitoyl transferase CPT-1

Counts per minute ..... CPM

Cyclic adenosine monophosphate ..... cAMP

Cyclooxygenase-2 ..... COX-2

Cystic fibrosis ..... CF

Cystic fibrosis transmembrane conductance regulator ..... CFTR

Dihydroethidium ..... DHE

Distal intestinal obstruction syndrome ..... DIOS

Docosahexaenoic acid ..... DHA

Docosapentaenoic acid ..... DPA

Eicosapentaenoic acid ..... EPA

Electron transport chain ..... ETC

Elongase 2 ..... Elovl2

Elongase 5 ..... Elovl5

Endoplasmic reticulum ..... ER

Epithelial sodium channel ..... ENaC

Fatty acid methyl ester ..... FAME

Forced expiratory volume in 1 second ..... FEV_1

Forced vital capacity ..... FVC

Free fatty acid ..... FFA

Gas chromatography - mass spectrometry ..... GC-MS

High-performance liquid chromatography ..... HPLC

Human bronchial epithelial ..... HBE

Human serum albumin ..... HSA

Intermediate density lipoprotein ..... IDL

Krebs henseleit buffer ..... KHB

Krebs ringer hepes buffer ..... KRB-Hepes

Leukotriene B_4 ..... LTB_4

Linoleic acid ..... LA

Linolenic acid ..... LNA

Liver kinase B1 ..... LKB1

Low density lipoprotein ..... LDL

Messenger RNA ..... mRNA

Microsomal prostaglandin E_2 synthase-1 ..... mPGES-1

Mito-TEMPO ..... mT

Monoacylglycerol ..... MAG

N-acetyl cysteine..... NAC

Nitric oxide ..... NO

Non-steroidal anti-inflammatory drugs..... NSAID

Nucleotide binding domain..... NBD

Omega 3 ..... n-3

Omega 6 ..... n-6

Oleic acid ..... OA

Palmitic acid..... PA

Peroxisome proliferator-activated receptor alpha ..... PPARα

Peroxynitrite..... $ONOO^-$

Polyunsaturated fatty acid..... PUFA

Prostaglandin E_2 ..... PGE_2

Protein kinase A ..... PKA

Quantitative real-time PCR..... qRT-PCR

Reactive oxygen species ..... ROS

Reduced glutathione.....GSH

Regulatory domain.....R

Small interfering RNA..... siRNA

Specific pathogen free.....SPF

Sterol regulatory element-binding protein 1 .....SREBP-1

Superoxide dismutase .....SOD

Transmembrane domain.....TMD

Triglyceride..... TG

Trolox..... TX

Unfolded protein response .....UPR

Very low density lipoprotein .....VLDL

CHAPTER I

INTRODUCTION

Objective

Cystic Fibrosis (CF) is the most common lethal autosomal recessive disease in the Caucasian population, occurring in roughly 1 in 2000-3000 live births [1]. In African Americans, CF occurs in approximately 1 in 15,000-20,000 births [2], whereas in Asian populations, disease incidence has been estimated to be as rare as 1 in 350,000 births [3]. CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, leading to lack of functional CFTR protein at the apical surface of secretory epithelia [4-6]. There is currently no cure for CF, and in spite of improved screening and treatment, the US Cystic Fibrosis Foundation estimates the life expectancy of CF patients to be only 37 years [7].

Numerous studies have revealed the presence of alterations in polyunsaturated fatty acid (PUFA) composition in CF patients as well as in CF mouse and cell culture models [8-16]. From these findings, several reproducible fatty acid changes have emerged as dominant: decreased linoleic acid (18:2 n-6, LA), decreased docosahexaenoic acid (22:6 n-3, DHA) and variably increased arachidonic acid (20:4 n-6, AA) (Figure 1). These PUFA alterations are independent of nutritional status and pancreatic insufficiency [12, 17-20]. Additionally, the magnitude of these changes has been shown to correlate with disease severity. For example, LA and DHA levels tend to be lower in CF patients with more severe disease [21, 22]. Previous studies have also shown that high-dose DHA

treatment leads to normalization of fatty acid levels in the lung, pancreas and intestine of two different CF mouse models, and reverses the pathological manifestations of CF [13, 14]. It is therefore believed that CF fatty acid alterations play a key role in pathophysiology of the disease. However, the mechanism(s) of the PUFA changes, their connection to CFTR gene mutations and how they contribute to a CF phenotype is currently unknown. We hypothesized that the fatty acid alterations seen in CF are caused by altered PUFA metabolism, contributing to increased levels of AA and increased metabolism of AA to pro-inflammatory eicosanoids. The research presented in this dissertation focuses on understanding the mechanisms underlying PUFA alterations in CF, and the role they play in the pathogenesis of disease.

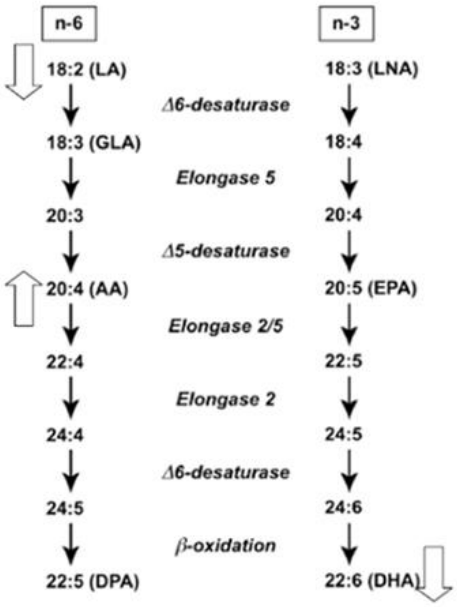

Figure 1: Diagram of polyunsaturated fatty acid metabolism through the n-6 and n-3 pathways. Specific omega-6 (n-6) and omega-3 (n-3) fatty acid changes have been described in cystic fibrosis patients, mice and cell culture models. These include decreased levels of linoleic acid (LA) and docosahexaenoic acid (DHA), and increased levels of arachidonic acid (AA).

Molecular structure and function of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

CF is a life-shortening genetic disorder that affects multiple organs in the body. It is caused by a defective gene encoding a protein known as the cystic fibrosis transmembrane conductance regulator (CFTR) [4-6]. The CFTR gene encompasses approximately 180,000 base pairs and is located on the long arm of chromosome 7. It encodes a 1,480 amino acid membrane protein that functions as an epithelial chloride ion channel and regulates the absorption and secretion of salt and water in various tissues such as the lung, sweat glands, gastrointestinal tract and pancreas [23].

CFTR is a member of the adenosine triphosphate (ATP)-binding cassette transporter superfamily of proteins, which utilize nucleotide hydrolysis to transport substrates across the membrane bilayer [24]. CFTR protein is composed of five distinct domains: two homologous transmembrane spanning domains (TMD1 and TMD2), each containing six transmembrane segments that form the hydrophilic channel through which anions are transported; two nucleotide binding domains (NBD1 and NBD2) that are exposed to the cytosol and are involved in ATP binding and hydrolysis; and a regulatory R domain whose phosphorylation by protein kinase A (PKA) regulates channel gating [25] (Figure 2). Chloride transport by CFTR requires interaction between the multiple domains. Phosphorylation of the R domain by cAMP-dependent PKA is necessary for channel activation and induces a conformational change in the protein, leading to reduced interaction between the R domain and NBD1[26]. This allows for the NBDs to dimerize and interact in a head-to-tail manner, enclosing two ATP molecules within the interfacial composite sites. ATP binding signals conformational changes in the TMDs and causes

the ion channel to open. Following channel opening, hydrolysis of one ATP molecule disrupts the NBD interface, triggers NBD dimer dissociation and leads to closing of the channel [27].

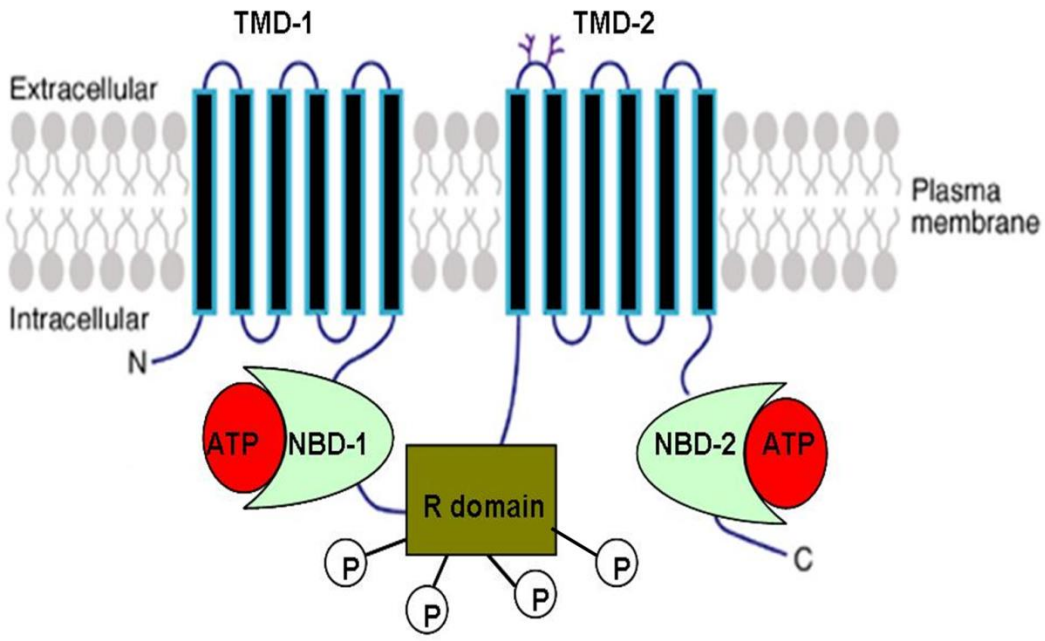

Figure 2: Diagram showing the different domains of the CFTR protein. CFTR is a member of the ATP-binding cassette (ABC) superfamily of proteins. It is made up of five domains: two six-membrane spanning domains that form the chloride channel (TMDs), two nucleotide binding domains (NBDs) that bind and hydrolyze ATP, as well as a unique regulatory domain (R domain) that can be phosphorylated by protein kinase A. Diagram adapted from the cystic fibrosis mutation database, 2012.

CFTR possesses other functions, in addition to being a chloride channel. CFTR is known to act as an inhibitor of the epithelial sodium channel (ENaC) [28]. The absence of functional CFTR in the apical membrane leads to unregulated, excessive sodium and water absorption. This promotes dehydration of the airway surface liquid, causing a collapse of the periciliary layer, loss of mucociliary clearance and concentration of mucus within the airway surface [29]. CFTR can also transport bicarbonate (HCO_3^-) ions [30]. This function is important in regulating mucus thickness, as well as the pH of the external environment of epithelial cells and of intracellular organelles. Highly compacted mucins found in intracellular granules are held together by high concentrations of calcium (Ca^{2+}) and hydrogen (H^+) cations. HCO_3^- can complex with, and sequester, the Ca^{2+} and H^+ cations away from the mucin anions, thus promoting mucin unfolding and expansion [31]. In CF, loss of HCO_3^- transport through CFTR leads to a HCO_3^- poor extracellular milieu. This results in impaired Ca^{2+} removal, hinders normal mucin expansion and promotes the accumulation of mucus on luminal surfaces and in ducts of CF-affected organs. Lack of HCO_3^- transport also decreases local pH and impairs bacterial killing [32].

Furthermore, CFTR is involved in the transport of larger anions, such as the antioxidant glutathione (GSH) and thiocyanate. CFTR-mediated GSH transport regulates redox reactions at the airway surface and can reduce mucus viscosity by disrupting disulfide bonds in mucin proteins [33]. Thiocyanate, on the other hand, is needed for the production of antimicrobial hypothiocyanite. Failure to transport thiocyanate anions to the surface of airway epithelium, as seen in CF, leads to a shortage of hypothiocyanite and allows for *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacterial

colonization [34]. Lastly, CFTR can regulate other membrane channels, including the outwardly rectifying chloride channel, inwardly rectifying potassium channels and ATP channels [35-38].

CFTR gene class mutations

More than 1800 CFTR gene mutations have been described to date, all with the potential to cause disease [39]. The most common mutation is the F508del-CFTR, which is caused by deletion of a phenylalanine codon at position 508 and accounts for approximately 70% of all CF cases in northern European and North American populations [2]. Besides F508del, only four specific mutations reach a frequency of 1% to 4%, including G551D, W1282X, G542X and N1303K [39]. The majority of the remaining CF mutations are extremely rare and have not been fully functionally characterized. CFTR gene mutations can be divided into six separate classes based on the primary mechanism responsible for reduced CFTR function.

Class I mutations

Class I mutations are caused by nonsense, frameshift or splice-site mutations that result in premature stop codons. This leads to the termination of mRNA translation and consequently, complete absence of CFTR protein. These stop mutations are indicated by an X. The W1282X is the most common class I mutation and accounts for slightly >1% of worldwide CF mutations but is very frequent in the Israeli Ashkenazi Jewish population, where it accounts for approximately 50% of CF cases [40]. Previous studies have shown that aminoglycoside antibiotics such as gentamicin induce read-through of

premature stop codons, resulting in formation of functional full-length CFTR protein. Short-term topical application of gentamicin to the nasal epithelium in CF patients with class I mutations has been reported to improve CFTR function [41]. Nevertheless, the safety and efficacy of long-term aminoglycosides as a CF therapy still needs to be evaluated.

Class II mutations

Class II mutations are associated with defective protein processing. Normal CFTR protein is folded and glycosylated in the endoplasmic reticulum (ER) and Golgi, allowing the protein to traffic to the apical cell surface. Class II mutant CFTR protein fails to complete these processes correctly and instead, is degraded in the ER. These mutations result in little or no mature CFTR at the cell surface. The most common CF mutation, F508del, falls within this class. The F508del mutation impairs the conformational maturation of nascent CFTR and arrests it in an early folding intermediate. As a result, the mutated protein is misfolded, is recognized by the ER quality control system, and is targeted for degradation via the ubiquitin-proteasome pathway [42, 43]. A small fraction of F508del CFTR can escape the ER degradation pathway, exit the ER and make it to the cell surface. However, this mutated protein is not stable and has aberrant channel gating as reflected in reduced channel open probability [44]. The F508del mutation affects the majority of CF patients and is therefore the most important CF therapeutic target. Numerous attempts have been made to increase levels of functional F508del CFTR protein at the cell surface. These include: 1) Use of small molecules known as correctors to rescue the folding and/or trafficking of F508del CFTR and increase its cell surface

density. Such correctors include 4-phenylbutyrate (4-PBA) and curcumin [45-48]; 2) low temperature rescue to promote trafficking of F508del CFTR from the ER to the cell surface [49]; 3) suppression of the protein degradation process using either protein chaperones or deubiquitinating enzymes [50, 51]; and 4) use of pharmacological agents called potentiators, including sulfonamides, tetrahydrobenzothiophenes and phenylglycine, to increase stability and channel gating of mutant CFTR [52, 53].

Class III mutations

Class III mutations are characterized by defective CFTR regulation. The CFTR protein is properly processed and traffics to the plasma membrane, but cannot be activated by ATP or cAMP. G551D is an example of a class III mutation, and it occurs in approximately 3-4% of CF patients. Since this mutation involves impaired channel gating, CF potentiators are an attractive therapeutic option. Vertex Pharmaceuticals Inc. has developed a compound known as VX-770 that has been shown to increase CFTR open probability and improve clinical outcomes such as lung function and sweat chloride concentrations in G551D patients [54, 55]. This compound is now FDA approved under the names Ivacaftor or Kalydeco™.

Class IV mutations

Class IV mutations involve altered chloride ion conductance, leading to CFTR protein at the apical cell surface that exhibits a reduced rate of chloride transport. R117H is among the common class IV mutations and is found in approximately 0.5% of CF patients [39]. These mutations tend to result in mild disease manifestations.

Class V mutations

Class V mutations include promoter and splice-site mutations that affect the efficiency of normal mRNA splicing and reduce the amount of normally processed and functional CFTR at the cell surface. These mutations produce some correctly spliced mRNA transcripts. The levels of these transcripts vary among different CF patients and are inversely correlated with disease severity, such that lower levels of correctly spliced transcripts are associated with more severe disease and vice versa [56-58]. The 3849 + 10kb C to T mutation is an example of a class V mutation. Therapeutic options for these mutations include the use of antisense oligonucleotides or small molecules such as sodium butyrate to decrease abnormal splicing and increase the levels of correctly spliced transcripts [59, 60].

Class VI mutations

Class VI mutations are associated with defective CFTR stability at the cell surface and accelerated protein turnover.

Clinical features of Cystic Fibrosis

CF is a multi-organ disease that is characterized by elevated sweat chloride concentrations (which is the main diagnostic CF test), recurrent pulmonary infections and chronic bronchiectasis, pancreatic insufficiency, intestinal malabsorption, and male infertility [61]. The extent and severity of disease is inversely proportional to the degree of CFTR function in the affected organs.

Recurrent pulmonary infections that lead to respiratory failure are the primary cause of morbidity and mortality in CF patients. CFTR dysfunction in airway epithelia causes impaired chloride ion efflux, as well as excessive sodium and water reabsorption. This results in dehydration of the airway surface liquid, loss of mucociliary clearance and increased production of thickened, viscous mucus [29, 62]. These conditions are conducive for the growth and retention of bacteria, with bacterial colonization of airways in CF patients beginning shortly after birth [63, 64]. The most common infecting bacteria in infants with CF include *Haemophilus influenzae* and *Staphylococcus aureus,* while older patients tend to be colonized by *Pseudomonas aeruginosa* [65, 66]. Increased production of thickened mucus leads to airway obstruction and perpetuates a vicious cycle of phlegm retention, infection and inflammation. This leads to bronchiectasis, air trapping and progressive lung damage, and is ultimately responsible for at least 80% of all CF-related deaths [7].

Exocrine pancreatic insufficiency is another clinical manifestation of CF. It is present in roughly 85-90% of all CF patients [67], with symptoms such as failure to thrive, greasy and bulky stool, abdominal bloating and poor absorption of fat-soluble vitamins. Pancreatic insufficiency is triggered by destruction of pancreatic acinar cells as well as obstruction of pancreatic ducts by thick mucus secretions, resulting in an inability of the pancreas to supply digestive enzymes to the intestine [68, 69]. This brings about intestinal malabsorption and contributes to the malnutrition seen in CF patients. Additional gastrointestinal problems associated with CF include meconium ileus, distal intestinal obstruction syndrome (DIOS), and constipation. These are all consequences of increased viscosity of intestinal mucus and prolonged intestinal transit time [70-72].

Approximately 13-17% of all CF patients experience complete intestinal obstruction during the neonatal period and present with meconium ileus at birth [73], while DIOS and chronic constipation are commonly found in older patients [74, 75].

Approximately one third of CF patients will develop liver disease [76]. CF-related liver disease usually develops before puberty, but it is often asymptomatic and progresses slowly. The characteristic liver lesion in CF is focal biliary cirrhosis, caused by bile duct plugging/obstruction and progressive periportal fibrosis [77]. Although liver cirrhosis is not very common, it remains the single most important non-pulmonary cause of death among CF patients [78].

CF also affects the reproductive system. Infertility occurs in about 97% of all male CF patients and is attributable to congenital bilateral absence of the vas deferens, with subsequent obstructive azoospermia [79]. Female fertility may be reduced due to dehydrated cervical mucus that acts as a barrier to sperm passage [80], but reproductive function is largely normal.

Fatty acid abnormalities in Cystic Fibrosis

Fatty acid abnormalities in CF patients

Alterations in PUFA composition were first identified in CF patients in 1962, when serum chylomicrons of children with CF were found to contain decreased amounts of linoleic acid (LA) compared to healthy controls [8]. Subsequent reports confirmed this finding and reported decreased plasma LA levels in all the main lipid classes in CF patients [9, 81]. Lower LA levels were also observed in red blood cells, platelets [82], and in nasal tissue from CF patients [12]. These fatty acid changes were present in both

infant and adult CF patients [83]. In 1972, Underwood et al. reported that, in addition to decreased LA, low docosahexaenoic acid (DHA) concentrations were present in different tissues of CF patients [84]. Similar findings were shown in later studies describing decreased plasma DHA levels in pre-adolescent CF children compared to healthy children [18, 19]. One study found that serum phospholipid DHA concentrations were significantly lower in patients with severe CFTR mutations, suggesting a possible connection between the basic gene defect and abnormal fatty acid metabolism in CF patients [20]. These fatty acid changes are very consistent, such that measurement of plasma LA and DHA levels is adequate to distinguish CF from non-CF patients [85]. A third PUFA alteration that has been noted in CF patients is increased arachidonic acid (AA). This finding is less consistent than the decreased LA and DHA. In most reports, there is an increase in AA in association with a decrease in LA. For example, increased AA concentrations were described in nasal biopsy tissues from CF patients compared to healthy controls [12], and an increase in AA mole fraction was reported in most phospholipid classes in bronchial secretions of patients with CF [86]. However, some studies showed little or no increase in the amount of AA in CF patients [10, 20]. When these fatty acid changes were first described, it was believed that they were a consequence of fat malabsorption. However, studies comparing well-nourished CF patients to healthy controls found that the CF fatty acid alterations are independent of nutritional status [11, 18] and diet [21]. In addition to PUFA alterations, fatty acid changes involving monounsaturated fatty acids in the omega-7 (n-7) and omega-9 (n-9) pathways have been observed in red blood cells, platelets and plasma of CF patients. These include increased palmitoleic acid (16:1n-7), increased oleic acid (18:1n-9) and

increased mead acid (20:3n-9) [11, 18, 82], and are an indication of essential fatty acid deficiency.

Fatty acid abnormalities in CF model systems

Genetically modified CF mice provide a good model to study CF pathogenesis and experimental therapeutics. CF mice exhibit many features in common with CF patients, including failure to thrive and growth retardation [87], impaired gastrointestinal physiology leading to a high incidence of intestinal obstruction [88, 89], bacterial overgrowth [90], and inflammation [91]. Fatty acid abnormalities are also present in CF mouse models. Freedman et al. showed that there was a change in the fatty acid composition of $cftr^{-/-}$ knockout mice ($cftr^{tm1Unc}$), characterized by increased phospholipid-bound AA and decreased phospholipid-bound DHA. These changes were present in the lung, pancreas and ileum of the knockout mice compared to wildtype controls [13]. A further study using the same mouse model demonstrated a decrease in LA in phospholipids from pancreatic homogenates of $cftr^{-/-}$ mice, as well as an increase in fatty acid flux from AA to docosapentaenoic acid (DPA n-6) [92]. Lipid abnormalities including higher levels of AA and decreased LA have also been reported in the duodenum, jejunum, ileum and pancreas of CF mice homozygous for the F508del mutation [14]. Although the link between these PUFA changes and the pathophysiology of CF is unclear, there is evidence from a $cftr^{-/-}$ knockout mouse model to suggest that correcting the lipid imbalances can reverse the pathologic manifestations of CF [13, 93]. Daily treatment of CF knockout mice with large amounts of DHA resulted in increased DHA and decreased AA concentrations in lung, pancreas and intestine tissue. Moreover,

the treated animals exhibited reversal of CF-related pathology, including relief of pancreatic duct dilation, decrease in stimulated neutrophil accumulation in bronchoalveolar lavage (BAL) fluid, and normalization of ileal histology [13]. These findings imply that the use of lipid supplementation may represent a potential therapeutic avenue to correct the CF fatty acid alterations and alleviate disease symptoms.

In addition, fatty acid abnormalities have been described in cell culture models of CF. A study done in cultured pancreatic epithelial cells with or without the CFTR gene product revealed a decrease in LA levels in phospholipids of CF cells, particularly in phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine [94].This was accompanied by increased conversion of LA to AA, as well as increased flux of LA into triglycerides (TG). Cultured human respiratory epithelial cells also exhibit the characteristic fatty acid alterations [15, 16], indicating that the defect is intrinsic and not due to malabsorption.

Rationale for current studies

PUFA alterations have long been described in CF. Furthermore, several studies have demonstrated that the magnitude of these alterations correlates with disease severity [20, 82], suggesting that the fatty acid changes play an important role in the pathophysiology of CF. However, the precise mechanisms underlying the fatty acid abnormalities are unknown. The link between these fatty acid changes and CFTR mutations, as well as the mechanism by which DHA can reverse the alterations also remains unclear. We believe that improving our understanding of the mechanisms behind, and the role of, fatty acid alterations in CF may assist us in identifying novel drug

targets and lipid-based therapies for treatment of the disease. The research presented in this thesis is aimed at uncovering the mechanisms of PUFA alterations in CF, examining how DHA works to correct these fatty acid changes and reverse the CF phenotype in a knockout mouse model, and lastly, investigating the connection between fatty acid changes and CFTR gene mutations.

CHAPTER II

FATTY ACID CHANGES IN CYSTIC FIBROSIS RESULT FROM INCREASED EXPRESSION AND ACTIVITY OF DESATURASE METABOLIC ENZYMES

Introduction

Three main PUFA changes have been described in CF, including decreased LA and DHA, and increased AA (Figure 1). We hypothesized that these fatty acid changes are brought about by altered PUFA metabolism through the n-6 and n-3 pathways. Since mammals are unable to synthesize PUFAs from acetyl CoA, they must obtain essential fatty acids such as LA and LNA from their diet [95]. Once ingested, these essential fatty acids can be converted to longer and more desaturated products through parallel metabolic pathways that include desaturation, elongation, and β-oxidation (Figure 1). These modifications are carried out by the same set of desaturase and elongase enzymes in both pathways. Δ5 and Δ6-desaturase enzymes catalyze the addition of a double bond to the fatty acid chain, with the Δ number indicating the position at which the double bond is introduced. Δ6-desaturase has been shown to catalyze the first and rate-limiting step of PUFA synthesis [95]. Fatty acid elongation involves the addition of two carbon units to a fatty acyl-CoA, using malonyl-CoA as the donor and NADPH as the reducing agent. Elongase 2 (Elovl2) specifically elongates 22-carbon fatty acids while elongase 5 (Elovl5) is involved in the elongation of 18-20 carbon fatty acids [96]. The activities of the enzymes involved in fatty acid desaturation and elongation appears to be regulated primarily at the transcriptional level, and not by post-translational protein modifications

[97, 98]. Together, the desaturase and elongase enzymes generate end products of PUFA synthesis such as AA and DHA. We set out to determine whether changes in n-6 and n-3 metabolism could account for the fatty acid alterations in CF. To this end, we evaluated fatty acid metabolic flux in a cell culture model of CF. Additionally, we determined the expression (mRNA and protein) and activity of the PUFA metabolic enzymes in our cell culture model, as well as in a $cftr^{-/-}$ knockout mouse model.

Experimental Procedures

Materials

Human bronchial epithelial cells (16HBE cells) were a kind gift from Dr. Pamela Davis (Case Western University, Cleveland, OH). These cells were stably transfected with plasmids containing the first 131 nucleotides of CFTR in the sense or antisense orientation. Sense (WT) cells were shown to express normal, functional CFTR while antisense (CF) cells did not express CFTR and could not transport chloride [99]. IB3 and C38 bronchial epithelial cells were obtained from ATCC (Manassas, VA). Fatty acid methyl ester (FAME) standards were purchased from NuChek Prep (Elysian, MN) and 24:5 n-3 and 24:6 n-3 standards were purchased from Larodan Fine Chemicals (Malmö, Sweden). Radiolabeled fatty acids (^{14}C-LA, ^{14}C-LNA, ^{14}C-AA, ^{14}C-EPA and ^{14}C-22:5 n-3) were purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). All solvents used on the HPLC instrument were purchased from Fisher Scientific (Pittsburgh, PA), while the IN-flow 2:1 scintillation cocktail was purchased from LabLogic Systems, Inc (Brandon, FL).

Cell culture

16HBE cells were cultured as described previously [15, 16]. Briefly, cells were grown in 6-well plates pre-coated with LHC Basal media (Invitrogen, Carlsbad, CA) containing human fibronectin (10 μg/mL; Sigma), vitrogen (3 μg/mL; Angiotech Biomaterials, Palo Alto, CA) and BSA (0.1 mg/mL; Sigma). Sense and antisense cells were plated at a density of 3×10^5 and 1×10^5 cells/well respectively. IB3 and C38 cells were plated at a density of 1×10^5 cells/well. All cells were grown in minimum essential medium + glutamax (Invitrogen) with 100 μg/mL streptomycin, 100 U/mL penicillin, and 10% horse serum (Omega Scientific, Tarzana, CA). Horse serum was used because it contains a high concentration of LA, which allows for the PUFA changes in CF cells to be manifested. Media was changed every two days and experiments carried out in cells two days post-confluence.

Mice

CFTR heterozygous mice on a C57BL/6J genetic background (B6.129P2-$Cftr^{tm1Unc}$/J, stock number 002196) were purchased from The Jackson Laboratory (Bar Harbor, Maine). The mice were housed within a specific pathogen-free (SPF) barrier facility with a 12-h light/dark cycle. Cftr heterozygotes were bred to obtain both wild-type (WT) and $cftr^{-/-}$ (CF) mice. Ear-clip samples of 14-day old mice were used for genotype analysis. WT and CF mice were weaned at 23 days of age and placed on a liquid diet known as Peptamen (Nestle Clinical Nutrition, Deerfield, IL) with access to water *ad libitum* for 14 days. Peptamen is a complete liquid enteral formulation composed mainly of medium-chain triglycerides, carbohydrates and hydrolyzed protein.

The liquid diet was used to prevent intestinal obstruction in the CF mice. The mice were monitored daily for clinical signs that could indicate distress, including coat quality, posture, ambulation and porphyrin staining, and any mice that appeared severely distressed were euthanized before completion of the experiment. The mice also received fresh peptamen every day, at which time their body weight was measured. The mice were sacrificed two weeks post-weaning, and blood as well as various organs (lung, pancreas, ileum, liver, kidney, heart and brain) collected. All experiments were carried out under protocols approved by the Vanderbilt Division of Animal Care, and by the Institutional Animal Care and Use Committee.

Total fatty acid analysis

Fatty acids were extracted and methylated from cells two days post-confluence using a modified Folch method [100]. Briefly, the cells were washed twice in ice-cold PBS, scraped using a rubber policeman and pelleted by centrifugation at 100 g for 8 min. The pellet was resuspended in PBS and heptadecanoic acid (17:0) was added as an internal standard. Six volumes of chloroform-methanol (2:1) was added to the cells, vortexed and incubated on ice for 10 min. The mixture was then centrifuged at 1100 g for 10 min, and the lower organic phase transferred to a new glass tube and dried down completely under nitrogen gas. To methylate the fatty acids, 0.5 mL of 0.5 N methanolic NaOH (Acros Organics, Geel, Belgium) was added to the dried-down lipids, vortexed and heated at 100ºC for 3 min. Following this, 0.5 mL boron trifluoride (BF_3-methanol; Sigma) was added to the mixture and incubated at 100ºC for 1 min. The resulting FAMEs were extracted using 1 mL hexane, followed by 6.5 mL of saturated NaCl solution. The

mixture was vortexed and centrifuged at 500 g for 4 min, and the upper hexane layer transferred to a new glass tube. Total FAMEs contained in the hexane layer were analyzed by gas chromatography (GC) using an Agilent 7980A GC system (Agilent Technologies, Santa Clara, CA) equipped with a Supelcowax SP-10 capillary column (Supelco, Bellefonte, PA) coupled to a mass spectrometer (model 5975c, Agilent Technologies). The mass of the FAMEs was determined by comparing areas of unknown FAMEs to that of the 17:0 internal standard. Results were expressed as the molar percentage (mol %) of each FAME relative to the total FAME mass of the sample.

Fatty acids were also extracted from different mouse tissues and plasma. For lung tissue, cell suspensions were made to enrich for epithelial cells as previously described [13]. Briefly, the lung was flushed with Krebs-Henseleit buffer (KHB) containing 0.5% BSA and then minced and transferred to a tube containing 10 mL KHB with 2,000 units DNase (Sigma), 0.5 units thermolysin (Sigma) and 1,000 units collagenase (Sigma). The tissue was incubated in a shaker at 37^{o}C for 30 min. Following incubation, KHB containing 4% BSA was added to the cell suspension and centrifuged at 500 g for 10 min. The supernatant was removed and the cells washed once in KHB and resuspended in PBS. Pancreatic cell suspensions were prepared by mechanical dissociation and addition of collagenase as described by Bruzzone et al [101]. Briefly, Krebs-Ringer Hepes (KRB-Hepes) buffer, adjusted to pH 7.4, containing 12.5 mM Hepes, 135 mM NaCl, 4.8 mM KCl, 1.0 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM MgSO4, 5.0 mM $NaHCO_3$, 5 mM glucose and 0.01 mg of aprotinin / mL was used as the dissociation medium. The pancreas was chopped into small pieces and transferred to a glass tube. Two mL KRB-Hepes containing 1 mg collagenase per mL was added to the tube and shaken vigorously

until the tissue suspension appeared homogenous. Fresh KRB-Hepes with 0.1% human serum albumin (HSA) was added and the tissue centrifuged at 500 g for 5 min. The digested tissue was washed twice with repeated centrifugations, resuspended in fresh KRB-Hepes-HSA and filtered consecutively through a 300 μm and then a 70 μm filter. The pancreatic cells that passed through the filter were centrifuged and resuspended in PBS. For ileal cells, the ileum was rinsed with PBS, sliced open and cells obtained by scraping the inner mucosal surface. Additionally, tissue homogenates were prepared from the kidney, heart, liver and brain by mincing and homogenizing the tissue samples in PBS. FAMEs were prepared from the pancreas and lung cell suspensions, tissue homogenates and plasma as described above, and total lipid levels analyzed by GC-MS.

Fatty acid radiolabeling experiments

For fatty acid metabolic flux experiments, the cells were grown until two days post-confluence. ^{14}C radiolabeled fatty acids were dried down under nitrogen gas and resuspended in media containing 10% reduced-lipid fetal bovine serum (Hyclone, Logan, UT) by thorough vortexing and sonication. The final concentration of radiolabeled fatty acids was 4.1μM. The media supplemented with radiolabeled fatty acids was added to the cells for 4 h, washed off twice with PBS and replaced with complete media for an additional 20 h. The cells were then harvested and lipids extracted and methylated as described above. To measure metabolic flux, the extracted FAMEs were dried down under nitrogen, redissolved in 50 μL acetonitrile and analyzed by high-performance liquid chromatography (HPLC) (Agilent 1200 series; Agilent Technologies, Santa Clara, CA) using an Agilent Zorbax Eclipse XDB-C18 column, 4.6×250 mm, 5 μm. A guard

column of 4.6×12.5 mm, 5 μm was used in conjunction with the analytical column. Quantification of the radiolabeled peaks was performed by a scintillation detector (β-RAM Model 4, IN/US Systems) coupled to the HPLC. The counting efficiency of this detector is > 90% for ^{14}C with 5 CPM background. A binary solvent system was used to separate the fatty acids at a flow rate of 1 mL/min. Solvent A comprised HPLC grade H_2O with 0.02% H_2PO_4, and solvent B comprised 100% HPLC grade acetonitrile. For separation of n-3 fatty acids, the solvent program started with 76% solvent B and 24% solvent A for 0.5 min, followed by a linear gradient from 76% to 86% solvent B over 10 min, a hold for 20 min, an additional linear gradient from 86% to 100% solvent B over 2 min, and a hold for 18 min, followed by reconstitution of the starting conditions. For n-6 fatty acids, the solvent program began with 58% solvent B and 42% solvent A for 25 min, followed by a linear gradient from 58% to 61% solvent B over 2 min, a hold for 8 min, another linear gradient from 61% to 100% solvent B over 15 min, and a hold for 20 min, followed by reconstitution of the original conditions. The peaks were identified by ultraviolet detection at 205 nm and confirmed by comparison with retention times of unlabeled standards.

Quantitative real-time PCR

Total RNA was isolated from homogenized mouse tissues or from cultured cells using TRIzol reagent (Invitrogen), following the manufacturer's instructions. Complementary DNA was generated from 2 μg of total RNA with random hexamers using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Quantitative real-time PCR (qRT-PCR) was done on mouse cDNA using Taqman gene

expression assays, universal PCR master mix, and a CFX96 Real-Time PCR system (Bio-rad) with Taqman commercial primers and probes (Applied Biosystems). Data was analyzed using CFX Manager software (Bio-rad). The relative expression of each target gene was calculated using the comparative C_T method and normalized to a reference gene, GAPDH. For gene expression analysis of cultured cells, qRT-PCR was performed in a reaction containing 50 ng of reverse-transcribed total RNA, 156 nM forward and reverse primers, and 10 μL 2× SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 20 μL. Each PCR reaction was performed in triplicate in 96-well plates and RPLP0 was used as an endogenous control. Primer sequences used for the cell culture experiments are listed in Table 1.

Table 1: **Primer sequences used for quantitative real-time PCR**

Gene Name	Product	Sequence of Forward and Reverse Primers (5' to 3')	GenBank Accession No.
RPLP0	Ribosomal Protein, Large, P0	ATGGCAGCATCTACAACCC GACAGACACTGGCAACATTG	NM_001002
FADS1	Fatty acid Δ5-desaturase	CCTGGAAAGCAACTGGTTTGTG GAAGGCAGACTTGTGGACATTG	NM_013402
FADS2	Fatty acid Δ6-desaturase	GCCAAGCCTAACATCTTCCACAAG GTATTCGTGCTGGTGATTGTAGGG	NM_004265
ELOVL2	Fatty acid elongase 2	CTGCTCTCAATATGGCTGGGTAAC CACTGTAAGTTGTAGCCTCCTTCC	NM_017770
ELOVL5	Fatty acid elongase 5	TCCCTCTTGGTTGGTTGTATTTCC GCCCCTTTCTTGTTGTAGGTCTG	NM_021814
PTGS1	Cyclooxygenase-1 (COX-1)	CTTGACCGCTACCAGTGTG GTGAGTGAGCAGGAAGTGG	NM_000962
PTGS2	Cyclooxygenase-2 (COX-2)	TGAAACCCACTCCAAACACAG GCCATAGTCAGCATTGTAAGTTG	NM_000963
ALOX5	5-lipoxygenase (5-LOX)	CCCGAGATGACCAAATTCACATTC AGGGTTCCACTCCATCCATCG	NM_000698

Western blotting

Membrane fractions of 16HBE cells were prepared using a subcellular protein fractionation kit (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's recommendations. Protein samples were run on a 7.5% pre-cast SDS-PAGE gel and transferred to Immobilon-P PVDF filters. Blots were stained using a polyclonal Δ6-desaturase antibody (Santa Cruz) and a polyclonal anti-calnexin antibody (StressGen). After washing, bound antibodies were visualized with a peroxidase-conjugated goat anti-rabbit secondary antibody using the SuperSignal West Pico substrate system (Thermo Fisher Scientific) and exposed to CL-X Posure film (Thermo Fisher Scientific).

Eicosanoid ELISA

Culture media was collected from 16HBE cells two days post-confluence and the levels of prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) measured by ELISA (Cayman Chemicals, Ann Arbor, MI). Per cell eicosanoid production was calculated by dividing the eicosanoid level in the total media sample by the number of cells in the corresponding well.

Cyclooxygenase-2 (COX-2) inhibition

To inhibit COX-2, 16HBE cells were grown until almost confluent and then treated with a COX-2 specific small molecule inhibitor known as NS-398 (Sigma) or DMSO control (vehicle) for 48 h. Alternatively, COX-2 expression in the cells was abolished by use of a small interfering RNA (siRNA) directed against COX-2. For these

experiments, 16HBE cells were transfected with 250 nM silencer select COX-2 siRNA or 250 nM silencer select negative control siRNA (Invitrogen) using Lipofectamine RNAiMax transfection reagent (Invitrogen) according to the manufacturer's instructions. Following COX-2 inhibition or knockdown, the cells were either incubated with ^{14}C-radiolabeled precursor fatty acids for PUFA metabolic flux assays or harvested and RNA isolated for PUFA metabolic gene expression studies.

Results

To determine whether the characteristic PUFA abnormalities were present in our CF cell culture system, we measured the relative n-3 and n-6 fatty acid levels in sense (WT) and antisense (CF) cells. Antisense cells exhibited significantly lower levels of LA, with associated increases in the downstream metabolites 18:3 n-6, 20:3 n-6 and AA. In addition, levels of DHA were markedly decreased in antisense cells compared to sense cells (Figure 3). To confirm these findings, we measured the fatty acid composition in a cell line derived from a CF patient. The IB3 cell line is a compound heterozygote bronchial epithelial cell line from a CF patient with a ΔF508 mutation and a W1282X nonsense mutation [102]. The CF phenotype in the IB3 cells has been corrected in the C38 cell line using WT CFTR in an adenoviral vector. Similar to antisense cells, the IB3 cells were found to have decreased levels of LA and increased AA levels relative to C38 cells (Figure 4).

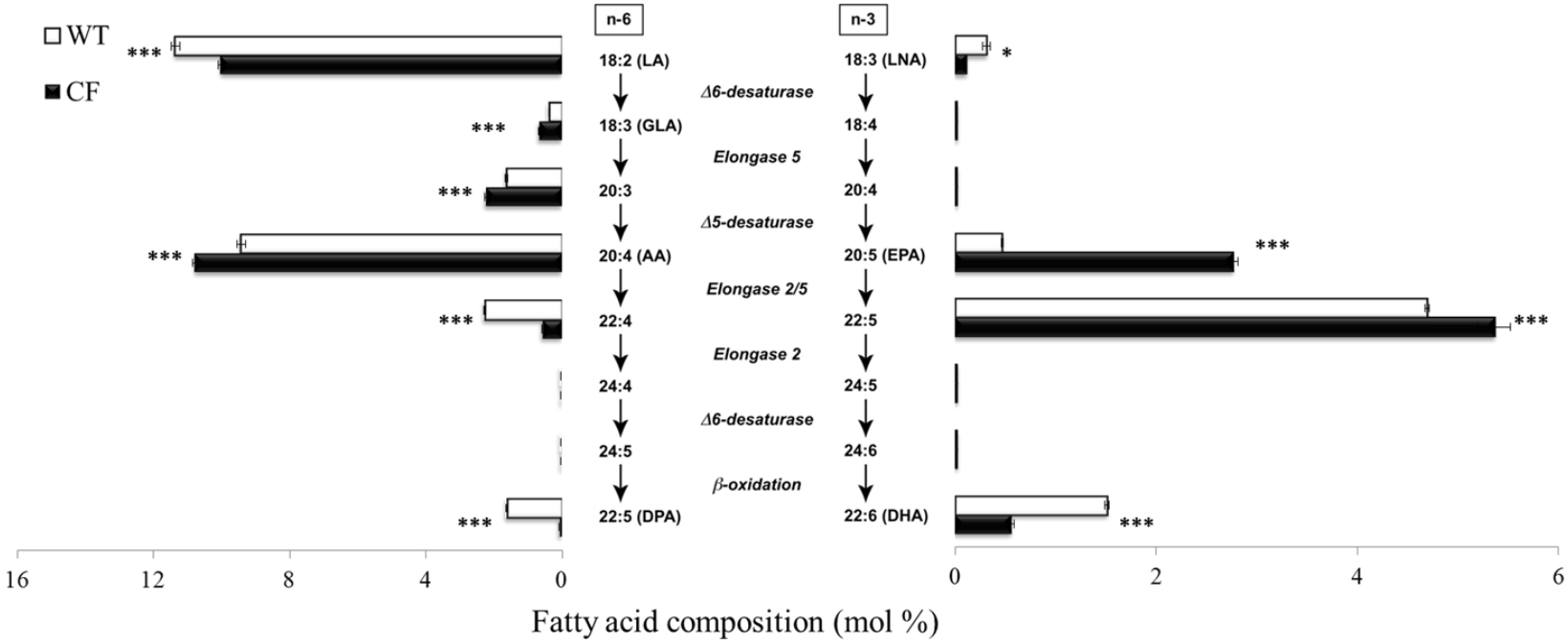

Figure 3: Fatty acid composition of Sense (WT) and Antisense (CF) bronchial epithelial cells. Total fatty acid levels were analyzed by GC-MS and data represented as molar percentage of total fatty acids (mol %). Levels of linoleic acid (LA) and docosahexaenoic acid (DHA) were significantly lower in CF cells, while levels of arachidonic acid (AA) were significantly higher in CF cells compared to WT cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ($n = 3$).

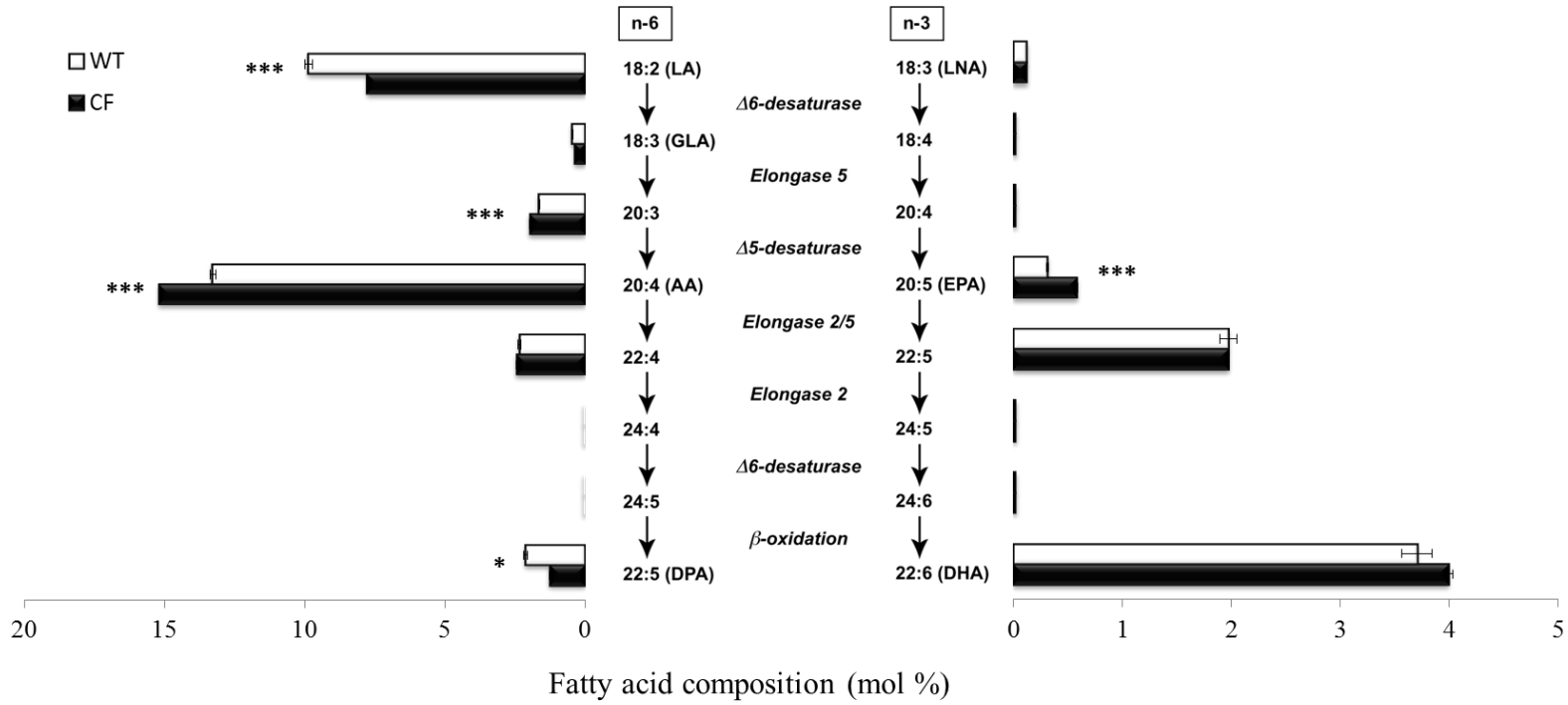

Figure 4: Fatty acid composition of C38 (WT) and IB3 (CF) bronchial epithelial cells. Total fatty acid levels were analyzed by GC-MS and data represented as molar percentage of total fatty acids (mol %). Levels of linoleic acid (LA) were significantly lower in CF cells, while levels of arachidonic acid (AA) were significantly higher in CF cells compared to WT cells. There was no difference in DHA levels between WT and CF cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ($n = 3$).

Higher levels of the downstream metabolites of LA in CF cells suggest that there is increased metabolism of LA to AA. To evaluate this hypothesis, sense and antisense cells were incubated with ^{14}C radiolabeled LA and incorporation into downstream metabolites measured. Significant increases in metabolism of LA to downstream products up to and including AA was observed in antisense cells compared to sense cells (Figure 5A). It is known that metabolism through the n-6 and n-3 pathways is carried out by the same set of enzymes [95]. Therefore we performed a parallel comparison of metabolic flux through the n-3 pathway. Sense and antisense cells were incubated with ^{14}C radiolabeled LNA and its conversion to downstream metabolites was measured. Similar to the n-6 pathway, there was a significant increase in metabolism of LNA to 18:4 n-3, 20:4 n-3 and EPA (Figure 5B). However, a closer look at the magnitude of conversion of LNA to EPA vs. LA to AA revealed greater metabolic flux when LNA was used as the substrate, resulting in a much higher EPA/LNA ratio than AA/LA ratio in both sense and antisense cells. This is consistent with previous studies reporting that PUFA metabolic enzymes have a preference for n-3 fatty acids over n-6 fatty acids [103-105].

The increased metabolism in the early steps of the n-3 and n-6 pathways provides a plausible explanation for the low LA and high AA levels in CF. However, this does not explain the low DHA levels present in CF. To investigate this, we incubated sense and antisense cells with ^{14}C radiolabeled AA and ^{14}C radiolabeled EPA and measured conversion to downstream metabolites. There was a significant decrease in metabolism of both AA and EPA to 22:5n-6 and DHA respectively (Figure 6), indicating a lower rate of DHA production from precursor fatty acids in CF cells.

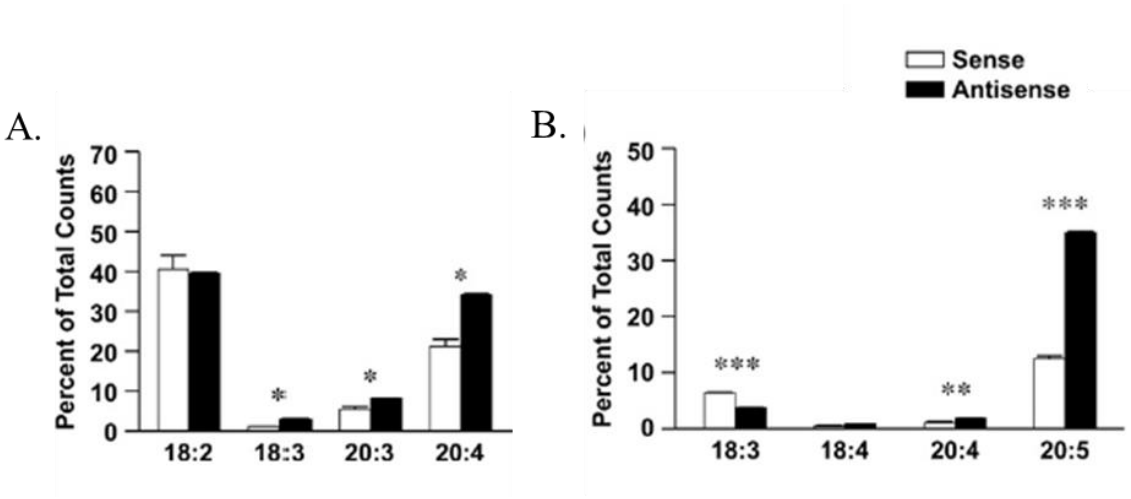

Figure 5: Metabolism of LA and LNA through the n-6 and n-3 pathways in 16HBE cells. Sense and antisense cells were incubated with 4.1 μM [^{14}C] LA (A) or [^{14}C] LNA (B) in reduced-lipid cell culture medium for 4 h as described in the experimental procedures. Levels of radiolabeled LA (18:2n-6), 18:3n-6, 20:3n-6 and AA (20:4n-6) (A) or radiolabeled LNA (18:3n-3), 18:4n-3, 20:4n-3 and EPA (20:5n-3) (B) were determined by HPLC and data presented as percent of total counts. Metabolism of both LA and LNA was shown to be revved up in antisense (CF) cells compared to sense (WT) cells. Bars represent mean ± SEM (n = 3). * P<0.05, ** P< 0.01, *** P<0.001 for sense vs. antisense cells.

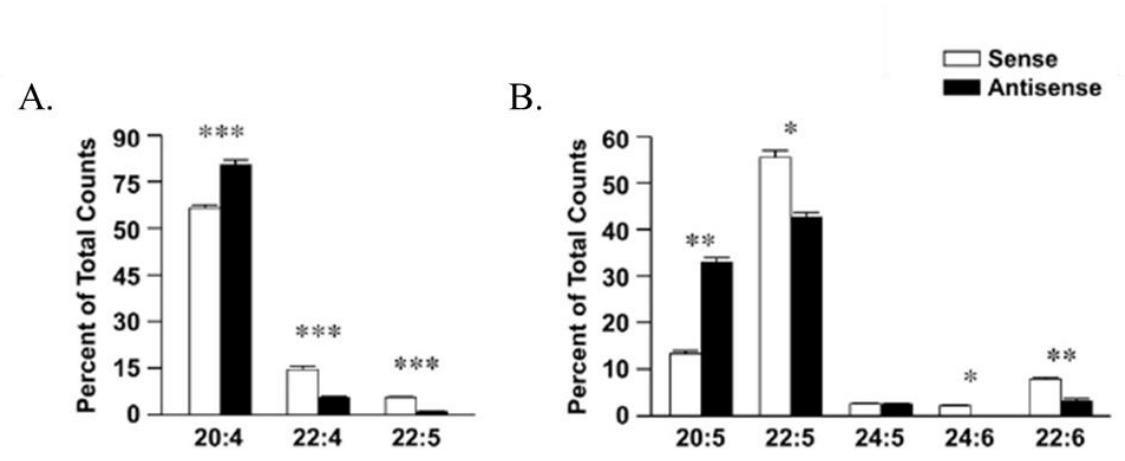

Figure 6: Metabolism of AA and EPA through the n-6 and n-3 pathways in 16HBE cells. Sense and antisense cells were incubated with 4.1 μM [^{14}C] AA (A) or [^{14}C] EPA (B) in reduced-lipid cell culture medium for 4 h as described in the experimental procedures. Levels of radiolabeled AA (20:4n-6), 22:4n-6 and 22:5n-6 (A) or radiolabeled EPA (20:5n-3), 22:5n-3, 24:5n-3, 24:6n-3 and DHA (22:6n-3) (B) were determined by HPLC and data presented as percent of total counts. Both AA and EPA metabolism was decreased in antisense (CF) cells compared to sense (WT) cells. Bars represent mean ± SEM (n = 3). * P<0.05, ** P< 0.01, *** P<0.001 for sense vs. antisense cells.

PUFA metabolism through the n-3 and n-6 pathways is controlled by desaturase and elongase enzymes (Figure 1), and regulation of these enzymes influences the production of end products of PUFA synthesis such as AA and DHA. Thus it is possible that differential expression of these enzymes in sense and antisense cells causes the PUFA alterations commonly found in CF. To test this, the mRNA expression of all four enzymes involved (Δ5-desaturase, Δ6-desaturase, elovl2 and elovl5) was measured by qRT-PCR. Both Δ5-desaturase and Δ6-desaturase were expressed at significantly higher levels in antisense cells (Figure 7A), while there was no difference in the mRNA levels of either elovl2 or elovl5 between sense and antisense cells. Protein levels of the rate-limiting enzyme, Δ6-desaturase, were also increased in antisense cells (Figure 7B). These findings were confirmed in the IB3/C38 cell line, which showed similar elevations in desaturase expression in CF cells (Figure 7C). This suggests that the altered levels of LA and AA in CF are brought about by differences in the transcriptional regulation of desaturase enzymes.

Although increased expression and activity of the metabolic enzymes can explain the differences seen in the early steps of PUFA metabolism, it does not explain the decreased metabolism of AA and EPA. A conceivable explanation for this is decreased β-oxidation at the second to last step in PUFA metabolism (Figure 1). However, this explanation cannot account for the decreased metabolism of earlier AA and EPA metabolites such as 22:4n-6 and 22:5n-3 (Figure 6).

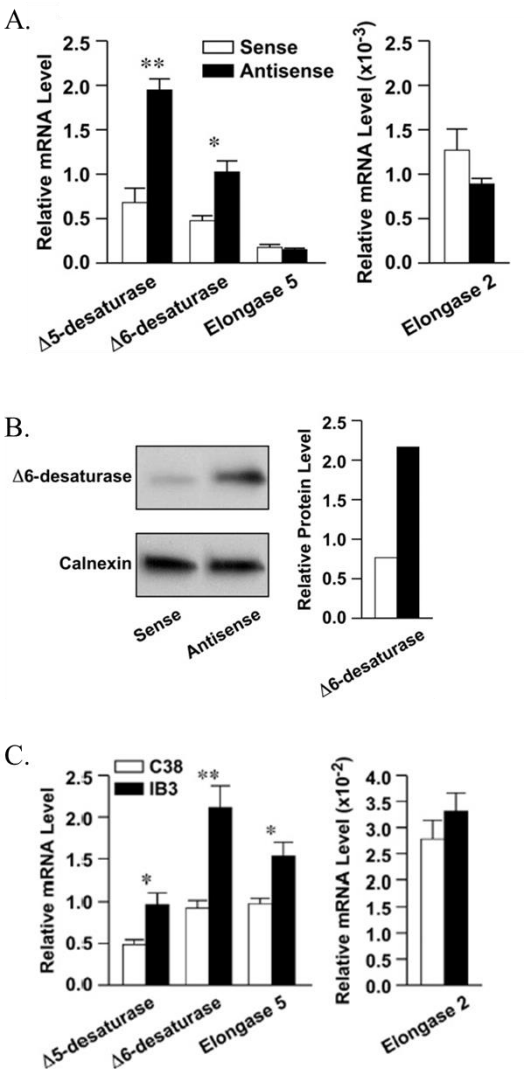

Figure 7: Relative mRNA and protein expression of PUFA metabolic enzymes in the n-6 and n-3 pathways. RNA was extracted and cDNA synthesized from sense and antisense cells (A) or from IB3/C38 cells (C) as described in the experimental procedures. qRT-PCR was performed using primers for the mRNA sequences of Δ5-desaturase (*FADS1*), Δ6-desaturase (*FADS2*), elongase 2 (*ELOVL2*), and elongase 5 (*ELOVL5*). Relative expression was determined by the $\Delta\Delta C_T$ method using ribosomal protein *RPLP0* as a control. Both Δ5-desaturase and Δ6-desaturase expression levels were significantly increased in CF cells (antisense and IB3) compared to WT cells (sense and C38). Bars represent mean ± SEM (n = 3). * $P<0.05$, ** $P< 0.01$, for sense vs. antisense cells. (B) Relative protein expression of Δ6-desaturase was determined in sense and antisense cells by western blot using an anti-FADS2 (Δ6-desaturase) polyclonal antibody (1:200). An anti-calnexin polyclonal antibody (1:1000) was used as a loading control. Data shown are representative of a minimum of three different experiments.

Another potential explanation is that AA and EPA are being metabolized via an alternative pathway, thereby reducing the amount of substrate available for conversion to 22:5n-6 and DHA. This hypothesis is supported by the fact that metabolism of ^{14}C-radiolabeled 22:5n-3 to DHA was approximately equal in sense and antisense cells (Figure 8). This indicates that decreased metabolism of EPA (and AA by inference) likely occurs at the first metabolic step and is perhaps due to metabolism of EPA and AA by a different pathway.

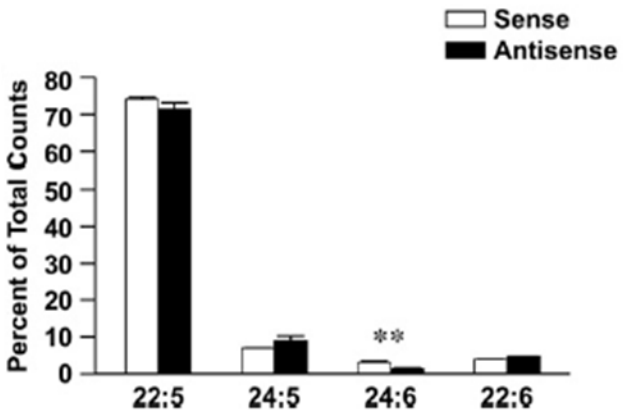

Figure 8: Metabolism of ^{14}C 22:5n-3 in 16HBE cells. Sense and antisense cells were incubated with 4.1 μM [^{14}C] 22:5n-3 in reduced-lipid cell culture medium for 4 h as described in the experimental procedures. Levels of radiolabeled 22:5n-3, 24:5n-3, 24:6n-3 and DHA (22:6n-3) were determined by HPLC and data presented as percent of total counts (cpm). 22:5n-3 was metabolized fairly equally in both sense and antisense cells, with the exception of 24:6n-3. Bars represent mean ± SEM (n = 3). ** $P< 0.01$ for sense vs. antisense cells.

A possible alternative pathway for AA and EPA metabolism is oxidation to eicosanoids such as prostaglandins and leukotrienes. This is facilitated by cyclooxygenase (COX-1 and COX-2) and lipoxygenase (5-LOX) enzymes. Several well studied prostaglandins produced from AA are pro-inflammatory, while several of those synthesized from EPA are by comparison less pro-inflammatory, or even anti-inflammatory (Figure 9). Previous studies have shown that pro-inflammatory eicosanoids including prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) are increased in CF patients [106-110]. This correlates with increased expression of cyclooxygenase and lipoxygenase in sinonasal tissue from CF patients [111, 112].

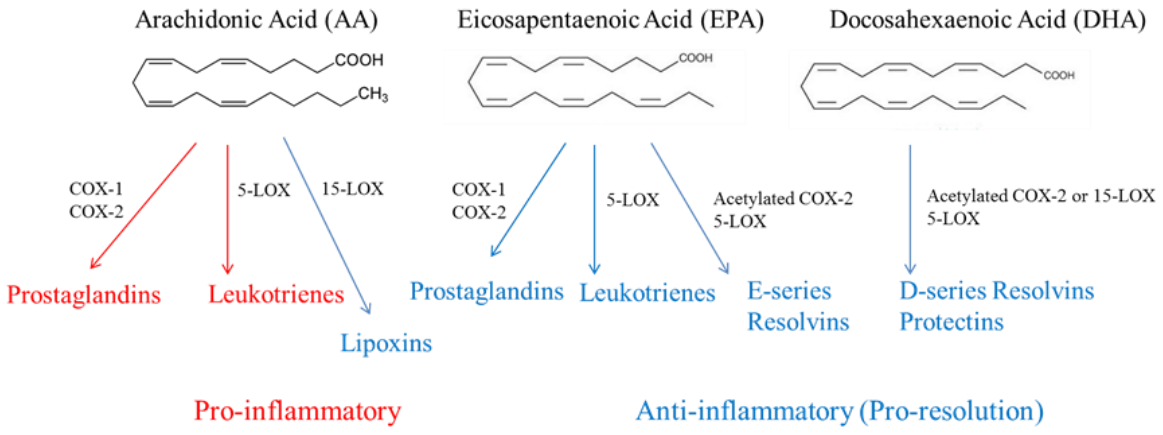

Figure 9: Diagram of the oxidative metabolism of Arachidonic Acid (AA), Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA). Eicosanoids produced from AA, with the exception of lipoxins, have pro-inflammatory effects. On the other hand, EPA and DHA-derived eicosanoids and resolvins have potent anti-inflammatory properties.

To determine whether this alternative eicosanoid synthesis pathway was upregulated in antisense cells, we measured both the expression and activity of COX and LOX enzymes (Figure 10). COX-1 is constitutively expressed and is responsible for housekeeping prostaglandin synthesis, and its mRNA levels were similar in sense and antisense cells. Conversely, both COX-2 and 5-LOX were markedly overexpressed in antisense cells and this was accompanied by increased production of PGE_2 and LTB_4 in antisense cells compared to sense cells. That the pathways leading to the synthesis of

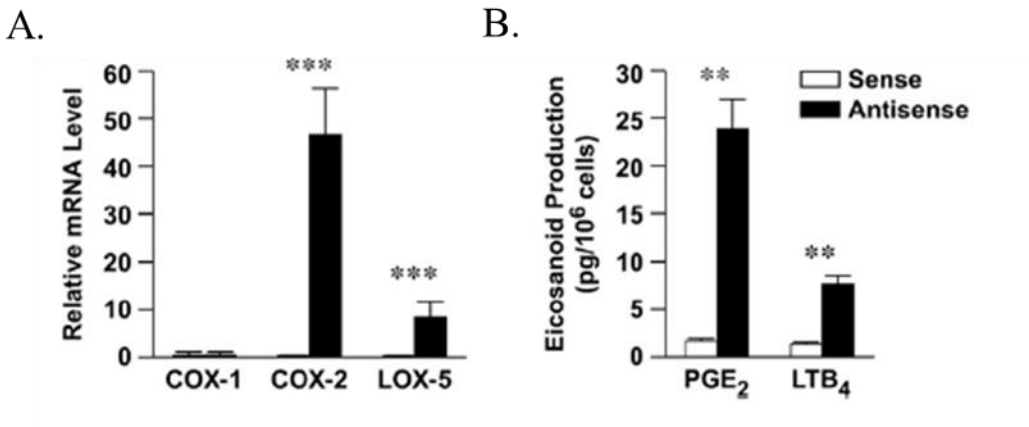

Figure 10: A. Relative mRNA expression of eicosanoid synthesis enzymes in 16HBE cells. RNA was extracted and cDNA synthesized from sense and antisense cells as described in the experimental procedures. qRT-PCR was performed using primers for the mRNA sequences of cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), and 5-lipoxygenase (LOX-5). Relative expression was determined by the $\Delta\Delta C_T$ method using ribosomal protein *RPLP0* as a control. Both COX-2 and 5-LOX expression levels were significantly increased in antisense cells compared to sense cells. **B. Eicosanoid production.** Prostaglandin E_2 (PGE_2) and Leukotriene B_4 (LTB_4) concentrations were measured in culture media from sense and antisense cells by ELISA. Results are expressed as total media eicosanoid per 10^6 cells. PGE_2 and LTB_4 production was significantly higher in antisense cells. Bars represent mean ± SEM (n = 3). ** $P< 0.01$, *** $P<0.001$ for sense vs. antisense cells.

eicosanoids from AA and EPA are increased in antisense cells suggests that perhaps these pathways divert some of the substrate available for metabolism to 22:5n-6 and DHA.

Since PGE_2 levels were increased in antisense cells, we decided to measure the expression levels of the corresponding PGE_2 receptors. PGE_2 exerts its actions by acting on specific G-protein coupled receptors including EP1, EP2, EP3 and EP4 receptors [113]. The mRNA expression levels of the EP2 receptor were greatly upregulated in antisense cells compared to sense cells (Figure 11). EP2 is linked to stimulation of cAMP and PKA signaling through activation of Gαs and its overexpression in CF cells may help promote the inflammatory actions of PGE_2.

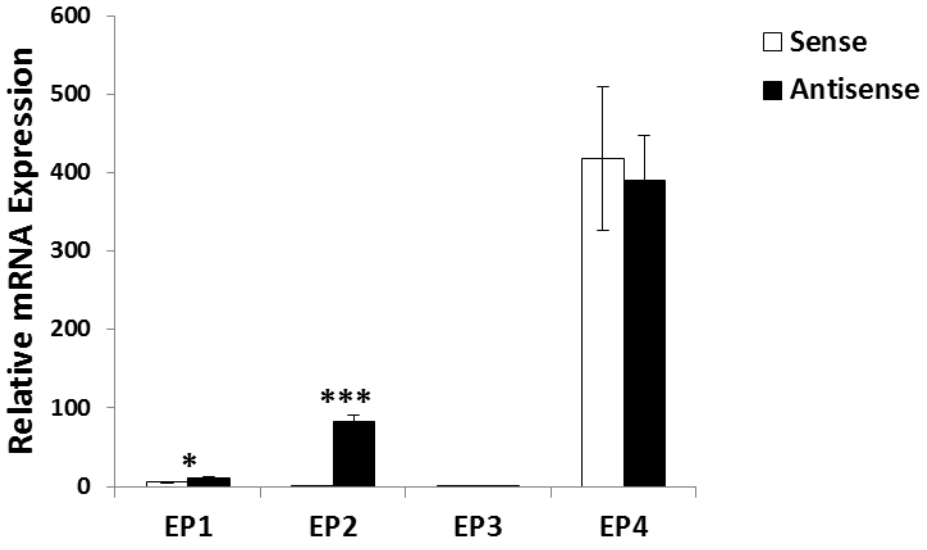

Figure 11: Relative mRNA expression of PGE_2 receptor subtypes in 16HBE cells. RNA was extracted and cDNA synthesized from sense and antisense cells as described in the experimental procedures. qRT-PCR was performed using commercially available Taqman primers (Applied Biosystems) for the EP1, EP2, EP3 and EP4 receptors. Relative expression was determined by the $\Delta\Delta C_T$ method using ribosomal protein *RPLP0* as an invariant control. The EP2 receptor was the most differentially expressed, with much higher expression in antisense cells compared to sense cells. Bars represent mean ± SEM (n = 3). * P<0.05, ** P< 0.01, *** P<0.001 for sense vs. antisense cells.

To determine whether increased eicosanoid production was responsible for the decreased metabolism of AA and EPA to 22:5n-6 and DHA, we chose to inhibit prostaglandin synthesis in our cells and determine if this would normalize PUFA metabolism. Sense and antisense cells were treated with a COX-2 specific small molecule inhibitor known as NS-398 [114], or with an siRNA directed against COX-2 (Figure 12).

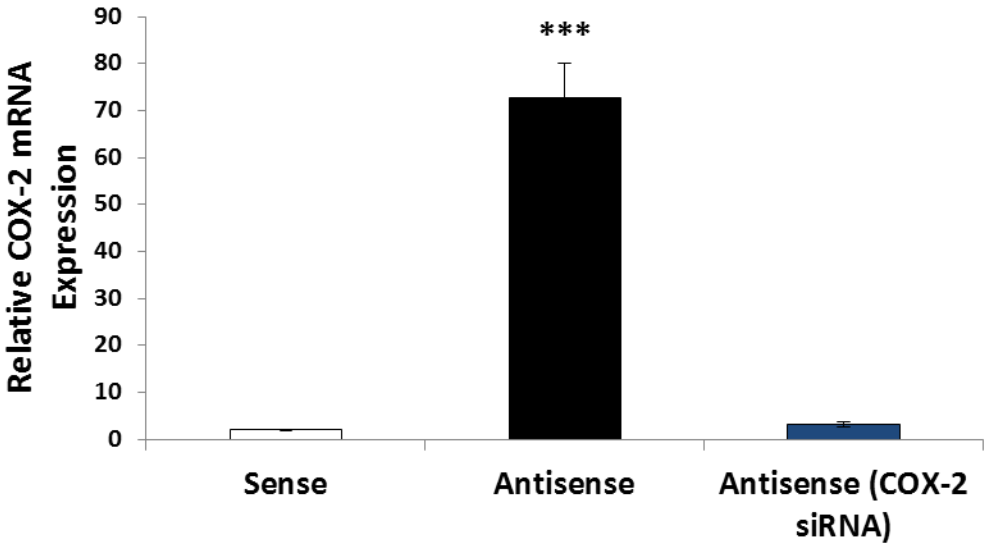

Figure 12: Relative COX-2 mRNA expression in 16HBE cells following COX-2 siRNA knockdown. RNA was extracted and cDNA synthesized from sense and antisense cells (control, treated with a scrambled siRNA) along with antisense cells treated with an siRNA directed against COX-2 as described in the experimental procedures. qRT-PCR was performed using primers for the mRNA sequences of cyclooxygenase-2 (COX-2). Relative expression was determined by the $\Delta\Delta C_T$ method using ribosomal protein *RPLP0* as a control. The COX-2 siRNA treatment was very effective and reduced COX-2 mRNA expression in the antisense cells to sense levels. Bars represent mean ± SEM (n = 3), *** $P<0.001$.

Metabolism of ^{14}C radiolabeled AA to downstream fatty acids was then measured. Neither COX-2 inhibition nor knockdown had any effect on AA to 22:5n-6 metabolism, which remained decreased in antisense cells compared to sense cells (Figure 13). Additionally, COX-2 inhibition did not attenuate the difference in metabolism of ^{14}C-EPA to DHA (Figure 14) or ^{14}C-LA to AA (data not shown) between sense and antisense cells. To determine whether the expression levels of the metabolic enzymes correlated with the observed PUFA metabolism, we measured desaturase mRNA expression following COX-2 inhibition. Both Δ5 and Δ6-desaturase remained overexpressed in antisense cells compared to sense cells even after inhibition of prostaglandin synthesis (Figure 15).

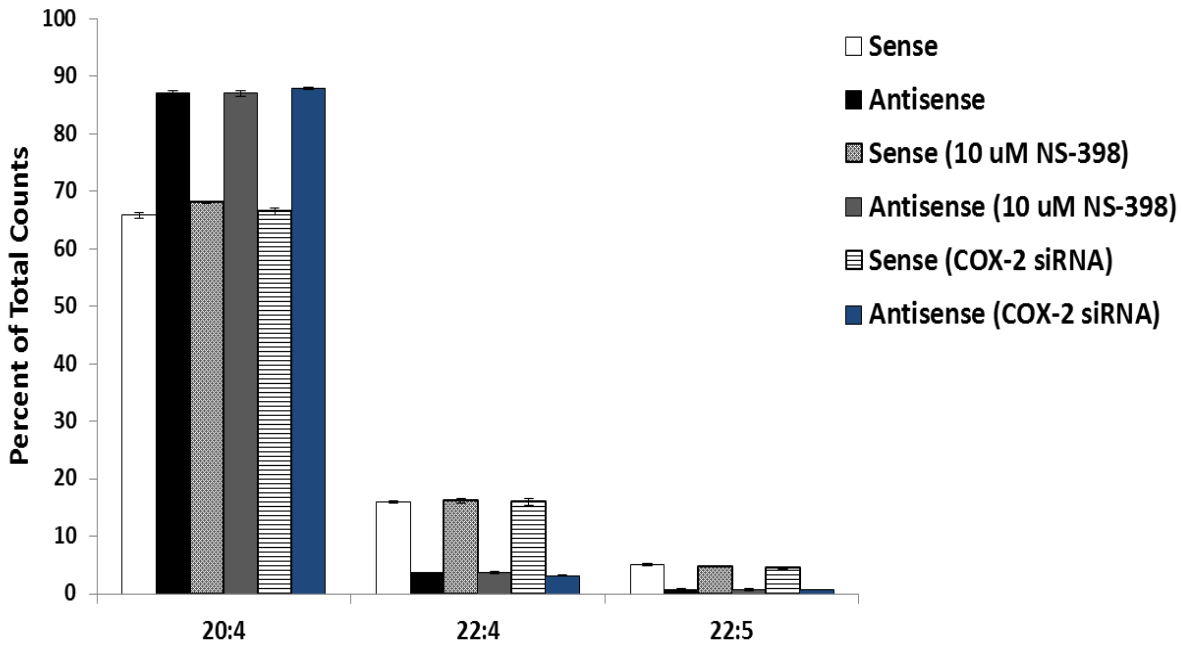

Figure 13: Metabolism of AA through the n-6 pathway in 16HBE cells following COX-2 inhibition. Sense and antisense cells were treated with vehicle (control), COX-2 inhibitor (10 uM NS-398) or COX-2 siRNA, and then incubated with 4.1 μM [^{14}C] AA in reduced-lipid cell culture medium for 4 h as described in the experimental procedures. Levels of radiolabeled AA (20:4n-6), 22:4n-6 and 22:5n-6 were determined by HPLC and data presented as percent of total counts (cpm). Conversion of AA to downstream fatty acids was decreased in the control antisense (CF) cells compared to sense (WT) cells, and this was unchanged by COX-2 chemical inhibition or siRNA knockdown. Bars represent mean ± SEM (n = 3).

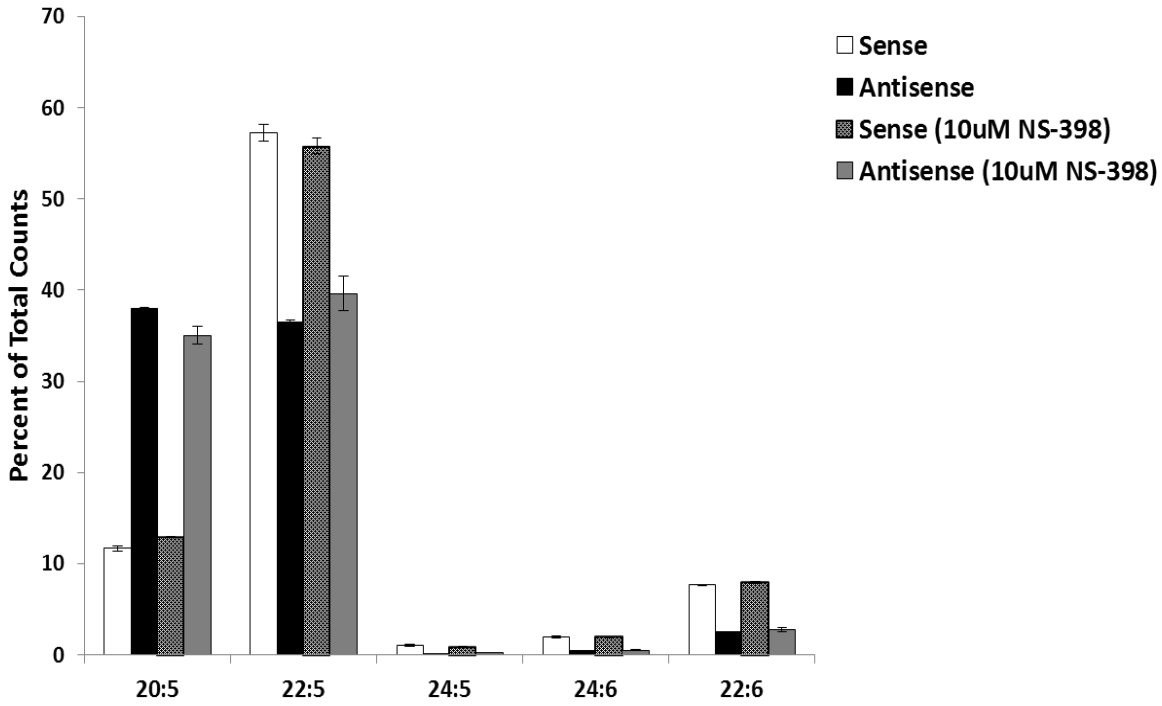

Figure 14: Metabolism of EPA through the n-3 pathway in 16HBE cells following COX-2 inhibition. Sense and antisense cells were treated with vehicle (control) or COX-2 inhibitor (10 uM NS-398), and then incubated with 4.1 μM [^{14}C] EPA in reduced-lipid cell culture medium for 4 h as described in the experimental procedures. Levels of radiolabeled EPA (20:5n-3), 22:5n-3, 24:5n-3, 24:6n-3 and DHA (22:6n-3) were determined by HPLC and data presented as percent of total counts (cpm). Conversion of EPA to downstream fatty acids was decreased in the control antisense (CF) cells compared to sense (WT) cells, and this was unchanged by COX-2 chemical inhibition. Bars represent mean ± SEM (n = 3).

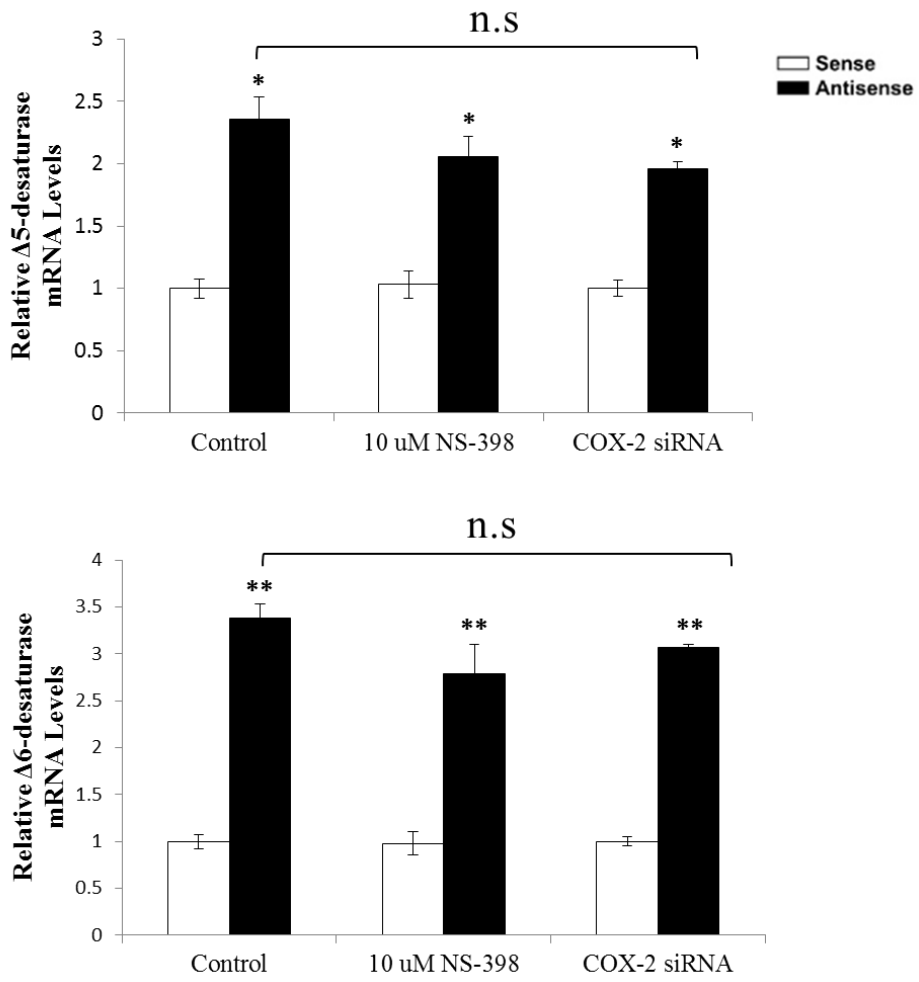

Figure 15: Relative mRNA expression of Δ5 and Δ6-desaturase enzymes in 16HBE cells following COX-2 inhibition. RNA was extracted and cDNA synthesized from sense and antisense cells treated with vehicle (control), 10 uM NS-398 or COX-2 siRNA as described in the experimental procedures. qRT-PCR was performed using primers for the mRNA sequences of Δ5-desaturase (*FADS1*) and Δ6-desaturase (*FADS2*). Relative expression was determined by the $\Delta\Delta C_T$ method using ribosomal protein *RPLP0* as a control. Both Δ5-desaturase and Δ6-desaturase expression levels were significantly increased in antisense (CF) cells compared to sense (WT) cells, even after COX-2 inhibition or knockdown. Bars represent mean ± SEM (n = 3). * P<0.05, ** P< 0.01 for sense vs. antisense cells. n.s (not significant).

To complement our in vitro cell culture experiments, we studied PUFA metabolism in a well-characterized CF knockout mouse model. This model ($cftr^{tm1Unc}$) was developed by introducing a stop codon in exon 10 of CFTR, thus disrupting the gene [115]. These mice share many features with human CF patients including failure to thrive, intestinal obstruction, small intestine bacterial overgrowth [90] and altered gastrointestinal motility [116], making them an appropriate model to use. Failure to thrive, indicated by consistently lower body weight and higher post-natal mortality, was noted in our CF knockout mice, compared to WT mice (Figure 16).

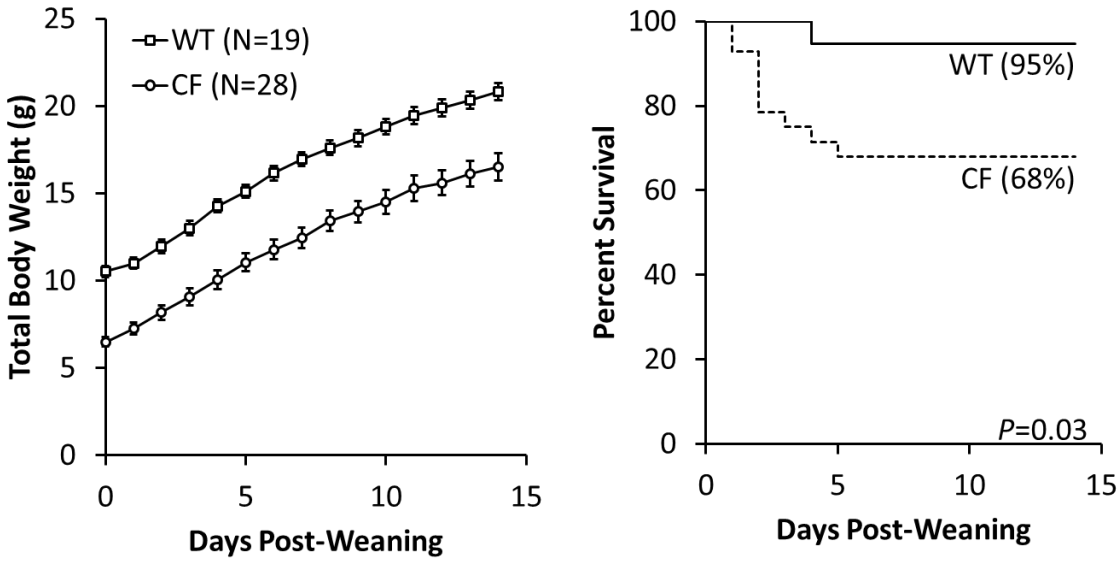

Figure 16: Total body weight and percent survival in $cftr^{tm1Unc}$ mice. CF mice weighed less than WT mice throughout the course of the study, indicative of failure to thrive. Additionally, CF mice had higher mortality rates within the first few days post-weaning compared to WT mice.

To examine if PUFA abnormalities existed in the CF mice, fatty acids were extracted and methylated from organs clinically affected in CF as well as from organs not affected in CF, and analyzed by GC-MS as described in the experimental procedures. Fatty acid alterations including decreased LA and DHA, and increased AA were found specifically in CF-related organs such as the lung, pancreas and ileum of CF mice (Table 2). This correlated with increased metabolism of LA to AA in these organs, as evidenced by the higher AA / LA ratios, and corresponded to increased mRNA expression of Δ5 and Δ6-desaturase and elovl5 enzymes in CF mice compared to WT (Figure 17). Further evidence that these PUFA alterations were caused by upregulation of PUFA metabolic enzymes was found in organs not commonly affected in CF such as the heart, kidney and liver. There was no difference in desaturase and elongase expression between WT and CF mice in these organs (Figure 18), and consequently PUFA levels were similar in WT and CF mice (Table 3). This suggests that PUFA abnormalities in CF are indeed associated with increased expression and activity of desaturase and elongase enzymes, and confirms the results from our two in vitro cell culture models. Moreover, it suggests that the PUFA abnormalities in CF mice may be related to loss of CFTR function since they occur specifically in organs that contain high levels of CFTR-expressing cells.

Table 2: Total fatty acid levels in the lung, ileum and pancreas of WT versus CF mice

	Lung		**Ileum**		**Pancreas**	
	WT	CF	WT	CF	WT	CF
Saturated						
14:0	2.34±0.12	2.29±0.15	1.11±0.07	0.84±0.12	1.07±0.07	0.74±0.06
16:0	30.12±0.62	31.16±0.36	22.20±0.33	19.66±0.06	28.65±0.32	27.45±0.31
18:0	13.67±0.25	15.51±1.03	15.38±0.60	21.16±1.18*	15.04±0.38	17.04±0.29*
20:0	0.19±0.01	0.20±0.01	0.19±0.01	0.35±0.05	0.08±0.00	0.09±0.00
22:0	0.19±0.02	0.18±0.02	0.15±0.02	0.39±0.10	0.04±0.00	0.05±0.01
n-3						
18:3	0.25±0.02	0.25±0.08	0.85±0.19	0.47±0.09	0.32±0.02	0.22±0.02
20:3	0.01±0.00	0.02±0.01	n.d.	n.d.	0.02±0.00	0.01±0.00
20:5	0.21±0.02	0.15±0.02	0.48±0.03	0.58±0.06	1.18±0.04	1.05±0.11
22:5	1.29±0.09	1.18±0.10	0.25±0.03	0.33±0.07	0.73±0.01	0.72±0.03
22:6	**4.32±0.17**	**2.24±0.14***	**4.01±0.13**	**2.06±0.14***	**3.00±0.08**	**1.40±0.05***
n-6						
18:2	**8.77±0.72**	**5.45±0.13***	**18.95±0.18**	**15.64±0.53***	**17.38±0.12**	**13.69±0.28***
20:2	0.32±0.05	0.43±0.08	0.28±0.04	0.30±0.10	0.34±0.02	0.46±0.08
18:3	0.27±0.02	0.20±0.04	0.22±0.02	0.24±0.01	0.21±0.01	0.32±0.05
20:3	1.39±0.09	1.25±0.16	2.03±0.26	2.88±0.13	1.56±0.04	2.20±0.32
20:4	**7.73±0.30**	**11.06±0.25***	**5.56±0.08**	**9.65±0.50***	**10.94±0.29**	**14.91±0.50***
22:4	2.09±0.14	2.30±0.32	0.34±0.05	0.59±0.07	0.38±0.01	0.53±0.05
22:5	0.32±0.02	0.47±0.08	0.09±0.01	0.16±0.02	0.28±0.01	0.44±0.05
n-7						
16:1	4.59±0.47	5.72±0.66	4.60±0.27	2.72±0.23*	2.97±0.22	1.82±0.22
18:1	4.32±0.57	2.74±0.07	4.34±0.37	6.78±0.92	2.95±0.06	3.47±0.09*
20:1	0.03±0.01	0.04±0.01	0.12±0.02	0.41±0.04	0.04±0.00	0.03±0.00
n-9						
18:1	16.79±0.44	16.41±1.35	18.12±0.57	14.11±0.55*	12.27±0.37	12.77±0.39
20:1	0.48±0.01	0.45±0.04	0.41±0.04	0.41±0.04	0.22±0.01	0.23±0.02
22:1	0.18±0.01	0.20±0.03	0.12±0.02	0.24±0.04	0.04±0.00	0.07±0.02
20:3	0.13±0.02	0.10±0.02	0.20±0.02	0.29±0.03	0.29±0.06	0.29±0.09

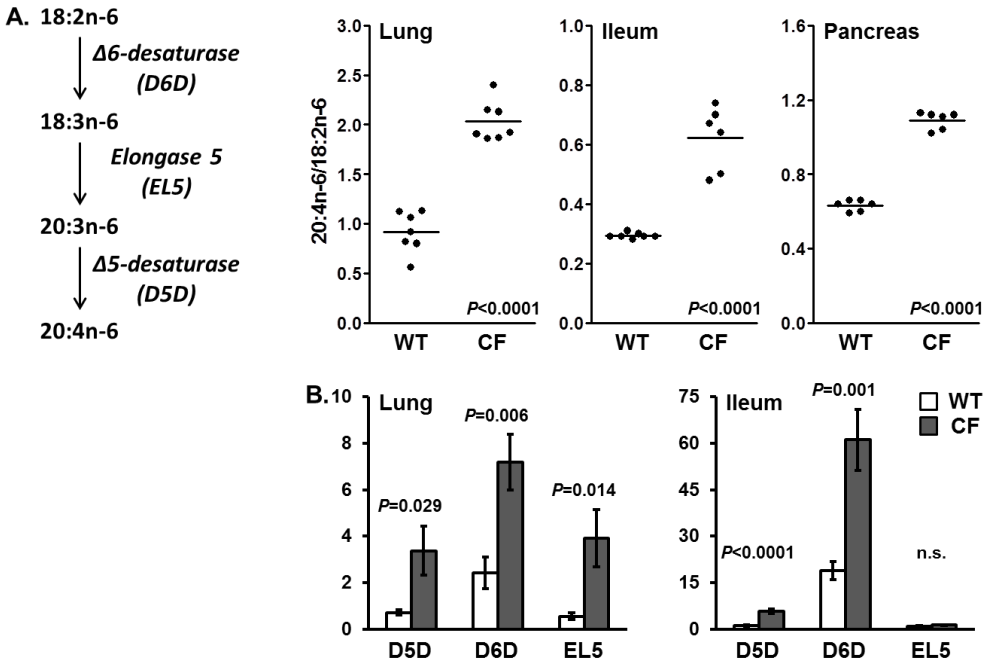

Figure 17: A. LA to AA metabolism in CF-related organs of cftr^{tm1Unc} mice. Increased metabolism of LA to AA was observed in the lung, pancreas and ileum of CF mice compared to WT mice. **B. Relative mRNA expression of PUFA metabolic enzymes in lung and ileum of cftr^{tm1Unc} mice.** RNA was extracted and cDNA synthesized from the lung and ileum of WT and CF mice as described in the experimental procedures. qRT-PCR was performed using commercial Taqman probes for the mRNA sequences of Δ5-desaturase (D5D), Δ6-desaturase (D6D) and elongase 5 (EL5) . Relative expression was determined by the $\Delta\Delta C_T$ method using GAPDH as a control. Consistent with the increased LA to AA metabolism, Δ5-desaturase, Δ6-desaturase and elongase 5 mRNA expression levels were increased in the lung and ileum of CF mice compared to WT mice. The expression levels of these enzymes was not studied in the pancreas due to insufficient amount of tissue for RNA extraction. Bars represent mean ± SEM (n = 6 or 7 mice).

Table 3: Total fatty acid levels in the liver, heart and kidney of WT versus CF mice

	Liver		**Heart**		**Kidney**	
	WT	CF	WT	CF	WT	CF
Saturated						
14:0	0.73±0.11	0.95±0.21	0.68±0.08	0.64±0.13	0.75±0.05	0.74±0.16
16:0	23.53±0.22	23.90±1.05	17.42±0.43	16.72±0.87	23.19±0.46	23.05±0.94
18:0	11.80±0.53	12.54±1.40	17.88±0.32	19.06±0.69	14.96±0.26	15.80±0.57
20:0	0.18±0.03	0.04±0.00*	0.15±0.01	0.11±0.01	0.20±0.01	0.19±0.01
22:0	n.d.	n.d.	0.05±0.01	0.04±0.00	0.05±0.00	0.05±0.00
n-3						
18:3	0.73±0.08	0.67±0.11	0.32±0.03	0.23±0.05	0.30±0.03	0.27±0.04
20:3	0.04±0.00	0.04±0.00	0.02±0.00	0.03±0.00	0.03±0.01	0.06±0.01
20:5	0.49±0.02	0.38±0.03	0.11±0.00	0.09±0.01	0.38±0.02	0.39±0.05
22:5	0.45±0.01	0.55±0.14	1.65±0.09	1.46±0.11	1.23±0.07	0.75±0.09*
22:6	**8.20±0.72**	**8.94±1.14**	**16.43±0.97**	**18.89±1.44**	**15.40±0.26**	**12.65±0.84**
n-6						
18:2	**14.15±0.39**	**13.94±0.42**	**15.38±0.48**	**14.62±0.42**	**10.21±0.13**	**10.62±0.39**
20:2	0.21±0.01	0.27±0.02	0.50±0.02	0.64±0.10	0.57±0.02	0.63±0.05
18:3	0.33±0.02	0.27±0.03	0.20±0.01	0.15±0.01*	0.15±0.01	0.12±0.01
20:3	1.65±0.09	1.21±0.11	1.27±0.06	1.04±0.06	1.54±0.09	1.10±0.07
20:4	**9.29±0.72**	**10.85±1.60**	**8.97±0.26**	**9.75±0.48**	**14.37±0.37**	**16.52±0.97**
22:4	0.20±0.01	0.34±0.05	0.57±0.02	0.67±0.03	0.42±0.02	0.46±0.05
22:5	0.19±0.01	0.32±0.04	1.04±0.06	1.40±0.13	0.44±0.03	0.41±0.04
n-7						
16:1	4.23±0.33	3.63±0.70	1.51±0.28	1.22±.046	1.88±0.21	1.82±0.44
18:1	2.76±0.12	2.60±0.19	3.45±0.06	3.41±0.09	2.88±0.04	3.00±0.02
20:1	0.09±0.01	0.03±0.01*	0.10±0.01	0.06±0.01	0.08±0.01	0.05±0.01*
n-9						
18:1	20.12±1.33	18.00±2.53	11.67±0.78	9.27±1.20	10.55±0.46	10.92±0.83
20:1	0.40±0.03	0.32±0.05	0.40±0.01	0.31±0.03	0.28±0.01	0.27±0.02
22:1	0.03±0.00	0.02±0.00	0.07±0.01	0.05±0.01	0.04±0.01	0.04±0.01
20:3	0.20±0.01	0.18±0.02	0.16±0.01	0.12±0.03	0.11±0.02	0.09±0.01

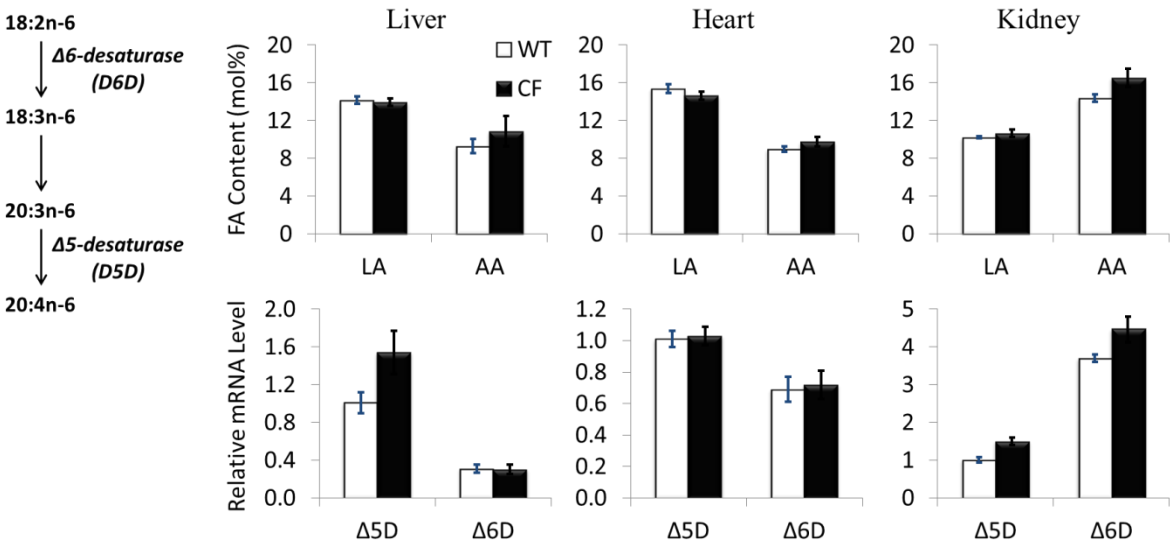

Figure 18: LA to AA metabolism in non CF-related organs and the corresponding PUFA metabolic enzyme expression in cftr^{tm1Unc} mice. Total LA and AA levels were measured in the liver, heart and kidney of WT and CF mice by GC-MS as described in the experimental procedures. There was no significant difference in the levels of these fatty acids in WT or CF mice. Relative mRNA expression of the enzymes involved in the metabolism of LA to AA was measured by qRT-PCR using commercial Taqman probes for the mRNA sequences of Δ5-desaturase (D5D) and Δ6-desaturase (D6D). Relative expression was determined by the $\Delta\Delta C_T$ method using GAPDH as a control. In each of the three organs examined, the expression levels of both Δ5 and Δ6-desaturase were similar in WT and CF mice. Bars represent mean ± SEM (n = 6 or 7 mice).

In addition to the classic PUFA alterations, changes in the levels of certain saturated and monounsaturated fatty acids were observed in CF mice compared to WT mice. The levels of 18:0 were higher in the ileum and pancreas of CF mice, leading to higher 18:0 to 16:0 ratios (Figure 19). 18:1n-7 was also increased in CF pancreas and ileum, although the increase was only statistically significant in the pancreas (Table 2). This suggests that the activity of elongase 6, which is the enzyme responsible for these reactions, is increased in the ileum and pancreas of CF mice. Conversely, the ratios of 16:1n-7 to 16:0 and 18:1n-9 to 18:0 were decreased in the ileum of CF mice, suggesting a decrease in the expression and activity of Δ9-desaturase.

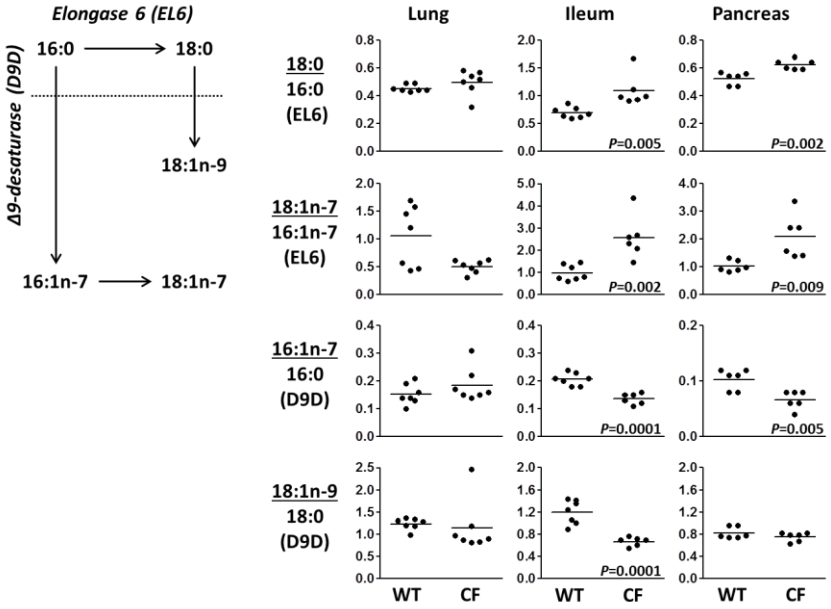

Figure 19: Metabolism of saturated and monounsaturated fatty acids in the lung, ileum and pancreas of cftr^{tm1Unc} mice. Increased metabolism of 16:0 to 18:0, and 16:1n-7 to 18:1n-7, was observed in the ileum and pancreas of CF mice, suggesting increased elovl6 activity. Metabolism of 16:0 to 16:1n-7 was decreased in the ileum and pancreas of CF mice, while 18:0 to 18:1n-9 metabolism was decreased in the CF ileum, indicating decreased Δ9-desaturase activity. No significant differences in saturated or monounsaturated fatty acids were detected in the lung of CF mice compared to WT mice. Data are expressed as mean ± SEM (n = 6 or 7 mice).

Analogous to CF bronchial epithelial cells in culture, lung epithelial cells from CF mice showed elevated expression levels of COX-2 and 5-LOX. Moreover, the mRNA expression of microsomal prostaglandin E_2 synthase-1 (mPGES-1) was upregulated in the lungs of CF mice compared to WT (Figure 20). mPGES-1 is an inducible terminal prostaglandin synthase that works in concert with COX-2 to produce inflammatory PGE_2. The fact that the expression of these enzymes is increased points to the possibility of increased inflammation in the lungs of CF mice. On the other hand, the mRNA expression of COX-2, 5-LOX and mPGES-1 did not differ between WT and CF mice in organs unaffected by CF including heart and liver (Figure 20). In fact, 5-LOX expression in heart tissue of CF mice was downregulated compared to WT mice while in the liver, mPGES-1 expression was lower in CF than in WT mice. This suggests that the production of pro-inflammatory mediators may be increased in CF mice, and specifically in CF-related organs that exhibit the characteristic PUFA abnormalities.

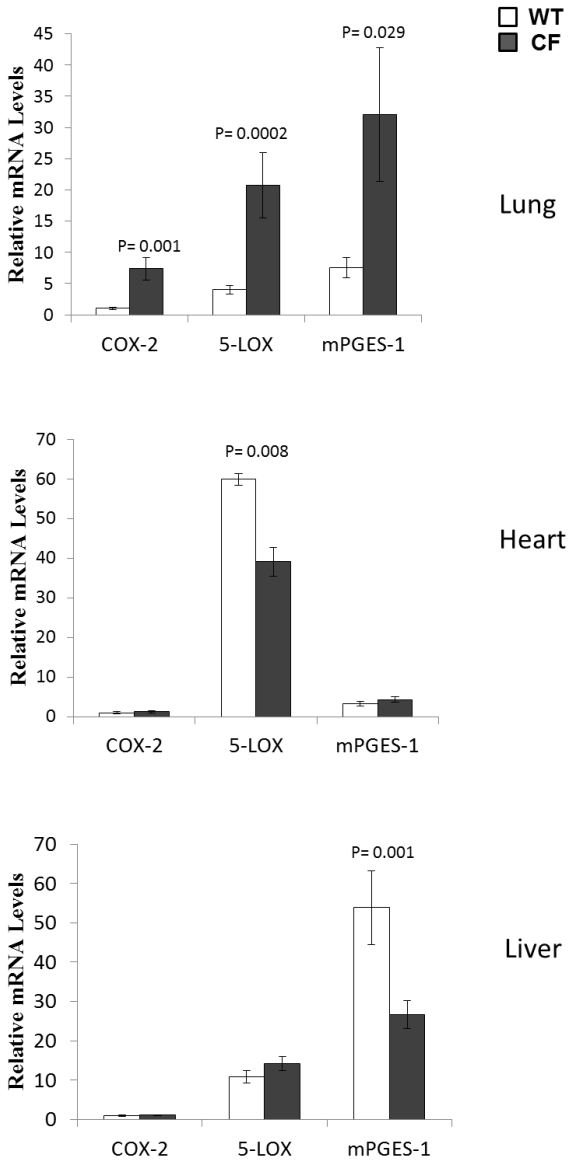

Figure 20: Relative mRNA expression of eicosanoid synthesis enzymes in cftr^{tm1Unc} mice. RNA was extracted and cDNA synthesized from the lung, heart and liver of WT and CF mice as described in the experimental procedures. qRT-PCR was performed using commercial Taqman probes for the mRNA sequences of cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX) and microsomal PGE_2 synthase-1 (mPGES-1). Relative expression was determined by the $\Delta\Delta C_T$ method using GAPDH as a control. COX-2, 5-LOX and mPGES-1 were all significantly overexpressed in the lungs of CF mice compared to WT mice. The expression levels of these enzymes was either similar or decreased in the heart and liver of CF mice compared to WT. Bars represent mean ± SEM (n = 6 or 7 mice).

Discussion

Mechanisms of PUFA alterations in CF

It is widely established that there are decreased levels of LA and DHA, and increased levels of AA in blood and tissue of CF patients, as well as in CF experimental models [8-16]. These fatty acid alterations are independent of nutritional status, and are consistent enough such that plasma fatty acid measurements can be used to differentiate CF versus non-CF patients [85]. Although PUFA abnormalities have been well studied and documented in the CF literature, the underlying mechanisms responsible for the PUFA changes are unknown. In this chapter, we show that these changes are caused by increased expression and activity of the enzymes that metabolize PUFAs, including Δ5 and Δ6-desaturase. Increased expression and activity of both Δ5 and Δ6-desaturase consume LA in its conversion to AA, leading to the lower LA and increased AA levels seen in CF. These findings have been confirmed in two different CF cell culture models (16HBE sense and antisense cells in which CFTR expression is modulated by expression of a sense or antisense transgene; and IB3 and C38 cells derived from a CF patient with the common ΔF508 mutation) and in vivo in the $cftr^{tm1Unc}$ knockout mouse model. In CF mice, PUFA abnormalities are found specifically in the organs most affected by CF including lung, pancreas and ileum and they correspond to higher desaturase and elongase expression (Figure 17). Moreover in organs that express little or no cftr protein such as heart, kidney and liver, the mRNA expression of Δ5 and Δ6-desaturase is similar in WT and CF mice (Figure 18). Consequently, there are no differences in PUFA levels in these organs. This further proves that the fatty acid alterations commonly found in CF result from dysregulation of PUFA metabolic enzymes.

Through our systematic, parallel comparison of the n-6 and n-3 metabolic pathways (Figure 5), we illustrate that metabolism from LA to AA and from LNA to EPA is increased in CF cells. The fact that the early metabolic steps in the n-3 pathway are upregulated excludes relatively decreased activity of the entire n-3 pathway as an explanation for the low DHA levels in CF. However, our experiments demonstrate that the later steps of PUFA metabolism from AA to 22:5n-6 and from EPA to DHA are significantly decreased in CF (Figure 6). While this may account for the low levels of DHA observed in CF, it cannot be explained by the PUFA desaturase and elongase expression profiles. The enzymes involved in metabolism in this lower part of the pathway are elovl2, elovl5 and Δ6-desaturase (Figure 1). Both elovl2 and 5 are expressed at similar levels in WT and CF cells, while Δ6-desaturase is upregulated in CF cells. Based on the enzyme expression levels we might expect to see higher DHA levels in CF and yet this is not the case.

To further assess the step at which metabolism from EPA to DHA is altered in CF, we studied metabolism of the fatty acid immediately downstream of EPA, 22:5n-3, to DHA. The overall metabolism of 22:5n-3 to DHA was equivalent in sense and antisense cells (Figure 8), suggesting that the decreased metabolism of EPA to DHA occurs at the EPA to 22:5n-3 step. In addition to EPA and AA serving as substrates that can be metabolized to longer, more desaturated PUFAs, they can also be metabolized to eicosanoids. Thus, we hypothesized that a portion of EPA and AA is diverted towards eicosanoid synthesis, reducing the amount of substrate to be converted to DHA and 22:5n-6. If this pathway leading to eicosanoid biosynthesis is upregulated in CF, that could explain why DHA levels are so low. Consistent with this hypothesis, we found that

the enzymes involved in eicosanoid synthesis, COX-2 and 5-LOX, were greatly overexpressed in the antisense cells and CF mice (Figures 10 and 20), leading to increased production of PGE_2 and LTB_4 in antisense cells. Furthermore, we observed that there was no difference in COX-2 and 5-LOX mRNA levels in the IB3 and C38 cell line (data not shown), and this correlated with no difference in DHA levels between IB3 and C38 cells. These results suggest a link between increased eicosanoid synthesis and decreased metabolism of AA and EPA. We chose to investigate this link and examine the effect of modulating eicosanoid synthesis on PUFA metabolism. Prostaglandin synthesis was blocked in our cells using either a COX-2 specific inhibitor or a COX-2 siRNA. Contrary to the hypothesis, we found that COX-2 inhibition did not correct the decreased metabolism of AA and EPA to 22:5n-6 and DHA in antisense cells (Figures 13 and 14). It is possible that the AA and EPA that is processed to eicosanoids is in a different metabolically active pool than the ^{14}C radiolabeled AA and EPA that gets metabolized to longer, more desaturated PUFAs. If this is the case, then although inhibition of COX-2 may have an effect on AA and EPA metabolism, it may not be observed because the fatty acids are in the "wrong" fatty acid pools for metabolism to eicosanoids. Alternatively, it is possible that COX-2 inhibition has no effect on PUFA metabolism, as some studies report that only a small percentage (<5%) of AA and EPA is actually converted to eicosanoids upon agonist stimulation [117]. Future studies designed to distinguish the different metabolically active pools of AA and EPA are needed to determine whether overproduction of eicosanoids contributes to the low levels of DHA in CF.

Role of PUFA alterations in CF pathophysiology

Several studies indicate that the degree of PUFA alterations in CF correlates with disease severity [21- 22, 118]. Moreover, it has been reported that correcting the PUFA imbalances in a CF knockout mouse model can reverse some of the pathologic manifestations of disease [13, 14]. These findings imply that PUFA alterations play a key role in CF pathophysiology. One of the common PUFA changes present in CF is increased AA levels. In our studies, we show that the increased AA, coupled with increased expression and activity of COX-2 and 5-LOX results in increased production of pro-inflammatory PGE_2 and LTB_4 in CF cells (Figure 21). Additionally, expression of the EP2 receptor is upregulated in CF cells, which could facilitate the inflammatory actions of PGE_2. In CF mice, we show that AA is increased in CF-related organs such as the lung, and that this is accompanied by increased COX-2, mPGES-1 and 5-LOX expression. This points towards increased production of PGE_2 and LTB_4 in the organs most affected in CF. We believe that the increased concentration of pro-inflammatory eicosanoids can contribute to CF development as these eicosanoids regulate numerous of the physiologic processes that lead to CF, including secretion, gut motility and inflammation. Elevated prostaglandin levels contribute to excessive mucus secretion [119], which is a hallmark of CF. This creates an environment that fosters abnormal bacterial overgrowth and alters gut function. In addition, increased PGE_2 levels are thought to cause uncoordinated smooth muscle contractions, leading to intestinal dysmotility [120-122]. Eicosanoids are also involved in the regulation of inflammation, which plays a vital role in CF disease. Early in life, CF patients begin to exhibit exaggerated inflammatory profiles, particularly in the lungs [123-125]. Over the course

of time, these responses become more persistent and eventually lead to a decline in pulmonary function, destruction of lung tissue and respiratory failure. The increased conversion of AA to pro-inflammatory eicosanoids may also be responsible for stimulating increased metabolism of LA to maintain AA levels, leading to the decreased LA and increased AA seen in CF.

Decreased DHA is another PUFA abnormality found in CF. DHA can be metabolized to potent anti-inflammatory mediators (Figure 9) [126]. That DHA levels are low in CF means there is less initial substrate to be metabolized into resolvins and protectins and this can further shift the inflammatory balance towards a more pro-inflammatory state and contribute to CF pathogenesis. Additionally, DHA levels can influence the eicosanoid profile in CF because DHA competes with pro-inflammatory AA for esterification at the *sn*-2 position of phospholipids. In this way, DHA can act as an anti-inflammatory agent by downregulating the levels of phospholipid-bound AA. We show that DHA levels are significantly decreased in cultured CF cells as well as in CF-related organs of CF mice compared to WT mice (Table 2). Further experiments are needed to determine the levels of anti-inflammatory eicosanoids synthesized from AA, and the levels of DHA-derived resolvins and protectins in CF versus WT mice, in order to verify whether these fatty acid abnormalities really do contribute to inflammation in CF. Experiments to test the effect of exogenous resolvin or protectin treatment, as well as COX-2 and 5-LOX inhibition, on the pathogenesis of CF in our mouse model would also help to answer these questions.

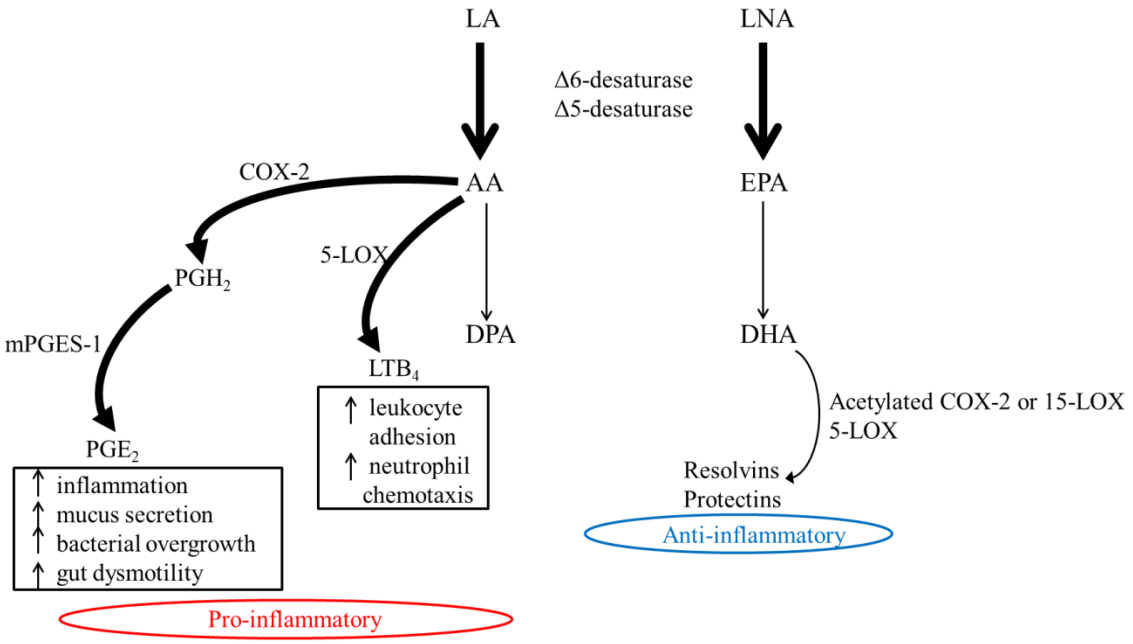

Figure 21: Diagram of polyunsaturated fatty acid and eicosanoid changes in CF. Increased expression and activity of Δ5 and Δ6-desaturase enzymes leads to increased metabolism of LA to AA in CF (dark arrows indicate increased metabolism while light arrows indicate decreased metabolism). This increase in AA levels, coupled with increased expression and activity of COX-2, mPGES-1 and 5-LOX, leads to increased synthesis of pro-inflammatory PGE_2 and LTB_4. This in turn promotes excessive mucus secretion, bacterial overgrowth and increased inflammation, all of which are hallmarks of CF disease. In addition, metabolism of EPA to DHA is decreased in CF, leading to low levels of DHA. This results in less substrate available for conversion to anti-inflammatory resolvins and protectins, as well as less DHA available to compete with AA for phospholipid esterification. Taken together, the above PUFA and eicosanoid alterations contribute to increased inflammation in CF, and may play an important role in CF pathophysiology.

CHAPTER III

EFFECT OF DHA AND EPA SUPPLEMENTATION ON PUFA METABOLISM AND THE PATHOGENESIS OF CYSTIC FIBROSIS

Introduction

DHA deficiency has been suggested to contribute to the pathophysiology of CF partly because supplementing CF mice with large amounts of DHA leads to reversal of CF-related pathology, including relief of pancreatic duct dilation, decrease in stimulated neutrophil accumulation into BAL fluid and normalization of ileal histology. DHA supplementation also corrects the fatty acid abnormalities in lung, pancreas and intestine tissue of CF mice [13]. In the same study, the authors show that this beneficial effect is only apparent when the mouse diet is enriched with DHA and not other PUFAs such as EPA or LNA. Therefore it is possible that certain phenotypic manifestations of CF result from decreased levels of DHA, and that rectifying this by increasing DHA levels and decreasing AA levels leads to normalization of CF phenotype. Nevertheless, the mechanism through which DHA corrects CF fatty acid abnormalities and ultimately reverses the CF phenotype in $cftr^{-/-}$ mice remains unknown. Additionally, it is unclear whether other fatty acids besides DHA can elicit a similar therapeutic effect.

The aim of the experiments described in this chapter was to investigate potential mechanisms by which DHA might correct PUFA alterations in CF and reverse the CF phenotype, and compare this effect to that of other PUFAs. We examined the effect of DHA supplementation on PUFA metabolism in 16HBE cells. The effect of additional fatty acids including EPA, AA, LNA, a monounsaturated fatty acid, oleic acid (OA) and a

saturated fatty acid, palmitic acid (PA) were also tested. To address the in vivo mechanism of DHA function, we studied the effect of DHA and EPA supplementation on PUFA metabolism and CF pathology in $cftr^{tm1Unc}$ mice. In these experiments, the mouse diet was enriched with DHA in free fatty acid (FFA) form or as a triglyceride (TG) in combination with EPA.

Experimental Procedures

Fatty acid supplementation

16HBE sense (WT) and antisense (CF) cells were seeded onto 6-well plates at a density of 3×10^5 and 1×10^5 cells/well respectively. The cells were grown until confluent, then supplemented with 0, 5, 10 or 20 μM fatty acid for 24 h. The fatty acids used for supplementation included FFA DHA, EPA, AA, LNA, OA and PA. To prepare the supplementation media, the FFAs were placed in a glass tube and dried down completely under nitrogen gas. The dried fatty acids were then resuspended in media containing 10% reduced-lipid fetal bovine serum by vortexing and sonication, and added to the cells. After 24 h incubation with or without fatty acid supplementation, the cells were harvested, lipids extracted and methylated and fatty acid composition analyzed by GC-MS. In addition, total RNA was isolated from fatty acid supplemented cells and qRT-PCR used to assess the expression levels of PUFA metabolic enzymes using the primers shown in Table 1. Fatty acid radiolabeling experiments were also performed in cells following FFA supplementation. In these studies, after 24 h fatty acid supplementation, 16HBE cells were incubated with 4.1μM ^{14}C-LA, ^{14}C-LNA, ^{14}C-AA or ^{14}C-EPA for 4h and then harvested. Metabolism of the radiolabeled fatty acids was analyzed by HPLC

coupled to a β-RAM scintillation detector. The methods used for fatty acid extraction, methylation, HPLC or GC-MS analysis and qRT-PCR are outlined in the previous chapter.

Mice

Cftr^{tm1Unc} WT and CF mice were weaned at 23 days of age and placed on one of the two fatty acid supplementation diets described below:

Diet 1: Peptamen + DHA diet (n = 6 or 7 per group). After weaning, the mice were maintained on Peptamen (Nestle Clinical Nutrition, Deerfield, IL) and had access to water *ad libitum* for seven days. On day 30, the mice were continued on the Peptamen diet with or without 40 mg per day of FFA DHA, prepared as a stable emulsion in Peptamen. The mice were maintained on the Peptamen + DHA diet for seven days and then sacrificed.

Diet 2: Peptamen AF diet (n = 6 or 7 per group). After weaning, the mice were placed on either the Peptamen diet (control) or on a variation of Peptamen containing a combination of TG DHA and EPA (Peptamen Advanced Formulation, AF) (Nestle Clinical Nutrition, Deerfield, IL) for 14 days then sacrificed.

WT and CF mice were housed individually to ensure each mouse received the correct dosage of the fatty acid supplement. Fresh peptamen was provided daily, at which time the weight of the mice as well as the volume of Peptamen consumed was measured. Mice were sacrificed two weeks post-weaning and various organs as well as plasma collected for fatty acid and gene expression analysis as described in the previous chapter.

Histopathology

For sample preparation, lung and ileum tissues were submerged in 10% neutral buffered formalin and fixed overnight. The tissues were processed routinely, embedded in paraffin, sectioned at 4 microns, and stained with hematoxylin and eosin. Slides were evaluated on an Olympus BX41 microscope by a pathologist blinded to the composition of the treatment groups. To analyze impaction of paneth cell secretions in the intestinal villi, the slides were scanned on an Aperio Scanscope CS2 (Aperio, Vista, CA) and scored according to the following criteria:

0 - no impaction of secretory products in the crypts

1 - impacted secretory material in the deep portion of the crypts in <10% of the crypts

2 - impaction of secretory material in the deep portion of the crypts in 10-30% of crypts, with rare accumulation of material in the bottom third of the villus

3 - impaction of secretory material in 30-70% of the crypts, with accumulation of material commonly observed up to the top third of the crypt

4 - impaction of secretory material in >70% of the crypts, with frequent dilation of the crypts and presence of material in the top third of the villus

Additionally, periodic acid schiff staining was performed to identify goblet cells in the small intestine. The presence (marked, moderate or mild), or absence of goblet cell hyperplasia was recorded.

Results

16HBE sense (WT) and antisense (CF) cells were treated with 20 μM DHA for 24 h and total fatty acid composition measured by GC-MS. The common PUFA alterations were present in untreated CF cells (Figure 22). These included decreased LA with increased 18:3n-6, 20:3n-6, AA and EPA, suggestive of increased metabolism of LA to AA and LNA to EPA. Levels of 22:4n-6, 22:5n-6 and DHA were also significantly decreased in CF cells compared to WT cells, indicating decreased metabolism in the later steps of the n-3 and n-6 pathways. The higher levels of 22:5n-3 observed in CF cells were likely explained by the fact that the initial substrate, EPA, was much higher in CF cells and not because of increased metabolism. Accordingly, the 22:5n-3/EPA ratio was markedly lower in CF cells than in WT cells (2.0 ± 0.03 versus 10.5 ± 0.6; $P < 0.001$). DHA-treatment caused an increase in DHA levels in both WT and CF cells, but more so in CF cells. Moreover, supplementing the cells with DHA led to a significant decrease in AA levels in CF cells, decreasing them to WT levels.

To assess whether this change in AA levels was caused by decreased metabolism of LA to AA, DHA-treated cells were incubated with ^{14}C-LA and metabolism of LA to AA measured (Figure 23A). DHA treatment caused a dose-dependent decrease in the production of radiolabeled AA, accompanied by a dose-dependent increase in LA. This was observed in both WT and CF cells, with a greater effect seen in CF cells and indicated that DHA could suppress the metabolism of LA to AA. Metabolism of ^{14}C-LNA to EPA was also decreased following DHA supplementation (Figure 23B).

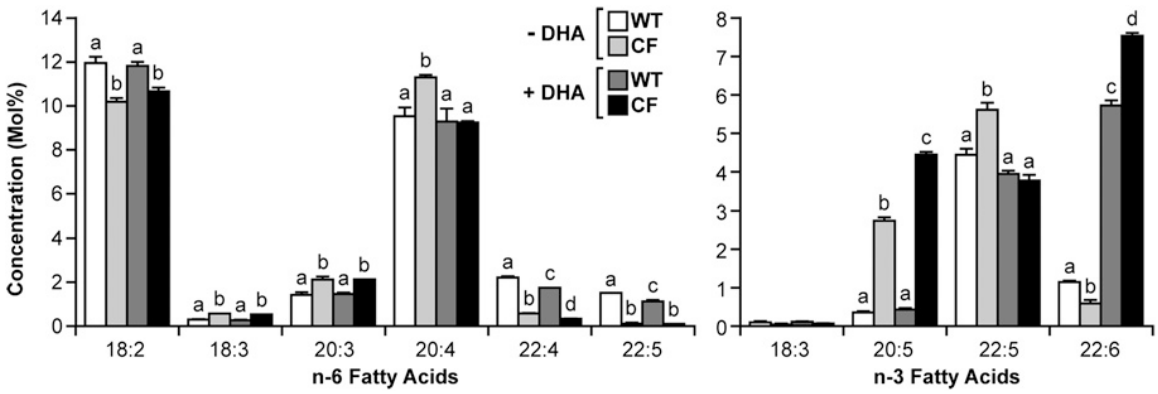

Figure 22: Total fatty acid composition of 16HBE cells with or without DHA supplementation. WT and CF cells were incubated with or without 20 µM DHA for 24 h, then harvested and total fatty acid composition measured by GC-MS as described in the experimental procedures. Individual fatty acid concentrations are expressed as molar percentage (mol %) of total fatty acids. Supplementation with DHA caused an increase in DHA levels in both WT and CF cells, as well as an increase in EPA levels specifically in CF cells. A significant decrease in AA levels was also noted in CF cells. Bars represent mean ± SEM (n = 3), and unlike letters indicate significant differences in pair-wise comparisons.

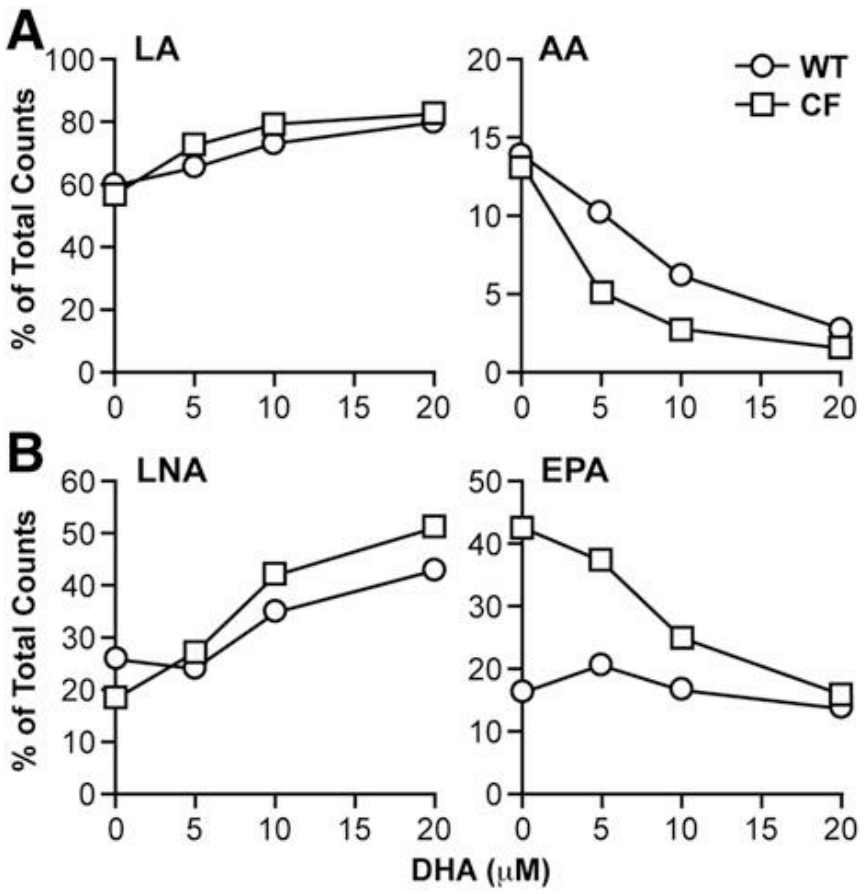

Figure 23: Metabolism of LA and LNA through the n-6 and n-3 pathways in 16HBE cells supplemented with DHA. WT and CF cells were supplemented with 0, 5, 10, or 20 μM DHA for 24 h. Following DHA supplementation, the cells were incubated with 4.1 μM ^{14}C-LA (A) or 4.1 μM ^{14}C-LNA (B) for 4 h and then harvested. Levels of radiolabeled LA and AA (A) or LNA and EPA (B) were determined by HPLC as described in the experimental procedures. Data are expressed as percentage of total counts (cpm). DHA supplementation suppressed metabolism of LA and LNA in WT and CF cells, but more so in CF cells. Each point represents mean ± SEM (n = 3).

DHA treatment did not correct the decreased metabolism of AA and EPA in CF cells. Instead it caused a minimal decline in the already low levels of 22:5n-6 (DPA) and DHA (Figure 24). Additionally, metabolism of ^{14}C-AA to DPA was significantly reduced in WT cells, with the greatest effect seen after treatment with 20 µM DHA.

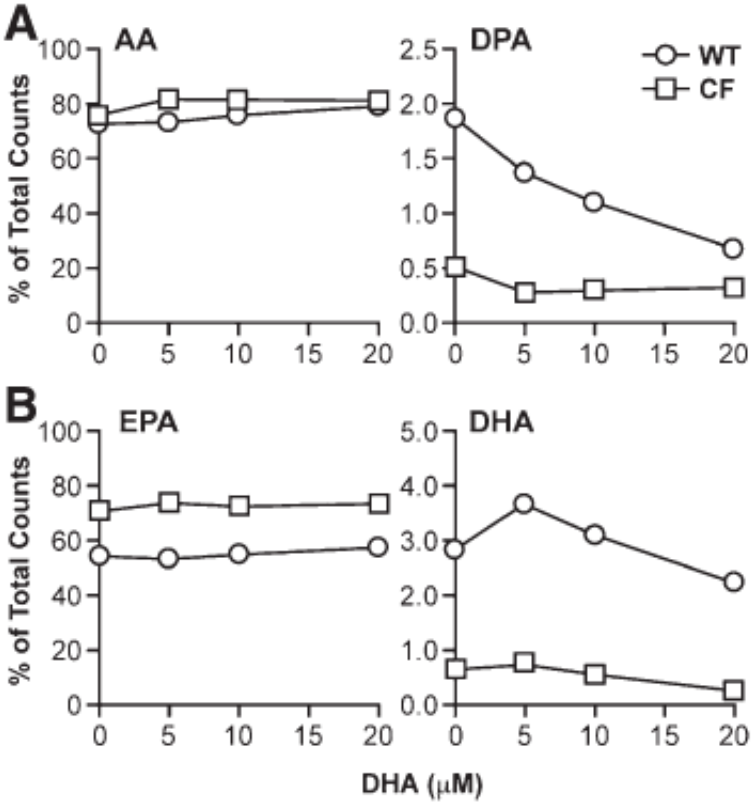

Figure 24: Metabolism of AA and EPA through the n-6 and n-3 pathways in 16HBE cells supplemented with DHA. WT and CF cells were supplemented with 0, 5, 10, or 20 µM DHA for 24 h. Following DHA supplementation, the cells were incubated with 4.1 µM ^{14}C-AA (A) or 4.1 µM ^{14}C-EPA (B) for 4 h and then harvested. Levels of radiolabeled AA and DPA (A) or EPA and DHA (B) were determined by HPLC as described in the experimental procedures. Data are expressed as percentage of total counts (cpm). DHA supplementation caused a modest decline in AA and EPA metabolism in CF cells while metabolism of AA to DPA was drastically reduced in WT cells. Each point represents mean ± SEM (n = 3).

Unexpectedly, we observed that the levels of EPA increased significantly in CF, but not in WT cells following DHA treatment (Figure 22). In contrast, in the corresponding n-6 part of the pathway, levels of AA were decreased following DHA treatment. The change in EPA levels could not be explained by increased metabolism of LNA to EPA as this part of the pathway was suppressed by DHA treatment (Figure 23B). Previous reports have described a process known as DHA retroconversion whereby DHA is converted to EPA through a series of enzymatic reactions [127-129]. To determine whether DHA retroconversion was responsible for the increased EPA levels, we measured retroconversion in our cells [ΔEPA / (ΔEPA + ΔDHA)] and found that this process was 20X greater in CF than in WT cells.

To investigate the specificity of the DHA effect, 16HBE cells were also supplemented with increasing concentrations of EPA and fatty acid composition and metabolism measured. EPA treatment led to an increase in EPA levels in both WT and CF cells, albeit more so in CF cells (Figure 25). This was accompanied by an increase in downstream metabolites of EPA including 22:5n-3 and DHA. However, these increases were more significant in WT compared to CF cells such that the levels of DHA remained lower in CF cells following EPA supplementation. EPA treatment also caused a decrease in AA levels in WT and CF cells. In terms of PUFA metabolism, EPA supplementation resulted in a dose-dependent decrease in conversion of ^{14}C-LA to AA and ^{14}C-LNA to EPA (Figure 26). This effect was greater in CF than in WT cells and was similar to what was observed following DHA treatment.

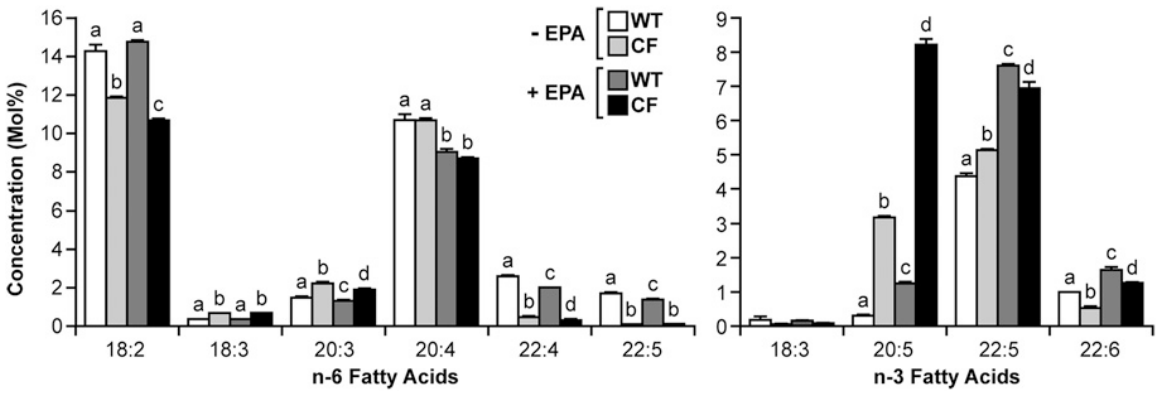

Figure 25: Total fatty acid composition of 16HBE cells with or without EPA supplementation. WT and CF cells were incubated with or without 20 µM EPA for 24 h, then harvested and total fatty acid composition measured by GC-MS as described in the experimental procedures. Individual fatty acid concentrations are expressed as molar percentage (mol %) of total fatty acids. Supplementation with EPA caused an increase in EPA levels in both WT and CF cells, as well as an increase in downstream metabolites of EPA including 22:5n-3 and DHA. A significant decrease in AA levels was also noted in both WT and CF cells. Bars represent mean ± SEM (n = 3), and unlike letters indicate significant differences in pair-wise comparisons.

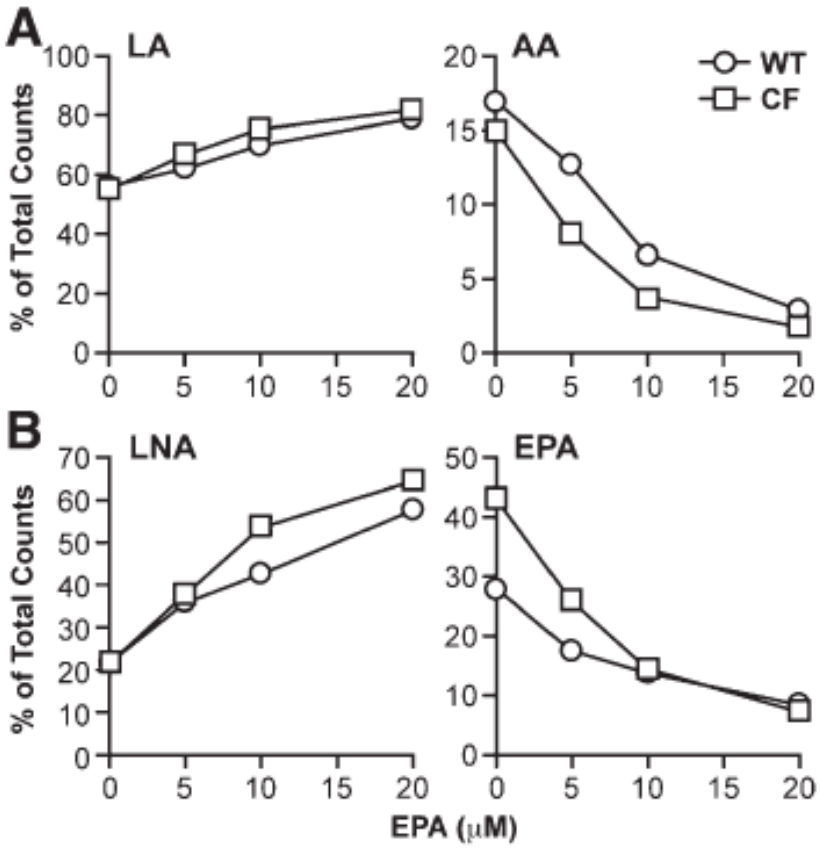

Figure 26: Metabolism of LA and LNA through the n-6 and n-3 pathways in 16HBE cells supplemented with EPA. WT and CF cells were supplemented with 0, 5, 10, or 20 μM EPA for 24 h. Following EPA supplementation, the cells were incubated with 4.1 μM ^{14}C-LA (A) or 4.1 μM ^{14}C-LNA (B) for 4 h and then harvested. Levels of radiolabeled LA and AA (A) or LNA and EPA (B) were determined by HPLC as described in the experimental procedures. Data are expressed as percentage of total counts (cpm). EPA supplementation suppressed metabolism of LA and LNA in WT and CF cells, but more so in CF cells. Each point represents mean ± SEM (n = 3).

Metabolism of ^{14}C-AA to ^{14}C-DPA was significantly reduced in EPA-treated WT cells, with only a modest decrease observed in CF cells (Figure 27). The effect of EPA supplementation on metabolism of ^{14}C-EPA to ^{14}C-DHA appeared to be concentration-dependent. Specifically, metabolism was increased in both WT and CF cells at low EPA concentrations (5 μM), but returned to baseline levels when the cells were supplemented with higher concentrations of EPA (20 μM). From these experiments, we concluded that there was little difference in the effect of DHA versus EPA on PUFA metabolism. The action of additional fatty acids (AA, LNA, OA and PA) on PUFA metabolism was tested. We discovered that AA and LNA supplementation had a similar effect on metabolism as DHA and EPA, but to a much lesser extent. In contrast, OA and PA did not have any effect on PUFA metabolism (data not shown). This suggests there is a hierarchy in terms of the effect of these fatty acids on PUFA metabolism. PUFAs such as DHA and EPA have the greatest ability to suppress LA to AA metabolism, followed by AA, LNA, OA and lastly, PA. This order [DHA and EPA > AA > LNA > OA > PA] implies that the effect of these fatty acids on PUFA metabolism may be regulated by the number of double bonds in the supplemented fatty acid.

We postulated that the mechanism through which DHA and EPA affected PUFA metabolism in CF cells was by acting on the expression and/or activity of PUFA metabolic enzymes. The mRNA expression levels of Δ5 and Δ6 desaturase, elovl2 and elovl5 were measured in WT and CF cells after DHA and EPA supplementation. In the absence of DHA or EPA, the expression levels of both Δ5 and Δ6 desaturase were upregulated in CF cells (Figure 28A and B). However, as little as 5 μM DHA or EPA was sufficient to reduce desaturase expression in CF cells to the level of WT cells, while

elovl2 and 5 expression levels were either slightly decreased or unchanged (Figure 28C and D). AA and LNA supplementation also reduced the expression of the desaturase enzymes but to a lesser degree than DHA and EPA (for instance, AA and LNA reduced Δ6-desaturase mRNA by 48% and 45% respectively, whereas DHA and EPA reduced expression by 68% and 73% respectively), while OA and PA had no significant effect on desaturase expression (Figure 28E). Thus we established that the suppression of PUFA metabolism following DHA and EPA supplementation is attributable to downregulation of Δ5 and Δ6 desaturase gene expression.

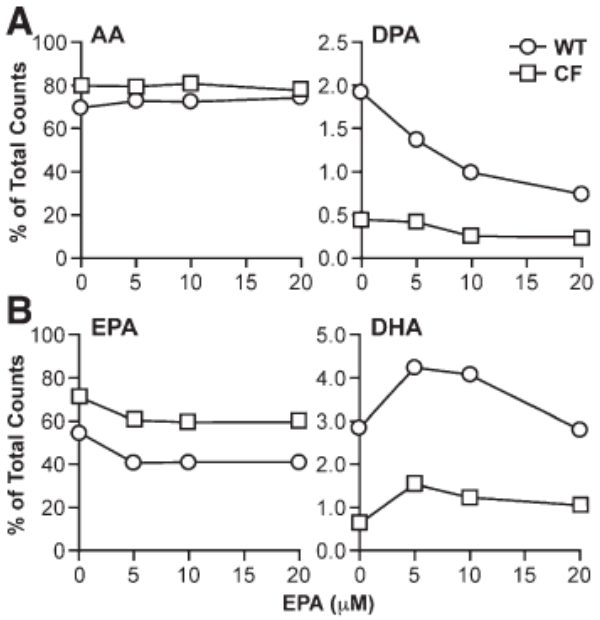

Figure 27: Metabolism of AA and EPA through the n-6 and n-3 pathways in 16HBE cells supplemented with EPA. WT and CF cells were supplemented with 0, 5, 10, or 20 μM EPA for 24 h. Following EPA supplementation, the cells were incubated with 4.1 μM ^{14}C-AA (A) or 4.1 μM ^{14}C-EPA (B) for 4 h and then harvested. Levels of radiolabeled AA and DPA (A) or EPA and DHA (B) were determined by HPLC as described in the experimental procedures. Data are expressed as percentage of total counts (cpm). EPA supplementation caused a minimal decline in AA and EPA metabolism in CF cells while metabolism of AA to DPA was significantly reduced in WT cells. Each point represents mean ± SEM (n = 3).

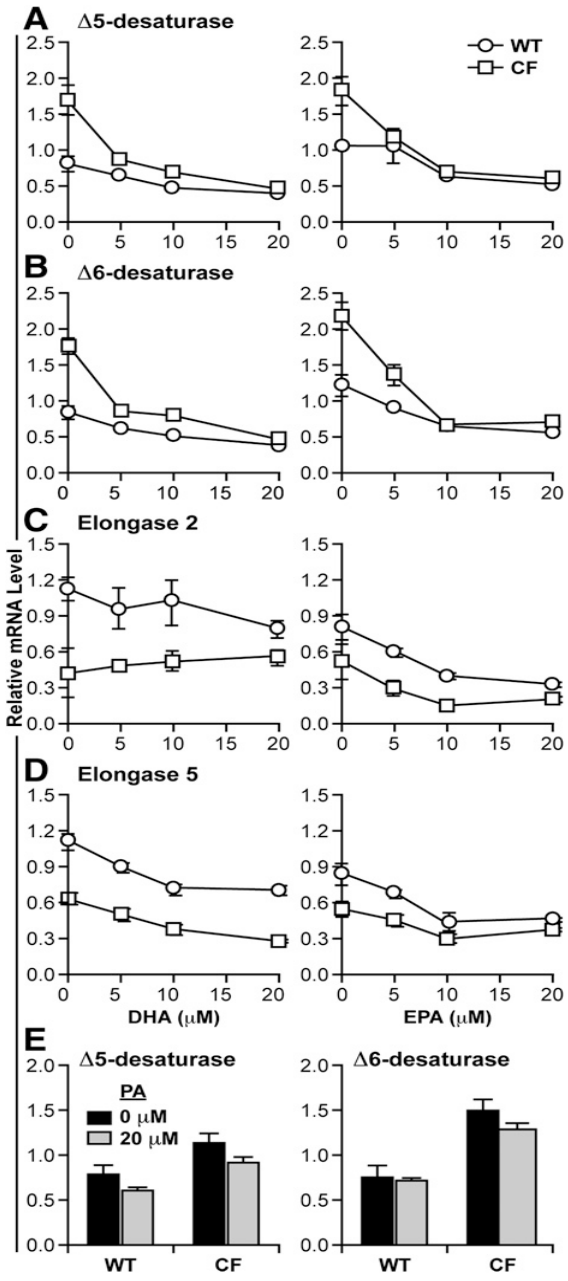

Figure 28: PUFA metabolic enzyme gene expression following DHA, EPA and PA supplementation. WT and CF cells were supplemented with 0, 5, 10 or 20 μM DHA (A-D, left) or EPA (A-D, right) or 20 μM PA (E) for 24 h. Total RNA was isolated and cDNA synthesized from the cells as described in the experimental procedures. qRT-PCR was performed using primers for the mRNA sequences of Δ5-desaturase (*FADS1*), Δ6-desaturase (*FADS2*), elongase 2 (*ELOVL2*), and elongase 5 (*ELOVL5*). Relative expression was determined by the $\Delta\Delta C_T$ method using ribosomal protein *RPLP0* as a control. Both DHA and EPA supplementation reduced the expression of desaturase genes in CF cells to WT levels. There was no significant effect on gene expression after PA supplementation. Data points represent mean ± SEM (n = 3).

In addition to our in vitro cell culture experiments, we studied the mechanism of DHA and EPA action on PUFA metabolism in the cftr^{tm1Unc} mouse model. Previous studies have shown that feeding CF mice a diet containing large amounts of FFA DHA can decrease AA and increase DHA levels in the mice [13]. We set out to investigate the mechanisms through which DHA worked to correct PUFA abnormalities in CF mice and reverse the CF phenotype. To minimize intestinal obstruction and maintain long-term viability, the mice were weaned onto a peptamen liquid diet. Peptamen served as the vehicle for fatty acid supplementation. In addition to FFA DHA (Peptamen + DHA), the cftr^{tm1Unc} mice were also supplemented with a combination of DHA and EPA in TG form (Peptamen AF). The main difference between the fatty acid composition of peptamen versus peptamen + DHA diet was the amount of DHA (0 % in peptamen, 32.5 % in peptamen + DHA). The peptamen AF diet contained much less DHA (3.8 %) than the peptamen + DHA diet, and it was the only diet out of the three that contained a significant amount of EPA (8.2 %) (Table 4).

Table 4: Fatty acid composition of the different mouse liquid diets

Feed	**Peptamen**		**Peptamen + DHA**		**Peptamen AF**	
	mMol/mL	Mol%	mMol/mL	Mol%	mMol/mL	Mol%
14:0	0.82	2.5%	0.73	1.7%	2.24	6.2%
16:0	5.22	16.1%	4.75	10.9%	5.81	16.0%
18:0	2.26	7.0%	2.05	4.7%	2.23	6.1%
18:3n-3	2.31	7.1%	2.14	4.9%	1.97	5.4%
20:5n-3	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**2.98**	**8.2%**
22:6n-3	**n.d.**	**n.d.**	**14.19**	**32.5%**	**1.38**	**3.8%**
18:2n-6	13.14	40.6%	12.12	27.5%	9.00	24.8%
16:1n-7	0.21	0.6%	0.23	0.5%	1.79	4.9%
18:1n-7	0.44	1.3%	0.40	0.9%	0.46	1.3%
18:1n-9	7.50	23.2%	6.79	15.5%	7.11	19.6%

The cftr^{tm1Unc} mice on the peptamen + DHA diet received peptamen for one week post-weaning followed by a week of peptamen supplemented with 40 mg/day FFA DHA. The peptamen contained antioxidants including 3 mg of vitamin E and 34 mg of vitamin C per 100 mL, which helped to minimize oxidation of the FFA DHA in the diet. Alternatively, some mice were placed on the peptamen AF diet for two weeks post-weaning then sacrificed. Fatty acid analysis was performed on cell preparations from the lung, pancreas and ileum, which are the principal organs clinically affected in CF. At baseline (peptamen diet), the characteristic PUFA alterations including decreased LA and DHA and increased AA were present in all three organs of CF mice (Figure 29). Supplementation with DHA led to a significant increase in DHA levels in both WT and CF mice such that the levels of DHA in the CF mice were at or above WT levels (Figure 29). A > 50% decrease in AA levels was also observed in the CF mice, normalizing AA to WT levels. Additionally, a slight increase in LA levels was observed in CF mice following DHA supplementation. To ensure there was no difference in intestinal absorption of DHA, we measured plasma DHA levels in mice receiving the peptamen + DHA diet. The levels of DHA in plasma of WT and CF mice were comparable, signifying normal intestinal absorption in the CF mice (data not shown). Similar to DHA supplementation, peptamen AF reversed PUFA abnormalities in the lung, pancreas and ileum of CF mice. Administration of peptamen AF increased DHA levels in CF mice to WT levels, although the overall increase was less than with the peptamen + DHA diet (Figure 29). A reciprocal decrease in AA and increase in LA levels was also observed. From these experiments we concluded that peptamen AF worked just as well as peptamen + DHA to normalize PUFA abnormalities in CF mice.

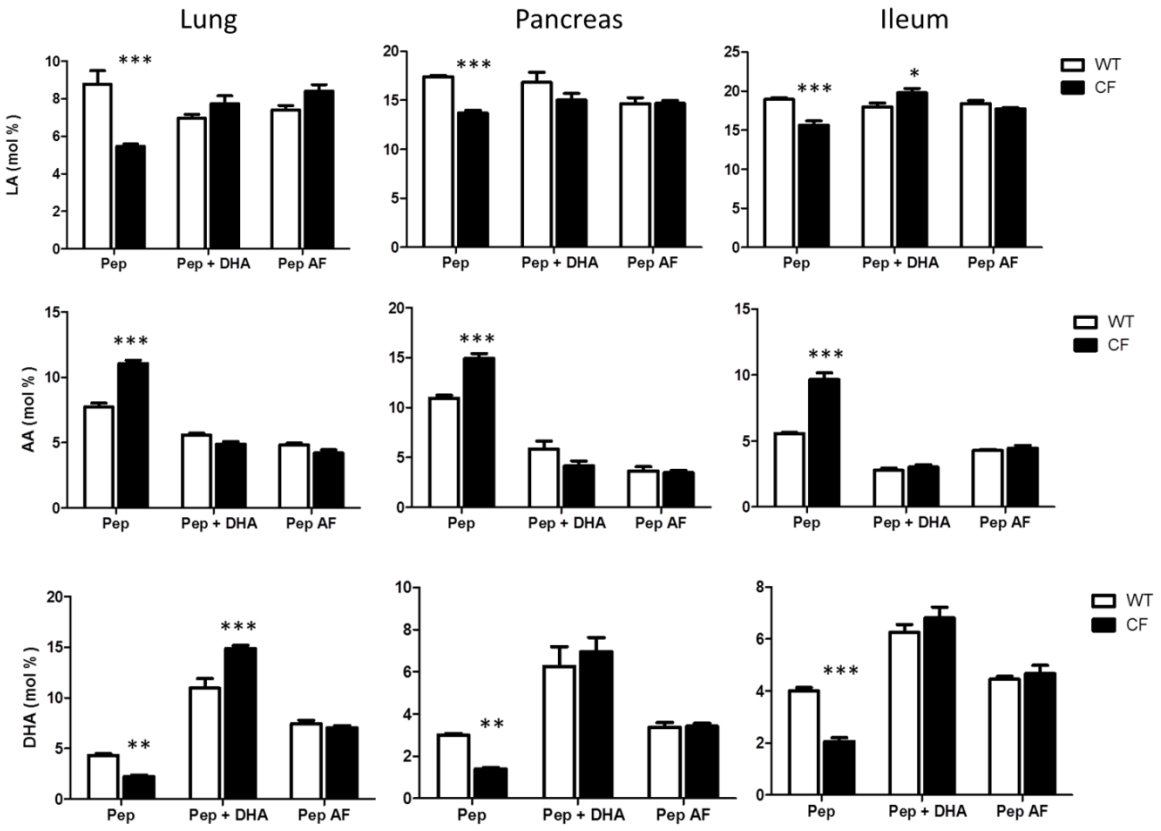

Figure 29: Fatty acid composition of CF-related organs in cftr^{tm1Unc} mice fed peptamen, peptamen + DHA or peptamen AF. Total LA, AA and DHA levels were measured in the lung, pancreas and ileum of WT and CF mice by GC-MS as described in the experimental procedures. Individual fatty acid concentrations are expressed as molar percentage (mol %) of total fatty acids. PUFA abnormalities in CF mice were reversed in the mice receiving the peptamen + DHA or the peptamen AF diet. Bars represent mean ± SEM (n = 6 or 7 mice). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Unlike the peptamen or peptamen + DHA diet, peptamen AF contained a significant amount of EPA (Table 4). Hence a substantial increase in EPA levels was seen in both WT and CF mice maintained on the peptamen AF diet (Figure 30). Interestingly, there was an increase in EPA levels in the lung and ileum of WT and CF mice fed the peptamen + DHA diet. This increase was brought about by retroconversion of the supplemented DHA to EPA. However, there was little difference in the amount of retroconversion between WT and CF mice, which was different from what we observed in cultured 16HBE cells, where retroconversion was 20X greater in CF compared to WT cells. It is possible that species or cell-type differences can explain these dissimilar observations. No retroconversion was observed in the pancreas of $cftr^{tm1Unc}$ mice.

The effect of peptamen + DHA or peptamen AF supplementation was also evaluated in organs not clinically affected in CF. An increase in DHA concentrations along with a decrease in AA levels was observed in the heart, kidney and liver of WT and CF mice, similar to what was seen in CF-related organs (data not shown). However, the brain of WT and CF mice appeared resilient to change, with AA levels remaining unchanged and only a minimal increase in DHA levels following supplementation (Figure 31).

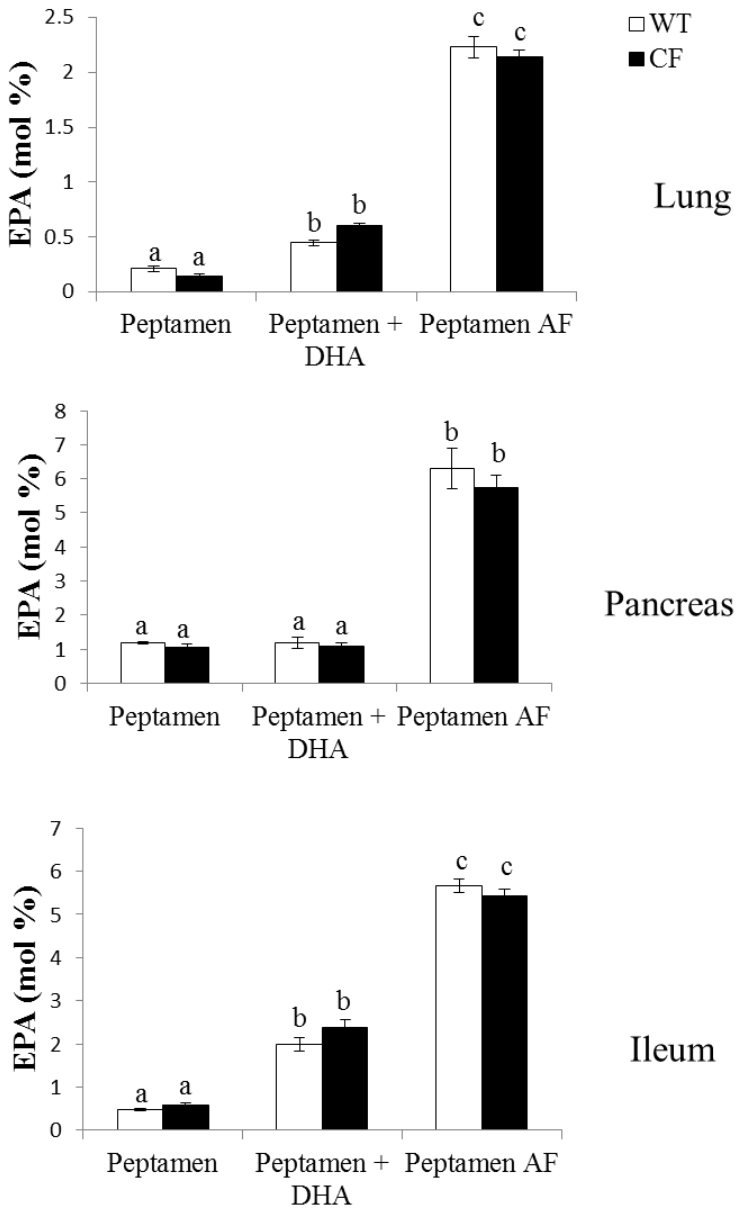

Figure 30: Levels of EPA in CF-related organs of cftr^{tm1Unc} mice fed peptamen, peptamen + DHA or peptamen AF. Total EPA levels were measured in the lung, pancreas and ileum of WT and CF mice by GC-MS as described in the experimental procedures. Fatty acid concentrations are expressed as molar percentage (mol %) of total fatty acids. There was no difference in EPA levels in WT and CF mice fed the control diet (peptamen). Supplementation with peptamen AF caused a significant increase in EPA levels in WT and CF mice while retroconversion of DHA to EPA was observed in the lung and ileum of mice fed the Peptamen + DHA diet. Bars represent mean ± SEM (n = 6 or 7 mice), and unlike letters indicate significant differences in pair-wise comparisons.

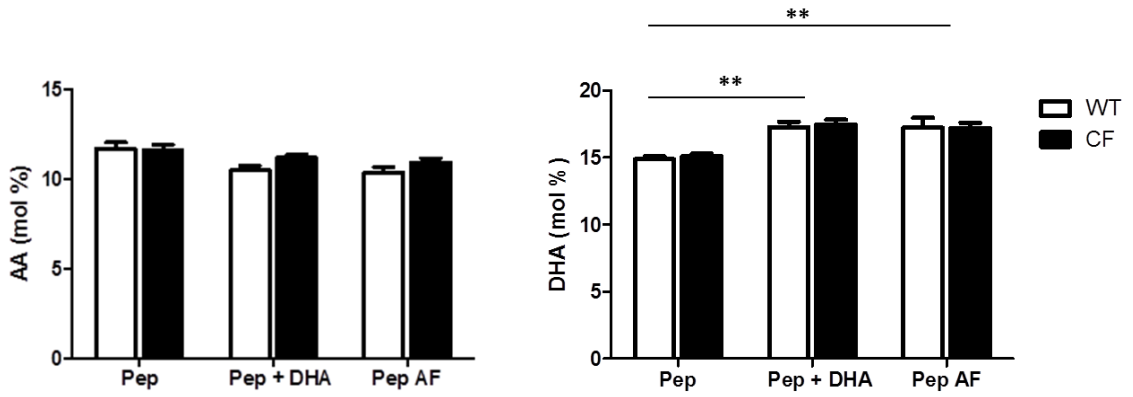

Figure 31: Brain fatty acid composition of cftr^{tm1Unc} mice fed peptamen, peptamen + DHA or peptamen AF. Total AA and DHA levels were measured in the brain of WT and CF mice by GC-MS as described in the experimental procedures. Fatty acid concentrations are expressed as molar percentage (mol %) of total fatty acids. There was no difference in AA or DHA levels in WT and CF mice fed the control diet (peptamen). Supplementation with peptamen + DHA or peptamen AF caused a significant increase in DHA levels in both WT and CF mice. No change was observed in AA levels following supplementation with either diet. Bars represent mean ± SEM (n = 6 or 7 mice). ** $P < 0.01$.

PUFA alterations in CF mice are associated with increased expression and activity of desaturase and elongase enzymes. We hypothesized that both the peptamen + DHA and peptamen AF diets worked to correct CF PUFA abnormalities by suppressing these enzymes. The mRNA expression levels of Δ5-desaturase, Δ6-desaturase and elovl5 were measured in the lung, ileum and liver of WT and CF mice with and without supplementation (Figure 32). All three enzymes were upregulated in lung tissue of CF mice fed the control peptamen diet compared to WT mice. Unexpectedly, supplementation with peptamen + DHA did not alter desaturase or elongase expression in the CF lung. Moreover, supplementation with peptamen AF led to a further increase in enzyme expression in both WT and CF mice. These results differed from what we observed in cultured 16HBE cells, where DHA and EPA supplementation led to downregulation of desaturase enzyme expression. In contrast, supplementation with peptamen + DHA or peptamen AF caused suppression of Δ5 and Δ6-desaturase in the ileum of CF mice, normalizing desaturase expression to WT levels. Elovl5 expression was also slightly decreased following supplementation. A similar pattern was seen in the liver, whereby supplementation with peptamen + DHA or peptamen AF had a suppressive effect on desaturase and elongase expression (Figure 32).

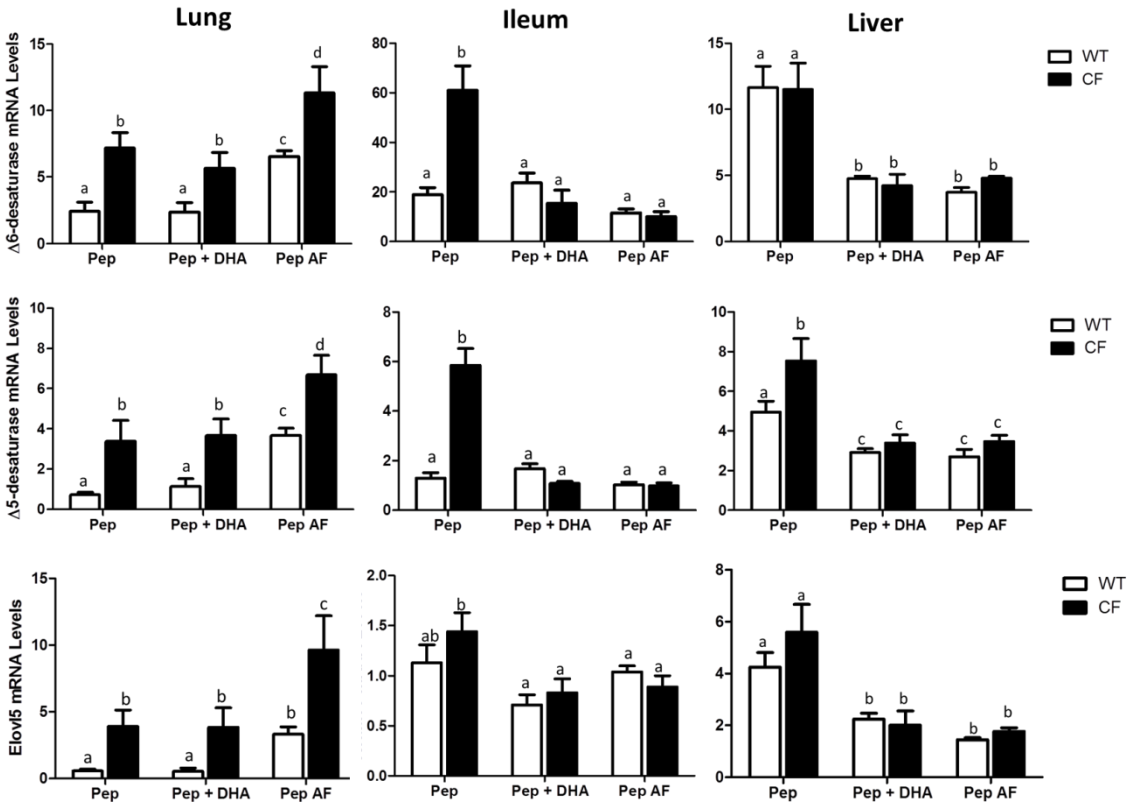

Figure 32: PUFA metabolic enzyme gene expression in lung, ileum and liver of cftr^{tm1Unc} mice fed peptamen, peptamen + DHA or peptamen AF. RNA was extracted and cDNA synthesized from the lung, ileum and liver of WT and CF mice as described in the experimental procedures. qRT-PCR was performed using commercial Taqman probes for the mRNA sequences of Δ5-desaturase , Δ6-desaturase and elongase 5 (Elovl5) . Relative expression was determined by the $\Delta\Delta C_T$ method using GAPDH as a control. Desaturase and elongase expression levels were increased in the lung and ileum of CF mice fed the control peptamen diet. While the peptamen + DHA and peptamen AF diets caused suppression of PUFA metabolic enzymes in the ileum and liver of the mice, the expression levels of these enzymes in the lung either remained the same or increased in mice on these two diets. Bars represent mean ± SEM (n = 6 or 7 mice), and unlike letters indicate significant differences in pair-wise comparisons.

The effect of peptamen + DHA and peptamen AF on the phenotypic expression of CF was also studied in cftr^{tm1Unc} mice. Histological analysis of the small intestine was performed using hematoxylin and eosin staining (Figure 33). WT mice maintained on peptamen, peptamen + DHA or peptamen AF displayed normal intestinal morphology, characterized by narrow crypts without apparent impaction of secretory products. In contrast, the crypts of peptamen-fed CF mice were dilated and filled with secretory material that appeared to emanate from paneth cells at the base of the crypts. Impaction of secretory material was found in 30-70% of the crypts and was present along the entire length of the villus. Supplementation with peptamen AF did not correct the intestinal phenotype in the CF mice, as accumulation of secretory material remained apparent in majority of the crypts examined. However, CF mice on the peptamen + DHA diet exhibited partial correction of the intestinal phenotype, with less accumulation of material in the crypts, no crypt dilation and fewer affected crypts (10-30%). Additional investigations of the intestinal phenotype revealed marked goblet cell hyperplasia in the CF, but not WT, mice (Figure 34). Supplementation with peptamen + DHA almost completely reversed the goblet cell hyperplasia in CF mice, while peptamen AF treatment caused only a slight decrease in goblet cell hyperplasia. Our results suggest that there is altered gut motility and secretion in CF compared to WT mice, and that supplementation with DHA partially corrects this intestinal phenotype while peptamen AF has little effect.

We found no evidence of altered lung morphology in CF mice fed the different diets compared to WT mice (Figure 35). This was an expected finding as previous studies have reported that CF mice show normal lung histology and do not develop spontaneous lung disease [13].

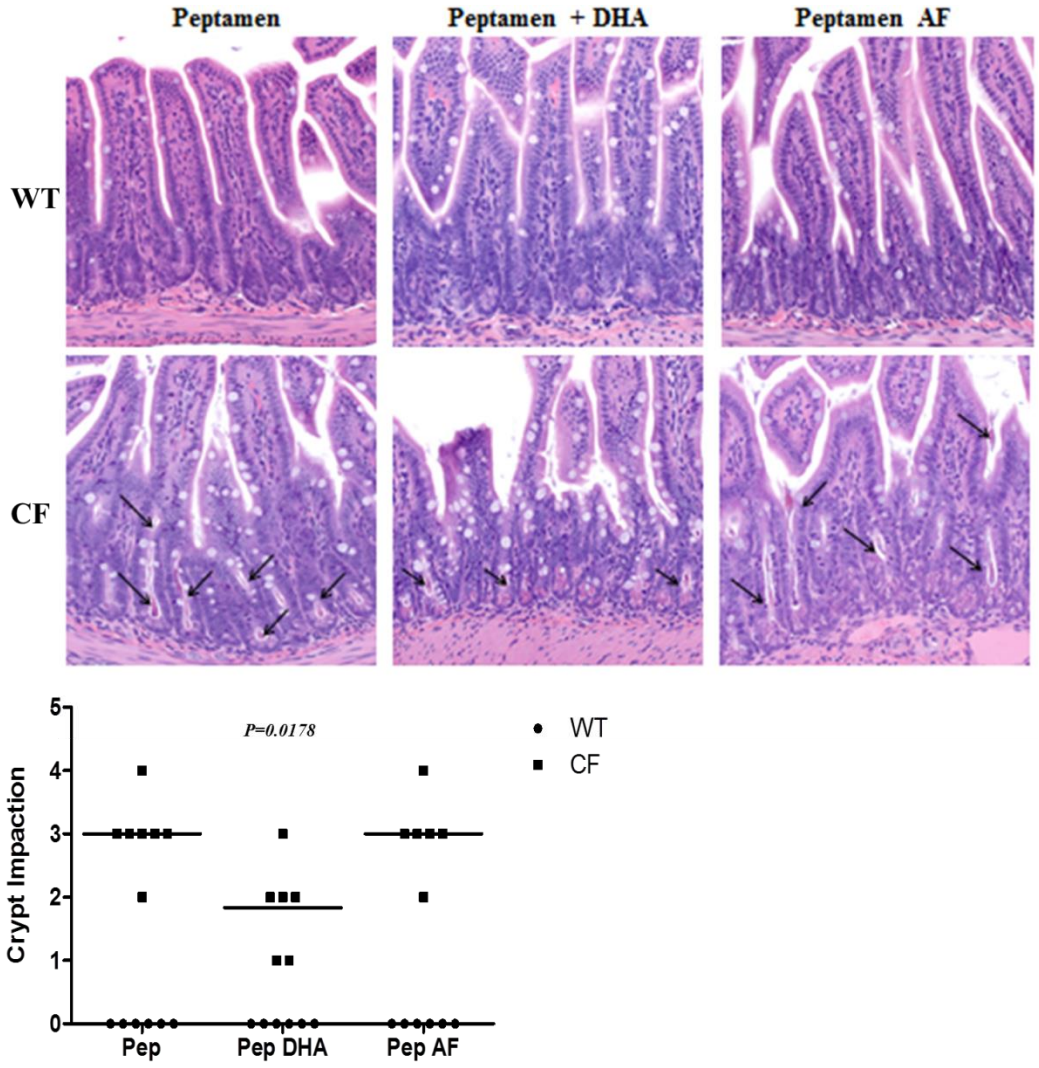

Figure 33: Histological appearance and morphometry of the small intestine of cftr^{tm1Unc} mice fed peptamen, peptamen + DHA or peptamen AF. Tissue was embedded in paraffin and stained with hematoxylin and eosin (representative images shown, n = 6 or 7 mice). There was no impaction of secretory products in the crypts of WT mice fed peptamen, peptamen + DHA or peptamen AF (top row). In contrast, CF mice on the peptamen or peptamen AF diet exhibited dilated crypts containing secretory product (arrows). Treatment of CF mice with DHA resulted in less accumulation of secretions in the deep portion of the crypts as well as a lower percentage of affected crypts. Crypt impaction was scored from 0-4:

0- no impaction of secretory products in the crypts.
1- impacted secretory material in the deep portion of the crypts in <10% of the crypts.
2- impaction of secretory material in the deep portion of the crypts in 10-30% of crypts, with rare accumulation of material in the bottom third of the villus.
3-impaction of secretory material in 30-70% of the crypts, with accumulation of material commonly observed up to the top third of the crypt.
4-impaction of secretory material in >70% of the crypts, with frequent dilation of the crypts and presence of material in the top third of the villus.

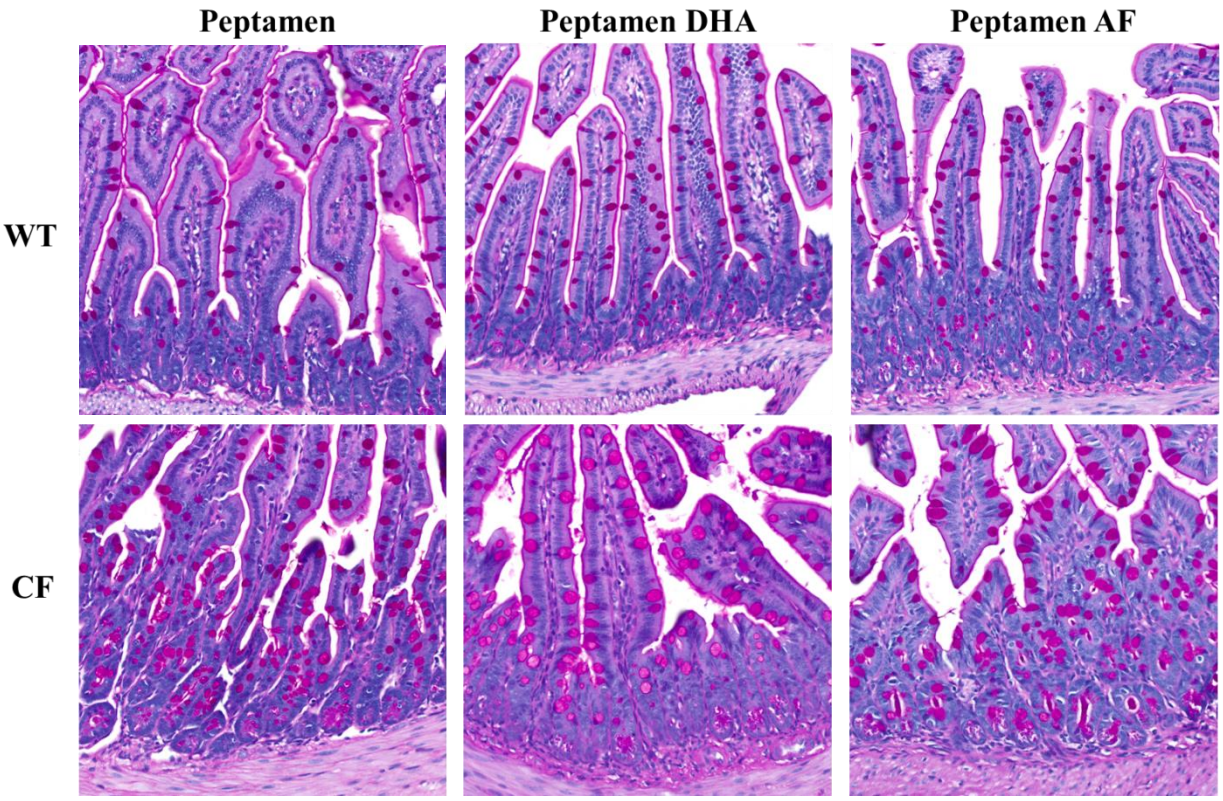

Figure 34: Detection of goblet cells in the small intestine of cftr^{tm1Unc} mice fed peptamen, peptamen + DHA or peptamen AF. Tissue was embedded in paraffin and periodic acid schiff staining used to detect goblet cells (bright pink). Goblet cell hyperplasia was scored as marked, moderate, or mild. WT mice fed the three different diets exhibited normal numbers of goblet cells (no goblet cell hyperplasia), while marked goblet cell hyperplasia was observed in the peptamen-fed CF mice. Treatment of CF mice with DHA resulted in mild goblet cell hyperplasia whereas supplementation with peptamen AF resulted in moderate goblet cell hyperplasia. Representative images shown (n = 6 or 7 mice).

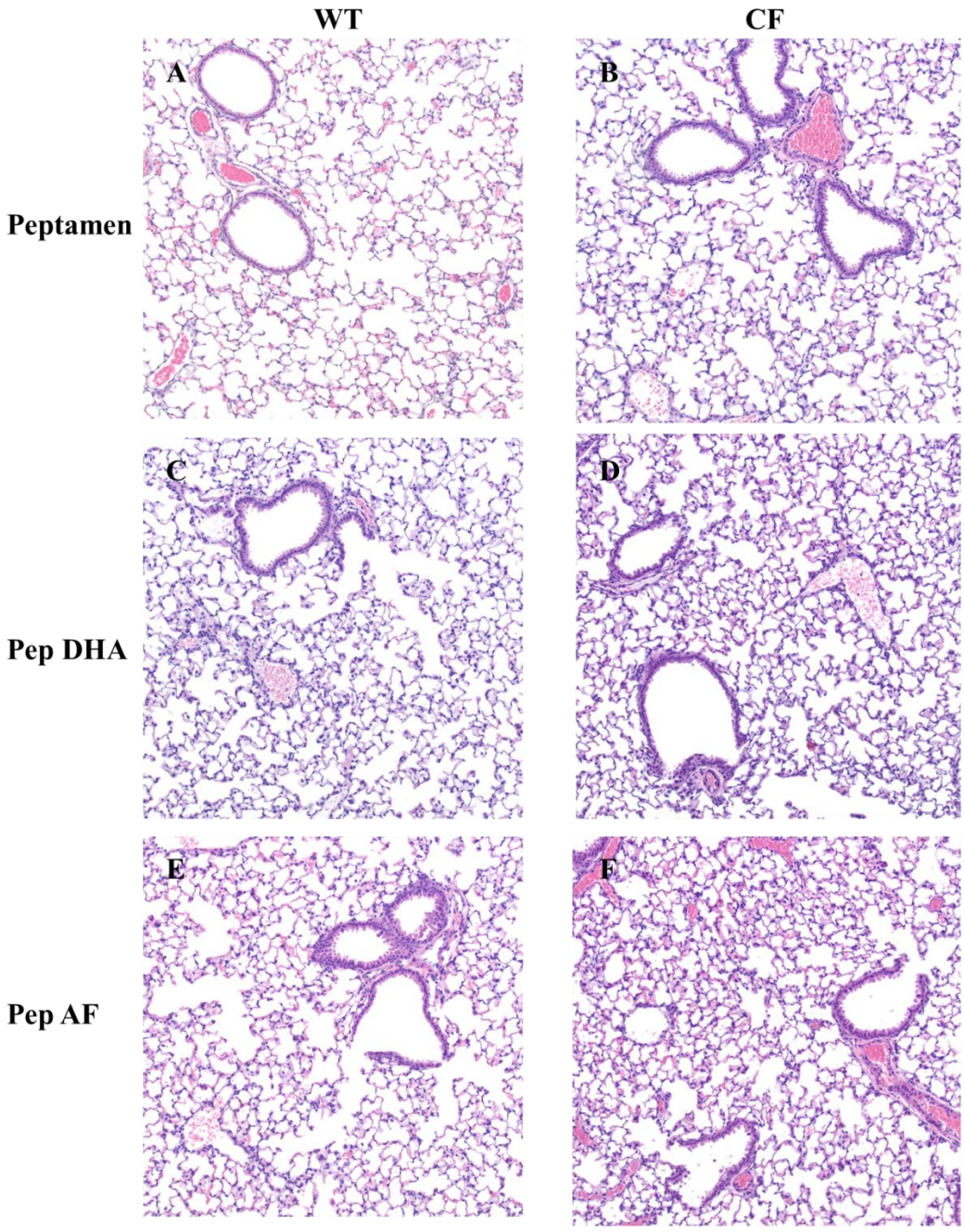

Figure 35: Hematoxylin and eosin-stained lung sections obtained from cftr^{tm1Unc} mice fed peptamen, peptamen + DHA or peptamen AF. A, C and E were obtained from WT mice, while B, D and F were obtained from CF mice, treated with peptamen, peptamen + DHA and peptamen AF respectively. Lung sections revealed normal architecture with no pathological abnormalities found in either the WT or CF mice fed the different diets. Representative images shown (20X magnification, n = 6 or 7 mice).

Discussion

DHA and EPA supplementation in cultured CF cells

Previous studies have shown that DHA treatment can correct PUFA abnormalities in CF patients and animal models [13, 14, 130-135]. However, the exact mechanism by which DHA works remains unclear. In this chapter, we show that supplementing cultured CF cells with exogenous DHA causes suppression of Δ5 and Δ6-desaturase expression and activity. This leads to reduced conversion of LA to AA, thus reversing the PUFA abnormalities. Moreover we demonstrate that a comparable n-3 fatty acid, EPA, has the same effect as DHA on PUFA metabolism. EPA supplementation leads to a decrease in desaturase gene expression and normalizes LA to AA, and LNA to EPA metabolism in CF cells to WT levels. These findings differ from those of Freedman et al [13], which showed a favorable effect of DHA treatment on CF pathology but no effect with EPA treatment. It is possible that our experiments produced different results because they were performed in cultured human CF cells and not in CF mice, as species differences may play a role. Additional PUFAs including AA and LNA also decrease desaturase activity in CF cells but not to the same extent as EPA and DHA, while monounsaturated and saturated fatty acids such as OA and PA do not have any effect on PUFA metabolism. This suggests that the degree of saturation of the supplemented fatty acid determines its effect on desaturase expression and PUFA metabolism. Since Δ5 and Δ6-desaturase are membrane-bound enzymes, it is possible that highly unsaturated fatty acids such as DHA and EPA effectively suppress these enzymes by altering membrane fluidity and flexibility. Further work needs to be done to evaluate the impact of exogenous DHA and

EPA on the physical properties of cell membranes and how this relates to desaturase activity.

In addition to reducing conversion of LA to AA and LNA to EPA, DHA and EPA also reduced metabolism of AA to 22:5n-6. This reduction was likely caused by decreased Δ6-desaturase and elongase activity and was seen mainly in WT cells, perhaps due to the fact that AA metabolism was significantly diminished in CF cells to begin with. Interestingly, DHA and EPA did not reduce metabolism of EPA in the n-3 pathway. At low concentrations (5 μM exogenous DHA or EPA), metabolism of EPA to DHA was actually increased. A plausible explanation for this is that the increased concentrations of these fatty acids may stimulate activity in their own metabolic pathway, which may effectively negate their suppressive effects on desaturase enzyme expression.

Our experiments demonstrate that DHA and EPA suppress desaturase gene expression in WT and CF cells, although the effect is much greater in CF cells. It is conceivable that this differential effect is caused by differences in uptake, as CF cells seem to incorporate higher amounts of both DHA and EPA compared to WT cells (Figures 22 and 25). Although the reason behind the increased uptake of exogenous PUFAs in CF cells is unclear, it could point to increased fatty acid transport into CF cells.

Retroconversion of DHA to EPA

The DHA supplementation experiments reveal a potential mechanism that can account for the increased EPA and decreased DHA levels in CF. This mechanism involves the retroconversion of DHA to EPA through a modified peroxisomal β-oxidation process in which DHA loses two carbons and becomes desaturated [127-129].

This is a regulated process that requires saturation of the Δ4 double bond by Δ4 enoyl CoA reductase and rearrangement of the double bond structure by Δ3, Δ2 enoyl CoA isomerase (Figure 36). We showed that treating CF cells with exogenous DHA led to an increase in EPA levels, and that the calculated retroconversion [ΔEPA / (ΔEPA + ΔDHA)] was much higher in CF cells (20%) than in WT cells (1%). Retroconversion of DHA to EPA was also observed in vivo, after supplementing cftr^{tm1Unc} mice with large amounts of DHA. In these experiments, we found that treating the mice with DHA led to an increase in EPA levels in several organs including lung and ileum. However, unlike our cell culture experiments, there was no difference in DHA retroconversion levels between WT and CF mice. DHA retroconversion is an important process that may have clinical implications for n-3 dietary therapies used in many diseases, including CF. Future investigation into the factors that affect retroconversion, including regulation of the enzymes that catalyze these reactions, may enhance our understanding of this process.

DHA and EPA supplementation in cftr^{tm1Unc} mice

PUFA alterations are present in CF mice and specifically in clinically affected organs such as the lung, pancreas and ileum. These fatty acid changes are associated with increased desaturase and elongase expression and activity. CF mice placed on either the peptamen + DHA or peptamen AF diet for two weeks exhibited reversal of PUFA abnormalities. Based on our in vitro cell culture experiments, we hypothesized that the mouse diets enriched with FFA DHA (peptamen + DHA) or a combination of TG DHA and EPA (peptamen AF) worked to correct PUFA abnormalities by suppressing desaturase expression and activity. To evaluate this, desaturase and elongase gene

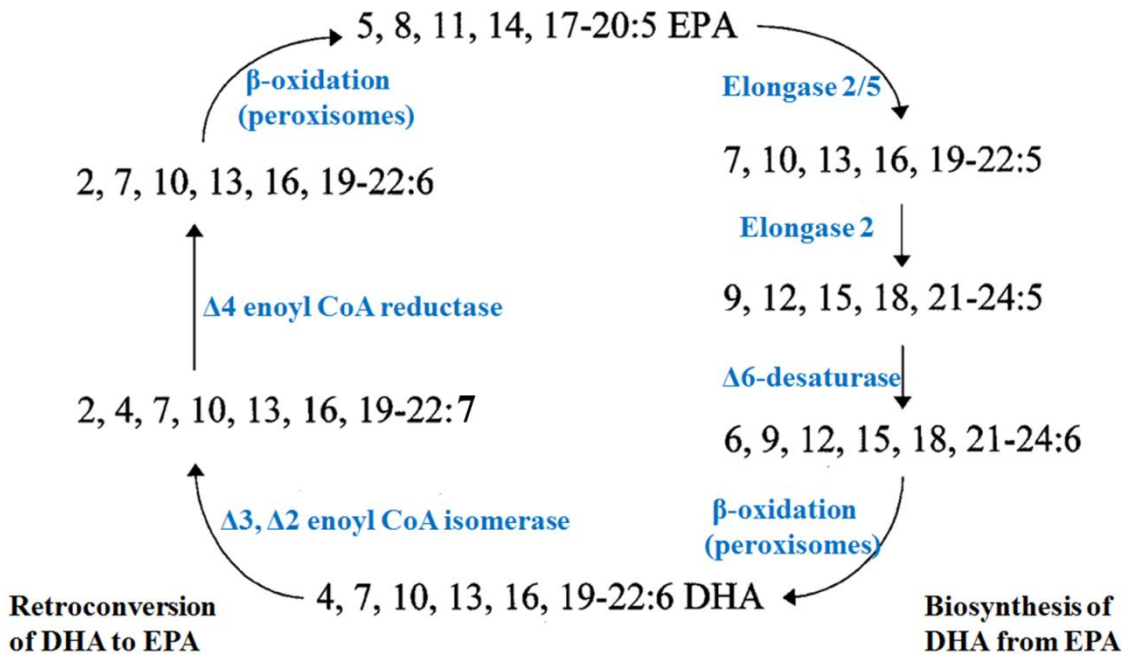

Figure 36: Diagram of the biosynthesis and retroconversion of docosahexaenoic acid (DHA). DHA is synthesized from EPA through a series of elongation and desaturation steps. DHA can also be retroconverted back to EPA through a modified β-oxidation process that involves a different set of enzymes.

expression was measured in the lung, ileum and liver of WT and CF mice following supplementation. In the ileum and liver, expression of Δ5 and Δ6-desaturase and elovl5 was decreased in CF mice to the level of WT mice. This corresponded with normalization of PUFA levels in the ileum of CF mice. Interestingly, even though PUFA alterations were reversed in the lungs of CF mice after DHA and EPA supplementation, the expression levels of desaturase and elongase enzymes still remained upregulated. This unexpected result can be explained first by noting the source of DHA or EPA that gets to the different organs, and second, by examining the overall principal source of PUFAs for each of these organs. Once ingested, the supplemented DHA and EPA is digested in the

small intestine of the mice. FFA DHA in the peptamen + DHA diet can be absorbed as is, whereas the TG DHA and EPA in peptamen AF has to be hydrolyzed by pancreatic lipase to release FFAs and monoacylglycerols (MAG). The FFAs and MAGs are absorbed into the enterocytes and resynthesized into TGs, which then get packaged into chylomicrons and released into the lymphatic system to be taken to different organs of the body. Therefore the source of DHA and EPA for the ileum is FFAs or MAGs. In the liver, the source is mainly intermediate density lipoproteins (IDL) or low density lipoproteins (LDL) which contain majority of their fatty acids as phospholipids. In the lung, the main source of DHA and EPA is as TGs from chylomicrons and very low density lipoproteins (VLDL). Thus, it is feasible that the ileum, liver and lung each receive a different pool of DHA and EPA, and this may explain why the effect of DHA and EPA on desaturase expression varies among these organs. Moreover, the primary source of PUFAs for these organs is different. Our results demonstrate that DHA and EPA supplementation reduces the expression of desaturase and elongase enzymes in the liver and ileum of CF mice, where most PUFA metabolism occurs. Therefore because desaturase and elongase expression is decreased, the synthesis of PUFAs including AA is also decreased in these organs. Unlike the liver and ileum, the primary source of PUFAs in the lung is fatty acid uptake from lipoproteins that originate in the GI tract (chylomicrons) and liver (VLDL). Thus, PUFA levels in the lung tend to be a reflection of metabolism and synthesis in the liver and ileum, irrespective of metabolic enzyme expression. This may explain why although desaturase and elongase expression is still increased in CF lung tissue, the overall AA to LA ratio is decreased.

It is also possible that cell-type differences can explain the differential effects of DHA and EPA supplementation in our in vitro and in vivo experiments. The cell culture experiments were performed in a population consisting of 100% 16HBE cells, whereas the mouse experiments were carried out in lung cell preparations enriched for epithelial cells. While the majority of the mouse lung cells may have been epithelial in nature, there were likely other cell types present. This mixed population of cells could dampen the suppressive effect on desaturase expression that would otherwise be observed in a pure epithelial population following DHA and EPA supplementation.

In contrast to the other mouse organs, minimal fatty acid changes were observed in brain tissue from mice fed the peptamen + DHA or peptamen AF diet. Previous studies have reported that the brain is more resistant to changes in dietary fatty acids and possesses inherent mechanisms to maintain lipid homeostasis in situ [136]. It has also been proposed that brain AA and DHA profiles are highly protected, perhaps by active transport of these fatty acids from the circulation or by increased chain elongation and desaturation to maintain the appropriate levels [137].

Additionally, the effect of peptamen + DHA and peptamen AF supplementation on the phenotypic expression of CF disease was evaluated in $cftr^{tm1Unc}$ mice. CF mice maintained on the peptamen diet exhibited intestinal disease, characterized by marked goblet cell hyperplasia and dilated crypts filled with secretory material. Interestingly, supplementation with peptamen + DHA appeared more effective at correcting the intestinal phenotype in CF mice than the peptamen AF diet (Figures 33 and 34). Peptamen AF contains a combination of EPA and DHA, with significantly less DHA than is found in the peptamen + DHA diet (Table 4). Therefore, although both peptamen +

DHA and peptamen AF work to decrease AA and increase DHA levels in the ileum, the increase in DHA is much greater in the peptamen + DHA group (Figure 29). In addition, the EPA present in peptamen AF may compete with DHA and actually decrease its incorporation. It is possible that the intestinal phenotype seen in CF mice is mainly a result of decreased DHA levels rather than increased AA levels, and this may explain why a beneficial effect is seen only in the CF mice on the peptamen + DHA diet. Further studies are needed to determine whether increasing the concentration of supplemented DHA and/or the length of time might help to completely reverse the CF intestinal phenotype.

There was no evidence of lung pathology in either the WT or CF mice receiving the different diets (Figure 35). This differs significantly from CF human disease, where the most significant impact in affected individuals is progressive lung disease that is characterized by mucus obstruction and goblet cell hyperplasia, excessive airway inflammation, spontaneous development of bacterial infection and progression to chronic infection [61]. Several factors may explain the relative absence of lung pathology in CF mice. It is believed that bacterial colonization of the airways of CF patients plays an important role in the development of significant lung disease. Thus, the failure of CF mice to develop lung pathology could be due, at least in part, to the fact that they are housed in a sterile barrier facility. It is possible that older CF mice raised in a less sterile environment may exhibit lung disease. In addition, these mice may have modifier genes which can directly or indirectly partially correct the abnormalities in epithelial ion transport. These include the presence of non-CFTR chloride conductance genes that can compensate for loss of CFTR function and prevent the development of lung pathology.

Ours is not the first study looking at the effect of DHA supplementation on the fatty acid profile and phenotype of CF mice. Freedman et al. [13] showed that feeding cftr^{tm1Unc} mice a diet supplemented with 40 mg/day free or esterified DHA for 7 days decreased AA levels and increased DHA levels in the lung, pancreas and ileum of the mice. DHA supplementation also caused a reversal of pancreatic duct dilation and ileal hypertrophy in the CF mice. A separate study examined the effect of supplementing F508del mice with liposomes containing glycerophospholipids enriched with DHA by gavage (60 mg DHA/kg daily, i.e. at maximum 1.4 mg DHA/d) for 6 wk. This treatment led to an increase in LA and DHA levels, a decrease in elevated AA, and an increase in (n-6)/(n-3) fatty acid ratio, depending on the tissue [14]. However, not all published studies reported a beneficial effect with DHA supplementation. Congenic B6 cftr^{tm1Unc} mice supplemented with 40 mg/day free DHA for 7, 30 or 60 days failed to show a significant improvement in lung, pancreas or ileum morphology [138]. A similar study using congenic B6 cftr^{tm1Unc} mice fed 40 mg/day free DHA showed no significant difference in the survival rates of WT and CF mice inoculated with *P. aeruginosa*-coated agarose beads [139]. Therefore, it is possible that the response to DHA is dependent, at least in part, on the genetic background of the various mouse strains used in the different studies.

CHAPTER IV

NEW FINDINGS AND CONCLUSIONS

Introduction

The data presented in the preceding chapters provides a mechanistic explanation for the PUFA alterations in CF and establishes that these alterations play an important role in CF pathophysiology. However, the connection between mutations in the CFTR gene and abnormalities in PUFA metabolism is still unknown. Preliminary experiments in our lab have shown that treatment of 16HBE sense (WT) cells with a small molecule inhibitor of CFTR ($CFTR_{inh}$-172, 20 μM) [140] leads to a significant increase in Δ5 and Δ6-desaturase gene expression compared to untreated cells, with no change in elovl2 and elovl5 expression. That similar effects on desaturase gene expression are present in both antisense cells and $CFTR_{inh}$-172-treated cells suggests that loss of CFTR function can regulate desaturase expression and lead to PUFA abnormalities. Moreover, studies in CF mice show that PUFA alterations are found solely in CF mouse organs that contain a significant number of CFTR-expressing cells including the lung, pancreas and ileum. This implies that loss of CFTR function is related to abnormalities in fatty acid metabolism. We hypothesize that CFTR mutations activate pathways leading to increased desaturase expression and activity, resulting in altered PUFA metabolism in CF. A potential pathway that can link CFTR mutations to altered desaturase activity involves the overproduction of reactive oxygen species.

CF is characterized by elevated oxidative stress and impaired antioxidant/oxidant balance [141]. Mutations in CFTR contribute to abnormal generation of reactive oxygen species (ROS), in part by altering fluid and electrolyte composition of secretions, leading to the production of thick, viscous mucus. The accumulation of viscous mucus in the CF lung causes airway obstruction, bacterial colonization and compromised pathogen clearance, leading to repetitive infections and chronic inflammation. The hallmark of chronic inflammatory lung disease in CF is the release of chemokines, including IL-8 [142, 143], and the excessive recruitment of neutrophils to the bronchial lumen [144]. These neutrophils, along with the bacterial pathogens such as *Pseudomonas aeruginosa* present in the lung, release large amounts of ROS, including the superoxide anion O_2^-, hydrogen peroxide H_2O_2, and the hydroxyl free radical OH. Elevated levels of lipid and protein oxidation products found in BAL fluid, exhaled breath condensate, and sputum of CF patients provide evidence of a pro-oxidative imbalance in CF airways [145-148]. In addition to increased oxidant production, antioxidant defenses are dramatically reduced in CF patients compared to healthy subjects [141, 149-151]. Exocrine pancreatic insufficiency is a common feature in CF which causes malabsorption of fat-soluble vitamins such as Vitamin A, E and carotenoids. Moreover, CFTR in addition to its role as a chloride channel can also transport reduced glutathione (GSH) [152-154]. GSH is an anionic tripeptide (γ-glutamyl-cysteinyl-glycine) that is considered one of the most important extracellular antioxidants in the lung [155, 156]. Defects in CFTR impair GSH transport and lead to markedly decreased GSH concentrations in CF patients and mouse models [157, 158]. Some studies have also shown that at the cellular level, CFTR mutations can cause mitochondrial depletion of GSH [159, 160]. Taken together, the

increased production of oxidants and decreased antioxidant protection bring about compromised redox homeostasis in CF. PUFAs have been reported to act as potent antioxidants [161], and therefore it is possible that in response to increased oxidative stress arising from CFTR mutations, cells respond by upregulating desaturase expression and activity in an attempt to generate more PUFAs to counteract the oxidative burden. We set out to determine whether ROS production was increased in our CF cell culture model and whether treating CF cells with various antioxidants could normalize PUFA metabolism.

Experimental Procedures

HPLC superoxide (O_2^-) measurements

16HBE sense (WT) and antisense (CF) cells were cultured until two days post-confluence as described in the previous chapters. On the day of the study, the cells were rinsed three times with 3 mL chilled KRB-Hepes buffer and exposed to 10 µM dihydroethidium (DHE) for 20 min at 37^{o}C in KRB-Hepes buffer containing 0.1% DMSO. DHE was then washed off from the cells to avoid absorption of any extracellular oxyethidium formed by autoxidation of DHE. The cells were incubated in KRB-Hepes buffer at 37^{o}C for an hour, after which the cells were harvested for HPLC analysis of O_2^- anions. The cells were scraped on ice and placed in cold methanol, homogenized and filtered using a 0.22 µm filter. DHE reacts with O_2^- to form oxyethidium and this can be separated from its parent DHE and ethidium by HPLC. The resulting peak intensity of oxyethidium reflects intracellular production of O_2^-. Separation of ethidium, oxyethidium and DHE was carried out using a Beckman HPLC System Gold model with a C-18

reverse phase column (Nucleosil 250, 4.5 mm; Sigma-Aldrich, St. Louis, MO), equipped with UV and fluorescence detectors. Fluorescence detection at 580 nm (emission) and 480 nm (excitation) was used to monitor oxyethidium production. UV absorption at 355 nm was used for the detection of DHE. The mobile phase was composed of a gradient containing 60% acetonitrile and 0.1% trifluoroacetic acid. DHE, ethidium, and oxyethidium were separated by a linear increase in acetonitrile concentration from 37% to 47% over 23 min at a flow rate of 0.5 mL/min.

Antioxidant treatment

16HBE cells were treated with three different antioxidants, namely N-acetyl cysteine (NAC), trolox (TX) and mito-TEMPO (mT). NAC is an antioxidant that effectively reduces free radical species directly and also facilitates GSH biosynthesis. TX is a water soluble derivative of vitamin E, while mT is a mitochondria specific superoxide scavenger. For NAC treatments, confluent cells were incubated with 10 mM NAC for 24 h. TX (400 µM) and mT (25 µM) treatments were carried out over a 5 day period beginning on day 2 post-seeding. Following antioxidant treatment, the cells were either harvested immediately for RNA isolation or incubated with 4.1 µM ^{14}C-LA for 4 h followed by a 20 h chase with complete media for metabolic flux experiments. Total RNA was isolated from antioxidant-treated cells and cDNA synthesized for assessment of desaturase, elongase and superoxide dismutase mRNA expression by qRT-PCR. Fatty acids were extracted and methylated from ^{14}C-LA radiolabeled cells, and conversion of radiolabeled LA to downstream metabolites measured by HPLC coupled to a β-RAM scintillation detector, as described previously.

Western blotting

Untreated and antioxidant-treated 16HBE cells were washed twice in ice-cold PBS and protein extracted using RIPA buffer. Samples were loaded on a 4-12 % gradient Bis-Tris SDS-PAGE gel (Invitrogen, Carlsbad, CA), run for 15 min at 100 V and 90 min at 120 V and transferred to Immobilon-P PVDF membranes. Blots were stained using a primary antibody to Cu/Zn-SOD1 (1:5000) (Cayman chemicals, Ann Arbor, MI), Mn-SOD2 (1:5000) (Abcam, Cambridge, MA) or β-actin (1:5000) (Sigma, St.Louis, MO). The bound antibodies were visualized with a secondary HRP-conjugated goat anti-rabbit antibody using the SuperSignal West Pico substrate system (Thermo Fisher Scientific) and exposed to CL-X Posure film (Thermo Fisher Scientific).

Results

To determine whether CF cells exhibited increased ROS production, we chose to measure O_2^- levels in WT and CF 16HBE cells. CF cells produced more than double the amount of O_2^- found in WT cells (Figure 37), indicating that there was increased oxidative stress in the CF cells. To assess if increased O_2^- production was driving overexpression of desaturase enzymes, 16HBE cells were pretreated with various antioxidants and mRNA expression of desaturase and elongase enzymes measured. Both Δ5 and Δ6-desaturase levels were higher in control CF cells (untreated) compared to WT cells. Treatment of CF cells with NAC significantly decreased Δ5 and Δ6-desaturase expression to WT levels (Figure 38). A similar decrease in desaturase mRNA levels was observed in CF cells treated with TX and the superoxide scavenger mT (Figures 39 and 40). Antioxidant treatment had no effect on elovl2 or 5 expression levels (data not

shown). The effect of antioxidants on desaturase activity was evaluated by measuring the metabolism of ^{14}C-LA to AA. Increased conversion of LA to AA was seen in untreated CF cells compared to WT cells, but treatment with NAC, TX and mT normalized LA to AA metabolism, resulting in increased levels of LA and decreased AA in CF cells.

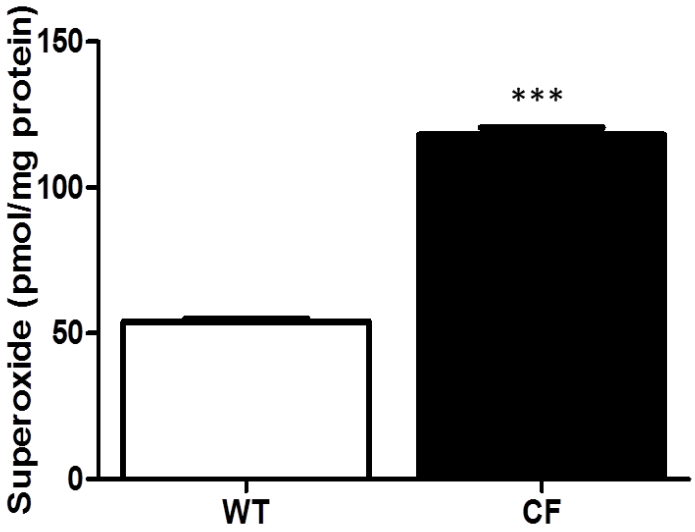

Figure 37: Detection of intracellular superoxide production in 16HBE cells. WT and CF cells were exposed to 10 µM DHE and levels of oxyethidium measured by HPLC as an indicator of superoxide levels, as described in the experimental procedures. WT cells produced much less superoxide compared to CF cells. Bars represent mean ± SEM (n = 3), *** P<0.001.

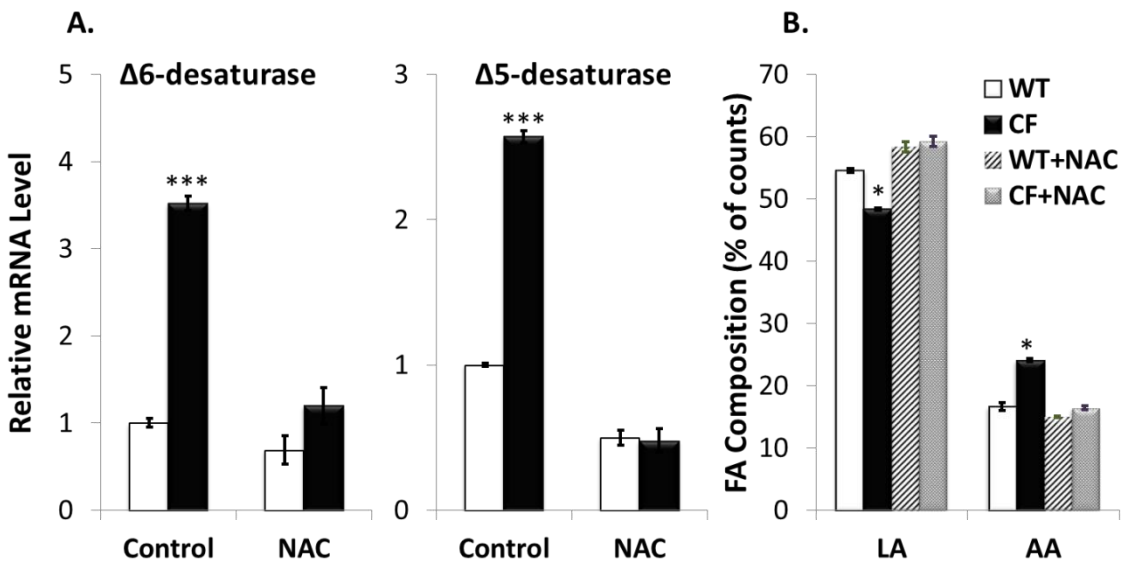

Figure 38: Relative desaturase mRNA expression and PUFA composition of 16HBE cells with or without NAC treatment. RNA was extracted and cDNA synthesized from WT and CF cells (A) as described in the experimental procedures. qRT-PCR was performed using primers for the mRNA sequences of Δ5-desaturase (*FADS1*) and Δ6-desaturase (*FADS2*). Relative expression was determined by the $\Delta\Delta C_T$ method using ribosomal protein *RPLP0* as a control. Both Δ5-desaturase and Δ6-desaturase expression levels were significantly increased in CF compared to WT cells at baseline. NAC treatment resulted in a significant decrease in CF desaturase expression, bringing Δ5 and Δ6-desaturase expression to WT levels. (B) Metabolism of LA to AA was determined in WT and CF cells. Treating CF cells with NAC antioxidant resulted in an increase in LA and decrease in AA levels, normalizing PUFA metabolism in these cells. Bars represent mean ± SEM (n = 3). * $P<0.05$, *** $P<0.001$ for WT vs. CF cells.

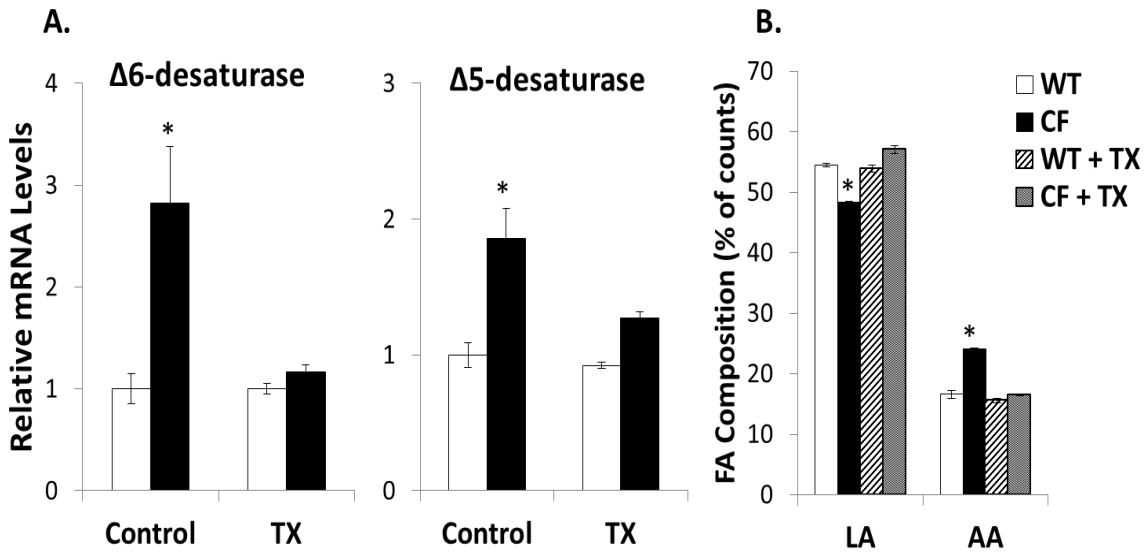

Figure 39: Relative desaturase mRNA expression and PUFA composition of 16HBE cells with or without trolox treatment. RNA was extracted and cDNA synthesized from WT and CF cells (A) as described in the experimental procedures. qRT-PCR was performed using primers for the mRNA sequences of Δ5-desaturase (*FADS1*) and Δ6-desaturase (*FADS2*). Relative expression was determined by the $\Delta\Delta C_T$ method using ribosomal protein *RPLP0* as a control. Both Δ5-desaturase and Δ6-desaturase expression levels were significantly increased in CF compared to WT cells at baseline. Trolox treatment resulted in a significant decrease in CF desaturase expression, bringing Δ6-desaturase expression to WT levels. (B) Metabolism of LA to AA was determined in WT and CF cells. Treating CF cells with trolox antioxidant resulted in an increase in LA and decrease in AA levels, normalizing PUFA metabolism in these cells. Bars represent mean ± SEM (n = 3). * $P<0.05$, for WT vs. CF cells.

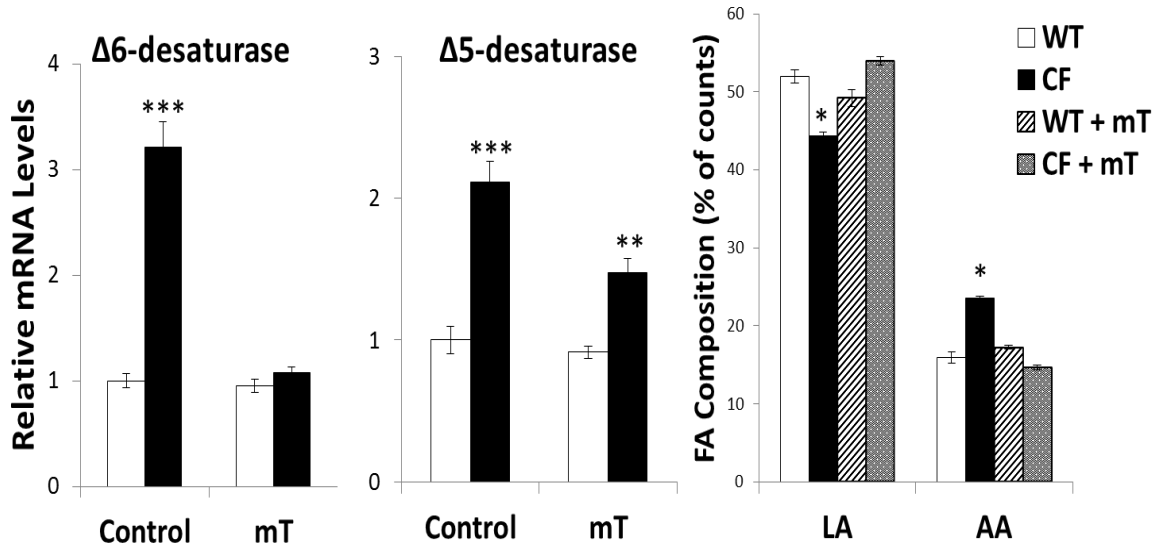

Figure 40: Relative desaturase mRNA expression and PUFA composition of 16HBE cells with or without mito-TEMPO treatment. RNA was extracted and cDNA synthesized from WT and CF cells (A) as described in the experimental procedures. qRT-PCR was performed using primers for the mRNA sequences of Δ5-desaturase (*FADS1*) and Δ6-desaturase (*FADS2*). Relative expression was determined by the $\Delta\Delta C_T$ method using ribosomal protein *RPLP0* as a control. Both Δ5-desaturase and Δ6-desaturase expression levels were significantly increased in CF compared to WT cells at baseline. mito-TEMPO treatment resulted in a significant decrease in CF desaturase expression, bringing Δ6-desaturase expression to WT levels. (B) Metabolism of LA to AA was determined in WT and CF cells. Treating CF cells with a mitochondrial-specific superoxide scavenger mito-TEMPO resulted in an increase in LA and decrease in AA levels, normalizing PUFA metabolism in these cells. Bars represent mean ± SEM (n = 3). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ for WT vs. CF cells.

Superoxide anions can be detoxified by enzymes known as superoxide dismutases (SODs). SODs are a family of metalloenzymes that efficiently catalyze the dismutation of the O_2^- anion to H_2O_2 and molecular oxygen [162]. Three forms of SOD enzymes are present in mammals, each having distinct metal cofactors. SOD1 complexes with copper and zinc and is found in the cytoplasm, while SOD2 binds manganese and is found in the mitochondria. SOD3 is extracellular and can bind to cell surfaces by its interaction with polyanions such as heparin sulfate. We measured the mRNA and protein levels of SOD1 and SOD2 in 16HBE cells and found that while SOD1 was expressed at similar levels in both WT and CF cells, SOD2 was overexpressed in CF cells (Figure 41). This is likely a result of the elevated O_2^- levels in CF cells, which may trigger increased SOD2 expression and activity in order to break down the O_2^- . Following antioxidant treatment, the levels of SOD2 in CF cells were significantly decreased.

These experiments suggest that there is a connection between PUFA alterations and increased O_2^- production in CF. When O_2^- levels are elevated in CF cells, there is increased conversion of LA to AA. However, the altered PUFA metabolism can be corrected by treating the cells with different antioxidants. We believe that loss of CFTR function leads to overproduction of ROS such as O_2^- in CF cells. This leads to activation of Δ5 and Δ6-desaturase expression and activity which in turn causes increased metabolism of LA to AA. Since Δ5- and Δ6-desaturases are regulated at the transcriptional level, it is plausible that increased ROS production triggers increased activity of a transcriptional activator of the desaturase genes. Several transcription factors are known to participate in the PUFA-mediated regulation of Δ5- and Δ6-desaturase expression [97, 163]. Two of these transcription factors, sterol regulatory element-

binding protein (SREBP)-1 and peroxisome proliferator-activated receptor (PPAR)α, exhibit altered expression and activity in CF [164-166]. Further experiments are needed to determine if these transcription factors play a role, as identification of the relevant transcription factor may help to identify specific signaling pathways linking altered PUFA metabolism to CFTR. Detailed studies looking at pathways such as the AMP-activated protein kinase (AMPK) signaling pathway, which is activated by ROS and is known to be involved in fatty acid metabolism, will allow us to develop a complete mechanistic framework linking CFTR mutations, ROS production and PUFA alterations in CF.

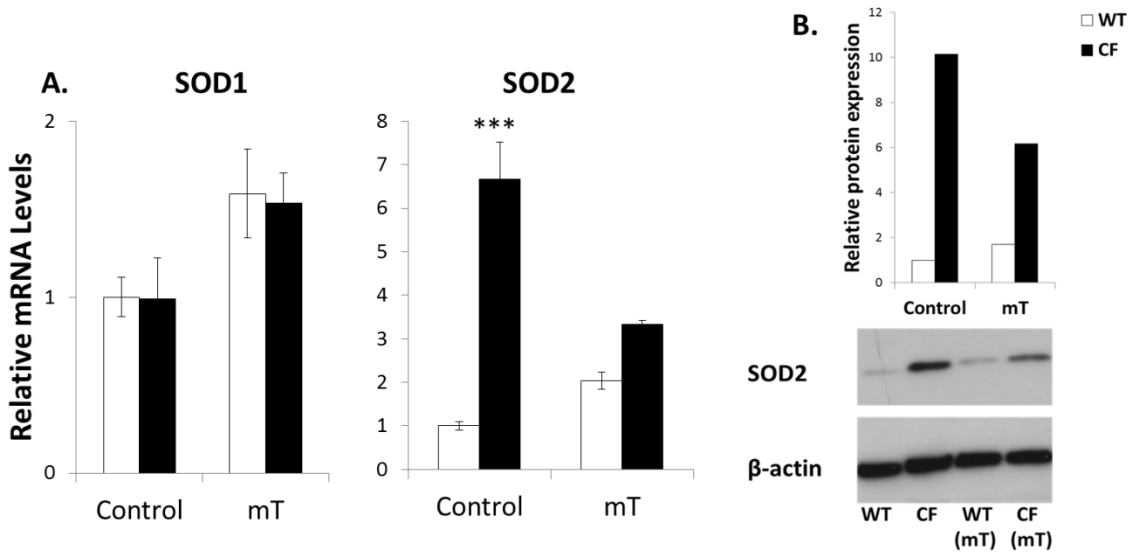

Figure 41: Relative SOD mRNA and protein expression in 16HBE cells with or without mito-TEMPO (mT) treatment. RNA was extracted and cDNA synthesized from WT and CF cells (A) as described in the experimental procedures. qRT-PCR was performed using primers for the mRNA sequences of SOD1 and SOD2. Relative expression was determined by the $\Delta\Delta C_T$ method using ribosomal protein *RPLP0* as a control. There was no difference in SOD1 levels between WT and CF cells with or without mT treatment. SOD2 mRNA levels were significantly higher in CF than WT cells at baseline, and mT decreased SOD2 levels in CF cells. Bars represent mean ± SEM (n = 3). *** $P<0.001$, for WT vs. CF cells. (B) SOD2 activity was determined by looking at protein expression levels. SOD2 protein levels were higher in untreated CF cells and mT treatment resulted in a significant decrease in SOD2, although not to WT levels. Data shown are representative of three different experiments.

Conclusions

The data presented in this dissertation focuses on understanding the mechanisms of PUFA alterations in CF, uncovering how DHA works to correct these alterations and determining the link between the PUFA changes and CFTR gene mutations. Previous studies have shown that three main PUFA alterations occur in CF, namely decreased LA and DHA and increased AA. In this dissertation, we provide a mechanistic explanation for the PUFA alterations in CF. We show that these changes are a consequence of increased expression and activity of the enzymes that metabolize PUFAs, including Δ5 and Δ6-desaturase. This leads to increased metabolism in the upper part of the n-3 and n-6 pathways, and consumes LA in its conversion to AA, resulting in the decreased LA and increased AA levels that are classically observed in CF. Our findings also demonstrate that there is greater conversion of LNA to EPA than there is of LA to AA, indicating that Δ6-desaturase has a higher affinity for n-3 fatty acids over n-6 fatty acids. That metabolism of LNA to EPA is increased in CF cells rules out the possibility of relatively decreased activity of the n-3 pathway specifically as an explanation for the low DHA levels in CF. The decreased DHA levels can potentially be explained by two things. First, only a small % of LNA that is incorporated into CF cells gets metabolized to DHA. This, coupled with the decrease in metabolism of EPA to DHA, may account for the low levels of DHA seen in CF. Second, DHA can undergo a process known as retroconversion wherein it gets shortened and desaturated back to EPA. DHA retroconversion is distinctly upregulated in CF cells compared to WT cells and this contributes to the increased EPA and decreased DHA levels in CF. Additionally, our studies show that the decrease in metabolism of EPA to DHA occurs at the first

metabolic step from EPA to 22:5n-3. It is feasible that reduced metabolism at this step stems from shunting of EPA to eicosanoid biosynthesis, thus reducing the amount of substrate available for metabolism to DHA. In support of this theory, both the expression and activity of the rate-limiting enzymes of eicosanoid synthesis, COX-2 and 5-LOX are increased in CF cells and in CF knockout mice. Further studies are necessary to confirm whether increased eicosanoid synthesis contributes to the decreased DHA levels in CF.

With regard to the mechanism by which DHA works to correct PUFA abnormalities and CF phenotype, we show that treatment of CF cells with exogenous DHA reverses the PUFA metabolic changes by suppressing expression and activity of Δ5 and Δ6-desaturase enzymes. This leads to decreased conversion of LA to AA and increases DHA levels, reversing all three PUFA abnormalities found in CF. Moreover, the related n-3 fatty acid EPA is just as effective as DHA in downregulating desaturase expression and activity and normalizing PUFA metabolism in CF. Similar effects on desaturase suppression are observed after supplementing CF mice with high levels of DHA or a combination of DHA and EPA. The noted benefit of n-3 fatty acid therapy in CF mice is likely due to its ability to normalize AA levels and subsequently reduce excessive production of pro-inflammatory eicosanoids. Additionally, n-3 fatty acids can be metabolized to potent anti-inflammatory resolvins and protectins to help combat the inflammation present in CF.

Proposed mechanism linking CFTR mutations to PUFA alterations

Loss of CFTR function as seen in CF can result in mitochondrial dysfunction, characterized by elevated oxygen consumption, increased activity of the mitochondrial

electron transport chain (ETC) and altered kinetics of complex I of the mitochondrial electron transport system [167,168]. Although the mitochondrial ETC is a very efficient system, electron leakage is common due to the nature of the alternating one-electron redox reactions that it catalyzes. Thus, electrons can pass to oxygen directly instead of to the next electron carrier in the chain, leading to the production of superoxide anions. It is commonly believed that mitochondrial generation of O_2^- represents the major intracellular source of ROS under physiological conditions, with complex I and III of the ETC producing majority of the O_2^- [169]. Previous studies report that increased activity of the ETC leads to increased ROS production while inhibition of certain ETC complexes decreases ROS production [169-172]. Consequently, increased activity of the mitochondrial ETC caused by dysfunctional CFTR can result in elevated intracellular ROS levels. This was confirmed in our CF cell culture system with CF cells producing much higher levels of O_2^- compared to WT cells. We hypothesize that increased ROS production, including increased mitochondrial O_2^-, may bring about the PUFA alterations commonly seen in CF through the mechanism outlined below (Figure 42).

Increased O_2^- levels can activate the AMPK signaling pathway by ROS-mediated opening of calcium release-activated calcium (CRAC) channels [173]. O_2^- triggers intracellular calcium release from the ER, which in turn initiates CRAC channel organization at the plasma membrane. This allows for extracellular calcium entry, leading to increases in cytosolic calcium that activate the calcium/calmodulin-dependent protein kinase kinase beta (CaMKKβ). CaMKKβ can then phosphorylate and activate AMPK. AMPK is a ubiquitous Ser/Thr kinase that exists as a heterotrimer composed of a catalytic α subunit and regulatory β and γ subunits [174]. The activity of AMPK increases

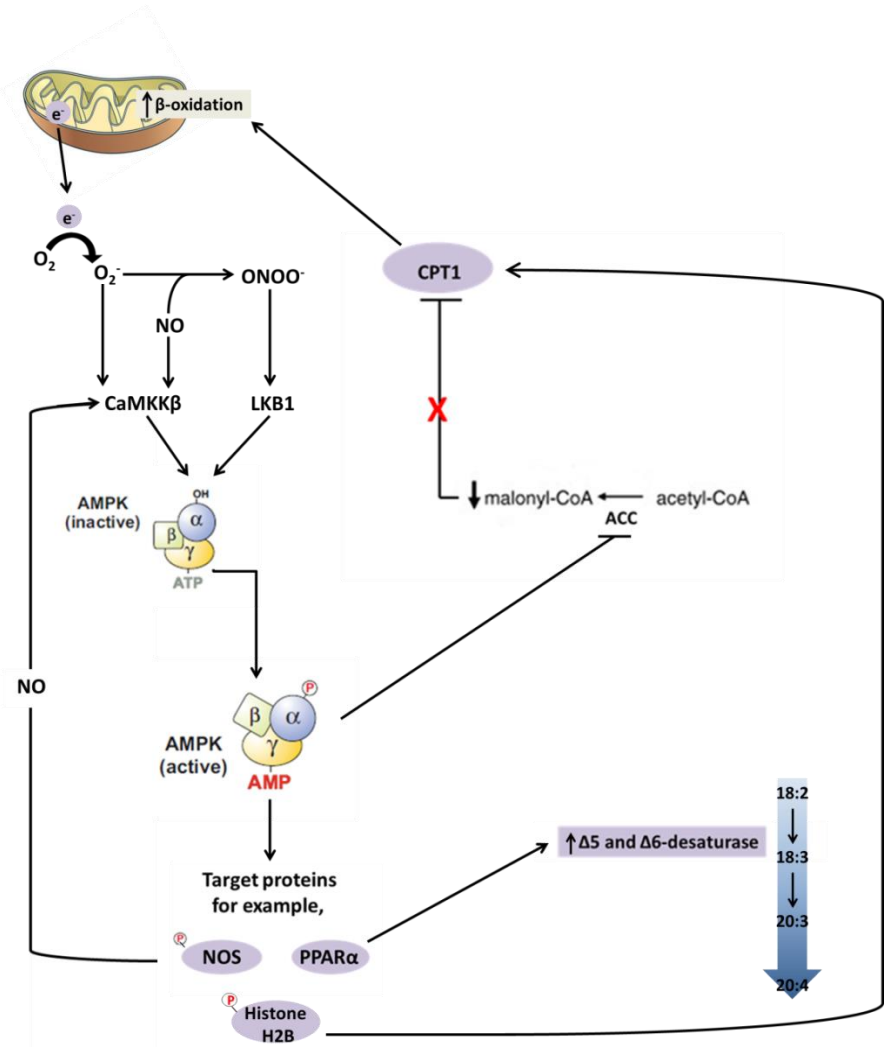

Figure 42: Proposed mechanism detailing ROS-mediated AMPK stimulation of Δ5 and Δ6-desaturase expression and activity. Mutations in CFTR can lead to increased ROS production through increasing mitochondrial respiration or initiating the unfolded protein response in the case of ΔF508 mutations. This leads to increased O_2^- production, which can activate AMPK via CaMKKβ-phosphorylation. In addition to this pathway, nitroxidative stress can also activate AMPK through NO and $ONOO^-$, which activate CaMKKβ and LKB1 respectively. When activated, AMPK can phosphorylate and inhibit ACC, thereby reducing malonyl-CoA levels and disinhibiting CPT-1. AMPK can also phosphorylate histone H2B, leading to increased transcription of CPT-1. This increases fatty acid oxidation and ROS production, generating a positive-feedback loop with AMPK. Active AMPK can also phosphorylate and activate downstream target proteins including NO synthase and PPARα. NO synthase phosphorylation induces NO production and creates a positive-feedback loop with NO through CaMKKβ. PPARα activation can result in increased transcription of Δ5 and Δ6-desaturase genes, leading to increased LA to AA metabolism as seen in CF.

during conditions of metabolic stress, in response to elevated intracellular [AMP]/[ATP] ratios [175,176]. Additionally, AMPK can be activated by phosphorylation of the α subunit at Thr-172 of its activation loop by upstream AMPK kinases, including liver kinase B1 (LKB1) and CaMKKβ [177,178]. As a cellular metabolic sensor, AMPK acts on a wide variety of substrates and cellular pathways, switching on catabolic pathways that generate ATP and switching off ATP-consuming processes.

In CF cells, CaMKKβ-activation of AMPK in response to increased ROS production can lead to increased mitochondrial fatty acid oxidation as well as increased mitochondrial biogenesis. Once activated, AMPK can phosphorylate and inactivate the mitochondrial-associated isoform of acetyl-CoA carboxylase (ACC2), resulting in decreased malonyl-CoA levels. Malonyl-CoA inhibits carnitine palmitoyl transferase (CPT-1), therefore a decrease in malonyl-CoA levels relieves CPT-1 inhibition and allows for long chain fatty acids to enter the mitochondria for β-oxidation [179]. AMPK can also phosphorylate histone H2B at serine 36, leading to transcriptional activation of CPT-1 [180]. With increased fatty acid β-oxidation, the delivery of reducing equivalents (NADH and $FADH_2$) to the ETC and the generation of ROS such as O_2^- is increased from either complex I or III of the ETC [181]. This creates a positive feedback loop in which increased ROS levels activate AMPK, which promotes mitochondrial biogenesis [182] and fatty acid oxidation, resulting in the production of more ROS and perpetuating the cycle. Activated AMPK also increases the transcriptional activity of PPARα [183], which in turn increases the expression of PPARα target genes including both Δ5 and Δ6-desaturase. In this way, we postulate that increased AMPK activation, via PPARα, leads to increased metabolism of LA to AA and results in the PUFA abnormalities found in CF.

The ROS measurements we performed focus solely on O_2^- production. However, it is important to mention that other pro-oxidant molecules may be involved in the activation of AMPK and subsequent upregulation of desaturase expression and activity. Nitro-oxidative stress generated by nitric oxide (NO) and peroxynitrite ($ONOO^-$) can also activate CaMKKβ and LKB1 respectively [184, 185], leading to AMPK activation. Conversely, AMPK can regulate the levels of reactive nitrogen species by inducing NO production through phosphorylation and activation of NO synthase [186], generating a positive feedback loop with NO through CaMKKβ. Several studies establish that the activity of NO synthase is elevated in lung tissue of CF patients [187], and the concentration of NO metabolites such as nitrate and peroxynitrite is increased in CF [188-190].

Mutations in CFTR can also lead to increased ROS production by initiating ER stress. This mainly applies to the ΔF508 mutation, which is the most common mutation in CF and is characterized by defective protein processing and misfolding. Deletion of phenylalanine at position 508 results in accumulation of misfolded ΔF508 protein in the ER and causes ER stress. The cells respond to the buildup of misfolded protein and adapt themselves to the stress condition by activating the unfolded protein response (UPR) [191]. The UPR integrates pathways aimed at improving the ER capacity to effectively process proteins, either by inducing the transcription of genes encoding ER chaperones, or by activating ERAD to degrade misfolded proteins [192-194]. It is well documented that ROS including mitochondrial O_2^- are generated during ER stress and the UPR, through enzymatic mechanisms involving mitochondrial electron transport, ER oxidoreductases and the Nox4 NADPH oxidase complex [195]. Therefore it is likely that

induction of the UPR in ΔF508 CF cells contributes to the production of ROS. This can then trigger phosphorylation of AMPK and result in the PUFA abnormalities seen in CF, as described above. However, the increase in O_2^- levels in our cell culture experiments is probably not due to the UPR since loss of CFTR expression in our culture system is not caused by ΔF508 protein misfolding.

If our hypothesis is correct and increased ROS production is central to the altered PUFA metabolism in CF, then reducing ROS levels should help correct the abnormal PUFA levels. In keeping with this, we observed that treatment of cultured CF cells with three different antioxidants reduced O_2^- levels and normalized LA to AA metabolism to WT levels. Additionally, it is possible that one of the ways through which dietary DHA and EPA supplementation reversed PUFA alterations in the CF mice was through decreasing O_2^- production in these animals. This theory is supported by previous studies that have reported n-3 fatty acid supplementation results in a decrease in O_2^- generation [196, 197].

In addition to altering cellular ROS levels by antioxidant or PUFA supplementation, there are numerous other ways to test this proposed mechanism. According to our hypothesis, activation of the AMPK signaling pathway by ROS is needed to initiate increased transcription of desaturase genes via PPARα. To determine if this is indeed the case, we can inhibit AMPK in CF cells using the small molecule inhibitor Compound C or siRNA against the AMPKα catalytic subunit. AMPK inhibition should result in normalization of Δ5 and Δ6-desaturase expression and activity in CF cells. Moreover, AMPK inhibition should decrease fatty acid oxidation and activation of NO synthase, and thus prevent the positive feedback loop responsible for augmenting

ROS production in CF cells. The reverse experiment involving the activation of AMPK in WT cells can be performed to assess if this contributes to altered PUFA metabolism. To determine if oxidant signals mediate an increase in AMPK activation via CaMKKβ, we can abrogate CaMKKβ expression using siRNA or pharmacological inhibition with STO-609. Knockdown of CaMKKβ should prevent activation of AMPK and abolish the AMPK-PUFA response. Alternatively, we can explore the role of CaMKKβ in ROS-mediated AMPK activation by modifying Ca^{2+} levels in CF cells. Because entry of extracellular Ca^{2+} is needed to activate CaMKKβ and phosphorylate AMPK, we would predict that removal of extracellular Ca^{2+} by growing CF cells in calcium-free media should prevent CaMKKβ activation and decrease AMPK phosphorylation. This would be consistent with a signaling pathway that requires Ca^{2+} influx for AMPK activation. The role of LKB1 in activating AMPK can also be evaluated using siRNA against LKB1. We also propose that AMPK activation leads to increased desaturase expression and activity via the PPARα transcription factor. This part of the mechanism can be assessed through siRNA-mediated PPARα knockdown in CF cells or by treating WT cells with the PPARα activator fenofibrate. If PPARα expression induces increased desaturase expression and activity, then knocking it down in CF cells should normalize PUFA metabolism while activating it in WT cells should result in increased LA to AA metabolism. The contribution of ER stress and the UPR towards increased ROS production and altered PUFA metabolism in ΔF508 cells can be tested by culturing the cells at low temperature (26^oC) or by treating the cells with 4-PBA. These strategies work to correct the folding defect and allow for ΔF508-CFTR to exit the ER and traffic to the cell surface. If ΔF508 misfolding leads to ER stress thus triggering the UPR and increasing ROS production,

then correcting the folding defect should decrease ROS levels and correct the PUFA abnormalities in ΔF508 cells.

More work is needed to validate and dissect our proposed model in order to determine whether the link between CFTR mutations and PUFA abnormalities involves ROS-mediated signaling. Further examination of the contribution of ROS and the AMPK signaling pathway to PUFA abnormalities and CF pathogenesis will allow us to determine if these are potential therapeutic targets for the disease and assist us in the development of better drug regimens to treat CF patients.

Potential impact on therapy

The data presented in this dissertation details in vitro experiments using n-3 fatty acid supplementation, antioxidants and COX-2 inhibitors to correct PUFA abnormalities in cultured CF cells. DHA and EPA were found to suppress desaturase expression and activity, and normalize PUFA metabolism in CF cells to WT levels. Antioxidant treatment using NAC, TX and mT was found to have a similar effect. In addition, DHA supplementation on its own or in combination with EPA was shown to drastically decrease AA levels and increase DHA levels in CF knockout mice. This data suggests that correction of PUFA alterations in CF using either of these treatments may be beneficial in ameliorating some of the pathologic manifestations of CF. Several clinical trials have been carried out to evaluate the effect of antioxidant therapy, non-steroidal anti-inflammatory drugs (NSAIDs), or n-3 fatty acid supplementation in CF patients (Tables 5-7). In these trials, pulmonary function tests including forced expiratory volume

in 1 second (FEV_1) and forced vital capacity (FVC) were the primary clinical measures used to assess the outcome of the various treatments.

Table 5: Clinical trials using antioxidant therapy for treatment of CF

Study	n	Duration	Antioxidant Intervention	Measured outcomes
Woods et al., 2003	46	8 weeks	Group A [low dose of supplement (10 mg vitamin E and 500 µg vitamin A)] Group B [high dose of supplement (200 mg vitamin E, 300 mg vitamin C, 25 mg β-carotene, 90 µg selenium, and 500 µg vitamin A)]	• Increased plasma vitamin E, β-carotene and selenium concentrations in group B • No change in FEV_1, FVC or $F_{2\alpha}$-isoprostane levels between group A and B
Homnick et al., 1995a	15	2 weeks	Control [placebo] Treatment [30, 90 or 300 mg β-carotene]	• No difference in shwachman scores or β-carotene intake between different treatment groups or control
Homnick et al., 1995b	20	14 months	Control [placebo] Treatment [60 mg β-carotene/day, increased throughout the course of the trial to a mean of 144 mg/day]	• Dose-proportional increase in plasma β-carotene levels in treatment group • No difference in shwachman scores between control and treatment groups
Portal et al., 1995a	27	5 months treatment, 1 month washout, 5 months other treatment	Treatment [2.8 µg selenium/kg/day] with a placebo control and inversion of treatment periods	• Improved lipid peroxidation markers in CF patients independent of selenium supplementation
Renner et al., 2001	24	6 months	Control [placebo] Treatment [1 mg β-carotene/kg/day (max 50 mg/day) for 3 months followed by 10 mg/day for 3 months taken once per day]	• Increased plasma β-carotene levels during 1st three months in treatment group • Decreased malondialdehyde levels and pulmonary exacerbations requiring antibiotic therapy in treatment group • No change in FEV_1 between control and treatment groups
Cobanoglu et al., 2002	15	6 months	Control [placebo] Treatment [0.69 ± 0.19 mg/kg β-carotene, three times per day]	• Increased plasma β-carotene and vitamin E • Decreased plasma TBFα and malondialdehyde • Improved FEV_1, FEF_{25-75} and shwachman scores in treatment group
Bishop et al., 2005	16	8 weeks	Control [placebo] Treatment [66 mg/kg buffered GSH, given across four inhalation sessions/day, spaced 3-4 h apart]	• Improved peak flow and greater subjective sense of improvement and cough frequency in treatment group • No significant improvement in FEV_1 or FVC in treatment group
Stafanger et al., 1989	31	3 months	Control [placebo] Treatment [Oral NAC, 200 mg X 3 daily (<30 kg) or 400 mg X 2 daily (>30 kg)	• No significant difference in lung function parameters between control and treatment for study group as a whole • CF patients with peak expiratory flow rate below 70% of predicted normal values showed an increase in FVC and FEV_1 in treatment group

Table 6: Clinical trials using NSAID therapy for treatment of CF

Study	n	Duration	NSAID Intervention	Measured outcomes
Konstan et al., 1995	84 [FEV_1 > 60% predicted]	4 years	Control [placebo] Treatment [High dose ibuprofen, 20-30 mg/kg to a maximum of 1600 mg twice daily: dose adjusted to reach peak plasma concentration of 50-100 µg/mL]	• Decreased annual rate of decline in FEV_1 % predicted • Decreased patient hospitalizations • Improved chest radiograph scores • Improved maintenance of body weight
Lands et al., 2007	142 [FEV_1 > 60% predicted]	2 years	Control [placebo] Treatment [High dose ibuprofen, 20-30 mg/kg to a maximum of 1600 mg twice daily: dose adjusted to reach peak plasma concentration of 50-100 µg/mL]	• Decreased annual rate of decline of FVC % predicted • Non-significant decrease in annual rate of decline in FEV_1 % predicted
Konstan et al., 2007	1365 Observational data from CF foundation patient registry	2-7 years	Treatment [ibuprofen, amount not specified]	• Less rapid decline in FEV_1 among ibuprofen-treated patients • Increased risk of gastrointestinal bleeding requiring hospitalization in ibuprofen-treated patients
Sordelli et al., 1994	41	12-19 months	Control [placebo] Treatment [single dose of between 5-20 mg piroxicam, determined by weight]	• No benefit for treatment group vs placebo in relation to lung function
Shmarina et al., 2004	47	1 year	Group A [cox-2 inhibitor nimesulide at 3 mg/kg] Group B [clarithromycin at 250 mg every other day]	• Decreased sputum TNFα in both groups • Decreased sputum IL-8 in group B • Higher efficacy of clarithromycin in anti-inflammatory treatment over nimesulide

The trials involving antioxidant therapy made use of antioxidants such as β-carotene, vitamin E, selenium, GSH and NAC (Table 5). Although most trials resulted in increased plasma antioxidant levels, there was no significant improvement in lung function parameters. Only one study by Cobanoglu et al. [198] reported improvement in FEV_1 after 6 months β-carotene treatment. Similarly, clinical trials involving NSAID use produced mixed results (Table 6). Some studies reported a decrease in the annual rate of FEV_1 and FVC decline, while other studies showed no benefit in relation to lung function. Konstan et al. [199] described some adverse effects including an increased risk of gastrointestinal bleeding in the CF patients receiving ibuprofen compared to placebo. In the clinical trials utilizing n-3 fatty acids, the supplemented fatty acids comprised

DHA, EPA or a combination of both DHA and EPA (Table 7). The majority of these trials described an increase in plasma n-3 fatty acid levels in the treatment group over the controls. However, these trials resulted in little to no improvement in lung function parameters. Consequently, we concluded that to the best of our knowledge, none of the CF clinical trials using antioxidants, NSAIDs or n-3 fatty acids have been very successful.

Table 7: Clinical trials using fish oil (DHA and EPA) therapy for treatment of CF

Study	n	Duration	n-3 fatty acid supplementation	Measured outcomes
De Vizia et al., 2003	30	8 months	1.28 g EPA and 0.93 g DHA daily	• Increased EPA and DHA and decreased AA in erythrocyte membrane phospholipids • Small improvement in FEV_1 • Decreased serum IgG and α-1 antitrypsin • Decreased use of antibiotics compared with previous 8 months
Kurlandsky et al., 1994	14	2 weeks	0.1 g ethyl ester EPA and DHA/kg/day	• Increased EPA and DHA in platelet phospholipids • Decreased serum LTB_4 • No effect on lung function parameters or shwachman score
Mengedhot et al., 1999	69	13 months	0.036 g/kg/day fish oil	• No effect on urinary LTB_4 excretion, disease score or infections • Small improvement in FVC
Lawrence et al., 1993	16	6 weeks	2.7 g EPA daily	• Improved FEV_1, FVC and shwachman scores • Decreased sputum volume
Katz et al., 1996	18	6 months	Control [150 mg/kg intravenous lipid emulsion enriched in linoleic and oleic acid] Treatment [150 mg/kg intravenous lipid emulsion enriched in EPA and DHA]	• Increased plasma EPA and DHA • No effect on lung function parameters
Jumpsen et al., 2006	5	6 weeks	70 mg/kg DHA daily	• Increased plasma and erythrocyte DHA levels • No effect on lung function parameters
Thies et al., 1997	5	1 year	6-9 capsules daily, each containing 300 mg EPA and 200 mg DHA	• No effect on lung function parameters • Treatment had no effect on number of hospitalization days

Treatment of CF is quite complex due to the fact that various factors can contribute to disease progression. Therefore instead of targeting a single causative factor, it may be more beneficial to target several factors at once. To this end, we propose that a combined drug 'cocktail' may be more efficacious than the use of single drug therapy as in the clinical trials described above. For example, based on our data we believe that excessive production of pro-inflammatory eicosanoids and ROS contribute to CF disease. Hence, we suggest the use of combination drug therapy that includes ibuprofen, n-3 fatty acids and antioxidants. The purpose of ibuprofen is to inhibit COX enzymes and decrease the production of AA-derived eicosanoids. Additionally, n-3 fatty acid supplementation can be given to decrease the production of pro-inflammatory eicosanoids by decreasing desaturase expression and activity and therefore decreasing AA levels. DHA and EPA can also compete with AA for esterification at sn-2 position of phospholipids, thereby decreasing AA levels and reducing the amount of substrate available to be converted to pro-inflammatory eicosanoids. DHA and EPA can themselves be metabolized to potent anti-inflammatory mediators such as resolvins and protectins. Lastly, antioxidants such as vitamin E, NAC or β-carotene can be given to neutralize the excessive production of free radicals and decrease the levels of ROS. Together, it is possible that the combination of these three drugs may have a greater effect at ameliorating CF disease symptoms and improving lung function parameters than any one of them singly.

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