Fenretinide's preventative effect on the development of osteoporosis in Cystic Fibrosis

By

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To Dominique,

For Being My Everything

ASTRACT

Cystic Fibrosis (CF) is the most common autosomal recessive disease affecting the Caucasian population. This devastating disease is caused by any one of 1500 mutations identified in the Cystic Fibrosis Transmembrane Regulator Conductance (*cftr*) gene. Chronic inflammation is a hallmark of CF and it affects all systems including respiratory, gastrointestinal, reproductive and skeletal. Although the exact molecular link between the CFTR dysfunction and various phenotypes remains to be delineated, many phenotypes seem to be linked to inadequate nutritional absorption of essential fatty acids and vitamins, which leads to an imbalance between the essential fatty acids docosahexaenoic acid (DHA) and arachidonic acid (AA). The skeletal system does not only serve as mechanical support, but also functions as an active organ that regulates balance and interactions between both local and systemic hormones, cytokines and prostaglandins. Previously our laboratory has shown that fenretinide [*N*-(4-hydroxyphenyl) retinamide] corrects the essential fatty acid imbalance. We hypothesized that correcting the DHA/AA ratio in the plasma of *Cftr*-KO mice could avoid the early-onset osteoporosis. This thesis presents our novel results describing how fenretinide prevents osteoporosis. We found that twice a week treatment with fenretinide over a period of four weeks dramatically increased trabecular bone volume and quality in *Cftr*-KO mice. The results of this thesis strongly suggest that fenretinide might have potential for the treatment of cystic fibrosis patients by preventing the reduction of trabecular bone mineral density.

RÉSUMÉ

La fibrose kystique (FK) est la maladie autosomique récessive la plus commune parmi la population Caucasienne. Cette maladie dévastatrice est causée par une des 1500 mutations identifiées dans le gène *cftr* (Cystic Fibrosis Transmembrane Regulator Conductance). L'inflammation chronique est une caractéristique incontournable de la FK et elle affecte tous les organes du corps incluant ceux dans le système respiratoire, gastro-intestinal, reproductif et squelettique. Malgré le fait que le lien moléculaire précis entre le dysfonctionnement du CFTR et les phénotypes de la FK n'est pas complètement expliqué, plusieurs de ces phénotypes semblent associés a l'absorption nutritionnelle inadéquate des acides gras et des vitamines. Ceci résulte dans un déséquilibre entre les acides gras essentiels acide docosa-hexanoïque (ADH) et acide arachidonique (AA). Le système squelettique n'est pas seulement un support mécanique mais il fonctionne aussi comme organe actif régulant l'équilibre et l'interaction locale et systémique de certaines hormones, cytokines et prostaglandines. Notre hypothèse est qu'en corrigeant le ratio d'ADH/AA dans le plasma sanguin des souris *Cftr*-KO, nous pouvons prévenir le début précoce de l'ostéoporose. Précédemment dans notre laboratoire, nous avons démonté que le fenretinide [N-(4-hydroxyphényl) retinamide] corrige ce déséquilibre d'acide gras essentiel. Cette thèse présente de nouveaux résultats qui décrivent comment le fenretinide prévient l'ostéoporose. Nos donnés prouve qu'un traitement de fenretinide deux fois par semaine étalé sur une période de quatre semaines hausse de façon dramatique le volume de l'os trabéculaire et améliores qualité. Les résultats de cette thèse suggèrent fortement que le fenretinide pourrait avoir un énorme potentiel dans le traitement des patients atteient de fibrose kystique en prévenant la réduction de la densité minérale osseuse trabéculaire.

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Which leads to the most significant person of my life, Dominique Marion, it is not possible to list the number of things I want to thank you for...most importantly for your unconditional love and our united future "happily ever after".

PRÉFACE

The author of this thesis has chosen to present it in the format of "manuscript-based thesis"; the following section indicates the "Contributions of Authors".

The Introduction chapter is based on a review entitled; "Novel Pharmaceutical Approaches for Treating Patients with Cystic Fibrosis" by **Zienab Saeed,** Gabriella Wojewodka, Dominique Marion, Claudine Guilbault, Danuta Radzioch, submitted to *Current Pharmaceutical Design.* The objective of this review was to determine what novel pharmaceutical approaches researchers are investigating for patients with cystic fibrosis. ZS, contributed to 90% of the literature search and 95% of the writing and correcting of the review. GW contributed approximately 10% of the literature search, DM contributed to the editing, CG contributed to approximately 5% of the writing of the manuscript.

Chapter II is based on a manuscript "Fenretinide prevents the development of osteoporosis in *cftr-KO* mice" by **Zienab Saeed**, Claudine Guilbault, Juan B. De Sanctis, Jennifer Henri, Dominique Marion, René St-Arnaud, Danuta Radzioch that has been prepared for submission. The objective of this manuscript was to determine if fenretinide could potentially be used as an effective treatment for the prevention of low bone mineral density in patient with cystic fibrosis using a mouse model. ZS was in charge of the design and planning of the experiments and all other experimental procedures*, data analyses, formatting and writing of manuscript. CG, provided assistance in data analysis and in training ZS, JBS performed lipid analysis, JH provided technical assistance with the animals, DM provided technical support through out all aspects of the experiments and the

editing of the manuscript, RSA laboratory assisted in vitamin D analysis and also in some histology.

Chapter III is a summary of the general conclusions of this thesis as well as potential future directions this project could lead to.

The research included in this thesis (the last 1.5 years) has been supported by a grant from the Canadian Cystic Fibrosis Foundation.

* Experimental procedures includes: experiment and harvest preparations, drug supplementation, experiment harvest, preparation of bones for microCT, and assisted in the analysis of the data collection afterwards, paraffin embedded histology (including quantification of osteoblast and osteoclast), immuno-histochemistry and statistical analysis.

KEYWORDS

Arachidonic acid

Bone Mineral Density

Ceramide

Clinical trials

Cystic fibrosis conductance transmembrane regulator (CFTR)

Cftr-KO mice

Cystic fibrosis

Docosahexanoic acid

Essential Fatty Acids

Fenretinide

Lipid Metabolism

Novel treatments

Osteoporosis

Trabecular bone density

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LIST OF ABBREVIATIONS

AA- Arachidonic acid
AI-Aztreonam Lysine for Inhalation
ATP-Adenosine triphosphate
B6- C57BL/6
BAL- Bronchoalveolar lavage
BHA- Butylated hydroxyanisole
BMC- Bucal mucosal cells
cAMP- Cyclic adenosine mono-phosphate
CBAVD- Congenital bilateral absence of the vas deference
CF-Cystic Fibrosis
Cftr- Cystic Fibrosis Transmembrane Conductance Regulator
CFU- Colony forming units
Cl- Chloride
CMV- Cytomegalovirus
COX2- Cyclooxygenase-2
DHA- Docosahexaenoic acid
DIOS- Disal intestinal obstructive syndrome
EFA- Essential fatty acid
ELISA-Enzyme-Linked ImmunoSorbent Assay
EPA- Eicosapentaenoic acid
ER- Endoplasmic reticulum
FDA- Federal Drug Administration
FEV_1- Forced Expiratory Volume in the first second
FEN-Fenretinide
GM-CSF- Granulocyte macrophage colony-stimulating factor
GSH- Glutathione
H&E- Haematoxylin and eosin
HPLC-High Pressure Liquid Chromatography
ICS- Inhaled corticosteroids
ICSI- Intracytoplasmic sperm into the cytoplasm
IgG- Immunoglobulin G
IL- Interleukin
KO- Knockout
MDA- Malonyl dialdehyde
Micro-CT- Micro Computed Tomography
MMA- Polymethylmethacrylate
MSD- Membrance spanning domain
NAC-*N*-acetylcysteine
NBD- Nucleotide binding domain
NIH- National Institutes of Health

NO- Nitric oxide
NOS- Nitric oxide synathase
NSAIDS- Non steroidal anti-inflammatory drugs
ORCC- Outward rectifying chloride channel
PA- *Pseudomonas aeruginosa*
PCR- Polymerase Chain Reaction
PEP-EUR- Eurand Pancreatic Enzyme Product
PERT- Pancreatic enzyme replacement therapy
PI- Pancreatic insufficiency
PMNs- Polymorphonuclear leucocyte
PUFA- Polyunsaturated fatty acids
QS- Quorum sensing
rAAV- Recombinant adeno-associated virus
rhDNase- Recombinant human deoxyribonuclease I
rhGH- Recombinant growth hormone
ROS reactive oxygen species (ROS)
RTC- Randomized controlled trial
SEM- Standard error of the mean
SNP- Single nucleotide polymorphism
SPF- Specific pathogen free
SLIT- Sustained Release Lipid Inhaled Targeting
TIP- Tobramycin Inhaled Powder
TLC- Thin Layer Chromatography
TLRs- Toll like Receptors
TN- Trabecular bone number
TNF- Tumor necrosis factor
TOBI- Tobramycin
TRAP- Tartrate-Resistant Acid Phosphatase
WT- Wild-type

Chapter I

Chapter I

Based in Part on Submitted Manuscript

To

Current Pharmaceutical Drug Design 2007

Novel Pharmaceutical Approaches for Treating Patients with Cystic Fibrosis

Saeed Z, Wojewodka G, Marion D, Guilbault C and Radzioch D

Abstract:

The advancement in drug discovery for cystic fibrosis promises improvements in both the quality and longevity of the lives of patients that live with this devastating disease. Before the cloning of the *Cftr* gene in 1989, there were relatively few options in treatments of the many phenotypes associated with the disease. The developments in areas of research such as immunology, molecular biology and pharmacology have provided new insights in the mechanism and evolution of CF. More than 40 systematic clinical trials evaluating new therapies for CF are registered with the NIH at the present time. A great deal of effort is focused on the main cause of mortality; chronic and persistent lung infections. Intestinal malabsorption, pancreatic insufficiency, reduced bone mineral density and reproductive abnormalities are other manifestations of this disease where the development of innovated treatments gives renewed hope to patients and their families of those affected. The following review is a summary of the novel pharmaceutical approaches for the treatment of cystic fibrosis.

1.1 Introduction:

1.1.1 CF Overview

Cystic Fibrosis (CF) was first described as a disease in the late 1930s, however literature from Germany and Switzerland in the 1700s warned "Woe is the child who tastes salty from a kiss on the brow, for he is cursed, and soon must die." CF remains the most common autosomal recessive disease affecting most frequently the Caucasian population. It affects over 3 400 children and adults in Canada and approximately 30 000 in the United States[1]. Based on the autosomal recessive mode of inheritance, researchers have estimated that the CF gene carrier frequency is about 1 in 25 among the Caucasian population (79, 198). The disease is however more prevalent in populations of Ashkenazi Jews, people from European descent and certain geographical areas, such as in the Saguenay-Lac-Saint-Jean (Quebec, Canada) region where the incidence is increased to 1 in 891 births (39, 182).

1.1.2 *Cftr* Gene

The *Cftr* gene is positioned on the long arm of chromosome 7 (7q31.3), comprised of 27 exons and is 250 kb long (30, 79). Over 1500 mutations[2] at the *cftr* gene have been detected to date. These mutations have been classified into 5 different classes of protein dysfunctions as shown in figure 1 (168):

- Class I- defective protein maturation (eg. nonsense G542X, frameshift 394delTT and splice junction 1717-1G →A)
- Class II- dsyregulation of the CFTR protein processing (eg. ΔF508 mutation)

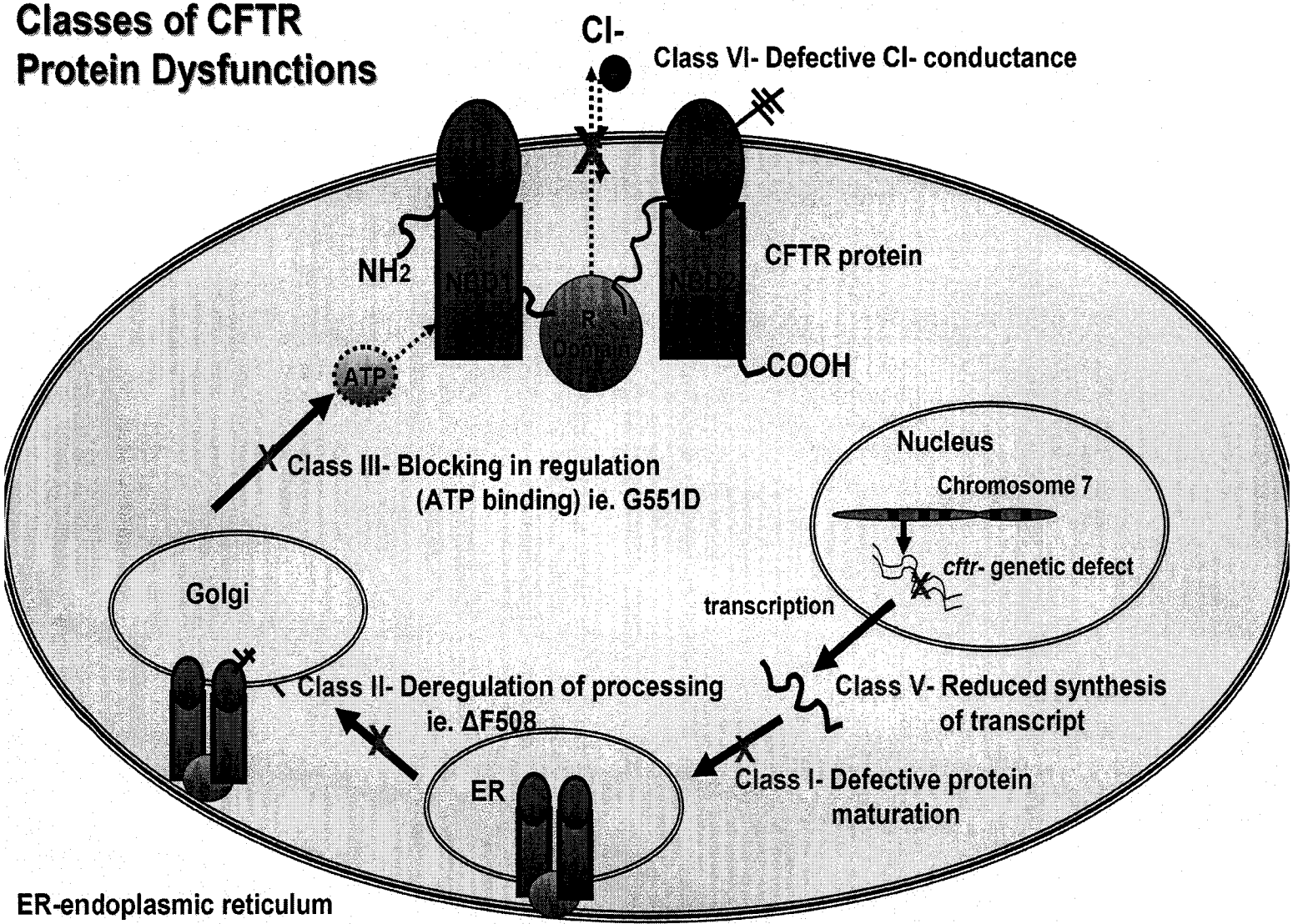
[1] www.cff.org

[2] A complete database composed of the 1529 *Cftr* mutations is available on the web site of the Hospital for Sick Children; http://www.genet.sickkids.on.ca/cftr/ accessed Feb 2/07

- Class III- blocking in regulation causing a disruption in activation and regulation of CFTR protein at the plasma membrane, for example the protein may be defective in respect to ATP binding and hydrolysis, or phosphorylation (G551D mutation).
- Class IV-defective chloride conductance or channel gating (ie. missense R117H)
- Class V- reduced synthesis of CFTR transcripts due to the promoter or splicing abnormalities (ie. missense A455E, alternative splicing 3849+10kbC→ T)

Figure 1.1 Classes of CFTR protein mutations. Class I is described when there is a defective protein maturation; Class II is a dsyregulation of the CFTR protein processing, (ΔF508 mutation found in 70-80% of CF patients); Class III occurs when there is a blocking in regulation causing a disruption in activation and regulation of CFTR protein at the plasma membrane, for example the protein may be defective in respect to ATP binding and hydrolysis, or phosphorylation (G551D mutation). Class IV is classified when there is a defective chloride conductance or channel gating; Class V refers to the reduction of *Cftr* transcripts synthesis due to the promoter or splicing abnormalities.

Classes of CFTR Protein Dysfunctions



ER-endoplasmic reticulum
ATP-adenosine triphosphate
NBD- nucleotide binding domain
MSD-membrane spanning domain
Cl- chloride
CFTR-Cystic Fibrosis Transmembrane Regulator

The most common mutation observed in CF patients is ΔF508. ΔF508 is a class II mutation which affects more than 70% of CF patients. It is characterized by the deletion of three base pairs in exon 10, resulting in the deletion of phenylalanine (38). More than 50% of Canadians with CF carry two copies of the most common mutation ΔF508; while, 85% of all individuals with CF in Canada carry at least one copy of ΔF508[3].

1.1.3 CFTR Protein

The CFTR protein is a transmembrane glycoprotein of 1480 amino acid residues that functions as a cyclic adenosine monophosphate (cAMP) dependent chloride conductance channel (38). The CFTR protein is a member of the adenosine triphosphate (ATP)-binding-cassette (ABC) membrane transporter superfamily. It is made of two homologous halves with each half containing a nucleotide binding domain that binds ATP and a membrane-spanning domain with 6 segments that helps form a channel pore spanning the cell membrane as shown in figure 1.1. The two halves are connected by a regulatory domain (R) that is phosphorylated by a cAMP-dependent protein kinase. CFTR appears to be located primarily in the apical membranes of the epithelial cells (2, 79, 89, 92). Originally recognized as a cAMP-dependent apical chloride conductance channel, it was then identified as a cAMP-dependent negative regulator of Na^+ channels (ENaC) (183). CFTR has also been postulated to have a role as a regulator of a separate chloride channel called the outward rectifying chloride channel (ORCC) (51, 70). Additionally, it has been identified to have a role in chloride transport across the membrane of mitochondrial organelles, as well as roles in regulation of the Cl^-/ HCO_3^- exchanger and the ROMK K^+ channel (114, 174).

[3] Data from the Canadian Cystic Fibrosis Foundation

An abnormal chloride level in sweat (more than 60 mmol/l) is a hallmark of CF and provides a good diagnostic test (181). The sweat test is the standard diagnostic test for CF by measuring the amount of salt in the sweat; high salt level indicates CF disease. Modern genetic diagnostic tools have also emerged like polymerase chain reaction (PCR) and single nucleotide polymorphism (SNP) analysis, to confirm the initial diagnosis obtained from the sweat test.

1.1.4 CF Mouse Model

Animal models represent a good surrogate for studying diseases such as CF. The animal models used to study CF include mouse, rat, ferret, rabbit, sheep, pig and cat (137, 172). Mice used as an animal model have several advantages; they share 78% homology with humans in the CFTR protein, the cost of their purchase and maintenance although very high is still relatively inexpensive compared to other larger animal models. They also reproduce faster than most other animal models and they can be easily genetically modified into transgenic, knockout or well-defined inbred strains. They also offer a significant advantage over other animal models in terms of the availability of experimental tools (specific antibodies, SNPs, microsatellites).

Approximately three years after the *Cftr* gene was identified and cloned, the generation of the first CF mouse model was reported (177). To date, eleven CF mouse models have been characterized and have been generated by the same general technique, using gene targeting in embryonic stem cells to disrupt the endogenous CFTR genotype as summarized in table 1.1.

Table 1.1 Mouse Model Summaries[4]

Researchers / Year	Mouse	Mutation	Uniqueness	Ref.
Snouwaert *et al.* Clarke *et al.* 1992	$CFTR^{tm1UNC}$ Mix genetic background (B6D2129, C57BL/6/129, BALB/C/129)	-*Cftr* gene contained a stop codon in the coding sequence of exon 10 -Absences of CFTR Protein	-First Model -Less then 5% survival	(28, 177)
Kent *et al.* 1997	$CFTR^{tm1UNC}$/ $CFTR^{tm1UNC}$	-Novel congenic strain was designated B6-KO -$CFTR^{tm1UNC}$ mice HZ at the *cftr* locus were backcrossed onto the B6	-Backcrossed 18 times to B6 background to ensure 100% homozygosity for B6 strain alleles (determined by microsatellite typing) -Suseptiable to S. aureus, B. cepacia, P. aeruginosa	(99)
Dorin *et al.* 1992	$CFTR^{tm1UNC}$ KO 1 (MF/129 genetic background)	-Targeting strategy used designated $CFTR^{tm1HGU}$, by insertional mutagenesis targeted to exon 10 of the *Cftr* gene	-Exon skipping and aberrant splicing produced about 10% normal *Cftr* mRNA, resulting in a much milder disease phenotype. -About 95% of the mice survived to maturity.	(46, 47)
Ratcliff *et al.* 1993	C57BL/6/129 (mixed genetic background of MF1/129)	-Replacement mutation of exon 10, resulting in a null mutation	-The phenotype of these mice, designated $CFTR^{tm1CAM}$, is very similar to that of the $CFTR^{tm1UNC}$ KO except that it further exhibits lacrimal gland pathology	(153)
O'Neal *et al.* 1993	$CFTR^{tm1BAY}$	-C57BL/6/129 CF mouse, designated by duplication of exon 3 in the *Cftr* gene	-These mice produce less than 2% of normal levels of WT mRNA, but exhibit a severe phenotype with a high mortality rate - 40% of the *Cftr*-KO mice survived past day 7	(139)
Hasty *et al.* 1995	$CFTR^{tm3BAY}$	-Replacement of exon 2, created another null mutation mouse	-40% survival rate at one month of age	(85)
Rozmahel *et al.* 1996	$CFTR^{tm1HSC}$ KO	-Disruption of exon 1 of the *Cftr* gene	-Mixed genetic background and displayed a severe phenotype with only 30% survival -Modified genes for meconium ileus	(169)
Van Doorninck *et al* 1995	$CFTR^{tm1EUR}$ (FVB/129 background) ΔF508 mutation	-ΔF508 mice model by inserting the mutation into exon 10 using a double homologous recombination ("hit and run") technique.	-No severe disease, possibly explained by the presence of the mutant CFTR protein at normal levels, which could provide enough residual function. -Several studies have shown that the ΔF508 CFTR protein exhibits partial function as a Cl^- channel,	(36)

[4] This table is in part based on a previously published review in 2007 entitled "Cystic Fibrosis Mouse Models" by Claudine Guibault, **Zienab Saeed**, Gregory P. Downey, and Danuta Radzioch

			with a similar conductance and a decrease in open channel probability	
Colledge *et al* 1995	$CFTR^{tm2CAM}$ (C57BL/6/129 background)	-ΔF508 knockout mice by replacement of exon 10	-20% survival, these ΔF508 mutants, designated exhibited approximately 65% survival. -30% of the normal level of mutant *Cftr* mRNA -Resistance to *S.typhi*	(153)
Zeiher *et al* 1995	$CFTR^{tm1KTH}$ (C57BL/6/129 background)	-ΔF508 knockout mice by replacement of exon 10	-40% survival rate of -Expressed nearly no mutant mRNA levels in the intestinal tract - Suseptiable P. aeruginosa	(200)
Delaney *et al* 1996	$CFTR^{tm1G551D}$	-Replacement of exon 11 in CD1/129 mice	-27% survival under normal conditions - Mutant *Cftr* mRNA expression resulted in 4% residual activity -Suseptiable to P. aeruginosa	(42)
Dickinson *et al* 2002	$CFTR^{tm2HGU}$	-Inserting the mutation in exon 10 using a double homologous recombination ("hit and run") technique	-Mimic the human mutation G480C -High rate of survival could be related to the use of the hit and run strategy -Normal levels of a mutant CFTR protein.	(44)

1.1.5 CF Phenotypes

CF causes a multi-organ malfunction that leads to chronic and persistent lung infections, pancreatic insufficiency, lipid malabsorption, reduced bone density, and reproductive abnormalities. Since the discovery of the *Cftr* gene in 1989 by a series of elegant experiments involving saturation mapping and chromosome walking and jumping techniques(100, 163, 164), advances in research have aided our understanding of the molecular mechanism of the various CF related phenotypes and these have led to the development of novel therapies improving the overall health status and life span of CF patients. The majority of newly diagnosed patients are expected to survive past their 37th birthday, which represents an amazing improvement when compared to the median survival rate of 4 years of age in the 1960's (www.ccff.com). Unfortunately, cystic fibrosis is still a lethal disease that shortens the lives of 100% of patients, mainly due to the progressive inflammatory lung damage.

1.1.6 Clinical Trials

Developing novel pharmaceutical agents is both a time consuming and an expensive endeavor. A successful new drug takes approximately 12 years to reach the market, from the time it's identified as a potential target. Additionally, it can cost anywhere from 50 million to 1.5 billion US dollars to develop (68). Clinical development of a pharmaceutical agent progresses through four Phases(68);

- Phase I- is the first time the drug is tested in human and this is usually after 2 different animal species have been tested

- Phase II- is called the "proof of concept" or "dose response" where a larger patient population is tested (100-300 subjects)
- Phase III- is aimed to evaluate the efficacy of the new agent on a target patient population (1000-5000 subjects)
- Phase IV- is the post approval stage where the drug has been approved to be put on the market how safety is still monitored.

Each Phase of clinical trials is approved by ethic review boards before it is allowed to proceed. Additionally, most clinical trials in the United States are required to be registered with the National Institute of Health's (NIH) clinical trials databases. This review will focus on the current and new pharmaceutical agents being developed to treat the multi-organ malfunctions associated with cystic fibrosis.

1.2 Lungs

Chronic and Persistent Infections

A hallmark of CF is the presence of a mucus secretion which clogs the bronchial tubes in the lungs, obstructing breathing. Mucus retention is thought to predispose CF patients to contract chronic bacterial infections. The most common pulmonary infection include: *Pseudomonas aeruginosa, Staphylococcus aureus, Haemophilus influenzae,* and *Burkholderia cepacia.* A more in depth summary on common CF infections has been extensively reviewed by Lyczak *et al.* (125). *P. aeruginosa* is the main culprit of reduced lung function ultimately leading to mortality for CF patients (58). *P. aeruginosa* often first colonizes CF lungs in the first decade of life. Once *P. aeruginosa* has established its residency, it causes infections resulting in epithelial surface damage and up regulation of inflammation via the influx of chemokines, cytokines and enzymes through neutrophil

recruitment (55). Although antibiotics can decrease the frequency and duration of these attacks, once the bacterium has infected the CF host and established a permanent home it can never be completely eliminated from the lungs.

1.2.1 Antibiotic Treatments

To develop novel anti-infective treatments for CF patients it is required to take into account the complexity of CF microbiology. Several eradication strategies using different combinations of oral, inhaled and intravenous antibiotics have been proven effective and are currently being prescribed; oral colomycin, ceftazidine, meropenem, nebulized ciprofloxacin, and/or nebulised tobramycin (TOBI), can prevent or at least delay chronic infections caused by mucoid *P. aeruginosa* (16, 32, 63, 115, 119, 120). These treatments are thought to be effective in two ways, first by eliminating/reducing bacterial growth and secondly by down regulating inflammatory mediators which leads to an overall improvement in lung functions. If infection persists after use of oral and nebulised antibiotics to eradicate *P. aeruginosa*, intervenous antibiotics are utilized to maximize antibacterial activities. Investigators have found when intravenous therapy is implemented two antibiotics which act mechanistically differently (e.g. aminoglycoside and ß-lactam) should be used. Unfortunately, patients with repeated exposures to *P. aeruginosa* frequenly develop antibiotic resistant strains of this bacterium, consequently altering membrane permeability, and resulting in plasmid-mediated resistance (1, 112). The study by Lechtzin and colleagues stresses the importance of infection control efforts to prevent patient-to-patient spread of *P. aeruginosa* (112).

Aztreonam Lysine is a monocyclic ß-lactam antibiotic, originally used as an intravenous antibiotic to treat *P. aeruginosa* infections and now it is being tested in aerosolized form.

Gilead Sciences is currently studying the efficacy of Aztreonam Lysine for Inhalation (AI) (Azactam®) in Phase 3 trials NCT00128492. This study is designed to assess if a 28 day on/off treatment with AI will increase the number of days patients are free of pulmonary exacerbation compared to placebo. In a recent press release Dec 19 2006, Gilead Science Inc. stated that preliminary results in 247 patients were very promising and long term (over 18 months) efficacy of the treatment is currently being tested[5].

Tobramycin is an aminoglycoside antibiotic used to treat various types of bacterial infections such as PA. Researchers from the Cystic Fibrosis Inhaled Tobramycin Study Group published reports from a two multicenter, double blind placebo controlled trials in 520 patients randomly assigned to receive either 300mg of inhaled tobramycin or placebo twice daily for 4 weeks, followed by four weeks with no study drug (total of 24 weeks)(150). These studies concluded that intermittent administration of inhaled tobramycin was well tolerated and improved pulmonary functions Forced Expiratory Volume in the first second (FEV_1) of 10% at week 20 compared to week 0, while patients on the placebo arm had a 2% decline. Colony forming units (CFU) per gram of expectorated sputum decreased 0.8 $\log_{10}$ CFU in the Tobramycin arm compared to the placebo where they noted a 0.3 $\log_{10}$ CFU increase (150). Long term benefits of inhaled tobramycin were also monitored by Richard Moss, where he concluded that inhaled tobramycin treatment improved pulmonary function and weight gain in 128 adolescent patients over a 2 year period (134). In the last six years there have been a number of studies that have all proven inhaled tobramycin statistically significant and more importantly clinically effective; Novartis Pharmaceuticals is currently participating in Phase 3 trials of

[5] (http://www.gilead.com/wt/sec/pr_943568) last accessed Jan 10th 2007

Tobramycin Inhaled Powder (TIP). This dry powder form is being tested to make inhaled Tobramycin treatments faster and more convenient (NCT00125346 & NCT00388505).

Another well-known antibiotic extensively studied is azithromycin. Long-term administration of azithromycin have shown to minimize lung deterioration in young patients with CF and also has proven anti-inflammatory effects that improve lung function and decrease frequency of hospitalizations (29, 147). Pfizer is completing Phase 4 study to ensure the safety of long-term use of this antibiotic Zithromax®, the registered name of Azithromycin.

Quorum sensing (QS) represents one possible mechanism whereby biofilm bacteria can tolerate lethal doses of antibiotics, and protect the bacteria against the bactericidal activity of polymorphonuclear leucocyte (PMNs). In a novel approach of aiding antibiotic treatment, Bjarnsholts and colleagues' demonstrated that QS was inhibited by garlic extracts (152), such that *P. aeruginosa* became more sensitive to tobramycin and phagocytosis by PMN. This led to an improved outcome of pulmonary infections in BALB/c mice, quantified by bacteriology, mortality, histopathology and cytokine production at 1, 3, and 5 days post infection (15). Studies are in progress to isolate and characterize the pure compound responsible for blocking the PA QS system and their successful completion might support new therapeutic avenues.

In addition there have been novel pharmaceutical strategies to improve delivery of antibiotic treatments. Recently Transave Inc. received orphan drug status by the FDA on their product the Sustained Release Lipid Inhaled Targeting (SLIT™). This product is an endogenous delivery vehicle for pharmaceutical therapeutics that is entirely made of lipids. Preliminary results have shown that using SLIT in conjunction with the antibiotic

amikacin, eradicated more efficiently P. *aeruginosa* bacteria (www.cff.org). SLIT vehicles enable antibiotics to spread more freely and work more efficiently.

Possible prevention of infection through vaccination is also being evaluated. Aerugen® is a conjugate vaccine against *P. aeruginosa* infections developed by Berna Biotech. Vaccination against *P. aeruginosa* bacteria has been shown to prevent or at least delay lung infections (35, 109, 110, 170). Using a cohort of 25 CF patients who were vaccinated yearly, researchers observed an elevated level of specific serum Immunoglobulin G (IgG) antibodies against the vaccine components over 10 years (201). The vaccine-induced IgG levels were lower compared with infection-induced IgG levels, but qualitative characterization showed that affinity and epitope specificity rather than the quantity of the antibodies mediated the protective effect of the vaccine(201). This polyvalent conjugate vaccine combines 8 prevalent *P. aeruginosa* serotypes and the bacterial exotoxin A and it is expected to be launched in 2007 in Europe and it is being evaluated in pre-clinical trials in North America.

1.2.2 Anti-inflammatory

The lack of functional CFTR channels in the airway of the epithelium contributes to the exuberant inflammatory response (26). Airway epithelial cells are in constant interaction with bacteria and phagocytes causing them to release pro-inflammatory cytokines. Macrophages respond to bacteria as well as early inflammatory cytokines such as interleukin 1ß (IL-1ß) and tumor necrosis factor (TNF) which in turn causes epithelial cells to produce IL-8, IL-6 and granulocyte macrophage colony-stimulating factor (GM-CSF) (98). IL-8 and GM-CSF specifically attract neutrophils to the inflammatory site. The host

response to bacteria is associated with release of oxidants and proteases by neutrophils and activated macrophages. If this process is prolonged, continuous stimulation causes damage to structural proteins leads to excessive mucin secretion (26, 98). The predisposition to an increase in pro-inflammatory cytokine production and/or a decrease in the level of anti-inflammatory cytokines such as IL-10 leads to dysregulation of the inflammatory response that causes tissue damage at the site of infection as observed in the CF lungs.

Anti-inflammatory therapy using inhaled corticosteroids (ICS) is a common approach to slowing down the degeneration of lung function in CF. Short term use of ICS shows the ability to slow the progression of lung disease by decreasing neutrophil counts from bronchoalveolar lavage (BAL). However, long term uses have many serious adverse effects such developmental impairments in weight and height (11, 41). Recently in a multi-center randomized controlled trial (RCT), researchers tested the safety of withdrawing from ICS therapies. Based on these studies, it was concluded that a reduction in unnecessary prescribing of inhaled corticosteroids is necessary and that no adverse consequences are expected in the decrease such as the early onset of acute chest exacerbations (11).

High doses of non steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen can inhibit neutrophil migration and adherence, and inhibit the release of lysosomal enzymes such as cyclooxygenase-2 (COX-2). Konstan and colleagues demonstrated among 85 CF patients who took ibuprofen for four years and had at least a 70 percent rate of compliance, the annual rate of change in FEV1 was (-1.48 +/- 0.69 percent vs. -3.57 +/- 0.65 percent in the placebo group, P = 0.03), and this group of patients also had a significantly slower rate of decline in forced vital capacity, the percentage of ideal body weight, and the chest-radiograph score (104).

CF patients additionally have an increased free radical load due to chronically stimulated immune cells and invading pathogens (176, 190). In fact, benefits have been attributed to the antioxidant and the free radical quenching activity of the carotenoids (lycopene, beta-carotene, phytoene, phytofluene) and tocopherols (vitamin E)(97). Antioxidant treatments (vitamins A, C, E, beta-carotene and linolenic acid) have been shown to improve neutrophil recruitment, and gas exchange in CF lungs that endure increased oxidative stress (97). Suggestions have been made that dietary modification can cause an up regulation of the antioxidant pool of the respiratory-tract lining fluid, and therefore decrease the oxidative stress (97). Back and colleagues studied the changes of the antioxidant concentrations in plasma, bucal mucosal cells (BMCs), and breathe condensate in a wide age group of CF patients. They found that as well as disease progression, patients age is also associated to a distinct vitamin deficits and elevated levels of oxidative stress (10). Rust and colleagues reported that a 1mg ß-carotene/ Kg body weight/ day for 12 weeks significantly increases ß-carotene concentrations and decreases malonyl dialdehyde (MDA) concentrations allowing for the correction of antioxidant capacity. In this study, plasma ß-carotene, ß-cryptoxanthin, and total lycopene were significantly lower in the 12 CF patients than in control subjects across all age groups (129). Additionally, plasma α-tocopherol and vitamin C concentrations as well as BMC α-tocopherol concentrations were significantly lower in CF patients and oxidative stress (C reactive protein) was significantly higher.

Glutathione (GSH) functions as an innate defense mechanism against the build-up of oxidants in the lung (21, 126). There has been reports that CF airways display a basal intracellular GSH deficiency (117). Herzenberg's laboratory recently reported treatment with *N*-acetylcysteine (NAC), a well known antioxidant GSH pro-drug, improved the redox

imbalance and inhibited the recruitment of neutrophils to CF airways (186). In 1999 a systematic review identified twenty-three studies, mostly uncontrolled clinical observations and three randomized controlled clinical trials on nebulized N-acetylcysteine to treat CF. Results from these studies did not show any beneficial effect on lung function (49). However, oral NAC is currently being studied in Phase 2 clinical trials (www.ccff.org).

EmphyCorp has been granted Orphan Drug status by the FDA to develop N115 (inhaled sodium pyruvate in 0.9% sodium chloride (saline) solution) for the treatment of CF and it is currently in the first Phase of clinical trials (NCT00308243). Sodium pyruvate is a reactive oxygen species (ROS) antagonist that has been shown to neutralize oxygen radicals, regulate the production and level of other inflammatory mediators, including synthesis of nitric oxide.

Some researchers have suggested that increase in Rho proteins could explain the low NO production and high inflammation seen in CF(106). Rho GTPases are molecules in the cells that lines the airways and decrease nitric oxide therefore lead to increased inflammation (106). The pharmaceutical company Merck has developed a drug, simvastatin (Zocor®) that blocks Rho protein (3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoAR) inhibitors). Simvastatin has previously been prescribed to lower cholesterol levels, and has good safety profile, with only very few side-effects and has been approved for use in children older then than 10 years. Phase 1 clinical trials are now underway to treat CF patients with simvastatin. Researchers primary outcome is to observe if an increase in NO production (exhaled NO), will cause a decrease in airway inflammation (NCT00255242). In addition to evaluating the anti-inflammatory effects of this statin, this study also presents an opportunity to evaluate an alternative method of measuring CF airway inflammation

using quantitative RT-PCR. Measurements of inflammatory cytokines (IL-6, IL-8 and NOS-2) in nasal epithelial cells can potentially be used as a surrogate marker of lower airway inflammation (NCT00255242).

Interestingly, Grasemann and colleagues from Toronto's Sick Kids Hospital reported a single inhalation of L-arginine acutely and transiently improves pulmonary function in CF patients through the augmentation of nitric oxide formation (75, 76). Animal experiments have shown that airway relaxation is significantly impaired in CF-KO mice and that this relaxation defect in CF airways could be reversed by an improvement of enzymatic NO formation through the addition of the NOS substrate L-arginine (76, 130). Researchers at the Vanderbilt University Medical Center in Nashville are conducting Phase 1 trials to evaluate inflammatory markers in sputum after a 4 week treatment with hydrochloroquine (NCT00311883). This drug has been used to treat chronic inflammatory diseases including lupus and pulmonary interstitial disease (56, 103).

1.2.3 Mucosal clearance

Mucus secretion in the airways represents the first-line of defense for the respiratory tract however hypersecretion of mucus is another pathophysiological feature of CF. Nebulised hypertonic saline has been shown to improve mucociliary clearance and it is thought to decrease destructive inflammatory process in the airways by the slow diffusion of saline from airway surfaces (53). Donaldson and colleagues concluded in the New England Journal of Medicine that hypertonic saline is an inexpensive, safe and an effective therapy in conjunction with bronchodilators. Both safety and effectiveness of the proposed treatment continues to be monitored through on going Phase 4 clinical trials (NCT 00271310) (53, 54).

Experiments almost 50 years ago revealed that a large concentration of DNA (3-14 mg/ml) (an extremely viscous polyanion) was present in infected lungs, and not present in uninfected lungs (175). Investigators' believe that this increase in DNA might also contribute to the increased viscosity of lung secretions that results in a reduced effectiveness of antibiotics (146). Pulmozyme is a recombinant human deoxyribonuclease I (rhDNase), an enzyme which selectively cleaves DNA (175). In adults and older children, studies have shown that daily use of Pulmozyme improves lung function and decreases the number of lung infections requiring hospital treatment by decreasing excessive mucus (161). Pulmozyme has been approved by the FDA for use in CF patients over 5 years old however is currently being studied in clinical trials for children younger then three years old (NCT00015756).

In concurrence to the pulmozyme studies, a Phase 2 crossover study by Pharmaxis is being conducted to determine the efficacy and safety of inhaled mannitol (Bronchitol™) (NCT00117208). Mannitol is a osmotic agent that has been shown to improve mucosal clearance in CF. A pilot study involving 12 CF patients studied the effect of inhalation dry powder Mannitol (300 mg), compared with its control (159). Results showed that post-intervention a significant improvement in BMC for the Mannitol group (8.7+/-3.3% versus 2.8+/-0.7%), also a significant improvement in cough clearance with the Mannitol (9.7+/-2.4%) compared with its control (2.5+/-0.8%) (159).

An additional method of treating CF lung disease is to attempt to correct the underlying ion transport defects in the airways. Normalizing the airway secretions, have been hypothesized to improve mucociliary clearance (43). P2Y2 receptor, has been shown to be capable of increasing airway surface hydration and improving mucociliary clearance.

Denufosol is a selective P2Y(2) agonist that stimulates ciliary beat frequency and Cl^- in airway epithelia cells. Denufosol (INS37217 Respiratory) is designed to promote the lung's innate mucociliary clearance through stimulation of the P2Y2 receptor, helping to keep the lungs of CF patients clear of thickened mucus (43). Inspire's recently began Phase 3 trials focusing on advancing denufosol as an early intervention therapy for treatment of CF patients with mild lung disease (NCT00357279).

2.4 Restoring CFTR protein malfunction

The Δ/Δ F508 mutation is the most common mutation associated to CF which is responsible for 70-80% of worldwide cases and causes defective trafficking of CFTR protein leading to abnormal retention and consequent loss of CFTR function. In 2005 studies showed that the PDE5 inhibitor sildenafil (Viagra) increases ΔF508-CFTR trafficking (48). In a more recent case report, Montgomery and colleagues reported that sildenafil improved pulmonary hemodynamics, exercise tolerance, and quality of life in a CF patient with advance lung disease (132). PTC Therapeutics, Inc. (PTC), a biopharmaceutical company focused on the discovery, development, and commercialization of small-molecule drugs targeting post-transcriptional control mechanisms, is currently participating in Phase 2 clinical trials of PTC124 (NCT00234663). Nonsense mutations that prematurely halt the translation process represent 10% of the CF population. PTC124 allows the cellular machinery to bypass the nonsense mutation and continue the translation process, restoring the production of full-length, functional proteins (9). PTC124 represents a unique opportunity to use a single small-molecule drug to address chronic and life-threatening diseases.

Egan and colleagues reported that curcumin (from the curry spice turmeric) can restore the CFTR protein (52). Curcumin was reported as a potent inhibitor of the endoplasmic reticulum (ER) Ca2+ pump, and lowers ER calcium concentration. It had been thought that curcumin might allow abnormal CFTR protein to function properly as a chloride channel and corrects the CFTR defect. After the initial studies by Egan and colleagues there was a great deal of hope but unfortunately further experiments have not provided sufficiently strong support to the postulated mechanism. Phase 1 clinical trials have been completed by SEER Pharmaceuticals, LLC (NCT00219882) however no results have yet been available.

1.2.5 Gene therapy

To date there have been over 140 CF patients that have been safely treated with recombinant adeno-associated virus (rAAV vectors) (60, 61). No dramatic therapeutic benefits have been observed, but new information has been gained both clinically and in preclinical studies allowing the development of new vectors. The major obstacle remains immune response mediated inflammation, gene uptake and steady state levels of gene expression (113, 116). The problems are in part due to the large size of the *Cftr*-coding sequence and studies are presently being conducted focusing on the use of more compact endogenous promoter elements such as cytomegalovirus (CMV) enhancer/chicken beta-actin hybrid (Cbeta). Another target of the study is to improve AAV capsid serotypes with greater tropism for the apical surface of airway cells(193).

1.3.1 Pancreatic/ Intestinal insufficiency

Excessive mucus obstructs the pancreatic ducts and prevents the delivery of crucial digestive enzymes in most individuals with CF. Duct obstructions and destruction of acinar cells leads to abnormal transmembrane fluid and electrolyte movement. A deficiency of pancreatic bicarbonate causing a more acidic pH that inactivates enzymes that are being secreted (86). Pancreatic enzyme replacement therapy (PERT), has been the basis for treating mal-digestion resulting from pancreatic insufficiency (PI) for over 50 years (72, 90, 136, 144, 149, 178). The three main enzymes supplemented are lipase, protease and amylase but there is a great deal of variation in their specific ratio among manufactures. A more in depth review of the different pancreatic supplementations currently utilized is available by Littlewood et al. (121). Treatment for (PI) remains largely unchanged, and there have been very few advances in the understanding and management of gastrointestinal problems. Currently Phase 3 trials are being carried out to determine the safety and efficacy of Eurand Pancreatic Enzyme Product (PEP EUR-1008) (NCT00297167). PEP is a new orally delivered pancreatic enzyme product consisting of approximately 14 enzymes, coenzymes and cofactors biologically similar to human pancreatic secretions. PEP is designed to treat patients for malabsorption of fats, proteins, carbohydrates and other essential nutrients. Recently Borowitz and colleagues published results on a novel pancreatic enzyme product, ALTU-135, a proprietary formulation of microbial derived lipase, protease, and amylase, to determine its efficacy and safety in treatment of pancreatic insufficiency (PI). ALTU-135 is marketed as TheraCLEA by Altus Pharmaceuticals Inc. during the 1-month study period at the dose of 25,000 units of lipase,

25,000 units of protease, and 3750 units of amylase studies were efficacious in treating PI (17).

The intestinal epithelium is exposed to a high level of microbes and microbial products, and therefore has a huge impact on the inflammatory response. Mucosal immunity has been linked to Toll like Receptors (TLRs) that line the polarized epithelium and accumulation within the lumen where large numbers of PMN migrate when there is an inflammatory response. The TLRs recognize microbial components and activate an innate inflammatory response causing mucosal inflammation. Suppression of TLR responses could reduce excessive inflammation in chronic diseases such as CF (154). To date there have been no therapies developed to modulate the expression of TLRs in the context of CF, however other inflammatory diseases such as asthma have profited from the research in this field (131). Bicarbonate deficiency from the pancreas reduces the duodenal pH resulting in abnormal bile salts and subsequently increasing fecal loss of bile salts (72). Recently Walker and colleagues investigated the effects of talniflumate (LOMUCIN^{TM}) in CF KO mice with disal intestinal obstructive syndrome (DIOS)(194). DIOS involves the insufficient hydration of mucus and debris at mucosal surfaces due to abnormal transepithelial electrolyte and water transport in the absence of CFTR activity. They found beneficial effects in the survival of CF mice, by decreasing small intestinal salt absorption through the inhibition of apical membrane Cl-/HCO_3^- exchangers (194). Genaera Corporation received the patent for LOMUCIN^{TM} as a mucoregulator (6, 737, 427 US) and Phase 2 clinical trials are underway with the support of the Cystic Fibrosis Foundation Therapeutics Inc., for the treatment of respiratory, sinus and gastrointestinal disorders associated with CF.

1.3.2 Lipid Malabsorption

Essential fatty acid (EFA) abnormalities in CF have been well-documented since the early 60's (108). EFA and their derivatives are divided into two group; alpha linolenic acid which is the precursor of n-3 polyunsaturated fatty acids (PUFA) and linoleic acid which is the precursor of the n-6 PUFA (20, 69, 155). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the n-3 PUFA metabolites that have been shown to have potent anti-inflammatory properties acting through transcription factors and gene expression, calcium fluxes, altered membrane fluidity, the regulation and secretion of digestive enzymes and hormones and they also play a role in decreasing susceptibility to inflammatory diseases, such as arthritis and asthma (25, 27, 122, 128, 157, 165, 166). DHA is found in sources such as fish oils and human breast milk and is available as over the counter nutritional supplement. In contrast, arachidonic acid (AA), an n-6 PUFA metabolite, stimulates pro-inflammatory reactions through various prostaglandins and leukotriennes pathways.

Increase levels of inflammation has been reported not only secondary to the lung infection but has been attributed directly to the mutation at the *Cftr* locus (64, 96, 142, 173, 189). Epidemiological, clinical, and biochemical studies suggest that the beneficial effects of consuming n-3 PUFA is generally considered to be due to the reduction of AA and its eicosanoid metabolites (133). DHA and AA are the "yin and yang" of fatty acid metabolism, and disruption of the n-3: n-6 EFA balance results in a number of systemic abnormalities (66). Freedman and colleagues analyzed the fatty acid profiles from plasma, nasal scrapings and nasal-and rectal-biopsy specimens of CF patients and a heterozygote population and compared them to healthy volunteers. They establish that the levels of DHA

and AA of the heterozygote population were directly in between the CF patients and the healthy volunteers (91). This provided proof that fatty acid abnormalities were not secondary to malabsorption and pancreatic insufficiency but are directly linked to the CFTR malfunction (64). Previously they had found that oral administration of DHA in CF-KO mice could correct this balance and normalize the histology of the ileum and the pancreas. Although there was no significant decrease in TNF levels in the BAL of CF-KO mice infected with PA, the number of neutrophils and eicosanoids were significantly decreased proving that DHA treatment could partially correct the membrane lipid defect (65). Clinical studies approved by the FDA evaluating DHA were initiated by Beth Israel Deacome Medical Center in collaboration with Genzyme in 2004; however, no results have been published to date. Investigators have not tried to correct the fatty-acid imbalance in patients as they have in mice because of the very high dose of pure DHA needed. If they were to extrapolate the dose to humans, it would be six to seven grams per dose of pure DHA, enough to cause major adverse effects such as uncontrollable bleeding (65, 123). Researchers in Belgium from the University Hospital Ghent are evaluating the overall effect of DHA-rich supplement over a 2 year study of 30 patients (NCT00297167).

1.4 Reduced Bone Density:

The most recently described phenotype associated with CF is reduced bone mineral density, which results in osteopenia and osteoporosis (18). The direct link between *Cftr* gene dysfunction and osteoporosis remains to be elucidated (22, 31, 160). Also the etiology of early-onset osteoporosis in CF patients has not been established and most likely involves complex interactions between several biochemical pathways (7, 8, 33, 34, 62). Aris and

colleagues described the origin of low bone density in patients with CF as the result of several factors including: malnutrition, pancreatic insufficiency, vitamin insufficiency, onset of diabetes, use of glucocorticoids, increase levels of inflammatory cytokines, and sex hormone insufficiency (6). A number of studies have documented a prevalence (40-70%) of decreased bone density in the CF population (18). A longitudinal study of 151 adults with CF showed that 34% of patients had a Z-score of -2 or less (180). Bianchi and colleagues reported that 66% (90/136) of children and young adults with CF (age 4-24) have low bone mineral density (14). A Canadian study of 40 CF patients of average age 28.7 +/- 8.4 showed total hip and lumbar spine BMD had decrease by 3.04% (95% CI -4.76 to -1.26) and 0.86% (95% CI –2.46 to 0.75) respectively after one year (19). Severe bone disease can lead to morbidity and exclusion from lung transplant, which is often a life-saving operation for individuals with CF(6). The problem is that despite recent clinical research, a number of questions still remain regarding the pathogenesis and management of CF related bone disease. Prevention of the early onset of osteoporosis in patients with CF involves a treatment regiment that consists of vitamin D, calcium, vitamin K supplementation (102).

The skeletal system does not only serve as mechanical support, but also functions as an active organ that regulates balance and interactions between both local and systemically circulating factors. Bone remodeling is regulated by a number of hormones, cytokines, growth factors, and prostaglandins (13). Gronowitz and colleges reported that the fatty-acid status of children with cystic fibrosis is directly associated with bone mineral density. Epidemiological studies and experiments using animals and humans have illustrated positive effects of n-3 essential fatty acids (EFA) on bone metabolism as well. It has been

suggested that an effective decrease in phospholipid-bound AA concentrations could consequently decrease the levels of prostaglandin production in bones which has been proven effective in periodontal disease in humans (156).

Currently two classes of experimental drugs are being examined in clinical trials. Human growth hormone (rhGH) is well known for its anabolic effects on protein synthesis and cell growth it has been demonstrated to decrease urinary nitrogen excretion, increased protein synthesis causing an increase in lean-body mass. Recently, Hardin and colleagues published results from a multi-center RCT were 61 patients with CF (7-12 years old) were administered 0.3mg/kg/week of rhGH. Their studies concluded that rhGH therapy improves height and weight, decreases the number of hospitalizations, improves quality of life in pre-pubertal children with CF and also an increased accrual of bone mineral (84). Anti-resorptive agents such as bisphosphates have been used in several uncontrolled observational studies and in few RCT for the treatment of low bone density in CF (6). Bisphosphonates have been shown to increase bone mineral density and decrease the risk of new fractures in post-menopausal women and in patients receiving long-term oral corticosteroids (94). Both pamidronate and alendronate (NCT00004489) are being studied in clinical trials and are showing promise for treating bone disease in CF despite early concerns that pancreatic insufficiency would limit absorption.

1.5 Reproductive Abnormalities:

Absence of the CFTR protein is directly linked to reproductive abnormalities for both sexes. The majority of men with CF have a congenital bilateral absence of the vas deference (CBAVD) leading to infertility. Sixty percent of CBAVD males are found to be

one carriers of the defective *Cftr* gene mutation (95). Reproductive abnormalities in males also include azoospermia, reduced volume, and increased acidity of the sperm (95). There are surgical method including; intracytoplasmic sperm into the cytoplasm (ICSI) where the sperm cells are retrieved, surgically artificially selected, and then the sperm is directly injected into the cytoplasm of a mature oocyte (140). Women with CF who have relatively good lung functions are expected to develop into sexually mature adults. However, some females reproductive systems abnormalities begin with delayed puberty and a late maturation of the reproductive endocrine system. Infertility is mainly caused by excessive mucus clogging access for sperm to enter the uterus (158).

1.6 Conclusion:

Through the hard work and dedication of many research scientists and physicians over the past 20 years, there has been significant progress in the development of new pharmaceutical agents used to treat the diverse CF pathologies. On many fronts research in the CF field has given much hope to the patients and families affected by this devastating disease. People with CF are now living longer healthier lives but the progress needs to continue to find a cure. This review illustrates exciting new treatments as summarized as table 1. Since none of the results from these clinical studies to date have reported a cure for CF, intensive basic research and well designed systematic clinical trials must continue so better approaches and treatments can be developed and an elusive cure might become a reality.

Table 1.2 Summary of pharmaceutical agents in clinical trials

Phases	Treatment	Sponsors	Reference
Pre-Clinical	Pioglitazone	University Hospitals of Cleveland	NCT00322868
	GSNO Nitrox	Nitrox LLC	www.cff.org
	VX-770	Vertex Pharmaceutical, Inc	www.cff.org
	INO 4995	Inologic, Inc	www.cff.org
	HE-2000	Hollis-Eden Pharmaceuticals	www.cff.org
	Aeugen Vaccine (against PA)	Berna Biotech	www.bernabiotech.ch
	L-arginine	Toronto's Sick Kids Hospital	(75, 76)
	sildenafil (Viagra)	University of Wales College of Medicine	(48)
	SLIT-amikacin	Transave, Inc.	www.cff.org
1	Hydroxychloroquine	Vanderbilt University	NCT00311883
	Phenylbutyrate/Genistein Duotherapy	National Center for Research Resources (NCRR)	NCT00016744
	Simvastatin	Merck &Co., Inc	NCT00255242
	Inhaled Sodium Pyruvate	University of Minnesota/ Emphy Corp	NCT00332215
	Compacted DNA	Copernicus Therapeutics, Inc.	www.cff.org
	Curcumin	Seer Pharmaceutical	NCT00219882
	Moli901	Lantibio, Inc.	www.cff.org
	Lomucin	Genarea Corp.	www.cff.org
	Inhaled N-acetylcysteine	Galephar Phar. Research	www.cff.org
	Oral N-acetylcysteine	BioAdvantex Pharma, Inc.	www.cff.org
	MP-610.205	MPEX Pharmaceutical, Inc.	www.cff.org
	Yasoo	Yasoo Health Inc.	www.cff.org
2	PTC124	PTC Therapeutics	NCT00234663
	Ciprofloxacin	NCRR	NCT00097773
	Pulmozyme in infants	Genentech	NCT0016352
	Salt Tablets	NCRR Australia/Monash University	NCT00163852
	DHA	University Hospital of Ghent (Belgiam)	NCT00221546
	Mannitol Dose	Pharmaxis	NCT00251056
	Inhaled 552-02	Paron Science	NCT0027431
	Azithromycin Infected With B. Cepacia	St. Michael's Hospital/Pfizer	NCT00298922
	SPI-8811	Sucampo Pharmaceuticals, Inc	www.cff.org
	Inhaled Cyclosporine	Novartis Pharmaceuticals	www.cff.org
	ALTU-135	Altus Pharmaceuticals, Inc	(17)
3	Aztreonam Lysine	Gilead Science	NCT00112359
	Pancreatic Enzyme Product PEP	Eurand S.p.A.	NCT00297167
	Denufosol	Inspire Pharmaceuticals	NCT00357279
	Tobramycin (TOBI) Inhalation Powder	Novartis Pharmaceuticals	NCT00125346
	Dornase Alfa Pulmozyme	Genentech	NCT00265434
4	Azithromycin	Pfizer, Inc.	www.cff.org
	Hypertonic Saline	University of North Carolina	www.cff.org

Preface to Chapter II

***Rational*:** Ceramide is a sphingolipid that serves as a secondary messenger. It is generated by the conversion of sphingomyelin by sphingomyelinase enzyme (sMase) and is regulated during cellular responses to extra-cellular signaling, such as TNF, LPS, IFNγ, and interleukins (23). Interestingly in the context for CF, Nieuwenhuis and colleagues (138) demonstrated that galactosyl ceramide treatment led to very efficient clearance of *P. aeruginosa*. These findings were further corroborated with more recent studies by Grassme *et al.*, (77) where they demonstrated that ceramide has been crucially important for *P. aeruginosa* internalization. Furthermore, failure to generate ceramide-enriched membrane rafts after infection was shown to result in an unabated inflammatory response consequently leading to release of pro-inflammatory interleukins and septic shock (77). From these studies, the regulation of ceramide levels seemed to be crucial in the maintenance of a proper homeostasis of cells and inflammatory response.

Fenretinide a semi-synthetic retinoid, has been extensively studied because of its chemo-protective and anti-tumor activities described when used on a variety of malignant cells, including non-small lung cancer, neuroblastoma, Kaposi's sarcoma, breast cancer and glioma (24, 57, 71, 118, 145, 148, 151, 192). Treatment with fenretinide leads to generation of ceramide which is subsequently converted to GD3 (disialoganglioside), GD3 induces lipoxygenase (LOX-12) activity; an enzyme that oxidizes arachidonic acid to be released from the phospholipid bilayer and converted to a less harmful metabolite 12-hydroxyeicosatetraenoic acid (12-HPETE/ 12-HETE) intracellularly. The exact mechanism of fenretinide action has not been elucidated yet and several laboratories, including our laboratory, are trying to characterize its effects in vivo and in vitro.We have established

that our *Cftr*-KO mice are ceramide deficient in all CF related organs including lung, liver, ileum, pancreas and plasma when compared to their WT littermate controls. Furthermore that there is also a lipid metabolism defect in all CF related organs where concentrations of phospholipid bound AA are significantly higher and phospholipid bound DHA are significantly lower (Manuscript submitted, Guilbault *et al* 2007). Interestingly, treatment with fenretinide completely corrects the fatty acid dysregulation in all CF related organs. Based on these encouraging results, we have started exploring the biological effects of fenretinide treatment on other CF-associated phenotypes, such as osteoporosis, asthma and infertility.

Objective/Significance: In the following chapter, we used our CF mouse model to characterize one of the CF-related phenotypes, the early onset of osteoporosis due to low bone mineral density. We hypothesized that low bone mineral density in CF might result from the exaggerated level of AA and that treatment with fenretinide could increase in bone formation, by stimulating production of ceramide and downregulation concentrations of phospholipid bound AA. The results generated from this study suggest that fenretinide treatment might protect CF patients against the early onset of osteoporosis and clinical studies are warranted.

Chapter II

Chapter II

Based on prepared manuscript

Fenretinide prevents the development of osteoporosis in Cftr-KO mice

Zienab Saeed, Claudine Guilbault, Juan B. De Sanctis, Jennifer Henri, Dominique Marion, René St-Arnaud, Danuta Radzioch

Abstract:

Cystic fibrosis is associated with chronic inflammation occurring in various internal organs, particularly the lungs. Inflammation also affects other sites including the gastrointestinal, reproductive and skeletal systems. Although the exact molecular link between the CFTR dysfunction and various phenotypes remains to be delineated, many phenotypes seem to be linked to inadequate nutritional absorption of essential fatty acids and vitamins, which leads to an imbalance between the essential fatty acids docosahexaenoic acid (DHA) and arachidonic acid (AA) and could contribute to the development of early-onset osteoporosis. In this study, we have characterized the protective effect of fenretinide [*N*-(4-hydroxyphenyl) retinamide], a vitamin A derivative, on the early onset of osteopenia and osteoporosis in *Cftr*-KO mice. Using micro computed tomography (microCT) we examined the effect of fenretinide on the bone composition and architecture in our *Cftr*-KO mouse model. These results were then correlated with histological analyses using von Kossa, H&E and TRAP staining. Interestingly we found that twice a week treatment with fenretinide over a period of four weeks dramatically increased trabecular bone volume. This remarkable increase in bone induced by fenretinide was also associated with an increased concentration of ceramide in the plasma that subsequently led to the

down-regulation of phospholipid-bound AA in *Cftr*-KO mice. To our knowledge, this is the first time that fenretinide has been demonstrated to have a protective effect against osteoporosis. The results of our study strongly suggest that fenretinide might have potential for the treatment of cystic fibrosis patients by preventing the early onset of osteoporosis.

2.1 Introduction

The life expectancy of patients with cystic fibrosis (CF) has dramatically improved in recent years. The majority of newly diagnosed patients are expected to survive past their 37th birthday, which represents an amazing improvement compared to the median survival of 4 years old in the 1960's in Canada. Unfortunately, cystic fibrosis is still a lethal disease that shortens the lives of 100% of patients, mainly due to the progressive inflammatory lung damage.

Essential fatty acid (EFA) abnormalities in CF have been well-documented since the early 60's (108). They have been shown to be very important in a number of metabolic processes including calcium fluxes, the regulation and secretion of digestive enzymes and hormones. They also play a role in decreasing susceptibility to inflammatory diseases, such as arthritis and asthma (91). Freedman and colleagues used *Cftr* knockout mice to show that CF-affected organs such as the pancreas, ileum and lungs have a significantly lower concentration of phospholipid-bound docosahexaenoic acid (DHA) and a significantly higher concentration of phospholipid-bound arachidonic acid (AA) (65). Recently, the same group demonstrated similar alterations in fatty acids profiles in 38 cystic fibrosis patients as compared to healthy controls (64).

DHA is a n-3 polyunsaturated fatty acid that regulates membrane functions and displays anti-inflammatory effects in CF (66). In contrast, AA is a n-6 polyunsaturated fatty acid that has been shown to regulate the production of prostaglandins and leukotrienes, therefore acting as an agonist in the inflammatory process (66). DHA and AA are the "yin and yang" of fatty acid metabolism, and disruption of the n-3: n-6 EFA balance results in a number of systemic abnormalities (91). It has been proposed that increased levels of AA

might contribute to the progression of CF lung disease, and that dietary supplementation with high doses of DHA could correct this imbalance (67). Investigators have not tried to correct the fatty-acid imbalance in patients as they have in mice because of the very high dose of pure DHA needed. If they were to extrapolate the dose to humans, it would be six to seven grams of pure DHA, enough to cause major adverse effects such as uncontrollable bleeding (65, 123).

A newly described phenotype associated with CF is reduced bone mineral density resulting in osteopenia and osteoporosis (59, 74). A number of studies have documented a prevalence (40-70%) of a decrease in bone density in the CF population (18). A longitudinal study of 151 adults with CF showed that 34% of patients had a Z-score of -2 or less (180). Bianchi and colleagues reported that 66% (90/136) of children and young adults with CF (age 4-24) have low bone mineral density (14). A direct link between *Cftr* gene dysfunction and osteoporosis remains to be elucidated (102). Osteoporosis literally means "porous bones" and is characterized by low bone mineral density and the structural weakening of bone tissue, which leads to an increased risk of fractures (187). Currently, the osteoporosis treatment regimen for patients with CF consists of a number of different medications including: vitamin D, calcium, vitamin K, sex hormones, anti-resorptive agents such as bisphosphates, anabolic agents such as parathyroid hormone (PTH) (extensively reviewed by Aris and colleagues (6)) and human recombinant growth hormones (hrGH) (88). Epidemiological studies and experiments using animals and humans have shown that n-3 EFA improve bone metabolism (87). Decreasing phospholipid-bound AA levels causes less competition for phospholipids, allowing DHA to incorporate into the phospholipids and increasing the DHA:AA ratio (66). However, the

effectiveness of this treatment is compromised in patients with CF due to the severe malabsorption of lipids displayed by the majority of CF patients. (143)

Fenretinide [*N*-(4-hydroxyphenyl) retinamide, 4-HPR], a semi-synthetic retinoid, has been extensively studied, both in the laboratory setting and in clinical trials, because of its chemo-protective and anti-tumor activities (12, 37, 199). Treatment with fenretinide leads to the generation of ceramide, a lipid secondary messenger (127, 184, 185), which subsequently increases 12-lipoxygenase (12-LOX), an enzyme that catalyzes the oxidation of AA, allowing it to be released from the membrane-bound phospholipids (83, 101). Furthermore, it has been shown that sphingomyelinase, the enzyme that catalyzes the formation of ceramide from sphingomyelin, plays a crucial role as an intercellular messenger in osteoblast differentiation from capillary endothelial cells, suggesting that ceramide has an important role in modulating bone metabolism (105). Recently, our laboratory has shown the beneficial effects of fenretinide-mediated ceramide production on clearing *Pseudomonas* infection from the lungs of *Cftr*-KO mice (Guilbault et al, 2007 submitted).

The studies described in this manuscript demonstrate a protective role of fenretinide against osteoporosis in CF. The data described in this manuscript show that eight treatments with fenretinide are sufficient for prevention of the low bone volume phenotype typically observed in mice with an ablated *Cftr* gene. This correction has been associated with an increase in ceramide levels and down regulation of phospholipid-bound AA in the blood. To our knowledge, this is the first time that a treatment effective in preventing the early onset of osteoporosis in CF animal model has been documented.

2.2 Methods

2.2.1 Mice: The inbred mice (11-14 week old) used in our study were bred in our animal facility (from C57BL/6J breeding pairs heterozygous (HZ) at the *Cftr* locus). Age- and gender-matched C57BL/6-*Cftr*$^{+/+}$ (WT) mice (n=16), and C57BL/6-*Cftr* $^{-/-}$ (*Cftr*-KO) mice (n=16) were maintained in a murine pathogen- and parasite- free environment. The number of mice represents a total of 3 independent experiments. The mice were kept in cages with sterile corn bedding (Andersom, Bestmonro, LA) and housed in ventilated racks (Lab Products). Mice were fed with either the NIH-31-modified irradiated mouse diet (Harlan Teklad, Indianapolis, IN) or a liquid diet starting at 14 days old (Peptamen, Nestle Canada, Brampton, ON). The liquid diet was provided in 50 ml centrifuge tubes (Sargledt Co, Canada, Montreal, PQ), which were freshly prepared every morning. Experimental procedures with the mice were conducted in accordance with the Canadian Council on Animal Care guidelines and with the approval of the Animal Care Committee of the McGill University Health Center, Montreal, Quebec, Canada.

2.2.2 Drug administration: Fenretinide powder was kindly provided by Dr. Robert Smith from the NIH (Bethesda, MD, USA). Fenretinide was resuspended in 95% ethanol to make a 2 μg/μL concentration. Approximately 40 μL of this preparation was incorporated into the Peptamen liquid diet (5 mg/kg per day per mouse). The prepared food containing fenretinide was then stored in the dark at 4°C for no more than 3 hours prior to being administered to the mice. To ensure that the entire drug dose was consumed, the mice were given 10 mL of fenretinide-containing Peptamen, which represents 2/3 of the daily mouse food consumption with the completed dose, in the late afternoon. The remaining 5 mL of

Peptamen (without fenretinide) was given the following morning. Mice were treated twice a week for 4 weeks. During the treatment period each mouse was kept in a separate cage (including WT mice) and was monitored to ensure that the entire amount of Peptamen was consumed whether containing the fenretinide or not. The diet for the mock-treated control group was prepared and administered similarly to that described above, by adding the same volume of ethanol to the Peptamen diet but omitting the fenretinide supplementation.

2.2.3 micro computed tomography (micoCT): Mice were euthanized using CO_2 inhalation and exsanguinated by cardiac puncture. Femurs, tibiae and vertebrae were extracted, stripped of soft tissue and fixed in 4% paraformaldehyde overnight. Micro computed tomography (microCT) was performed on the left femur after overnight fixation. The distal metaphysis was scanned with a Skycan 1072 instrument (Skyscan, Antwerp, Belgium). Image acquisition was performed at 100kV and 98μA, with a 0.9° rotation between frames. The two-dimensional images were used to generate three-dimensional reconstructions to obtain quantitative data using the 3D Creator software supplied with the instrument (ANT 3D Creator software, Skyscan, Antwerp, Belgium).

2.2.4 Histological analysis: After overnight fixation in 4% paraformaldehyde and rinsing in phosphate buffered saline (PBS), right femurs and tibiae were embedded in polymethylmethacrylate (MMA) or a mixture of 50% MMA and 50% glycolmethacrylate (GMA). Serial 4- to 6-μm sections were cut on a modified Leica RM 2155 rotary microtome (Leica Microsystems, Richmond Hill, Ontario, Canada). MMA-embedded tissues were stained with von Kossa and toluidine blue, while 4 μm MMA-GMA sections

were stained with tartrate-resistant acidic phosphatase (TRAP). Images were captured using a Carl Zeiss Microscope (Germany) equipped with an AxioCam MRc camera. The left femur and tibia and the lumbar were decalcified with 4% EDTA for paraffin embedding after 14 days. Serial 5 μm sections were cut and stained with hematoxylin and esosin (H&E). Osteoblasts were defined as a single-nucleated, rod shaped cells and were identified along the surface of the trabecular lamellae. Osteoclasts were defined as multi-nucleated cells that are much larger then osteoblasts and display typical macrophage morphology.

2.2.5 Fatty acid analysis: Plasma was suspended in 1mM butylated hydroxyanisole (BHA) in chloroform and methanol (2:1 vol) until the analysis was performed. Lipids were extracted from plasma with chloroform/methanol (2:1), as previously described (188). Phospholipids were identified by thin layer chromatography extraction. Fractionated lipids were dried and resuspended in heptane: methanol: sulfuric acid (5:1:1) for free fatty acid extraction. Diazomethane was used to esterify the fatty acids released and the esters were identified by GC/MS (Hewlett Packard5880A, WCOT capillary column (Supelco-10, 35 m x 0.5 mm, 1 μm thick)) using commercial standards (Sigma-Aldrich).

2.2.6 Ceramide/Sphingolipid analysis: Ceramide concentrations in the plasma of treated and untreated mice were determined by ELISA performed on lipid samples that had been separated by thin layer chromatography. The phospholipids from the dry silica were resuspended in ethanol and used to coat Nunc plates specific for lipid binding. Plates were then washed, incubated with blocking buffer for 1 hr at 37° C (PBS, 0.1% Tween 20, and

1% bovine serum albumin (BSA; Sigma, Oakville, ON), and incubated with murine anti-ceramide IgM (Sigma-Aldrich) antibody (Ab) for 1 hr at 37°C. The plates were then washed and incubated with peroxidase-conjugated anti-mouse IgM Ab for 1 hr at 37°C. Finally, the plates were incubated with the peroxidase substrate (TMB; Roche, Laval, QC). The intensity of the colorimetric reaction was determined by spectrophotometry at 405 nm. The levels of ceramide were calculated with reference to a standard curve prepared using purified ceramide (Sigma-Aldrich). Phosphate levels were assessed, as previously described, using the PiBlue^{TM} Phosphate assay kit (Boehringer Ingelheim, Chuao, Caracas), according to the manufacturer's instructions.

2.2.7 Statistical analyses: Data was analyzed using GraphPad Prism Version 4.03 software (GraphPad Software, San Diego, California, United States). All data was analyzed by parametric one way analysis of variance (ANOVA) followed by Bonferroni multiple comparison post-test between relevant groups. Significance was set at a two-tailed *P* value of ≤ 0.05.

2.3 Results:

It is well-established that CF patients develop early-onset osteoporosis (4, 5, 62, 73, 102, 167). Recently it was shown that sphingomyelinase, the enzyme that controls ceramide production, is crucial in the regulation of osteoblast differentiation (105). In our most recent study we demonstrated that our *Cftr*-KO mice have abnormal levels of ceramide, and that treatment with fenretinide corrected this deficiency (*submitted*). This study represents an extension of our investigations focusing on the protective effect of fenretinide on various phenotypes associated with *Cftr* gene mutations or *Cftr* gene ablation.

2.3.1 Trabecular bone density

Osteoporosis is characterized by a decrease in the quantity (amount of bone) and quality (structural integrity) of the trabecular bone. Figure 2.1A depicts the microCT analysis of the trabecular bones isolated from CF mice and their litter mate controls and it shows that the *Cftr*-KO mice display clear signs of osteoporosis. These results also clearly demonstrate that twice a week treatment with fenretinide over the course of four weeks (total 8 doses) is able to completely eliminate any signs of osteoporosis in the trabecular bone of *Cftr*-KO mice. Representative 3D reconstructions and 2D cross-sectional scans demonstrate that, before fenretinide treatment, *Cftr*-KO mice have virtually no trabecular bone (highlighted in black boxes evident in the cross-sectional image) as compared to littermate controls. After treatment with fenretinide there is a dramatic increase in trabecular bone in the *Cftr*-KO mice compared to their WT controls.

2.3.2 Bone composition and architecture

To address the osteoporotic changes from the composition and architecture, the trabecular bone was quantified. The following parameters: bone volume/ tissue volume (BV/ TV), structural model index (SMI) and trabecular separation, were calculated from the left femur. The data presented in figure 2.2A show a statistically significant ($p < 0.05$) reduction in bone volume fraction (BV/TV) in the *Cftr*-KO mice as compared to WT controls. Although osteoporotic changes were previously documented in 3 week old CF mice by Dif and colleagues (45), our study shows for the first time using microCT technology that adult *Cftr*-KO mice display a significant defect in BV/TV compared to their littermate controls. Interestingly, this difference disappears when *Cftr*-KO mice are treated with fenretinide (2.7 fold increase), which increases their BV/TV to a level comparable to the levels observed in the WT mice. This increase in BV/TV was associated with a statistically significant increase ($p < 0.05$) in bone volume (BV) and trabecular bone number (TN), as shown figures 2.2B and 2.2C. Fenretinide treatment increased BV, 3.1 fold and BN, 2.4 fold compared to *Cftr*-KO mice not treated. The SMI is an algorithm taking into account the change in surface area for the change in radial expansion of trabecular plate-like and rod-like structures, which is known as the "trabecular bone pattern factor" (196, 197). A score is given between 1 and 3; as the value approaches 3 the quality of the bone worsens. Figure 2.2D illustrates that SMI scores are significantly ($p = 0.026$) different between WT and *Cftr*-KO untreated mice. However, when these mice are treated with fenretinide, there is no significant ($p = 0.320$) difference between the WT and *Cftr*-KO. Additionally, trabecular separation was measured as shown in Figure 2.2E. *Cftr*-KO mice have a significantly ($p= 0.026$) higher degree of separation compared to WT controls.

When mice are treated with fenretinide there is no longer a significant difference between the trabecular separation ($p = 0.548$). These results suggest that fenretinide not only corrects the quantity of bone but also the quality of the bone structure as well.

The above results were confirmed by analyzing the lumbar vertebrae (L3-L5), which are rich in trabecular bone (data not shown). The microCT results were compared with histochemical staining of un-decalcified bone using von Kossa stain, which stains mineralized bone in black as shown as figure 2.1B.

2.3.4 Increased Osteoblast Formation

Osteoblasts lay down on new bone lamellae and are active in bone development and also in bone remodelling. In contrast, osteoclasts are involved in bone resorption, by digesting the adjacent bone matrix. To establish whether the increase in bone volume observed in the fenretinide treated *Cftr*-KO mice is the result of more efficient bone formation or of less efficient bone resorption, we counted the number of osteoblasts (bone forming cells) and osteoclasts (bone resorption cells) in the femur. Figure 2.3A shows a representative H&E stained slide used to quantify the number of osteoblasts and osteoclasts. Figure 2.3B shows the quantification of the average of 3 slides counted per animal in each group. Our analysis clearly demonstrates a striking difference in the number of osteoblasts between the *Cftr*-KO and WT mice ($p < 0.05$). Interestingly, our data also demonstrate that treatment with fenretinide leads to an increase in the number of osteoblasts for the *Cftr*-KO mice treated compared *Cftr*-KO mice that were not treated with fenretinide ($p < 0.05$). No significant difference in number of osteoclasts was found between the fenretinide treated and untreated C*ftr*-KO and WT, (figure 2.3C). This finding was corroborated using TRAP staining, as

shown as figure 3D, where the pink stain is positive for tartrate-resistant acidic phosphatase, an enzyme that is specific for osteoclast cells.

2.3.5 Essential Fatty Acid Profiles

Remarkably, after 8 treatments with fenretinide, the concentration of AA in the plasma of *Cftr*-KO mice was brought down significantly ($p<0.001$) as compared to the WT controls. We have previously found that 28 daily treatments with fenretinide completely normalize the lipid imbalance observed in *Cftr*-KO mice (Guilbault et al, 2007 *submitted*). The data in this study complement previous findings showing that only 8 (as opposed to 28) treatments with fenretinide effectively reduces the excessive amount of phospholipid-bound AA consistently found in the plasma of *Cftr*-KO mice. Indeed, in our study, the levels of AA observed in the treated *Cftr*-KO mice were no longer significantly different from those observed in the WT mice, as shown in figure 2.4A ($p>0.05$). When analyzing the concentration of phospholipid-bound DHA (figure 2.4B), we found a statistically significant increase ($p < 0.001$) after fenretinide treatment in both WT and *Cftr*-KO mice. A positive trend was seen with the fenretinide treatment where a 2 fold increase in phospholipid-bound DHA in *Cftr*-KO mice, compared to the control *Cftr*-KO mice. Hence, comparing control *Cftr*-KO animals to fenretinide treated *Cftr*-KO treated mice; there was a significant increase ($p < 0.05$) in the DHA: AA ratio, as shown in figure 2.4C.

2.3.6 Ceramide concentrations

Reports have suggested that sphingolipids and ceramide have an important role in modulating bone metabolism (105, 187, 191). Since we had recently found that ceramide levels are dramatically diminished in *Cftr*-KO mice, and that this impairment can be corrected with 28 days of fenretinide treatment (Guilbault et al, 2007 *submitted*), we

assessed whether 8 treatments with fenretinide applied in this study would be able to correct this impairment. Ceramide levels in the untreated *Cftr*-KO mice were statistically significantly lower as compared to their WT littermate controls ($p < 0.001$). After 4 weeks of twice a week treatment with fenretinide, the ceramide levels in the treated *Cftr*-KO mice increase 5.9 fold compared to untreated *Cftr*-KO mice ($p < 0.001$) as shown in Figure 2.5. The increased concentration of ceramide is associated with the observed protective effect of fenretinide treatment against osteoporosis in these mice. These results suggest that up-regulating ceramide levels in the plasma might be promoting an increase bone mineralization, which could lead to an increase in bone volume.

2.4 Discussion

The etiology of early-onset osteoporosis in cystic fibrosis patients is not yet established and most likely involves complex interactions between several biochemical pathways (7, 33, 34, 62). Aris and colleagues described the origin of low bone density in patients with CF as the result of several factors including: malnutrition, pancreatic insufficiency, vitamin insufficiency, diabetes, glucocorticoids, inflammatory cytokines, and sex hormone insufficiency(6). It seems logical that the lipid imbalance, one of the hallmarks of CF, would also play an important role in the etiology of osteoporosis. There has been no direct link established between the *Cftr* gene mutations and reduced bone volume. All of these factors are interconnected which makes identification of a causal relationship between a single factor and osteoporosis a difficult task.

Research in the CF field was greatly facilitated when the *Cftr* gene was first cloned, which led to the creation of CF mice (100, 163, 164). Since the first generation of CF mice, there have been multiple successful attempts to improve this model, to make them better suited to the study of CF phenotypes and their treatment (82). Overall, CF mice still represent the most useful and cost-efficient animal model available to study such a multifaceted and devastating disease. We have previously reported that the *Cftr*-KO mice model that we developed by backcrossing *Cftr*-KO^{UNC} mice to the C57BL/6 strain for more than 20 generations, is unlike the original 129/J genetic background knockout mice that does not express any alternative chloride channel, and ours develops spontaneous lung disease (50, 80, 81, 99). In addition, we have recently demonstrated that they also exhibit an imbalance between DHA and AA, as do CF patients (Guilbault *et al*, 2007 *submitted*). Therefore, it may also represent a suitable model for studying other CF related phenotypes.

From all the studies using mouse models, there has only been one report by Dif and colleagues in 2004 that demonstrated severe osteopenia in 3 week old mice (45). In this present manuscript we used our cystic fibrosis mouse model to characterize in a detailed manner the early onset of osteoporosis and assess the efficiency of fenretinide in treating this condition. Our study shows that these *Cftr*-KO mice develop the osteoporosis phenotype, showing significant decrease in bone volume and structural abnormalities. It is worth noting that using the mouse model to study the etiology of this condition might be superior to performing human studies because *Cftr*-KO mice, in contrast to CF patients, are not treated with antibiotics and glucocorticoids. Additionally there is no 1, 25 vitamin D deficiency present in our *Cftr*-KO mice (data not shown), which allows us to rule out these factors as playing a role in the development of bone disease.

Based on previous findings and on the findings presented in this manuscript, we hypothesized that up regulating ceramide levels in the plasma would increase bone mineralization and therefore would cause an increase in bone volume. As shown in figure 2.5, ceramide levels do increase 5.9 times after only 8 treatments with fenretinide. Subsequently, when analyzing the microCT data, we observed that fenretinide treatment also significantly increased the trabecular bone volume and quality of the bone, as shown in figures 2.1 and 2.2. Matrix vesicles, are cell-derived microstructures involved in the initiation of bone mineralization, cartilage and dentin (191). They are rich in 5-AMP-ase (3) and sphingomyelin (105), both of which are concentrated in plasma membranes. These membranes are saturated with phospholipids which act as trap for calcium ions (141). During normal calcification, there is a major influx of calcium and phosphate ions into the cell accompanied by cellular apoptosis and matrix vesicle formation (191) . Matrix vesicles

separate from the plasma membrane at sites where there are interactions with the extra cellular matrix, to nucleate mineral formation (44). These processes occur to coordinate the mineralization during bone development. Kozawa and colleagues hypothesized that sphingomyelinase played a crucial role as an intercellular messenger in osteoblast differentiation from mesenchymal stem cells cells (105). These findings strongly suggest that ceramide plays an important role in modulating bone metabolism. Interestingly, we show that the corrective effect of fenretinide treatment on ceramide concentrations also prevents osteoporosis in *Cftr*-KO mice. We have also proposed a possible mechanism through which Fenretinide may be acting to exert this effect as summarized in figure 2.6.

A skeletal system not only serves as mechanical support, but also functions as an active organ that regulates balance and interactions between both local and systemic circulating factors. Bone remodeling is regulated by a number of hormones, cytokines, growth factors, and prostaglandins (13). Defective fatty acid metabolism has been linked to inflammatory diseases such as asthma and rheumatoid arthritis as well to osteoporosis in elderly people (162, 179). The balance between DHA and AA has been suggested important for patients with CF to fight infections and improve absorption. Even more recently Gronowitz and colleges reported that the fatty-acid status of children with cystic fibrosis influences bone mineral density, indicating that fatty-acid status would be important for bone growth (8). It has been previously suggested that an effective decrease in phospholipid-bound AA concentrations could consequently lead to an increase in the DHA:AA ratio and a decrease in the level of prostaglandin production in bones (156). Furthermore, increases prostaglandin synthesis has been linked to increasing bone formation and improving osteoblast cell function in cell cultures and in periodontal disease

in humans (156). Tight regulation of AA metabolism is of crucial importance. AA is a very powerful EFA because of its ability to up regulate inflammatory mediators when the body tries to react to trauma and injury (40).

Fenretinide has been shown to catalyze the oxidation of AA so that it can be moved from the phospholipid membrane and converted to a less harmful metabolite 12-Hydroxyeicosatetraenoic acid (12-HPETE/ 12-HETE) via LOX-12 (124). Ichikawa and colleagues demonstrated that the regulation of the human *LOX12* gene is closely associated with bone mineral density (93). As shown in Figure 2.4A, our results demonstrate that treatment with fenretinide is able to normalize the levels of AA in the plasma of *Cftr*-KO mice. DHA concentrations were also significantly increased in fenretinide-treated animals, which positively affected the abnormal DHA: AA ratio typically observed in the *Cftr*-KO. Taken together, these results demonstrate not only that fenretinide has a protective effect on bone density in *Cftr*-KO mice, but also provide clues regarding its mechanism of action. Overall, treatment with fenretinide represents a unique method of directly increasing ceramide levels, which directly or indirectly decreases excessive phospholipid-bound AA and consequently increases trabecular bone volume, as deplicted in figure 2.6.

Based on our previous results and the results presented in this manuscript, it is quite clear that fenretinide could potentially be a beneficial treatment for CF patients, due to its demonstrated activity against lung disease (Guilbault *et al,* 2007 *submitted*) and early-onset osteoporosis. It is especially promising since this drug has been reported to have only very mild side effects and has already been approved and currently in clinical trials to treat other disorders, such as neuroblastoma and other cancers.

2.5 Acknowledgements

This work was supported by Canadian Cystic Fibrosis Foundation grants (216714(DR)). We would like to thank Dr. Marie-Christine Guiot for allowing us to use her histology laboratory and Dr. Janet Henderson and her technical staff of the J T N Wong Laboratories for Mineralized Tissue Research for their assistance with microCT and histological analyses. We would also like to thank Pierre Camateros for his assistance with statistical analysis and Kalyn Eadie for her critical review of our manuscript.

Figure 2.1A micro-CT image of femur. Bones were dissected free of soft tissue, fixed overnight before being scanned on a Skyscan 1072 static instrument equipped with 3D Creator analytical software. Representative 3D reconstructions and 2D cross-sectional scans demonstrate a clear difference between control WT and *Cftr*-KO mice. Cftr-KO mice have much less bone volume then their WT controls. Areas of the bone where micro CT was used to scan and display fenretinide's positive effect in increasing *Cftr*-KO bone volume are highlighted by black boxes.

A

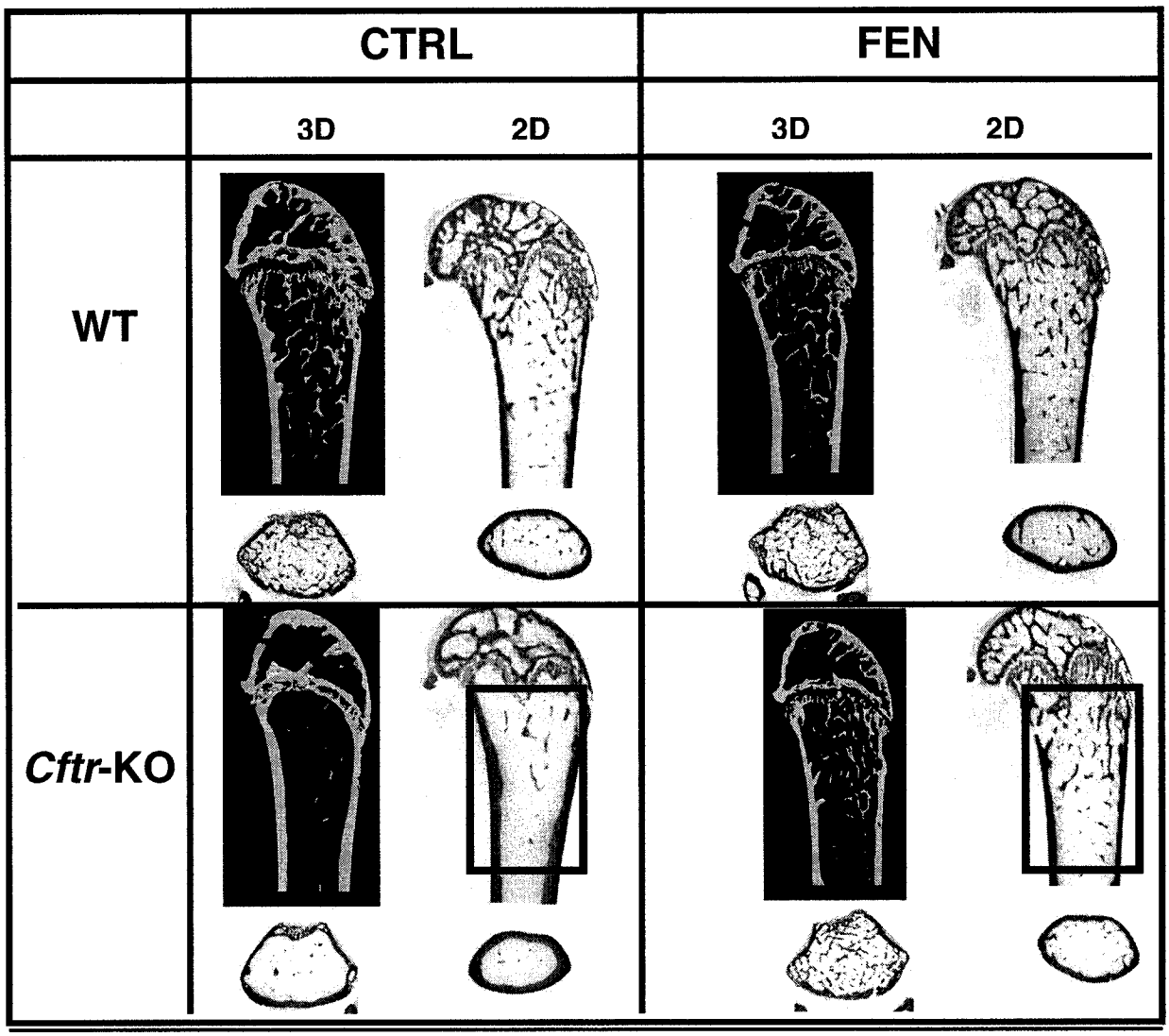


Figure 2.1

Figure 2.1B von Kossa stains of femur. Bones were embedded in MMA and stained with von Kossa. These were used to confirm the amount of mineralized bone (black stain) measured in the micro CT images. Area of the bone where micro CT was used to scan and displays fenretinide's positive affect in increasing *Cftr*-KO bone volume is highlighted by black boxes. A representative slide is shown.

B

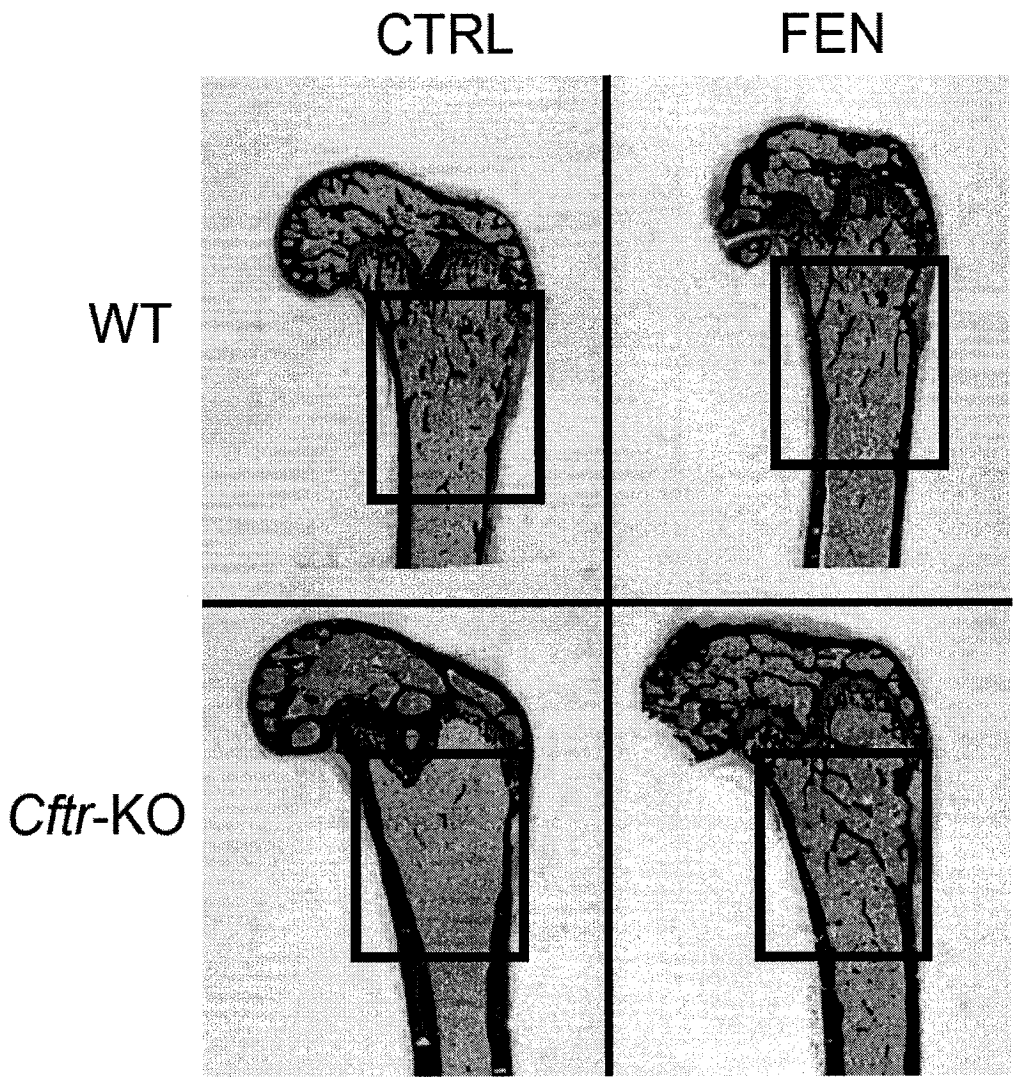


Figure 2.1

Figure 2.2 Quantitative microCT of Trabecular Bone Composition and Architecture. The following parameters were calculated on the left femur of 5 to 6 mice per group using 3D Creator software supplied with the Skyscan instrument. Lines represent the mean (+/- SEM). Significance is set at $p<0.05$. (*) indicates significance between untreated WT and untreated *Cftr*-KO and (#) indicates a significant difference found between the *Cftr*-KO control and treated groups. **A. Bone volume/tissue volume. B. Bone volume, C. Trabeculae bone number D. Structure model index, E. Trabeculae Separation ;** A clear difference is observed between the WT and Cftr-KO control groups, as shown through panels A to E, fenretinide is then shown to increase the BV/TV, BV, TN, SMI and Trb. Sp to the level of the control groups.

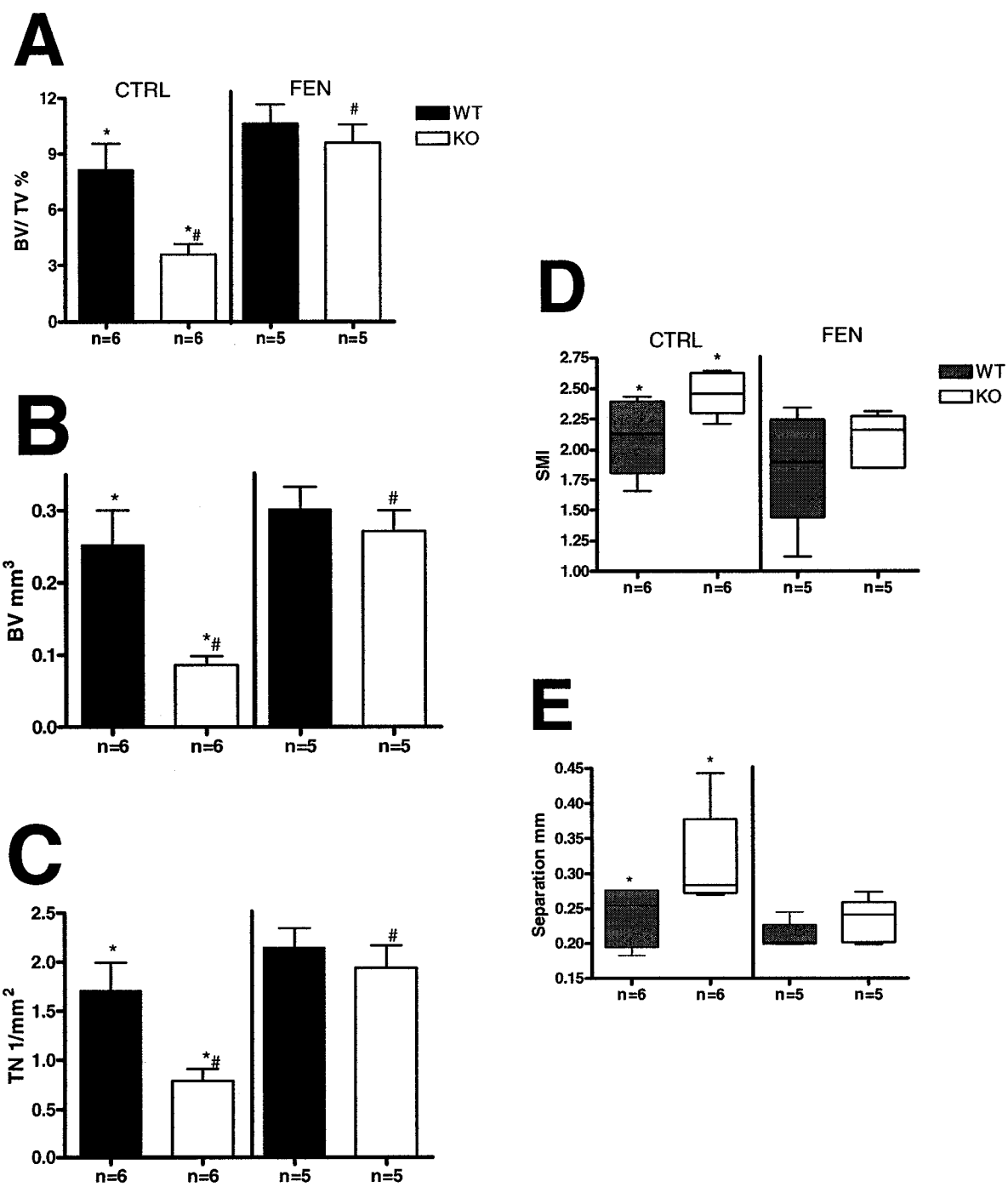

A
CTRL
FEN
WT
KO
BV/ TV %
12
9
6
3
0
n=6
n=6
n=5
n=5
B
BV mm3
0.3
0.2
0.1
0.0
C
TN 1/mm2
2.5
2.0
1.5
1.0
0.5
0.0
D
SMI
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
E
Separation mm
0.45
0.40
0.35
0.30
0.25
0.20
0.15

Figure 2.2

Figure 2.3A Osteoblast and Osteoclasts Quantification in Femur. Bones were decalcified; embedded in paraffin and stained with H&E. Multiple slides were used to count the number of osteoblasts and osteoclasts (a representative slide is shown). Slides were counted at 400X magnification. Osteoblasts were identified as single-nucleated, rod shaped cells attached to the trabecular bone as shown by black arrow. Osteoclasts were defined as large multinuclear round (macrophage type) cells attached to the trabecular bone as shown by arrows.

B. Quantification of the Counted Slides. Data is shown as the mean +/- the SEM, (*) indicates significance between untreated WT and *Cftr*-KO mice and (#) indicates a significant difference between the *Cftr*-KO untreated and treated groups. Data shown are representative of an average of 3 slides counted per animal

C. TRAP Staining. Bones were embedded in MMA and stained with TRAP. Multiple slides were analyzed to identify and quantify osteoclasts present in each slide (a representative slide is shown at 200X magnification).

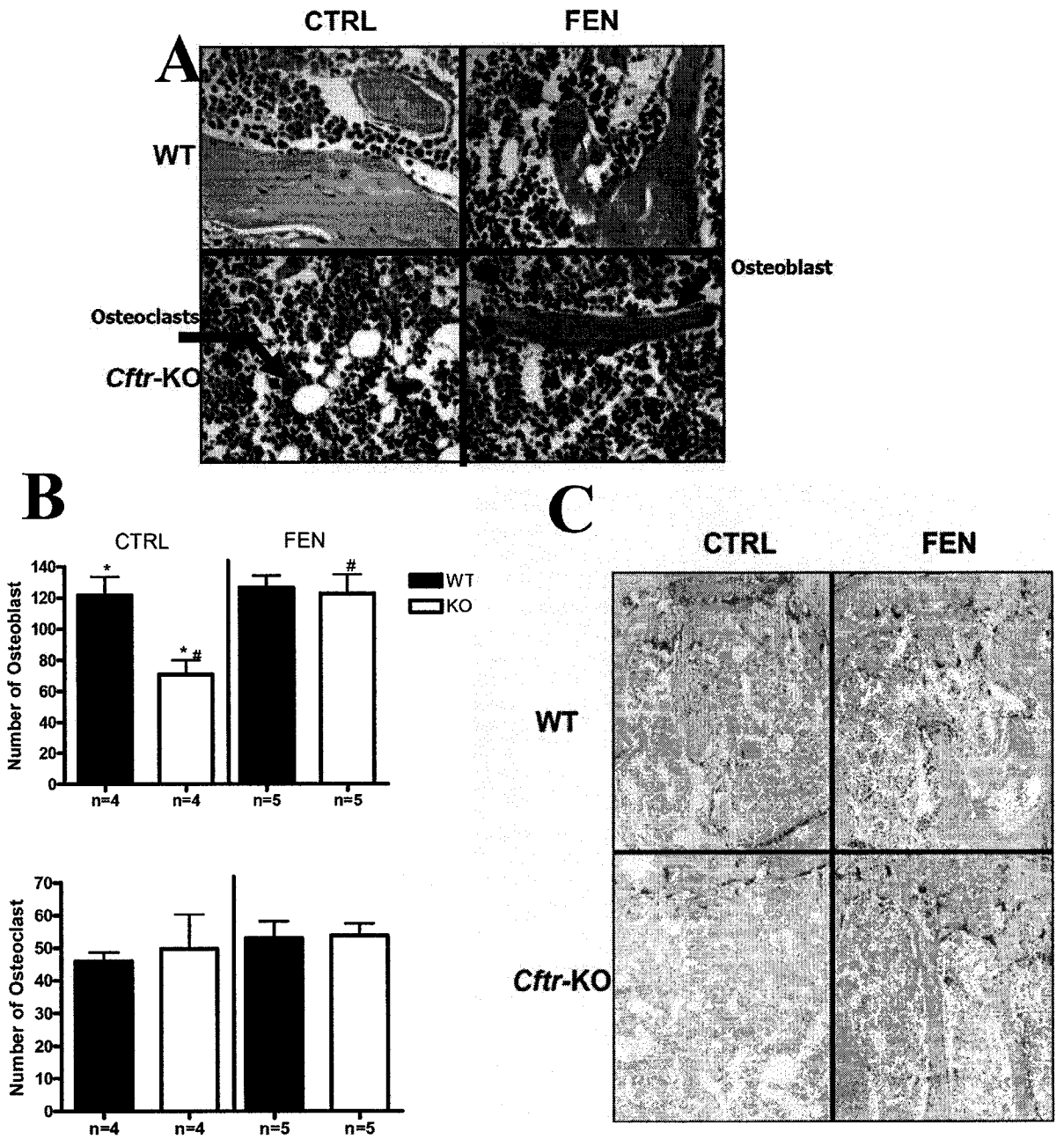

A
CTRL
FEN
WT
Osteoblast
Osteoclasts
Cftr-KO
B
CTRL
FEN
WT
KO
Number of Osteoblast
140
120
100
80
60
40
20
0
*
*#
#
n=4
n=4
n=5
n=5
Number of Osteoclast
70
60
50
40
30
20
10
0
n=4
n=4
n=5
n=5
C
CTRL
FEN
WT
Cftr-KO

Figure 2.3

Figure 2.4 Lipid Profile of *Cftr*-KO and WT mice untreated and treated with Fenretinide

A. Phospholipid-bound Arachidonic acid. The levels of phospholipid-bound arachidonic acid were quantified in the plasma of WT and *Cftr*-KO mice. Data is shown as the mean +/- the SEM (*) indicates significance between untreated WT and untreated *Cftr*-KO and (#) indicates a significant difference found between the *Cftr*-KO untreated and treated groups. After 4 weeks of twice a week treatment with fenretinide (total of 8 treatments), phospholipid-bound arachidonic acid in the *Cftr*-KO mice was decreased significantly ($p<0.001$) to the level observed in the WT. A significant difference between treated *Cftr*-KO animals and WT animals was no longer detectable ($p>0.001$).

B. Phospholipid-bound DHA. Concentration of DHA bound in phospholipids was assessed in WT and *Cftr*-KO mice. (*) indicates significance between untreated WT and untreated *Cftr*-KO and (#) indicates a significant difference found between the *Cftr*-KO untreated and treated groups. After 4 weeks of twice a week treatments with fenretinide, DHA bound in phospholipids in WT and *Cftr*-KO mice increased 2 folds, illustrating a positive trend.

C. Phospholipid-bound DHA/AA. The DHA/AA ratio was assessed in WT and *Cftr-KO* mice. (*) indicates significance between untreated WT and untreated *Cftr*-KO and (#) indicates a significant difference found between the *Cftr*-KO untreated and treated groups. The DHA: AA ratio is statistically different between WT and *Cftr*-KO mice ($p<0.001$). After 4 weeks of twice a week treatments with fenretinide, the ratio of phospholipid-bound DHA and phospholipid-bound AA increases significantly ($p < 0.05$).

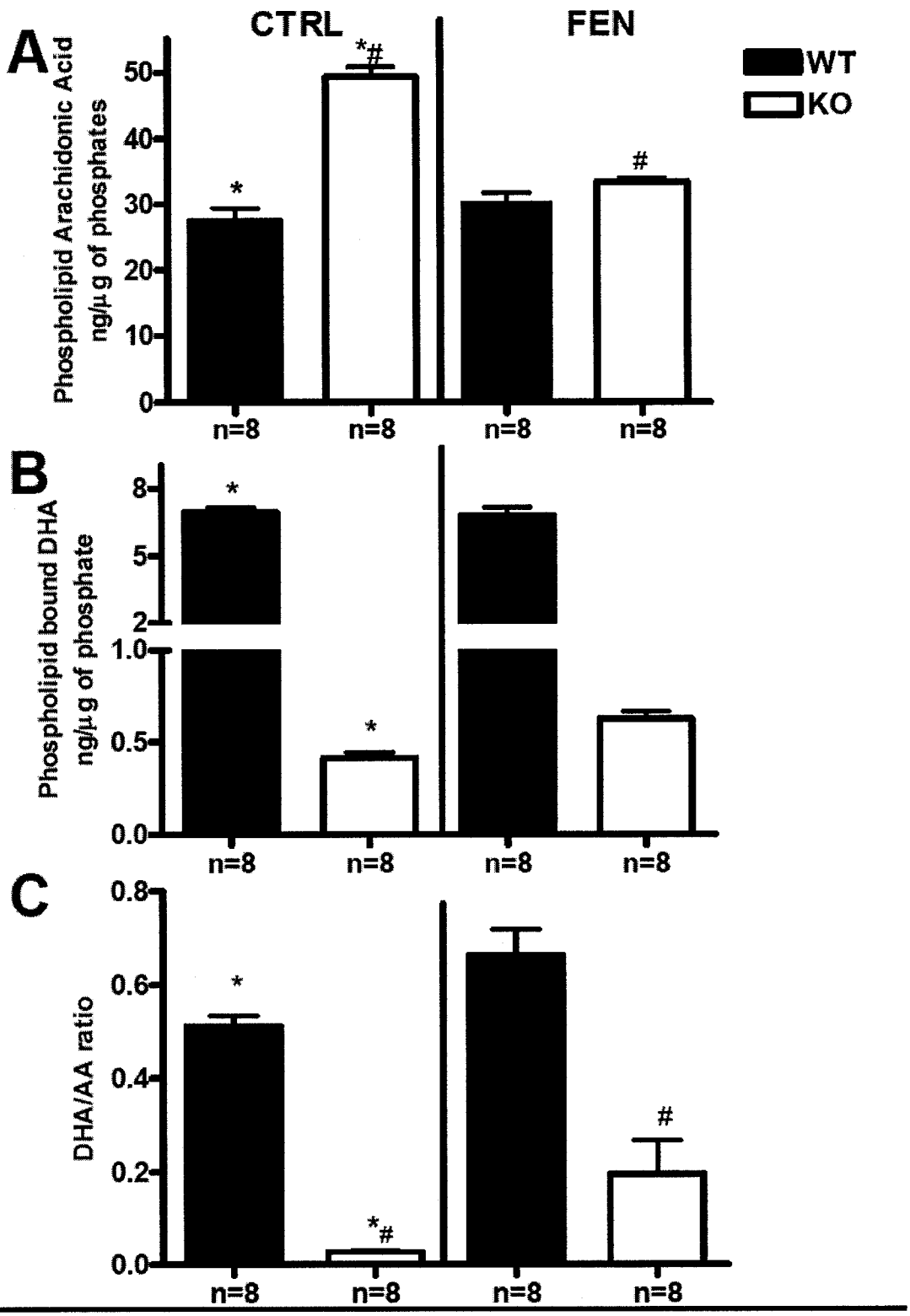

A
CTRL
FEN
WT
KO
Phospholipid Arachidonic Acid
ng/µg of phosphates
50
40
30
20
10
0
*
*#
#
n=8
n=8
n=8
n=8
B
Phospholipid bound DHA
ng/µg of phosphate
8
5
2
1.0
0.5
0.0
*
*
n=8
n=8
n=8
n=8
C
DHA/AA ratio
0.8
0.6
0.4
0.2
0.0
*
*#
#
n=8
n=8
n=8
n=8

Figure 2.4

Figure 2.5 Ceramide-sphingolipids levels in *Cftr*-KO and WT mice untreated and treated with Fenretinide. Ceramide levels were assessed in plasma isolated from WT and *Cftr*-KO mice. The ceramide levels in the plasma samples were statistically different ($p < 0.001$(*)) between WT and *Cftr*-KO mice. Following 4 weeks of twice a week treatment with fenretinide, the ceramide levels in the *Cftr*-KO mice increased significantly [$p<0.001$(#)].

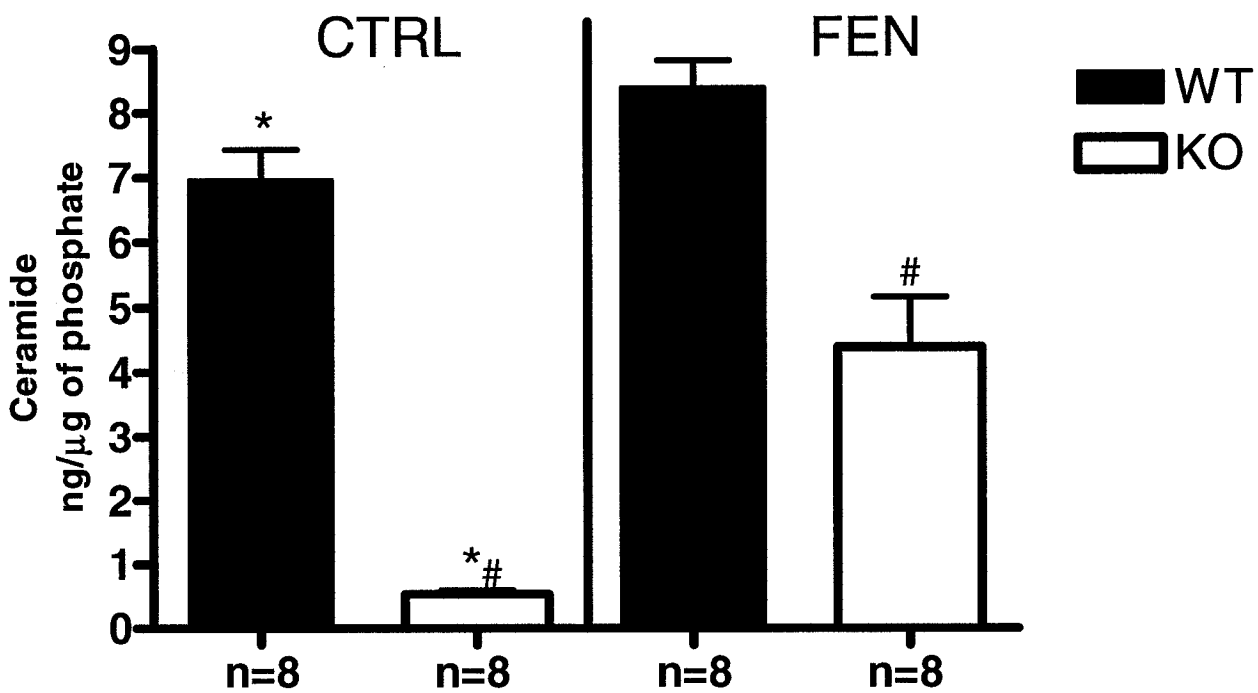

CTRL
FEN
WT
KO
Ceramide
ng/μg of phosphate
9
8
7
6
5
4
3
2
1
0
*
*#
#
n=8
n=8
n=8
n=8

Figure 2.5

Figure 2.6 Fenretinide induced up-regulation of phospholipid-bound AA
The diagram depicts biochemical links that might be involved in a fenretinide-induced increase of bone formation in the *Cftr*-KO mice. Fenretinide stimulates the production of sphingomyelin that subsequently increases ceramide concentrations. Increasing ceramide (a lipid secondary messenger) stimulates lipoxygenase-12 (LOX-12), an enzyme that catalyzes the oxidation of arachidonic acid (AA) to leave the phospholipid bi-layer and become a less harmful metabolite 12-Hydroxyeicosatetraenoic acid (12-HPETE/ 12-HETE) intracellular.

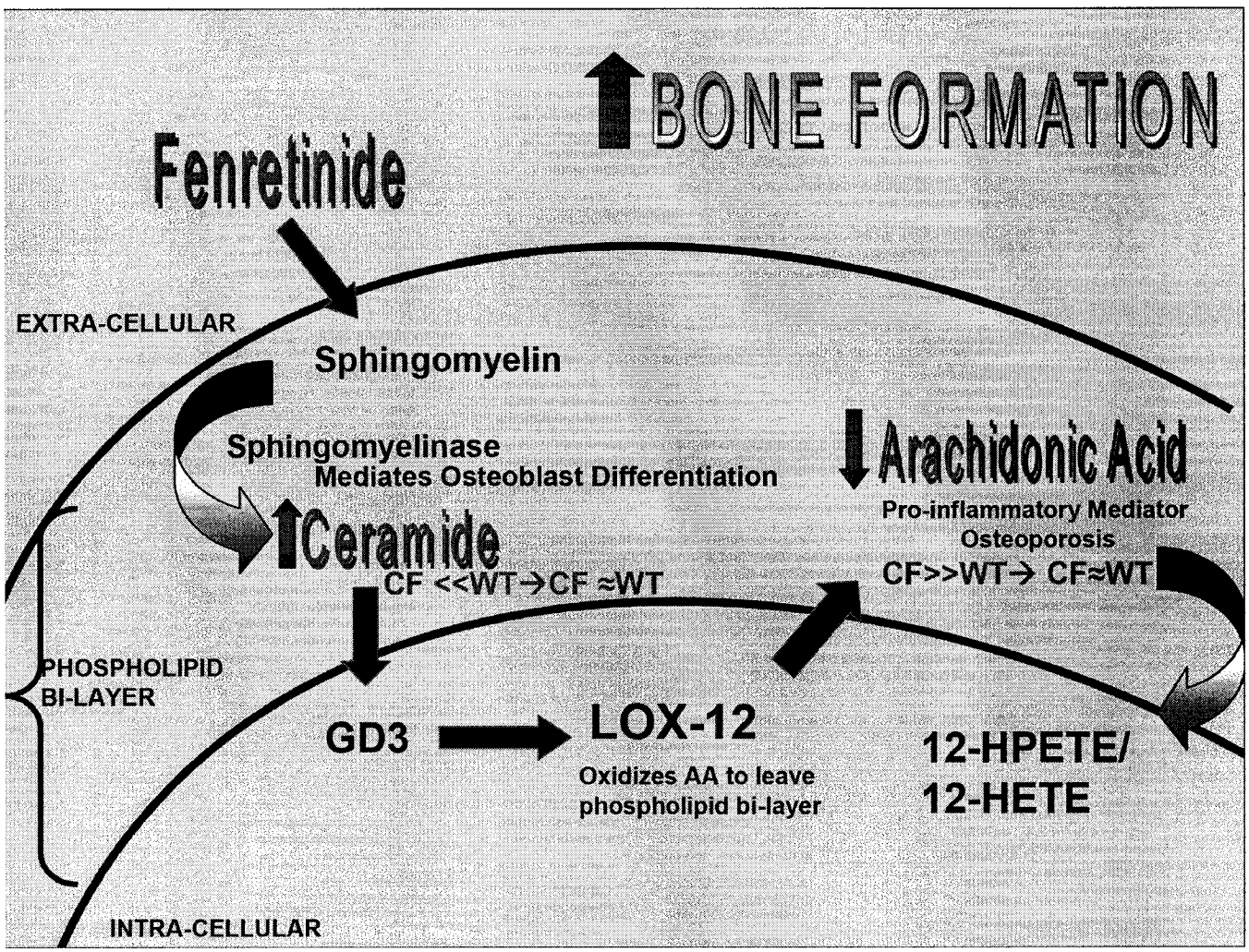

BONE FORMATION
Fenretinide
EXTRA-CELLULAR
Sphingomyelin
Sphingomyelinase
Mediates Osteoblast Differentiation
Ceramide
CF <<WT→CF ≈WT
Arachidonic Acid
Pro-inflammatory Mediator
Osteoporosis
CF>>WT→ CF≈WT
PHOSPHOLIPID
BI-LAYER
GD3
LOX-12
Oxidizes AA to leave
phospholipid bi-layer
12-HPETE/
12-HETE
INTRA-CELLULAR

Figure 2.6

Chapter III

GENERAL DISCUSION:

3.1 Summary of Results

The life expectancy of patients with CF has dramatically improved in recent years. The majority of newly diagnosed patients in Canada are expected to survive past their 37th birthday. Low bone density compromises the quality of life of patients with CF who are living longer than ever because of the advances in research aimed at preventing chronic lung infections. However, because CF bone disease has only recently received attention, most therapeutic trials are either ongoing or have only been published as preliminary observations. The current preventative actions for optimal bone health is vitamin and mineral supplementations including(6): calcium (32-37 mmol/day), vitamin D (400-800 IU/day), vitamin K (2.5-5 mg/week), vitamin E (200-400 IU/day), vitamin A (10,000 IU/day). This is aimed to combat the innate deficiency observed in CF patients, however the problem remains.

CF bone disease pathology can be caused by a number of different factors including; significantly higher levels of circulating pro-inflammatory cytokines, increased levels of respiratory acidosis, both of which are associated with severe lung disease, pancreatic insufficiency which has been linked to poor nutritional status, essential mineral & vitamin deficiencies (calcium, vitamin D, vitamin K) additionally an increased prevalence of diabetes, delayed puberty, cumulative corticosteroid dose, and physical inactivity. A number of studies have documented a prevalence (40-70%) of decreased bone density in the CF population (14, 18, 19, 180). Severe bone disease can lead to morbidity and exclusion from lung transplant, which is often a life-saving operation for individuals with CF(6). The

problem remains that despite recent clinical research, a number of questions still remain regarding the pathogenesis and management of CF related bone disease.

The skeletal system not only serves as mechanical support, but also functions as an active organ that regulates the balance and interactions between both local and systemic circulating hormones, cytokines, growth factors, and prostaglandins. Modulation of prostaglandin synthesis has been linked to increasing bone formation and improvement of osteoblast cell function in cell cultures and in periodontal disease in humans. Previous studies suggest that tight regulation of AA metabolism is of crucial importance and the addition of n-3 EFA in the diet could decrease prostaglandin production (156). Gronowitz and colleges are the most recent to report that the fatty-acid status of children with cystic fibrosis is directly associated with bone mineral density (78). Skeletal development begins in *utero* and continues throughout childhood and adolescence. At this point, bones reach their maximum strength and density, or what is known as the peak bone mass (135). It is logical that the lipid imbalance, one of the hallmarks of CF, would also play an important role in the etiology of osteoporosis. However, all of these factors are intertwined making it difficult to know the exact cause, especially when there has been no direct link established between the *Cftr* gene mutations and reduced bone volume.

This thesis documents the results of experiments to assess the effect of fenretinide on the early onset of osteoporosis due to low bone mineral density. Although the mechanism of action of fenretinide remains unclear, our previous studies suggest that this drug has anti-inflammatory effects mediated through ceramide production and the down regulation of phospholipid bound AA that could be beneficial to *Cftr* deficient mice. Ceramide is

upregulated through the production of sphingomyelinase, that was previously shown to play a crucial role as an intercellular messenger in osteoblast differentiation from capillary endothelial cells, suggesting that ceramide has an important role in modulating bone metabolism (105).

The data described in this thesis uses our cystic fibrosis mouse model to characterize in a detailed manner the early onset of osteoporosis and assess the efficiency of fenretinide in treating this condition. Our study shows that these *Cftr*-KO mice develop the osteoporosis phenotype; using microCT scans we are able to show extremely low bone volume and structural abnormalities, in which the *Cftr*-KO mice show a significantly lower SMI score then the WT controls. We were then able to conclude that a treatment with fenretinide is able to correct the low bone volume and structural abnormalities observed in mice with cystic fibrosis. This correction is associated with; an increase in osteoblast activity quantified by histology, an increase in ceramide concentrations quantified by TLC-ELISA, and a down regulation of phospholipid-bound AA quantified by HPLC. Taken together the results in this thesis are a stepping stone in a potential treatment for CF patient with low bone mineral density and also provide clues into the possible mechanism of bone metabolism.

3.2 Future Directions

Although Fenretinide has been studied extensively in many disease models, its mechanism of action has not been defined in the cystic fibrosis model. It would be interesting to study the specific biochemical interaction through which this drug produces its pharmacological effect; by identifying the specific ligands it binds to, and the exact signaling pathways it activates. One future direction can be to elucidate parts of the mechanism

specific to bone disease in CF. Biochemical markers such as insulin, osteoprotegerin (OPG), activator of nuclear factor-kappaB ligand receptor (RANKL), osteocalcin, leptin, IL-β, IL-6 and TNF are all important in bone metabolism and play an important role in the assessment of bone diseases such as osteoporosis. Insulin is a hormone that regulates the number of osteoblast, promotes osteoclastic bone absorption, and has been shown to suppresses the expression of transcription factor gene that differentiate osteoblasts (107). OPG, also known as osteocalstogenesis inhibitory factor (OCIF) is a soluble member of the TNF receptor superfamily. OPG regulates osteoclast differentiation and activation; it is secreted by osteoblastic lineage cells and immune cells such as dendritic cells and B cells. OPG acts as a decoy receptor for its ligands, RANKL (195). RANKL is a member of the TNF superfamily as well, it is secreted by osteoblastic lineage cells and activated T lymphocytes and is a key regulator of osteoclastogenesis(195). Osteocalcin is synthesized by osteoblasts during the process of bone formation. Osteocalcin is used as a marker for bone turnover. Leptin is a hormone that plays a key role in regulating energy intake and energy expenditure, including the regulation of appetite and metabolism. It has also recently gotten attention by the CF community to assess the correlation between leptin, proinflammatory cytokines and nutritional status with regard to clinical status in homozygous delta F508 cystic fibrosis patients, researchers found that there was physiological regulation of leptin in more advanced states of disease with significantly lower body mass index than controls (171). Cytokines like TNF, IL-6 and IL-1β have all been associated with a heightened inflammatory response observed in bone disease. LINCO provides a kit that is called "The Mouse Bone multiplex" that enables the simultaneous analysis of these particular bone biomarkers from plasma. Once the particular proteins have been identified it would also be interesting to analyze the

gene expression using real time PCR from RNA isolated from crushed bone samples specifically.

Patients with CF have been shown to have a significantly lower concentration of glutathione (GSH). SMase has been shown to be inhibited by glutathione (GSH) (111). Therefore, it is possible, that low levels of GSH observed in cystic fibrosis cells (117) might negatively affect the generation of intracellular ceramide. To study this hypothesis, acid-sphingomyelinase knock out mice (ASMase KO) are available from Dr. E. Schuchman laboratory at the Institute of Molecular Biology, Roche Research Center in New Jersey. Using these KO mice we can detect wether fenretinide is specifically involved in this hypothesized pathway and if the same physiological outcomes are observed resembling our *Cftr*-KO mice. Furthermore, fenretinide's effect on glutathione production in our *Cftr*-KO model would also be interesting to examine.

Fenretinide's ability to correct fatty acid metabolism by significantly decreasing AA through the induction of ceramide is the major finding of our laboratory. With the encouraging results summarized in this thesis on fenretinide's positive effect on bone disease it would also be informative to examine if other CF related phenotypes, such as pancreatic insufficiency, inflamed villi in the ilium, liver abnormalities and reproductive abnormalities would also see positive results as well. Experiments are underway to examine these other phenotypes.

We have recently finished a pilot study where we were able to correlate low ceramide concentrations found specifically in CF patients to the high concentration of AA in approximately 70 patients with CF compared to healthy controls. Based on these results and

previous ones, it would be interesting to explore if ceramide concentrations from plasma can be used as a possible diagnostic technique for the detection of CF instead of the conventional sweat test. In addition we could examine if low ceramide concentration correlate with low bone scans, as seen in the *Cftr*-KO mice.

Overall the work in this thesis may provide hope to the patients and families affected by this devastating disease.

CLAIMS TO ORIGINALITY

From the studies on fenretinide described in this thesis, major findings are exposed:

Fenretinide treatment using bi-weekly dietary supplementation of Peptamen diet for *Cftr*-KO mice for a period of one month prevents development of osteoporosis:

- Increases bone mineral density by significantly:
    - Increasing Ceramide concentrations
    - Decreasing Arachidonic Acid
    - Increasing Osteoblast functions

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APPENDIX I

List of other published manuscripts

Guilbault C, **Saeed Z**, Downey GP, & Radzioch D. Cystic Fibrosis Mouse Models. *Am J Respir Cell Mol Biol.* (2007) 36:1-7

Guilbault C, Novak JP, Martin P, Boghdady ML, **Saeed Z**, Guiot MC, Hudson TJ, & Radzioch D. Distinct Pattern Of Lung Gene Expression in the Cftr-KO mice Developing Spontaneous Lung Disease Compared to their Littermate Controls. *Physiol Genomics.* 2006 25(2):179-93.

APPENDIX II

Certificates of compliance

Animal use protocol

Radioactivity certificate