The Effects of Fenretinide, a Vitamin A Derivative, on Phenotypes of Cystic Fibrosis

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January - 2014

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

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ACKNOWLEDGEMENTS

The work described in this thesis could not have been completed without the help of many people. I am grateful to my supervisor Dr. Danuta Radzioch for her invaluable experience and support throughout my graduate studies. Her commitment to research and her fighting spirit have taught me skills for all aspects of life.

I would like to thank my supervisory committee members Dr. Feige Kaplan, Dr. Larry Lands and Dr. Basil Petrof for their council and support throughout my graduate studies. A big thank you to great collaborators, Dr. Elias Matouk for being so encouraging and kind over the years, and to Dr. Juan B. De Sanctis for all his valuable expertise, help and support.

To the members of the Radzioch lab, past and present, Cynthia, Daniel, Xu, Mitra, Claude, Thusanth, Pierre, Rafael, Tom, Jennifer, Claudine, Sahar, Dominique, Marie-Linda, and to everyone on L11 thank you for all the help, discussions, friendships and good memories. A special thank you to Cynthia for being a great lab mate and an even better friend.

To my friends and family who have been the greatest support system anyone could ask for, thank you for listening to my frustrations, celebrating my successes, believing in me, not being too upset when I postponed things, and for always cheering me on. A special thanks to David Dubois who has given me a glimpse of how difficult life can be with cystic fibrosis. The way he lives his life is inspirational and, whether he knew it or not, he was a great motivation throughout my graduate studies.

This thesis is dedicated to my parents. To my Mom for being an example of courage, strength and perseverance. And to the memory of my Dad for his faith in me and his great sense of humour.

Gabriella

LIST OF ABBREVIATIONS

°C	degree Celsius
µg	microgram
µL	microlitre
µM	micromolar
AA	arachidonic acid
ABC	ATP-binding cassette
Abx	antibiotics
ANOVA	analysis of variance
AP-1	activator protein-1
ASL	airway surface liquid
ATP	adenosine triphosphate
BALF	bronchoalveolar lavage fluid
BHA	butylated hydroxyanisole
BMI	body mass index
CaCC	calcium-activated chloride channel
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CF	cystic fibrosis
CFRD	cystic fibrosis related diabetes
CFTR	cystic fibrosis transmembrane conductance regulator protein
CFTR	cystic fibrosis transmembrane conductance regulator gene (human)
Cftr	cystic fibrosis transmembrane conductance regulator gene (mouse)
CFU	colony forming unit
cm	centimeter
COX	cyclooxygenase
CRP	C-reactive protein
C_t	threshold cycle
CYP	cytochrome P450
DAG	diacylglycerol
DHA	docosahexaenoic acid
DHC	dihydroceramide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease (rh: recombinant human)
DP1-2	prostaglandin D2 receptor 1 and 2
ED	early diagnosis
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ENaC	amiloride-sensitive epithelial sodium channel
EP1-4	prostaglandin E2 receptor 1 to 4
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum

ERK	extracellular signal-regulated kinase
FABP	fatty acid-binding protein
FEF	forced expiratory flow
FEV_1	forced expiratory volume in 1 second
FP	prostaglandin F2 alpha receptor
FVC	forced vital capacity
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	gas chromatography
h	hours
HC	healthy control
HPLC	high pressure liquid chromatography
HZ	heterozygote
IgM/G	immunoglobulin M/G
IL	Interleukin
IV	intravenous
IP	prostacyclin receptor
JNK	c-Jun N-terminal kinase
kb	kilobase
KC	keratinocyte chemoattractant
kg	kilogram
KO	knock-out
LA	linoleic acid
LC	liquid chromatography
LD	late diagnosis
LOX	lipoxygenase
LPS	lipopolysaccharide
$LTA_4/B_4/C_4/D_4/E_4/B_5$	leukotriene A4/B4/C4/D4/E4/B5
LXA_4/B_4	lipoxin A4/B4
m	meter
MaR1	maresin
MDA	malondialdehyde
mg	milligram
min	minute
MIP-1β	macrophage inflammatory protein 1 beta
mL	millilitre
mm	millimeter
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MUC	mucin
NF-κB	nuclear factor kappa B
nm	nanometer
nmol	nanomole
NO	nitric oxide

NO_2	nitrogen dioxide
NSAIDS	non-steroidal anti-inflammatory drugs
NT3	nitrotyrosine
p38	p38 mitogen activated protein kinase
PA	*Pseudomonas aeruginosa*
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCL	periciliary layer
PCR	polymerase chain reaction
PD1	protectin D1
PEx	pulmonary exacerbation
pg	picogram
$PGD_2/E_2/F_{2\alpha}$	prostaglandin D2/E2/F2 alpha
PGI_2	prostacyclin
PLA_2	phospholipase A2 (c: cytosolic, s: secreted, i: calcium-independent)
PP2A	phosphatase 2A
PUFA	polyunsaturated fatty acid
RvD1-6	resolvin 1 to 6
QOL	quality of life
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBP	retinol binding protein
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RXR	retinoid X receptor
S100A8	S100 calcium binding protein A8, calprotectin
SA	*Staphylococcus aureus*
SD	standard deviation
SMase	sphingomyelinase (a: acidic, n: neutral, alk: alkaline)
Smpd1	sphingomyelin phosphodiesterase 1 (mouse)
STRA6	stimulated by retinoic acid 6
TLC	thin layer chromatography
TLR	toll-like receptor
TNF	tumour necrosis factor
TP	thromboxane receptor
TXA_2/B_2	thromboxane A_2/B_2
VEGF	vascular endothelial growth factor
vol	volume
WT	wild-type
yr	year

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ABSTRACT

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the CF Transmembrane Conductance Regulator (*CFTR*). Individuals are usually diagnosed in infancy and are burdened with extensive medical treatments throughout their lives. The treatments currently available attempt to preserve lung function by addressing bacterial infections and physiological defects of the CF lung. There are many CF phenotypes not addressed by current therapies such as high levels of inflammation, high oxidative/nitrosative stress, abnormal levels of polyunsaturated fatty acids (PUFA) and defects in ceramide. Based on our preliminary studies, we hypothesized that fenretinide could address these gaps in CF treatment.

Our previous studies demonstrated the protective effects of fenretinide, a derivative of vitamin A, in CF mice. The drug treatment in CF mice improved clearance of lung infections and normalized the levels of ceramide, which were abnormally low in CF. In this thesis, we show that the abnormal levels of PUFAs, arachidonic acid (AA) and docosahexaenoic acid (DHA), are associated with the defects in ceramide in CF patients. Fenretinide treatment in CF mice improved the PUFA imbalance systemically and in CF-related organs, lung, ileum, pancreas and liver. The drug also reduced the expression of inflammatory genes, IL-1β and S100A8, in the lung of CF mice.

Fenretinide had prolonged normalizing effects on PUFA and ceramide levels in mice with one dose improving the lipids up to 90 hours after treatment. It also reduced the levels of malondialdehyde, a marker of lipid peroxidation, and nitrotyrosine, a marker of nitrosative stress. High levels of lipid peroxidation and nitrosative stress were correlated with greater defects in lipids. Lipids from leukocytes isolated from CF patients responded to fenretinide

treatment in similar ways with a reduction in AA, and increased DHA and ceramide. Lipid peroxidation also decreased in CF cells. We also tested different formulations of the drug in monkeys and demonstrated a drug effect in yet another animal species.

CF patients periodically experience worsening of their pulmonary symptoms and these events are called pulmonary exacerbations (PEx). These events impact the progression of lung disease however they are nearly impossible to prevent. We followed a cohort of CF patients during stable disease to evaluate markers associated with the risk of a future PEx. We determined that at stable disease, a worse clinical picture and a lower score on the patient's quality of life assessment were related to a higher risk of a PEx. After treatment for PEx, patients with high levels of inflammation had rapidly re-exacerbated. We discovered that the aggressive treatments used during PEx decreased inflammatory markers but also improved the defects in PUFA by decreasing AA and increasing DHA. Additionally, treatments for PEx reduced the levels of malondialdehyde and nitrotyrosine, however they are not feasible for chronic use unlike fenretinide.

Fenretinide has been used in long-term cancer prevention trials. The reported side-effects were minimal and reversible with short drug-free intervals. The results in this thesis show the impact of fenretinide on improving lipid defects, reducing inflammation and normalizing high levels of lipid peroxidation and nitrosative stress. These phenotypes are not currently addressed in routine CF therapy thus fenretinide is a good candidate for future clinical trials.

RÉSUMÉ

La fibrose kystique (FK) est une maladie génétique, à transmission autosomique récessive, causée par une mutation du gène *CFTR* (Cystic Fibrosis Transmembrane Conductance Regulator). Son dépistage se fait généralement à l'enfance et les traitements requis représentent un lourd fardeau que les patients portent toute leur vie. Présentement, les traitements disponibles cherchent à maintenir le fonctionnement des poumons en ciblant les infections pulmonaires et les problèmes physiologiques du poumon FK. Plusieurs phénotypes, dont les niveaux élevés d'inflammation, le stress oxydatif/nitrosatif élevé, les niveaux anormaux d'acides gras polyinsaturés (AGPI) et de céramide, ne bénéficient pas de ces thérapies. Suite à nos études préliminaires, nous avons émis l'hypothèse que la fenrétinide pourrait combler ce manque dans l'arsenal thérapeutique de la FK.

Nos études précédentes ont démontré les effets de la fenrétinide, un dérivé de la vitamine A, chez les souris ayant la FK. Le traitement avec fenrétinide chez les souris FK avait amélioré l'élimination des infections pulmonaires et avait normalisé les niveaux de céramide, qui sont réduits dans la FK. Dans cette thèse de doctorat, nous démontrons que des niveaux anormaux d'AGPI, notamment l'acide arachidonique (AA) et l'acide docosahexaénoïque (ADH), sont associés avec une carence en céramide chez les patients FK. Le traitement avec fenrétinide chez les souris FK a amélioré le déséquilibre en AGPI dans le plasma et tous les organes liés à la FK, soit les poumons, l'iléon, le pancréas et le foie. Ce médicament a aussi réduit l'expression des gènes proinflammatoires, IL-1β et S100A8, dans les poumons des souris FK.

Une dose unique de fenrétinide avait des effets de longue durée sur les AGPI et céramide chez les souris, soit jusqu'à 90 heures après administration du traitement. Les niveaux

de malondialdéhyde, un indice de peroxydation des lipides, et de nitrotyrosine, un indice de stress nitrosatif, ont également été réduits. Les niveaux élevés de peroxydation et de stress nitrosatif étaient corrélés avec de plus grosses carences lipidiques. En utilisant des leucocytes isolés des patients FK, nous avons démontré que les lipides des cellules humaines peuvent être modifiés par la fenrétinide de la même façon que chez les souris, soit avec une réduction de l'AA et une augmentation de l'ADH. La peroxydation des lipides et les niveaux de nitrotyrosine sont aussi réduits par le traitement. Nous avons essayé différentes formulations de fenrétinide chez les singes, qui démontrent l'effet du médicament sur les AGPI dans un autre modèle animal.

Les patients FK souffrent de façon intermittente d'une détérioration de leurs symptômes nommée exacerbation pulmonaire (EP). Ces événements ont un impact important sur la progression de la maladie, mais sont par contre presque impossible à prévenir. Nous avons suivi une cohorte de patients FK pendant des périodes de maladie stable pour évaluer des marqueurs associés à un risque d'EP future. Nous avons déterminé, lors de ces périodes stables, qu'un pire état clinique et des scores de qualité de vie inférieurs sont associés à un risque d'EP plus élevé. Après traitement de l'EP, les patients avec plus d'inflammation ont rapidement redéveloppé une EP. Nous avons constaté que les traitements agressifs prescrits aux patients lors des EP ont non seulement réduit les marqueurs d'inflammation, mais ont aussi amélioré les défauts dans les AGPI, réduisant l'AA et augmentant l'ADH. Ces traitements ont aussi réduit les niveaux de malondialdéhyde et de nitrotyrosine. Par contre, ces améliorations ne sont que transitoires et la nature agressive de ces traitements n'en permet pas l'usage quotidien.

La fenrétinide a été utilisé dans des études à long terme pour le cancer. Les effets secondaires sont mineurs et réversibles avec de courts intervalles d'interruption du traitement. Les résultats présentés dans cette thèse démontrent l'impact de la fenrétinide sur l'amélioration des défauts lipidiques, la réduction de l'inflammation, des hauts niveaux de peroxydation et du stress nitrosatif. Ces phénotypes ne sont pas traités par les thérapies actuellement disponibles pour la FK, donc la fenrétinide représente un bon candidat pour des essais cliniques futurs.

PREFACE

This thesis is presented in the manuscript based format for a Doctoral Thesis, as described in the Thesis Preparation Guidelines by the Department of Graduate and Postdoctoral Studies. The studies described here were performed under the supervision of Dr. Danuta Radzioch. Chapters containing experimental results are preceded by paragraphs linking the chapters together.

Chapter 1 contains a review of the literature covering topics such as cystic fibrosis, polyunsaturated fatty acids, ceramide, retinoids and fenretinide. The sections on CF animal models and ceramide are adapted from the review "Ceramide in cystic fibrosis: a potential new target for therapeutic intervention" by Gabriella Wojewodka, Juan B. De Sanctis, Danuta Radzioch published in the *Journal of Lipids, 2011*. Contribution of the authors: the thesis author researched and wrote the manuscript. JBS and DR reviewed and edited the manuscript.

Chapter 2 is adapted from the manuscript: "Fatty acid imbalance in CF correlates to ceramide deficiency and can be corrected with fenretinide" by Claudine Guilbault, Gabriella Wojewodka, Zeinab Saeed, Marian Hajduch, Elias Matouk, Juan B. De Sanctis and Danuta Radzioch published in *Am JRespir Cell Mol Biol*, 2009. Contribution of authors: CG and GW (thesis author) are listed as co-primary authors. The thesis author was involved in experimental design, processing of human samples, data analysis and figure preparation, writing of the manuscript. CG contributed to experimental design, processing of animal samples, data analysis, writing of the manuscript. ZS contributed to collection of animal samples. MH contributed to experimental design. EM recruited patients. JBS analyzed lipids. Figure 2.4 of this chapter is extracted from "Fenretinide corrects newly found ceramide deficiency in cystic

fibrosis" by Claudine Guilbault C, Juan B. De Sanctis, Gabriella Wojewodka, et al. *Am J Respir Cell Mol Biol*, 2008. Contribution: For the results presented in this figure, the thesis author processed the mouse samples, performed the experiments for gene expression analysis, analyzed the data, and wrote the methods, description and discussion pertaining to these results.

Chapter 3 is adapted from the manuscript in preparation: "Fenretinide reduces lipid peroxidation and nitrosative stress in cystic fibrosis" by Gabriella Wojewodka, Juan B. De Sanctis, Marian Hajduch and Danuta Radzioch. Contribution of authors: the thesis author participated in experimental design, preparation of drug formulations for animal treatments, sample collection and processing from mouse experiments, processing of monkey samples, all data analysis and figure preparation, writing of manuscript. JBS quantified lipids, malondialdehyde and nitrotyrosine. MH isolated and treated patient leukocytes. Steve Nuara from the McIntyre Animal Facility treated the monkeys and collected blood samples. Fenretinide concentrations were analyzed by Dr. Barry Maurer (HPLC) and by the company Eliapharma (LC-MS).

Chapter 4 is adapted from the manuscript: "Candidate markers associated with the probability of future pulmonary exacerbations in cystic fibrosis patients." by Gabriella Wojewodka, Juan B. De Sanctis, Joanie Bernier, Julie Bérubé, Heather G. Ahlgren, Jim Gruber, Jennifer Landry, Larry C. Lands, Dao Nguyen, Simon Rousseau, Andrea Benedetti, Elias Matouk and Danuta Radzioch in PLoS One, 2014. Contribution of authors: Thesis author participated in study design, quantification of inflammatory markers, data analysis, table and figure preparation, writing of manuscript. JBS completed lipid, peroxidation and nitrotyrosine analysis

of all samples. J. Bernier was the clinical coordinator for the study. J. Bérubé processed blood samples for storage. HGA, JG and JL participated in study design. LCL, DN and SR participated study design and manuscript review. EM participated study design, clinical assessment of patients and manuscript review.

Chapter 5 contains a general discussion of the results. It includes a section on ceramide in cystic fibrosis which is adapted from the review "Ceramide in cystic fibrosis: a potential new target for therapeutic intervention" mentioned above.

Claims to originality

- Treatment schedule of 28 days with fenretinide reduces arachidonic acid levels to normal values and increases docosahexaenoic acid in cystic fibrosis mice.
- Greater defects in ceramide levels are correlated to greater imbalanced in arachidonic acid and docosahexaenoic acid in cystic fibrosis patients and mice.
- One dose of fenretinide reduces arachidonic acid, increases docosahexaenoic acid and improves their ratio in mice and monkeys.
- Fenretinide normalizes the defects polyunsaturated fatty acids and ceramide levels in leukocytes isolated from cystic fibrosis patients.
- Fenretinide reduces the expression of inflammatory genes in the lungs of cystic fibrosis mice.
- One dose of fenretinide decreases lipid peroxidation in mice and human leukocytes.
- One dose of fenretinide decreases nitrotyrosine, a marker of nitrosative stress, in mice and human leukocytes.

- During periods of stable disease, worse clinical markers such as FEV_1% and FVC% and patient quality of life are associated with increased risk of pulmonary exacerbations. Patients who experienced a rapid re-exacerbation had higher levels of inflammation at the end of treatment for their pulmonary exacerbation.
- Malondialdehyde and nitrotyrosine levels were correlated with polyunsaturated fatty acids in mice and CF patients suggesting that defects in lipids are associated with increased lipid peroxidation and nitrosative stress.

INTRODUCTION

Cystic fibrosis (CF) is a fatal genetic disease affecting approximately 70,000 individuals worldwide (Cystic Fibrosis Foundation www.cff.com). There is no cure for CF and life expectancy differs around the world depending on the care available to patients. The most exasperating facet of CF is the lung disease with recurring infections leading to progressive respiratory failure. Presently, the lung pathology in CF is the most important target for new treatments in order to delay or prevent the need for lung transplantation, the final recourse available for patients.

Using a knock-out mouse model of CF, previous work in our laboratory showed the benefits of a drug called fenretinide on lung pathology by improving infection clearance. This treatment also prevented osteoporosis, another phenotype of the disease. Additionally, our group showed that the sphingolipid ceramide is low in CF patients compared to healthy controls. Ceramide levels were also low in our CF mouse model and were normalized with fenretinide treatment. We hypothesized that fenretinide may impact other phenotypes of CF such as the polyunsaturated fatty acid (PUFA) imbalance or high levels of inflammation.

Chapter 2 of this thesis explores the relationships between ceramide and the defects in PUFAs found in CF patients. We show that the severity of the defects in PUFAs, high arachidonic acid (AA) and low docosahexaenoic acid (DHA), are linked to lower ceramide levels and to worse disease severity in patients. Treatment with fenretinide improves the defects in PUFAs seen systemically and in CF-related organs in our mouse model. These improvements are correlated with increased levels of ceramide. Additionally, fenretinide reduces the expression of inflammatory genes in the lungs of CF mice. Overall, the results presented in this chapter

further demonstrate that fenretinide may benefit CF patients by affecting the expression of inflammatory genes and improving PUFA levels towards an anti-inflammatory state.

The data presented in Chapter 3 continue to describe the effects of fenretinide by assessing the changes of a single dose on PUFA levels in mice and in monkeys. We hypothesized that fenretinide may act as an antioxidant to reduce lipid peroxidation. We show that one dose of fenretinide is sufficient to cause improvements in PUFA levels with prolonged effects. Using leukocytes from CF patients, we also demonstrate that fenretinide treatment can improve lipids in human tissues similarly to our animal models. The bioavailability of the drug using different formulations is presented along with a potential explanation for the improvements in PUFA levels following fenretinide treatment in mice: a reduction in lipid peroxidation. Additionally, we show the decrease in nitrotyrosine following fenretinide treatment in mice and in human leukocytes. This chapter demonstrates that fenretinide may affect other phenotypes of CF, high lipid peroxidation and high nitrosative stress.

Chapter 4 looks at which markers of pulmonary exacerbations (PEx) are associated with higher risk of future exacerbations in CF patients. As PEx are important events in the progression of CF disease, we hypothesized that inflammatory markers and lipids in conjunction with clinical assessments could allow for a better assessment of patients at risk of PEx. Additionally, this study enabled us to characterize clinical, inflammatory and lipid markers in CF patients and assess how fenretinide may be of use. This study also serves as a model for future clinical trials in terms of which clinical and molecular markers should be assessed. Using survival models (Cox proportional hazards and Kaplan-Meier), we show that worse clinical and quality of life assessments at periods of stable disease are associated with a higher risk of a future PEx.

We show that patients with unabated inflammation at the end of PEx treatment may experience a rapid re-exacerbation. Importantly, the changes in PUFA levels throughout PEx are presented and we find that the deficient ratio between AA and DHA lipid improved with aggressive PEx treatment, but this effect was transient. The observed improvements are associated with reduction in lipid peroxidation and nitrosative stress. Ceramide levels were unchanged with treatment for PEx, which reinforces fenretinide as a candidate for CF therapy.

Together, the results we present in this thesis demonstrate that fenretinide can correct multiple phenotypes of CF: defects in PUFAs, abnormally heightened inflammation and high levels of lipid peroxidation and nitrosative stress. However, our study on PEx in CF shows that systemic PUFA defects and high peroxidation of lipids can only be corrected in patients using aggressive antibiotic treatment which is not feasible for long term therapy. Using cells from CF patients, we confirm that fenretinide has a corrective effect in human cells, demonstrating that this drug is a candidate for future clinical trials in CF patients as a chronic therapy to correct multiple phenotypes of CF disease.

CHAPTER 1. LITERATURE REVIEW

Includes sections adapted from "Ceramide in cystic fibrosis: a potential new target for therapeutic intervention." Gabriella Wojewodka, Juan B. De Sanctis, Danuta Radzioch. *J Lipids*, 2011:674968

Cystic fibrosis (CF) is the most common autosomal recessive disease in Canada where one in 3600 births is affected by this disease [1]. CF treatment begins in infancy and continues throughout the lives of patients. The burden of CF is felt not only physically but has tremendous psychological impacts on patients and families alike. Daily treatments last for hours each day and lives are disrupted with countless days in hospital. The latest data from 2011 indicates the median age of survival of CF patients in Canada has reached 48.5 years which is a testament to the significant advances in therapies and clinical care for patients [2]. However, the road leading to these 48.5 years is harsh. Ultimately, there is no cure for CF and the last resort for patients is lung transplantation which comes with its own complications.

1. CYSTIC FIBROSIS

1.1. CYSTIC FIBROSIS: GENE DEFECT

In 1989, a group led by Tsui and Collins identified the gene responsible for CF: Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) [3-5]. The gene maps to chromosome 7q31.2 and is 180 kb pairs in length. It is involved in chloride secretion and is expressed in most epithelial cells including those of the digestive and respiratory systems consequently the lung, liver, pancreas and intestines are affected organs in CF. The degree to which a mutation in *CFTR* will affect an organ depends on its protein expression, its importance in the functioning of the organ and whether other types of proteins are present in cells which can compensate for the lack of *CFTR*.

Over 1800 *CFTR* mutations have been found however, only 23 mutations are most commonly detected in affected individuals [6]. The most frequent disease causing mutation is a deletion of phenylalanine at position 508, called F508del. Close to 92% of CF patients in Canada

have at least one copy of this mutation [2]. The various disease causing *CFTR* mutations have been categorized as follows:

- Class I mutations: nonsense mutations, frame-shift mutations, and abnormal mRNA splicing resulting in a truncated CFTR protein
- Class II mutations: folding and maturation defects resulting in premature degradation of CFTR protein (e.g. F508del)
- Class III mutations: defective nucleotide binding, low levels of channel gating activity (e.g. G551D)
- Class IV mutations: defective conductance preventing free flow of ions through CFTR
- Class V mutations: *CFTR* transcripts are normally produced, but translation is defective resulting in a low level of normal protein.
- Class VI mutations: abnormally high turnover of CFTR at the membrane

Class I, II and III mutations generally cause a more severe presentation of CF disease with more lung and pancreatic disease. Class IV, V and VI are associated with milder forms of CF disease. Patients with two severe mutations will most likely have pancreatic insufficiency, while those with at least one mild mutation are generally pancreatic sufficient.

1.2. CYSTIC FIBROSIS: DIAGNOSIS

Individuals with CF have many symptoms such as respiratory infections leading to severe lung pathology, defective digestion due to fibrosis of the pancreas, intestinal blockage and malabsorption of nutrients. Often patients experience exacerbations which are worsening of pulmonary symptoms requiring aggressive antibiotic treatment. With time, some may develop co-morbidities such as CF-related diabetes (CFRD), liver disease and osteoporosis.

Some newborn infants with CF develop meconium ileus which requires surgical intervention and can be the earliest symptom of the disease. Prior to the identification of the disease causing gene mutation, a CF diagnosis was uniquely based on a sweat chloride test as individuals with CF have saltier sweat due to defective chloride absorption in the sweat gland. This test still remains the gold standard in CF diagnosis and can be used to detect changes in CFTR function for clinical trials [7,8]. Nowadays, a diagnosis of CF is confirmed by genotyping mutations in *CFTR*. More and more, newborn screening programs include testing for mutations in *CFTR* in hopes that early diagnosis will allow for earlier intervention, leading to a better preservation of lung function and slowing down disease progression.

1.3. CYSTIC FIBROSIS: PROTEIN DEFECT

The CFTR protein contains 1480 amino acids with 5 domains: 2 membrane spanning domains which form the channel, 1 regulatory domain and 2 nucleotide binding domains. The CFTR protein is a member of the ATP-binding cassette (ABC) transporter family and is located in cellular membranes. It is mainly known for being a chloride channel but it is also involved in fluid secretion [9]. It negatively regulates sodium transport by acting on the amiloride-sensitive epithelial Na^+ channel (ENaC) [10]. Consequently, there has been evidence of increased activity of ENaC in CF [11].

1.4. CYSTIC FIBROSIS: LUNG PATHOGENESIS

Being in contact with the outside environment, the lung must act as a barrier to maintain sterility within the host. To this effect, the lung has various mechanisms to defend against pathogens. For a physiological barrier, the airway epithelial cells are lined with a thin

layer of fluid called the airway surface liquid (ASL). The ASL is composed of two parts. On the apical side of the epithelia lies the periciliary layer (PCL) which enrobes the cilia, hair-like structures located on the surface of the epithelium. The low viscosity and the fluidity of the PCL enable proper ciliary movement. The PCL is essential in lubricating the cell surface to enable effective cough clearance. It also acts as a barrier between the epithelial cell surface and the mucosal layer entrapping foreign particles before they reach the cell surface. Above the PCL rests a layer of mucus which is formed of glycosylated proteins encoded by the *MUC5AC* and *MUC5B* genes [12,13]. They form a mesh network whose rheology is dependent on water content, ion concentration and pH of the ASL [14]. The mucus layer forms the first barrier against pathogens and foreign particles. Other mucins, MUC1 and MUC4, are expressed on the epithelial cell surface and form brush-like structures in the PCL. These anchored mucins act as an extra physical barrier against pathogens [15]. The cilia move in a coordinated fashion to shift the mucosal layer resting on top. Normal ciliary beating together with coughing enables mucocilliary clearance, which plays a large part in maintaining the sterility of the lung.

It can take approximately six hours for effective mucus clearance to occur with coughing [16], which would allow bacteria large amounts of time to replicate; however, the lung has additional defenses in place. They mainly lie in the chemical artillery of the ASL which is abundant in antimicrobial factors (e.g. defensins, lactoferrin), enzymes (e.g. lysozyme), proteases (e.g. neutrophil elastase), protease inhibitors (e.g. secretory leukocyte protease inhibitor, α1-antitrypsin) [17], oxidants and anti-oxidants (e.g. glutathione). These antimicrobial factors can interrupt bacterial growth within 24 hours of exposure [17]. Thus with normal coughing and antimicrobials, bacteria can essentially be cleared. When bacteria persist in the

lung, the host's innate immune defenses, neutrophils and macrophages, are recruited to the ASL following pathogen detection.

There are several different hypotheses regarding the link between CFTR dysfunction and lung infections in CF. The "high salt hypothesis" or "compositional hypothesis" suggests that mutated CFTR causes higher than normal salt levels in the ASL which would inactivate antimicrobial factors. This hypothesis was supported by *in vitro* studies using human airway epithelial cells which demonstrated the ability of healthy cells to kill bacteria while CF cells did not. The authors determined the bactericidal components of ASL were similar in both cell types, as was the volume of the fluid. Increasing the salt concentration of the ASL from non-CF cells caused inhibition of bacterial killing. The ASL obtained from nasal epithelium of CF patients had higher concentrations of salt compared to those from healthy individuals. Applying fluid with low salt concentrations to the surface of CF cells restored normal bacterial killing [18]. Recently, studies using the CF pig model determined that the NaCl content was similar in both healthy and CF pigs. However lower pH levels were observed in ASL from CF pigs with reduced levels of bicarbonate which led to impaired bacterial killing [19]. Following these recent studies, the "high salt hypothesis" was revised to include an overall altered composition of the ASL, leading to it being renamed the "compositional hypothesis".

Alternatively, the "low volume hypothesis" suggests that the over-activation of ENaC in CF leads to increased sodium absorption which induces increased chloride absorption into epithelial cells via alternate chloride channels, such as the calcium-activated chloride channel (CaCC). In turn, this increased flux of ions into the cells induces osmosis pressure towards the epithelium, thus reducing the height of the ASL [20]. The mucosal layer becomes thickened due

to dehydration. The ciliary beat is restricted by the low volume of the PCL and mucociliary clearance is impaired [20]. Matsui and colleagues demonstrated that the salt content of the ASL is not higher in CF. Instead, they showed that fluid from the ASL was absorbed into CF airway epithelial cells but not in normal cells. Their study also demonstrated that mucocilliary movement is impaired in CF [20]. This decrease in PCL causes the mucosal layer mucins (MUC5AC and MUC5B) to interact with those anchored to the cell surface. The mucins "glue" together making mucus clearance even more difficult [14]. This thick layer of stagnant mucus provides a perfect environment for pathogen colonization.

1.5. CYSTIC FIBROSIS: PULMONARY BACTERIAL INFECTIONS

CF patients are plagued with persistent lung infections by pathogens which are innocuous to healthy individuals. Chronic lung infections cause the greatest burden for CF patients in terms of treatments required, clinic visits and hospitalizations, and eventually lead to lung function decline into respiratory failure. *Pseudomonas aeruginosa* (PA) is prominent among respiratory bacterial pathogens in CF and causes the most damage to the lung. *Staphylococcus aureus* (SA), *Haemophilus influenzae*, *Burkholderia cepacia* are also common pathogens isolated from lungs of patients [21]. Typically, pediatric patients become infected with SA and *Haemophilus influenzae*, although not permanently. They may experience infections with PA throughout their childhood when bacteria can still be cleared from the lungs relatively well. However, it can eventually colonize the lung permanently and by adulthood most CF patients test positive for PA colonization. Recent data show approximately 50-60% of Canadian CF adults are colonized with PA [2].

The thick mucus layer in the CF lung offers a favourable environment for PA colonization where it can remain out of reach from host defense mechanisms and antibiotics. While a large amount of bacteria can be eradicated from initial infections, some colonies can remain and adapt to the CF lung by modifying their gene expression. Studies have shown that over time PA shifts to a mucoid phenotype with increased expression of genes involved in alginate production allowing for survival in hypoxic regions in the thick mucosal layer. Also, mutations are acquired over time which in turn confer antibiotic resistance [22]. Virulence factors are suppressed such as the type III secretion system and flagella in order to evade the host's immune response [22]. Bacteria in the CF lung eventually form aggregates, called biofilms, where they switch to starvation mechanisms to survive in nutrient depleted environments. Biofilms are notoriously resistant to antibiotic treatments [23,24], adding to the difficulty in eradicating bacteria in CF.

1.6. CYSTIC FIBROSIS: DEFECTS IN INFLAMMATION

CF is characterized by an abnormally activated inflammatory response occurring prior to bacterial infections [25-27]. In particular, NF-κB, a transcription factor for many inflammatory markers such as IL-1β, IL-6, IL-8 and TNF, was shown to be highly activated in cells with defective CFTR [28-31] or inhibited CFTR [29]. NF-κB also regulates the expression of *CFTR* [32]. High levels of IL-1β were found to induce *CFTR* mRNA expression via the activation of NF-κB in non-CF cells [32,33]. In turn, CFTR was found to be a negative regulator of NF-κB phosphorylation [29]. Therefore, this pathway may be constitutively activated in CF cells since insufficient CFTR protein is formed to quench the signal.

As a consequence of the over-activated NF-κB, cells with defective CFTR secrete more IL-8, a neutrophilic chemoattractant [28,29,34]. IL-8 levels were found to be increased in bronchial alveolar lavage fluid (BALF) and sputum of CF patients [35,36]. The high levels of IL-8 induce the recruitment of neutrophils even in the absence of pathogens [25]. IL-1β stimulates the production of IL-6 which is also exaggerated in CF [37-39]. TNF was also found elevated in sputum and BALF from CF patients [40,41], while the anti-inflammatory IL-10 was found to be reduced in CF [40,42].

Normally, the increased inflammation during infections induces high levels of oxidation in the airways which assists in bacterial killing. Healthy lungs have antioxidant mechanisms to prevent tissue damage due to oxidative stress once the infection has been cleared. Glutathione is a potent antioxidant in lungs however it was found to be reduced in CF airways [35]. Interestingly, the levels of glutathione were found to be normal in CF lung tissues [43,44]. Thus it was concluded that CFTR is involved in regulating the export of glutathione from epithelial cells into the airway lumen [43]. With defective CFTR, glutathione remains trapped inside epithelial cells unable to counter the effects of reactive oxygen species in the airways, causing further lung destruction.

There is no clear explanation for the heightened inflammation in CF. As there is evidence it occurs prior to bacterial colonization [26,45], the gene defect must be a contributor. Certain mutations in CF, such as F508del, result in misfolded CFTR protein which trigger the unfolded protein response in cells [46]. The misfolded CFTR was found to accumulate in the endoplasmic reticulum (ER), causing stress and over-activation of MAPK signalling, which in turn induced IL-6. The combination of the unfolded protein response and ER stress also caused an

overactive response to Toll-Like Receptor 5 (TLR5) activation by flagellin [47]. In fact, mutations resulting in normal protein trafficking and no ER stress, such as G551D, caused less NF-κB activation than F508del mutations. However, even when no protein accumulated in the ER, blocking chloride secretion itself also induced some inflammation [28]. The high expression of IL-8 was correlated with reduced conductance of chloride through CFTR in epithelial cells. The high levels of inflammatory markers were reduced when an alternative chloride channel was activated pharmacologically [48]. Thus inflammation in CF is due to defective chloride conductance and may be exacerbated by mutations causing protein accumulation in the ER.

Despite the high recruitment of neutrophils and the active inflammatory response, bacterial infections in CF are not properly eradicated. One hypothesis would be that neutrophils have limited contact with pathogens due to the thick mucosal layer. Additionally, neutrophils in CF were found to behave differently than normal neutrophils. For instance, the expression of CD14 and CD16 phagocytic receptors on the surface of neutrophils from CF patients was decreased compared to controls, reducing the phagocytosis of pathogens [49]. CFTR was found in the membrane of mature phagosomes in neutrophils, allowing for chloride to enter the organelle to form hypochlorus acid, which is needed to kill pathogens. Defective CFTR, such as with the F508del mutation, significantly reduced CFTR recruitment to phagosomes which impacted pathogen killing [50]. When phagocytosis does occur, neutrophils in CF have impaired apoptosis and instead undergo necrosis, leaking their intracellular contents into the airways [51]. Evidence of this can be found in the thick mucosal layer which has a high content of DNA [52]. The removal of neutrophils by macrophages is also defective in CF. The amount of apoptotic cells is increased in CF sputum compared to patients with chronic bronchitis. It was

found that the signal for macrophages to clear apoptotic cells was blocked by the high levels of neutrophil elastase (NE) secreted by the large number of neutrophils [53]. In fact, neutrophils in CF were found to persist longer than control cells after TNF stimulus, and their apoptosis was delayed [54].

Macrophages also play a role in the heightened inflammation in CF. When stimulated with LPS, alveolar macrophages from CF mice had higher production of IL-6 and KC, a murine homolog of IL-8 [39]. Similar findings were observed in macrophages isolated from BALF of patients [37]. The ability of macrophages in CF to kill bacteria was lowered despite that their ability to phagocytose bacteria was similar to normal cells [55]. The conditional knockdown of *Cftr* in myeloid derived cells in mice caused a delay in bacterial clearance and prolonged inflammation compared to WT mice [56].

1.7. CYSTIC FIBROSIS: MULTIORGAN PATHOLOGIES

Other organ pathologies are also associated with CFTR dysfunction. Thick mucus in the pancreatic ducts prevents the release of digestive enzymes. Pancreatic insufficiency is found in the vast majority of CF patients and they are treated with pancreatic enzyme replacement therapy (i.e. digestive enzymes) to ensure proper digestion of food. A major complication in CF disease is the development CFRD due to progressive fibrosis of the pancreas, leading to the reduction in insulin production.

CF patients have known malabsorption of fat due to abnormal pH levels in the intestine [57] and high levels of inflammation [58]. Thick mucus layers in the intestines also have a role in malabsorption as CF mice with *MUC1* ablations were found to have less mucus in their intestines and better nutrient absorption [59]. Undernourished patients, defined as having

low body weight and body mass index (BMI), have more severe CF lung disease and higher risk of mortality [60]. Despite these challenges, nutritional programs and dietary supplements have helped the majority of patients to increase their weight and BMIs over the years to reflect healthy norms [2].

The thick mucosal layer impacts the development of the vas deferens in males with CF, which impairs fertility. This defect, called congenital bilateral absence of the vas deferens, can be discovered in infertile men carrying mild *CFTR* mutations who frequently receive a first diagnosis of CF when treated for infertility [61].

1.8. CYSTIC FIBROSIS: MOUSE MODELS

The first attempts at generating a knock-out (KO) mouse came from the University of North Carolina by inserting the neomycin resistance gene into exon 10 of *Cftr* [62]. These mice had severe intestinal obstruction but failed to produce any lung disease. A mixed genetic background was at fault and researchers began to backcross the mice to a C57BL/6J background [63]. These mice produce a strong inflammatory pulmonary phenotype in the absence of infections. Reports have described severe lung disease with the destruction of lung structure [64], thick mucus lining the distal airways [64], infiltration of inflammatory cells around the airways [65] and increases in inflammatory gene expression in the lungs [65]. Additionally, severe pathology of the pancreas was observed with acinar atrophy and increased inflammation [64]. Intestinal phenotypes were also observed with chronic intestinal obstruction. These mice require a liquid diet (Peptamen, Nestle, Brampton, Ontario, Canada) not only to avoid intestinal blockage, a phenotype which can occur in CF patients, but to improve the absorption of nutrients without having to supplement the diet with enzymes. This

diet allows CF mice to thrive and to maintain a healthy weight [66]. Other mouse models were generated to express specific *Cftr* mutations such as F508del [67] and G551D [68].

To escape the need for special diets, CF mice were generated which could express CFTR locally in the gut. These "gut-corrected" *Cftr*^{tm1unc}-Tg$^{(FABPCFTR)}$ mice express CFTR in the intestines under the control of a human fatty acid binding protein (FABP) promoter. Although these mice show evidence of impaired alveolar macrophage function without severe intestinal pathology there is however no description of chronic lung disease in these mice (Jackson Lab site www.jax.org). The B6.129P2(CF/3)-*Cftr*$^{TgH(neoim)Hgu}$ mouse model has low levels of CFTR expression which allow these mice to survive on dry pellet food. It is evident that different mouse models present different phenotypes of CF disease [69]. For example, defects in omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) were shown in the B6.*Cftr*^{tm1unc} KO mouse [70,71] but not in other models [72]. Some mouse models have failed to demonstrate lung disease and it was speculated that disease modifier genes such as alternate chloride channels could exist in certain mouse backgrounds [63,73]. It was verified that the B6.*Cftr*^{tm1unc} KO mouse does not express alternative chloride channels; however, this has not been studied in most mouse models, including the *Cftr*^{tm1unc}-Tg$^{(FABPCFTR)}$ described above [63]. The latter mouse model was generated using a mixed background of 129P3/J, C57BL/6 and FVB/NJ (see www.jax.org). The differences between the various CF mouse models are important to consider when comparing results from various studies.

1.9. CYSTIC FIBROSIS: TREATMENTS

The treatment regimens for CF patients involve numerous pills, supplements and inhaled medications to address chronic infections, inflammation, thick mucus, malabsorption

and digestion issues. Chest physical therapy is also used to induce mucus clearance. Despite numerous therapies developed to combat the various pathologies in CF, patients still experience respiratory failure and ultimately the only recourse left is lung transplantation which comes with its own risks and complications. There still remains much room for improvement in CF therapy.

1.9.1. Correcting the gene defect

Directly correcting a mutated *CFTR* gene by inserting a normal copy into the lungs is believed to be the cure for CF. Since the discovery of the gene mutation in 1989, there was much hope that a cure would be rapidly found. However, almost 25 years later, gene therapy is still in research stages as the lung has proven to be a hard organ to target due to its defense mechanisms. This is even more problematic in CF where the thick mucosal layer and viscous PCL make it difficult for this therapy to reach epithelial cells. The safety of viral vectors was an issue in gene therapy which now may be bypassed with the discovery of non-viral delivery systems [74,75]. New research has demonstrated that delivering *CFTR* mRNA may prove to be more successful than attempting to insert the large *CFTR* DNA fragment [76]. However, gene therapy still seems to require many more years of development before it can become therapeutically available to CF patients.

1.9.2. Correcting the protein defect

Researchers are examining how the mutated CFTR protein can be modified by using molecules to assist in protein folding, called “correctors”, or to improve channel gating with “potentiators”, depending on the mutation. Recently, a breakthrough was achieved with ivacaftor, a potentiator for the G551D mutation, which improved the defect in channel gating.

This drug restored significant function to mutated CFTR and improved lung function of patients [8]. The major caveat of this drug is that it can only help 4 - 5% of CF patients who have this specific mutation in *CFTR.* It remains to be seen whether it will be effective in all patients with this mutation such as in patients with more advanced disease. Its long term effectiveness has not been assessed and potential adverse events have not been well elucidated. Additionally, this drug has an enormous cost (more than 300 000 US$ per year per patient) which unfortunately may limit its availability to patients [77]. Drugs able to modulate the most common mutation, F508del, are still being developed and it has recently been determined that a combination of potentiators and correctors may be necessary to restore protein function [78]. Until a solution is found for all genotypes, the therapies currently available to CF patients focus on eradicating or controlling bacterial infections in the lung, and attempting to improve mucus clearance by increasing the ASL and reducing mucus viscosity.

1.9.3. Anti-infective

Tobramycin, an aminoglycoside, is the recommended anti-pseudomonal antibiotic to treat CF patients in nebulised form [79]. More recently, the use of the monobactam aztreonam has shown positive effects on improving lung function [80]. Other antibiotics such as macrolides have shown moderate benefits in improving lung function and decreasing pulmonary exacerbation events. The use of azithromycin in particular has been recommended for CF patients [79].

1.9.4. Restoring the ASL volume

Inhaled hypertonic saline has emerged as a standard treatment for CF. The hypothesis behind this treatment is based on the activation of ENaC in the presence of high salt levels in

the airways. In healthy lungs, hypertonic saline induces a high absorption of sodium through ENaC and chloride through CFTR. Due to the rapid absorption of the ions, there is little osmosis that occurs. When CFTR is defective, chloride absorption occurs at a slower rate through alternate chloride channels. This reduced rate of ion absorption provokes a greater osmotic gradient inducing water to flow out of epithelial cells into the airway surface, increasing the volume of the ASL [81]. This treatment was found to improve lung function and reduce the number of pulmonary exacerbations [82].

1.9.5. Mucus breakdown

The thick mucosal layer in CF lungs contains high amounts of DNA, derived mostly from necrotic neutrophils. This DNA is targeted by inhaled recombinant human deoxyribonuclease (DNase), dornase alfa, which helps break down the excessive amounts of DNA in mucus and has been proven to benefit CF patients [83]. With treatment, patients had a reduced rate of lung function decline compared to non-treated patients, and the number of pulmonary exacerbations was reduced [84].

1.9.6. Anti-inflammatory

There are no anti-inflammatory drugs routinely prescribed to CF patients and there is a large gap in this area for therapeutic options. Anti-inflammatory drugs such as corticosteroids have been found ineffective as chronic treatments with substantial adverse events in children. Their routine use has not been recommended in CF, even for adults [79]. They are used however to treat pulmonary exacerbations for short periods of time.

Non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, reduce the levels of pro-inflammatory prostaglandins derived from the PUFA arachidonic acid (AA) by inhibiting

the enzyme cyclooxygenase-2 (COX-2). They also have an effect on infection eradication as rats infected with PA were found to have less bacteria in their lungs after ibuprofen treatment compared to controls [85]. This treatment for CF is still debated. Many studies describe benefits of ibuprofen in slowing lung function decline in CF patients [86-89]. Others describe no benefit to ibuprofen administration [90]. The danger of gastrointestinal bleeding is always a factor although a rare event in CF patients [87,88]. Retrospective analysis of the CF Foundation Patient Registry suggest that ibuprofen treatment in CF patients is of significant benefit [91]. A recent meta-review of ibuprofen use in CF has concluded that it can reduce the rate of decline of lung function particularly in children [89]. Despite, these positive studies, ibuprofen is not administered readily in CF and its use has declined within the last decade [92].

Treatments targeting the lipooxygenase (LOX) pathway which converts AA into leukotrienes were proposed for CF. Trials with montelukast, a leukotriene receptor antagonist, improved symptoms in children with CF specifically lung function scores, decreasing cough, wheezing and inflammation [93]. A long term study showed an improvement in lung function with a decrease in PA burden when montelukast was combined with antibiotics [94]. Another leukotriene receptor inhibitor, zafirlukast, did not improve respiratory function in adults but did improve the perception of well-being, radiography scores and physical appearance [95]. There are a few drugs currently being tested for inflammation in CF such as the protease inhibitor α1-antitrypsin to counter the effects of neutrophil elastase. So far trials have shown its ability to reduce inflammation but with no effect on lung function [96].

1.9.7. Antioxidant

As with anti-inflammatories, there is no routine treatment for CF to reduce the levels of oxidative stress. Currently, CF patients obtain antioxidants from their diet and supplementation with vitamins. Vitamin supplementation is required to ensure normal levels of fat soluble vitamins such as A, D, E and K, but is not specifically prescribed for antioxidant benefits. Even with adequate vitamin levels, oxidative stress remains high in CF patients [97]. N-acetylcysteine, an oral antioxidant, is on trial to counter the effects of oxidative damage by increasing the amount of glutathione in the airways. Few studies have been conducted so far but there is evidence that it can reduce neutrophil counts [98] and increase glutathione levels in sputum, although its effects on inflammatory markers is unclear [99]. A recent trial studying the effects of inhaled glutathione in CF patients concluded that there was no benefit to this treatment on oxidation, inflammatory markers or lung function [100]. There is much to be improved in this area of CF therapy but not many viable options have been studied to date.

2. POLYUNSATURATED FATTY ACIDS

2.1. POLYUNSATURATED FATTY ACIDS: ARACHIDONIC ACID

Omega-3 and omega-6 PUFAs are lipids with important roles in the immune response. The “omega” name indicates where the first double bond in the molecular structure occurs from the methyl end. For example, an omega-6 PUFA will have its double bond at the sixth carbon from the end. These PUFA can alternatively be designated by “n-3” for omega-3 and “n-6” for omega-6. AA is an omega-6 PUFA derived from linoleic acid (LA), an essential fatty acid which must be obtained from food, consequently the diet is the principal regulator of AA levels. LA is converted to γ-linolenic acid through the action of the enzyme Δ6-desaturase (Figure 1.1).

Further conversion by elongase forms dihomo-γ-linoleic acid. The generation of a 20 carbon chain with four double bonds known as AA (20:4) results from a final conversion step by Δ5-desaturase. The omega-3 PUFAs compete with the omega-6 for the same elongation and desaturation enzymes.

AA is bound to phospholipids in the plasma membrane and is released under certain stimuli such as growth factors, hormones or cytokine signalling. Under these conditions, AA is cleaved from the membrane by phospholipase A_2. There are three main categories of phospholipase A_2: cytosolic PLA_2 ($cPLA_2$), secreted PLA_2 ($sPLA_2$) and calcium independent PLA_2 ($iPLA_2$). $cPLA_2$ and $iPLA_2$ exert their functions within the cell while $sPLA_2$ acts extracellularly. Each of these three categories of PLA_2 contains multiple isoforms of the enzyme [101]. Once removed from the cell membrane AA is further metabolised into eicosanoids which continue to influence the cell signalling process.

2.2. POLYUNSATURATED FATTY ACIDS: AA DERIVED EICOSANOIDS

Cyclooxygenases (COX-1, COX-2) convert AA into prostanoids. More specifically, they convert AA into prostaglandins (prostaglandin E_2, PGE_2; prostaglandin D_2, PGD_2; prostaglandin $F_{2\alpha}$, $PGF_{2\alpha}$), prostacyclin (PGI_2), and thromboxanes (thromboxane A_2, TXA_2; thromboxane B_2, TXB_2). COX-1 is expressed constitutively while COX-2 is induced following an inflammatory response [102]. These two enzymes are the targets of NSAIDs. Aspirin inhibits the eicosanoid metabolism by competing with AA for COX-1 and COX-2 binding sites. Other NSAIDs, such as ibuprofen, act mainly on COX-2 with varying levels of COX-1 inhibition [103].

Lipoxygenases (5-LOX, 12-LOX, 15-LOX) convert AA into leukotrienes, lipoxins and hepoxilins. Each lipoxygenase enzyme produces specific metabolites. 5-LOX will produce

leukotriene A_4 (LTA_4) which is further converted into cysteinyl-leukotrienes (cysLTs: LTC_4, LTD_4 and LTE_4), leukortiene B_4 (LTB_4) and lipoxins (lipoxin A_4, LXA_4; lipoxin B_4, LXB_4). Five lipoxygenase activating protein (FLAP) is necessary to activate 5-LOX in order to produce leukotrienes. 12-LOX converts AA into 12-HETE, whereas 15-LOX converts AA into LXA4 and LXB4. While eicosanoids are generally referred to as pro-inflammatory molecules, lipoxins are known to have anti-inflammatory effects by stimulating pathways involved in the resolution of inflammation by reducing cytokine production, phosphorylation intracellular kinases (ERK, p38), and the activity of transcription factors (NF-κB, AP-1) [104]. Eicosanoids perpetuate the inflammatory response by binding to membrane receptors. There are 9 receptors known to bind prostanoids. PGE_2 binds to receptors EP1 to EP4, PGD2 binds to DP1 and DP2, $PGF_{2\alpha}$ binds to FP, prostacyclin to IP and TXA_2 to TP. All cell types produce eicosanoids, although the whole spectrum of eicosanoids are not necessarily produced by each cell type.

2.3. POLYUNSATURATED FATTY ACIDS: DOCOSAHEXAENOIC ACID

The metabolism of omega-3 PUFAs results in the conversion of α-linolenic acid, an essential fatty acid, into two potent PUFAs: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). AA and DHA are often studied together and are considered opposites of each other. AA is thought to be a pro-inflammatory PUFA while DHA is anti-inflammatory; increasing the levels of one, decreases the levels of the other.

DHA is released from phospholipids via the action of $iPLA_2$ [105]. DHA was found to inhibit $cPLA_2$ and thus limit the release of AA [106]. When released from the membrane, DHA metabolizes to anti-inflammatory protectins (protectin D1, PD1), D-series resolvins (RvD1 to RvD6) and maresins (MaR1). They participate in the resolution of inflammation by enhancing

the phagocytosis of apoptotic neutrophils by macrophages, improve efferocytosis and limit the infiltration of neutrophils [107,108]. M2 macrophages produce the anti-inflammatory MaR1 and LXA4 during the resolution phase, and have lower levels of LTB_4 than classically activated M1 macrophages [109]. DHA supplementation can decrease the binding of NF-κB to DNA and lower the production of cytokines such as TNF, IL-1β and IL-6 in LPS stimulated macrophages [110,111].

AA and its precursors can be found in oils (corn, peanut, sunflower) and nuts. Omega-3 fatty acids are found in canola, olive and linseed oils and various fish [112]. The Western diet contains high levels of AA and consequently it has been attributed to increased incidences of disease such as cardiovascular disease, cancers and other inflammatory related diseases [113,114]. Noteworthy, CF was characterized as a disease with defects in the levels of PUFA long before the discovery of the disease-causing gene mutation. It was even hypothesized that these defects were the root of the disease [115-118].

2.4. POLYUNSATURATED FATTY ACIDS AND CYSTIC FIBROSIS

The PUFA defect in CF, also referred to as a fatty acid imbalance, is characterized by low levels of the omega-6 essential fatty acid LA and reduced levels of the omega-3 DHA [117,119-121]. Many studies have also found high levels of AA in CF [117,119,122] although this was not a universal finding [120,123,124]. Often this imbalance is represented as the AA/DHA ratio which is higher than normal in CF [119,125].

The explanation behind the PUFA defects in CF has not been confirmed. Fat malabosorption may be part of the issue since CF patients have difficulties in absorbing long chain fatty acids [126], however the deficiency was confirmed in well-nourished patients [127].

The imbalance is likely linked to the genetic defect as carriers of *CFTR* mutations have intermediate PUFA levels between non-CF and CF patients [119]. Additional evidence comes from *in vitro* studies where cells lacking *CFTR* also have PUFA defects [128-130]. Studies have demonstrated a rapid turnover of LA into AA [128] which may explain why LA is low in CF [131] and AA is high [125]. The desaturase enzymes were found to be increased in CF epithelial cells, which may explain the rapid AA synthesis. However since both omega-3 and omega-6 pathways used the same enzymes, this does not explain why DHA is low. The rapid processing of LA into AA can be halted with DHA treatment as DHA reduces the expression of the desaturases. Since the desaturases have an affinity towards the omega-3 pathway, this may explain why increasing DHA levels reduces AA [128,129,132]. How this relates to the *CFTR* gene defect is still uncertain.

The explanations behind the defect in DHA are even less well understood than AA. One hypothesis is based on defective membrane recycling in CF. There are four classes of phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine. PC is synthesized from choline which is obtained from the diet. Choline uptake is increased in CF cells yet PC levels are normal, which suggests a higher turnover of PC. Interestingly, cells from CF carriers were found to have intermediate rates of PC turnover [133]. When choline sources are sparse, PC can be converted from phosphatidylethanolamine and this process was found to reduce DHA levels [134]. Thus, the high turnover of PC in CF would necessitate more choline than is available in the diet, causing a switch to PC synthesis from phosphatidylethanolamine, ultimately reducing DHA levels [135].

Alternatively, low DHA levels may be due to abnormal conversion of EPA to DHA which was found to be reduced in CF [129]. It is also possible that DHA may be transforming back to

EPA via the retroconversion pathway, a process which was found to be elevated in cells with defective CFTR [132].

2.5. EICOSANOIDS AND CYSTIC FIBROSIS

In the context of CF, COX-2 was found to be upregulated in patients [136-138] and in cells lacking CFTR [129]. Polymorphisms in the COX genes have been associated with modifying disease severity in CF. More specifically, those causing a reduction in COX-2 expression tend to reduce the severity of symptoms [139]. The interaction between PA and the lung can stimulate COX-2 production. To increase its virulence, PA produces a group of molecules called acylated homoserine lactones. These molecules were found to increase the expression of COX-2 in human lung fibroblasts and this may have major impacts for CF [140]. The prostaglandin PGE_2 was found increased in sputum [141,142], urine [143], BALF [144], saliva [145] and exhaled breath condensate in CF patients and even higher in those with pulmonary exacerbations [146]. High levels of PGE_2 in the CF airway may be due to the over stimulation of epithelial cells by cytokines and LPS [147]. Additionally, PGE_2 was found to be involved in chloride secretion [148]. Since chloride transport is impaired in CF, PGE_2 levels may be increased in attempts to boost chloride transport, without success. Bacterial killing was inhibited in macrophages treated with PGE_2 [149] which might add to the list of potential reasons explaining the reduced clearance of bacterial infections in CF. Other prostaglandins are also upregulated in CF such as $PGF_{2\alpha}$ [144,145,150], which can stimulate CFTR activation [151].

The expression of the 5-LOX gene was shown to be increased in human bronchial epithelial cells lacking CFTR and consequently LTB_4 levels were significantly higher compared to cells expressing normal levels of CFTR protein [129]. LTB_4 is over expressed in sputum from

patients [141,152] and its levels are inversely correlated to lung function scores [152]. Patients colonized with PA were found to have the highest levels of LTB_4 followed by patients with SA. Non-colonized patients had the lowest levels compared to infected CF patients, although they were still higher than controls [153]. LTB_4 was increased in exhaled breath condensate of CF patients with pulmonary exacerbations. These levels decreased after antibiotic treatment [154]. The high levels of LTB_4 might be explained by the results of the study conducted by Lawrence and colleagues which demonstrated a decrease in chemotaxis of neutrophils from CF patients to LTB_4 stimulus [155]. The high levels of chemoattractants found in the CF lungs, such as LTB_4, might result from inadequate neutrophil function in CF and LTB_4 is increased in attempts to recruit more neutrophils to clear bacteria. Alternatively, the high levels of LTB_4 may be directly linked with higher concentrations of AA in CF patients, as LTB_4 is metabolized from AA by LOX.

2.6. PUFA SUPPLEMENTATION IN CYSTIC FIBROSIS

Despite the long history of CF and PUFA defects, the frequency of fatty acid supplementation studies declined as the focus turned towards gene therapy. However, so far gene therapy has failed to generate the anticipated curative therapies. Interest in correcting the lipid defects was reignited following a study by Freedman and colleagues where DHA treatment of CF mice reversed pathology in the pancreas and lung [70]. In other mouse studies, omega-3 supplementation decreased the pulmonary bacterial load in CF mice [156]. Treatment with DHA in CF mice also improved liver pathology [157]. DHA supplementation in mice did not decrease cytokine expression following exposure to aerosolized LPS, but resulted in reduced infiltration of inflammatory cells [158]. Clinical trials in CF patients have conflicting results explained by different doses and types of omega-3 supplements (DHA and EPA simultaneously,

only EPA, only DHA, α-linolenic acid), the variety in the treatment lengths and populations studied (Table 1.1). An eight month omega-3 supplementation study led to improved inflammatory responses and lung functions with a reduction in antibiotic use [159]. A six month omega-3 supplementation study in adults and children resulted in a reduction of the ratio between LTB_4 to the anti-inflammatory LTB_5 [160]. No changes in lung function were observed in four other studies despite reductions in AA levels and increases in DHA [160-163]. A year-long algal DHA supplementation study in children showed a trending decrease in IL-8 levels, albeit not statistically different from placebo treatment [122]. Another year-long trial of PUFA supplements, including both omega-3 and omega-6, showed improved lung function, reductions in antibiotics and number of pulmonary exacerbations, although no changes were noticed in the patient quality of life scores [164]. It is important to note that an intravenous lipid supplementation (Intralipid) used by certain clinics to correct the lipid deficiencies can improve symptoms of CF patients in terms of lung functions, BMI and, most importantly, their survival [135].

The timing of fatty acid supplementation might influence the results of studies for example in early disease stages, before severe lung damage has occurred, treatment with omega-3 PUFAs might be more effective than in patients with very advanced lung disease. Most patients enrolled in these studies are from a broad range of age. This may dilute the observed effect of omega-3 supplementation. The genotypes of patients may also play roles since those with mutations causing severe disease phenotypes have greater lipid imbalances [131]. One caveat of many of the studies showing no changes in lung function is the short treatment protocol. The studies where omega-3 supplementation lasted six months or less resulted in no

improvement in lung disease (defined by less pulmonary exacerbations and antibiotic use) [160-163] while some lasting more than six months showed improved lung disease [159,164], with the exception of the oldest omega-3 supplementation study lasting six weeks resulting in improved lung function [165].

3. CERAMIDE

3.1. CERAMIDE: MOLECULAR STRUCTURE, SYNTHESIS AND FUNCTION

Ceramide is a lipid molecule from the sphingolipid family involved in membrane rigidity and permeability, apoptosis, and can act as a secondary messenger [166]. The molecular structure of ceramide is defined by the presence of a sphingoid base in its backbone, which is the name given to the carbon chain containing two to three OH groups and often a double bond at the fourth carbon [166]. All ceramide species have sphingoid backbones of 18 carbons; however, they can differ in the number of carbons in the fatty acid moiety. The sphingoid backbone contains a double bond in ceramide which when removed forms dihydroceramide (DHC), which is often indistinguishable from ceramide when using assays detecting total ceramide levels [167].

There are two pathways for ceramide synthesis (Figure 1.2). The *de novo* pathway occurs in the ER and begins with the conversion of serine and palmitoyl-CoA to sphinganine by serine palmitoyl transferase. Ceramide synthase then transforms sphinganine into dihydroceramide which is converted to ceramide by dihydroceramide desaturase. Alternatively, ceramide can be synthesized through a recycling pathway with the conversion of sphingomyelin to ceramide by sphingomyelinase (SMase) [166]. There are several types of SMase categorized based on pH optima and localization in the cell: acidic (aSMase), neutral (nSMase) and alkaline

(Alk-SMase) [168]. Generally a pH of 4.5-5.0 is optimal for aSMase and it is localized mainly in lysosomal compartments in the cells as well as in the plasma membrane [169]. Neutral SMases have a pH optima of 7.4 [169] and can be located in the plasma membrane [170]. Alk-SMase is mainly found to be expressed in the intestines [171].

Ceramide is an important component of the phospholipid cell membrane and can be found in microdomains. Membrane microdomains, also known as lipid rafts, are lipid aggregates in plasma membranes comprised of cholesterol, phospholipids and sphingolipids. They are involved in signal transduction by forming platforms for activated receptors. Microdomains spatially organize proteins and protect them from enzymes which could prevent downstream signalling (e.g. phosphatases) [172].

Ceramide was shown to be generated under certain stimuli such as ultra-violet light, heat, cytokines, oxidative stress and LPS, and in turn it can regulate the expression of cytokines. The pre-treatment of 10 μg/mL of C8 ceramide reduced the expression of IL-5, IL-10 and IL-13 in mast cells in response to LPS stimulation, while TNF and IL-6 levels were unaffected. C8 ceramide did however inhibit TNF and IL-6 in macrophages but did not alter IL-10 levels. In both cell types, the addition of C8 ceramide inhibited the transcription of cytokines in response to LPS however the inhibited cytokines were different per cell type [173]. LPS administration in aSMase KO mice resulted in the increase of serum TNF, which was normalized by ceramide treatment [174]. The inhibition of ceramide synthesis was found to also cause increases in IL-8 [175] and IL-6 levels [176].

3.2. CERAMIDE AND CYSTIC FIBROSIS

3.2.1. Signalling following bacterial infections

A study by Grassme and colleagues showed the importance of ceramide in membrane microdomains and infections with PA. The bacteria were shown to interact with sphingolipid membrane platforms following tracheal infection of mice. The disruption of microdomains prevented apoptosis following PA infection and caused an 8-fold increase of IL-1β release compared to when microdomains remained intact. When aSMase was inhibited, human nasal epithelial cells failed to internalize the bacteria and IL-1β release was increased 10-fold compared to when aSMase was active. The addition of C16 ceramide was able to correct the defects seen with aSMase inhibition; internalization of bacteria, cytokine secretion and apoptosis returned to normal [177]. Although not performed in the context of CF, this important study demonstrated the role of ceramide in regulating the downstream effects of bacterial infections, especially with PA, and provided important insight into possible abnormalities in ceramide levels in CF.

Signalling following infection occurs mainly through TLRs found in plasma membranes of cells. TLR4 is a receptor for LPS, which is found on the surface of gram negative bacteria. TLR4 expression was found to be reduced in a CF bronchial epithelial cell line, located mainly intracellularly in endosomes and absent from the plasma membrane. This abnormal localization allows for partial TLR4 signalling, but overall the response to LPS is quite decreased in CF [178]. However when the CFTR defect was corrected in epithelial cells, levels of TLR4 increased on the cell surface restoring proper signalling [178]. In healthy tissue, TLR4 expression is translocated to membrane microdomains in the presence of pathogens. This translocation was found to be

dependent on the generation of ceramide via aSMase. Blocking the ceramide response, such as by inhibiting aSMase, reduced the presence of TLR4 in microdomains [179]. With ceramide being low in CF [180], this may explain why TLR4 is abnormally expressed on the cell surface.

Kowalski and Pier described the presence of CFTR in membrane microdomains. They postulated that in normal situations, CFTR was a receptor for LPS and induced the internalisation of PA, the inflammatory response and apoptosis. During infection with PA, the presence of CFTR increased in microdomains; however, the F508del mutation prevented this localization and bacteria were not cleared [181]. Another member of lipid microdomains is caveolin-1, which was shown to be involved in endocytosis and cell signalling [182]. In a study using caveolin-1 KO mice, PA infection was modeled as an acute infection with intranasal inhalation of PA, or as a chronic oropharyngeal infection where mice were given water with PA. When caveolin-1 KO mice were infected with PA using both methods, increased bacterial colonization was observed compared to wild-type (WT) mice, with higher mortality rates, indicating that caveolin-1 is an important component in fighting infection [182]. Caveolin-1 was found to co-localize with CFTR and was increased in CFTR deficient macrophages inducing an inflammatory phenotype [183]. In summary, the components of microdomains in CF seem to be abnormal, including TLR4, CFTR and ceramide, which may be responsible, at least in part, for impaired bacteria clearance in CF.

3.2.2. Apoptosis

As mentioned previously, cells lacking functioning CFTR display defective control of apoptosis [42,184]. PA infection of normal epithelial cells was found to induce apoptosis and bacterial internalization, whereas infection of CFTR defective cells did not result in apoptosis

[185]. Glutathione, which is high in CF cells, was found to inhibit nSMase preventing the generation of ceramide following oxidative stress [186]. It was postulated that low levels of ceramide prevent the completion of apoptosis even when high DNA fragmentation can be observed [187]. Increases in apoptosis of various tumour cells were seen following the treatment of mice with ceramide analogs [188]. Cells were incapable of apoptosis when ceramide induction was inhibited, such as by blocking aSMase activity [189]. Thus an inhibition or low levels of ceramide can prevent apoptosis, which may explain in part the defective apoptosis in CF.

3.2.3. Inflammatory response

Ceramide was shown to activate protein phosphatase 2A (PP2A) [190], a negative regulator of both NF-κB and MAPK pathways. Using human alveolar epithelial cells, Cornell and colleagues demonstrated an increase in IL-8 levels when PP2A is inhibited. The authors found that PP2A inhibition increased signalling through the MAPK pathway by prolonging the activation of JNK, p38 and ERK, and enhanced the stability of IL-8 mRNA. Similarly, inhibition of ceramide synthesis led to a reduction in PP2A activation and consequently an increase of IL-8 levels. The authors concluded that ceramide is needed to activate PP2A in order to control and, consequently, down regulate expression of IL-8 [175]. The activation of p38 MAPK in lungs of CF patients is greater compared to non-CF controls. Following exposure to PA, cells expressing the F508del mutation experienced a much greater increase in IL-6 due to the over-activation of p38 MAPK compared to cells expressing normal CFTR [38]. Additionally, decreased ceramide levels were found to lead to the activation of p38 MAPK which resulted in increased IL-6 production. Treatment of alveolar epithelial cells with C6 ceramide prevented this increase in IL-6 [176].

These studies demonstrate that proper ceramide levels are important for regulated cytokine expression and that defects in the CF inflammatory response may involve defects in ceramide levels.

Azythromycin has been shown to improve clinical symptoms of CF such as lung function and improvement in bacterial clearance. While the mechanism of this drug is still under investigation, it was found to induce the expression of genes involved in the cholesterol pathway [191]. An older study demonstrated that total cell phospholipid content was increased following azythromycin treatment of primary fibroblasts [192] suggesting that manipulation of cell membrane lipids may benefit the outcome of infections in CF.

The results of these studies demonstrate the importance of ceramide in regulating excessive inflammatory responses and that a reduction in ceramide levels can result in an uncontrolled production of cytokines which is resolved by supplementation of ceramide.

3.2.4. Ceramide in disease

The role of ceramide has been investigated in the context of various diseases. In oncology, the role of ceramide as an inducer of apoptosis has been exploited to kill tumour cells in neuroblastoma [193], breast cancer [194], head and neck cancer [195], and colon cancer [196]. Defects in aSMase have been shown to cause Niemann-Pick disease, a progressive neurodegenerative disorder. KO mice for *Smpd1*, the gene encoding for aSMase, were found to be resistant to radiation, apoptosis and TNF stimulation due to the reorganization of membrane microdomains following the disruption of ceramide, sphingomyelin and cholesterol [197]. Interestingly, following infection with PA, 90% of aSMase KO mice died while the control mice were able to clear the infection [177].

4. FENRETINIDE

4.1. RETINOIDS: METABOLISM AND FUNCTIONS

Fenretinide is a synthetic derivative of vitamin A which is an umbrella term given to the family of retinoids. Retinoids have several forms: retinol, retinal, retinoic acid, retinyl ester and the provitamin carotenoids such as β-carotene (Figure 1.3A). The metabolism of retinoids as they travel from the digestive system into the circulatory system is a complicated process involving many steps of esterification and hydrolysis. The basic conversion pathway is illustrated in Figure 1.3A. Retinoids are ingested from the diet as retinyl esters (as retinyl palmitate mostly) from animal sources or as carotenoids from plants. Retinyl ester is biologically inactive and must be converted to retinol to be used. Once ingested, retinyl ester is hydrolysed by pancreatic enzymes into retinol before absorption into enterocytes. In these intestinal cells, retinol is esterified into more stable retinyl esters and packaged into chylomicrons for transport to the liver through the lymphatic system. Retinoids are stored as retinyl esters in the liver and are converted back to the active form retinol when needed. Retinol binds to retinol binding protein (RBP) and the retinol-RBP complex is bound to transthyretin for transport into blood. It was shown that the binding with transthyretin prevented the loss of the retinol-RBP complex through renal filtration since RBP is small in size [198] and this complex may also prevent the nonspecific release of retinol [199]. Once at the cell surface, the retinol-RBP complex binds to STRA6 which enables the cellular uptake of retinol. Retinol can be converted to retinal, which is the active retinoid in the eye, and then to retinoic acid for further effects in cells (Figure 1.3A).

Retinoids are involved in cell growth, differentiation and apoptosis. They impact vision, development, reproduction and immune function. These functions of retinoids were exploited in the treatment of cancer cells *in vitro*. They were found to induce apoptosis in leukemia [200,201]. Retinoic acid inhibited cell growth at the G1 cell cycle stage and prevented bronchial epithelial cells from transforming after carcinogen exposure [202]. It also induced differentiation of neuroblastoma cells and reduced the level of oncogene expression [203]. Clinical trials involving retinoids showed potent effects on cancer cells [204] but their use in patients was limited due to the risk of toxicity [205]. Additionally, some cancer cells may have undergone mutations in the retinoic acid pathways which render them resistant to retinoid treatment [206]. β-carotene is thought to be less toxic than retinoic acid because there is no dose dependant accumulation in the liver [207]. It was assessed as a preventative treatment for cancer but the results showed no effect on tumour incidence in patients. In some cases, it was found to be more detrimental than no supplementation at all [208,209].

4.2. FENRETINIDE: A RETINOID DERIVATIVE

Variants of retinoic acid were synthesized to overcome the limitations of dietary retinoids. One such derivative, fenretinide, [N-4-hydroxyphenyl retinamide], was proven to be effective *in vitro* against cancer cells [210-212] and was found to have limited toxicity [213]. The induction of apoptosis in cancer cells by fenretinide was found to involve in part the increase in ceramide levels [193,214-219]. The longest study to date involved daily fenretinide treatment over a 5-year period in breast cancer patients as a prophylaxis against secondary breast cancers [220]. The most common adverse reactions to the drug after four weeks of treatment were minor visual side effects such as diminished dark adaptation which were reversed when a three

day drug interruption was added into the treatment schedule. Additionally, fenretinide was able to bypass retinoic acid resistance and induced more cell death than retinoids in neuroblastoma cells [221]. Fenretinide was a strong candidate for cancer therapy however it quickly became apparent that for some forms of cancer (e.g. neuroblastoma) higher doses of the drug would be needed to generate an effect in patients [222]. The available formulation was a large capsule with fenretinide powder suspended in corn oil which unfortunately had low absorbance. This formulation was adequate for some trials such as for macular degeneration [223] where low doses were sufficient. However, the size of the capsule also had a great impact on patient compliance, especially when multiple capsules were needed to achieve a high dose. Many current studies are focused on reformulating the drug to increase its bioavailability [224,225]. Part of this thesis project involved examining the bioavailability of fenretinide using different formulations in different animal models to assess the best option for clinical trials in CF patients.

4.3. FENRETINIDE: MECHANISM

The fact that most common side effect of fenretinide after weeks of uninterrupted treatment involved visual impairments led researchers to believe it was interfering with retinol pathways. In cancer patients, the drug treatment caused a reduction in the plasma retinol and RBP levels [226]. However, adding fenretinide directly into plasma samples showed no interaction between the drug itself and retinol [227]. Fenretinide did not inhibit the absorption of retinol [228], yet somehow its levels were decreased after drug treatment. Just like retinol, fenretinide binds to RBP because both of the molecules have identical cyclohexane rings in their structure (Figure 1.3B). However, the fenretinide-RBP complex does not bind with

transthyretin due to the modification of the hydroxyl group in fenretinide [229]. It is thought that the fenretinide-RBP complex is being eliminated through renal filtration, leading to a decrease in circulating RBP and interfering with the transport of retinol, thus explaining the decrease in retinol levels with uninterrupted fenretinide treatment [229,230]. This rapid filtration may also explain why fenretinide has a lower toxicity than retinoic acid.

In the cell, retinol is converted to retinoic acid and binds to nuclear receptors, called retinoic acid receptors (RARs). There are several isoforms of RARs, α, β and γ, which bind both all-trans retinoic acid and 9-cis retinoic acid. The RARs bind to retinoic acid receptor elements (RAREs) on DNA to regulate the transcription of hundreds of genes [231]. Retinoid X receptors (RXR) α, β and γ bind only the cis form of retinoic acid. The regulation of gene expression by retinoids is quite complex as it involves specific RAR isoforms binding to specific RAREs. Fenretinide was found to have higher affinities for RARβ and γ for gene activation, and can act through RARα and β to repress genes [232]. Its effects are not identical to retinoic acid and the genes expressed can be different even when the same receptor is targeted [233]. Not all cells express the spectrum of receptors, for instance macrophages express RARα and β, and RXRα and β [234]. Thus the effects of fenretinide may be specific for each cell type. The mechanism of action on tumour cells is also different between fenretinide and retinoic acid. As mentioned previously, retinoic acid induces cell cycle arrest and differentiation of cancer cells while a high dose of fenretinide was found to kill cells via apoptosis [235,236]. Fenretinide can also act independently of RAR on cancer cells, affecting breast cancer cells which were resistant to retinoic acid treatment [237].

Treatment with fenretinide increased ceramide levels in alveolar epithelial cells with the F508del mutation and reduced the IL-8 response to TNF stimulation [238]. The study published from our group in 2008 described for the first time defects in ceramide levels in CF patients and in a CF mouse model [180]. Treatment with fenretinide corrected total ceramide levels to normal WT values in CF mice. This treatment also enabled CF mice to clear lung infections with PA similarly to WT mice. These results propelled the studies presented in this thesis which will demonstrate the effects of fenretinide on manifestations of CF such as inflammation, lipid peroxidation, nitrosative stress, the PUFA imbalance and ceramide defects which may ultimately impact the eradication of PA in CF patients and prevent lung deterioration.

Figure 1.1. Metabolism of omega-3 and omega-6 polyunsaturated fatty acids. The metabolic pathways of omega-3 and omega-6 fatty acids begin with the modifications of the essential fatty acids procured from the diet. Both pathways compete for the desaturase and elongase enzymes. It is thought that by increasing the levels of PUFAs in one pathway, for example with dietary supplementation, this may diminish the activity of the other pathway.

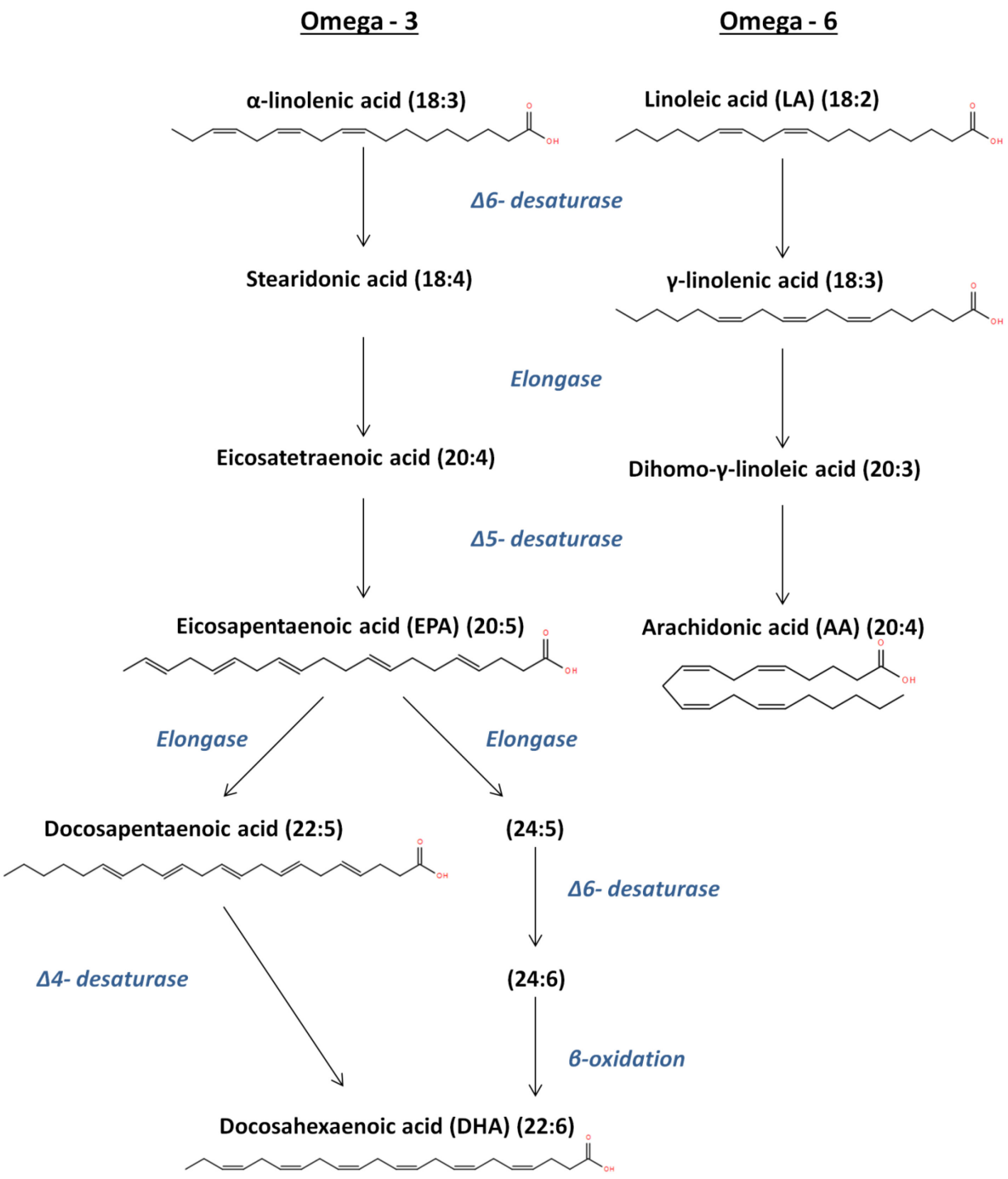

Omega - 3
Omega - 6
α-linolenic acid (18:3)
Linoleic acid (LA) (18:2)
Δ6- desaturase
Stearidonic acid (18:4)
γ-linolenic acid (18:3)
Elongase
Eicosatetraenoic acid (20:4)
Dihomo-γ-linoleic acid (20:3)
Δ5- desaturase
Eicosapentaenoic acid (EPA) (20:5)
Arachidonic acid (AA) (20:4)
Elongase
Elongase
Docosapentaenoic acid (22:5)
(24:5)
Δ6- desaturase
Δ4- desaturase
(24:6)
β-oxidation
Docosahexaenoic acid (DHA) (22:6)

Figure 1.2. Synthesis pathways of ceramide. Two pathways exist for ceramide synthesis: *de novo* pathway and recycling pathway. The *de novo* pathway occurs in the endoplasmic reticulum while the recycling pathway occurs in vesicular and cell membranes.

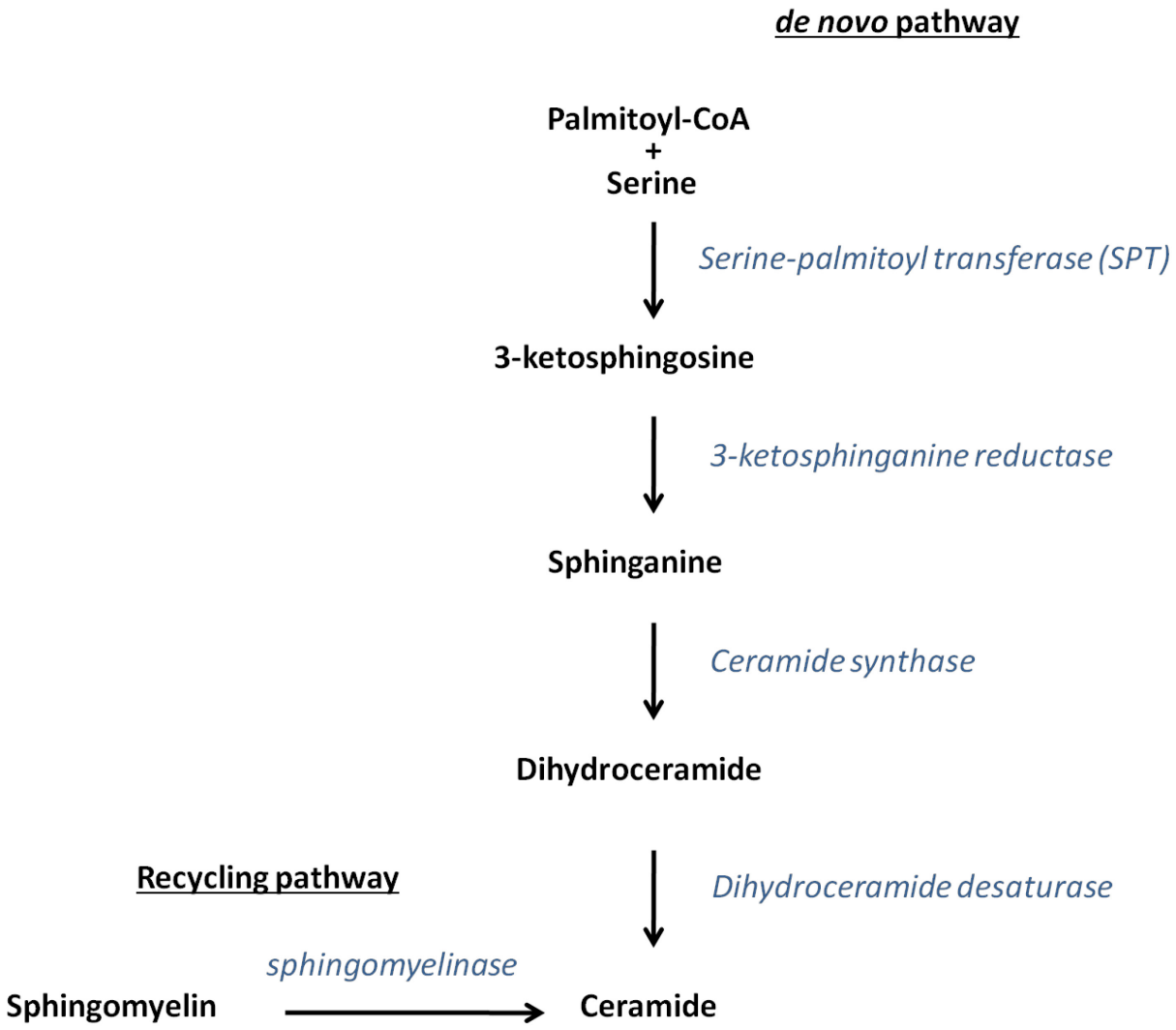
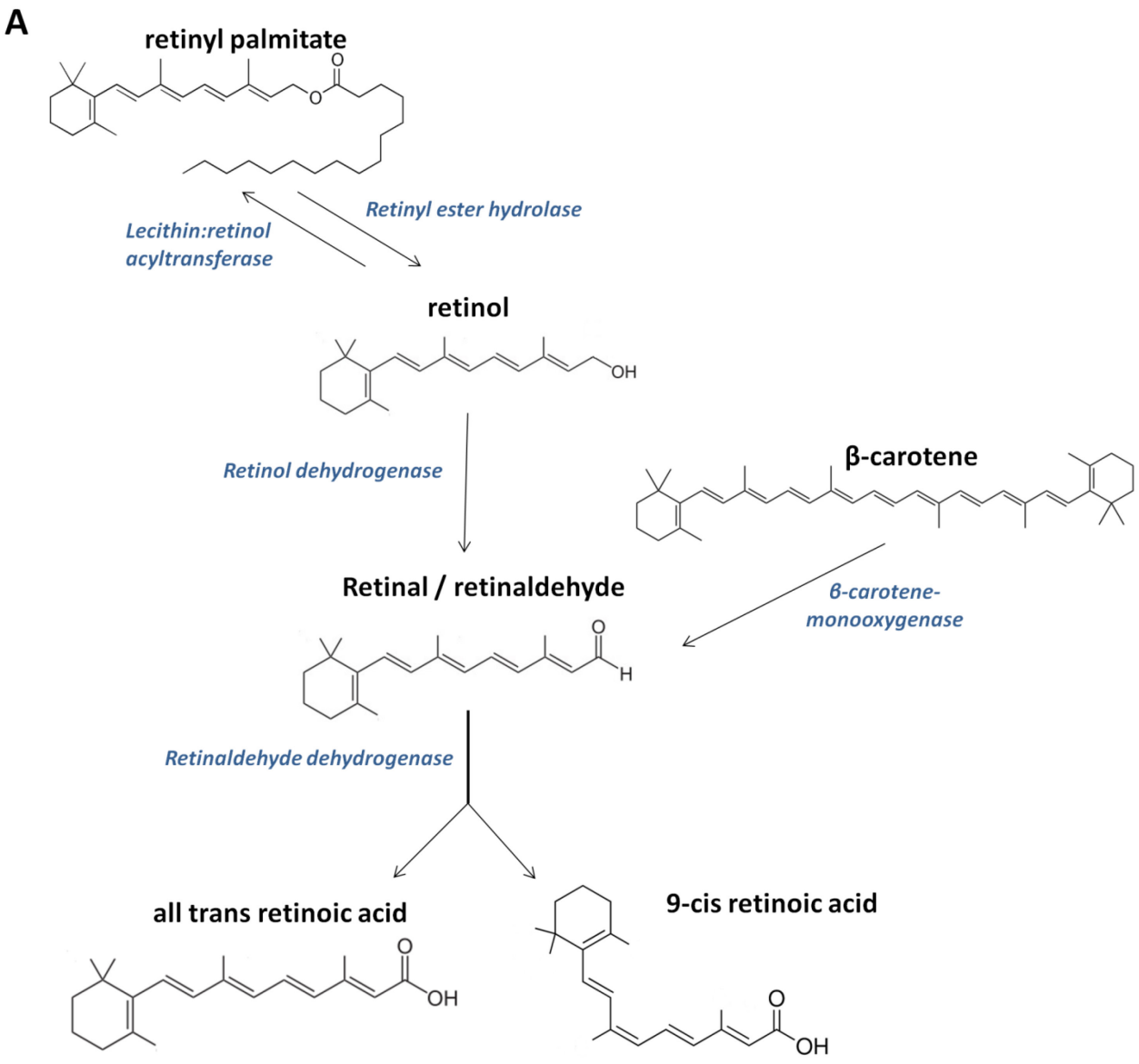

de novo pathway
Palmitoyl-CoA
+
Serine
Serine-palmitoyl transferase (SPT)
3-ketosphingosine
3-ketosphinganine reductase
Sphinganine
Ceramide synthase
Dihydroceramide
Recycling pathway
Dihydroceramide desaturase
sphingomyelinase
Sphingomyelin
Ceramide

Figure 1.3. Retinoids. A) Pathway of conversion between dietary retinoids into active retinoic acid. B) Molecular structure of fenretinide. It has a similar structure to all-trans retinoic acid with a modification on the hydroxyl group (*highlighted in blue*). Binding to retinol binding protein (RBP) occurs at the cyclohexane ring, common to both retinoic acid and fenretinide. The modification present in the structure of fenretinide with the extra cyclohexane ring (*in blue*), prevents proper binding to transthyretin.


A
retinyl palmitate
Lecithin:retinol acyltransferase
Retinyl ester hydrolase
retinol
Retinol dehydrogenase
β-carotene
Retinal / retinaldehyde
β-carotene-monooxygenase
Retinaldehyde dehydrogenase
all trans retinoic acid
9-cis retinoic acid

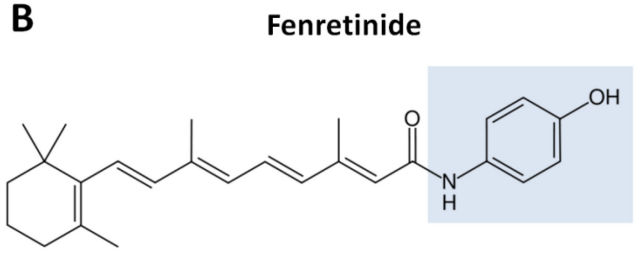

B
Fenretinide

Table 1.1. Summary of omega-3 PUFA supplementation trials in CF patients.

(on following page)

Study	EPA dose	DHA Dose	Control	Duration of treatment	age group	# CF patients treated	Lung Function	Sputum	PEx[1]	Abx[2]	Inflammation	QOL[3]
Lawrence 1993	2.7 g	unknown	Olive oil - placebo group vs. treated group	6 weeks	adolescents/ young adults	9	higher in treated	down	N/A	N/A	N/A	N/A
Henderson 1994	3.2 g	2.2 g	Olive oil - placebo vs. treated (also included non-CF controls)	6 weeks	children/ adolescents	7	N/A	N/A	N/A	N/A	N/A	N/A
Kurlandsky 1994	1.5 g average	0.8 g average	Safflower oil (high omega-6) - patients received both treatments after a washout period	6 weeks	children/ adolescents	14	N/A	N/A	N/A	N/A	LTB4 down	N/A
Katz 1996	0.1 g average	0.2 g average	Low Omega-3 solution - placebo group	1 month	adolescents/ adults	6	no change	N/A	N/A	N/A	N/A	N/A
De Vizia 2003	1.3 g average	0.9 g average	Patient history	8 months	children/ adolescents/ young adults	30	no change	N/A	N/A	down	α-1-antitrypsin down	N/A
Lloyd-Still 2006	none	1 – 4.2 g	Corn/soy oil - placebo group	6 months	children/ adolescents	9	no change	N/A	N/A	N/A	N/A	N/A
Panchaud 2006	0.2 – 0.6 g	0.1 – 0.3 g	Olive oil - crossover study	6 months	adolescents/ young adults	16	no change	N/A	N/A	N/A	LTB4/LTB5 down	N/A
Van Biervliet 2008	None	40% of PUFA is DHA	Sunflower seed oil - placebo group	1 year	children/ adolescents/ young adults	8	no change	N/A	N/A	N/A	N/A	N/A
Olveira 2010	0.3 g	0.2 g	Patient history and healthy controls	1 year	adolescents/ adults	17	no change	N/A	down	down	N/A	no change
Keen 2010	21.27 mol%	6.99 mol%	Low PUFA or high AA mixtures - two placebo groups	3 months	children to adults	12	no change	N/A	N/A	no diff	IL-8 down	N/A
Alicandro 2013	< 0.01g	0.25 g	LA acid and oleic acid (omega-9)	1 year	children	21	no change	N/A	no change	N/A	no diff (IL-8,TNF,CRP)	N/A

[1] PEx = number of pulmonary exacerbations; [2] Abx = antibiotic use; [3] QOL = patient quality of life based on questionnaire. N/A: data not available

Previous studies in our laboratory demonstrated that ceramide levels are low in CF patients and in CF mice. We assessed ceramide in 10 patients using two methods: liquid chromatography – mass spectrometry and thin layer chromatography (TLC) – ELISA. While the former method is more specific and can distinguish the various ceramide species as opposed to total ceramide levels by ELISA, the results from both methods correlated well. In mice, ceramide levels were normalized following fenretinide treatment for 28 days. This treatment also improved bacterial clearance from the lungs of CF mice. These results prompted us to analyze whether the PUFA defects in CF could also be corrected. In addition, we sought to test whether fenretinide had an effect on the heightened levels of inflammation in CF. The results presented in following chapter show that fenretinide can correct the defects in PUFA and abnormal expression of inflammatory markers in CF mice. We also demonstrate for the first time that defects in ceramide levels are correlated with abnormal levels of PUFA, which are associated with more severe CF disease symptoms.

CHAPTER 2: FATTY ACID IMBALANCE IN CF CORRELATES WITH CERAMIDE DEFICIENCY AND CAN BE CORRECTED WITH FENRETINIDE

Adapted from the published manuscripts

Guilbault C*., Wojewodka G*., Saeed Z., Hajduch M., Matouk E., De Sanctis J. B., Radzioch D. Fatty acid imbalance in CF correlates to ceramide deficiency and can be corrected with fenretinide. *Am JRespir Cell Mol Biol*, 2009 Jul;41(1):100-6

* These two authors equally contributed to this manuscript

Guilbault C., De Sanctis J.B., Wojewodka G., Lachance C., Skinner T. A., Vilela R. M., Kubow S., Lands L. C. , Hajduch M., Matouk E., Radzioch D. Fenretinide corrects newly found ceramide deficiency in cystic fibrosis. *Am J Respir Cell Mol Biol*. 2008 Jan;38(1):47-56.

ABSTRACT

Cystic fibrosis (CF) patients and *Cftr* knock-out mice (CF mice) display an imbalance in polyunsaturated fatty acids with high arachidonic acid (AA) and low docosahexaenoic acid (DHA) concentrations. Our recent studies demonstrated defects in another class of lipids, ceramide, in CF patients and CF mice. This study investigates the relationship between ceramide, AA, DHA and the correction of lipid imbalances in CF mice following treatment with fenretinide. Concentrations of AA, DHA and ceramide were assessed in plasma from 58 adult CF patients and 72 healthy controls. Following 28 days of treatment with fenretinide, the same lipid analysis was performed in wild-type and CF mice from plasma and organs (lung, ileum, pancreas and liver). Low ceramide levels were associated with high AA and low DHA concentrations in CF patients. No correlation was observed in healthy controls. Greater lipid imbalances were seen in CF patients who were diagnosed before the age of 18, specifically with statistically significant higher levels of AA. Treatment with fenretinide (N-4-hydroxyphenyl retinamide) normalized the high levels of AA, the low levels of ceramide and increased DHA in CF mice. As in CF patients, greater deficiencies in ceramide levels correlated with higher AA and lower DHA levels in plasma of CF mice. Fenretinide treatment reduced AA to normal levels and increased DHA. The drug treatment reduced the expression of inflammatory genes in lung tissue of CF mice. We propose that fenretinide treatment might improve the lipid defects and heightened inflammation in CF patients.

INTRODUCTION

Patients with cystic fibrosis (CF) have imbalances in polyunsaturated fatty acids (PUFA) meaning they exhibit low concentrations of docosahexaenoic acid (DHA) and high arachidonic acid (AA). DHA, an omega-3 fatty acid, is produced from the essential fatty acid α-linolenic acid. DHA metabolizes to anti-inflammatory resolvins and protectins [239]. AA, an omega-6 fatty acid, results from the essential fatty acid linoleic acid. AA metabolites are pro-inflammatory eicosanoids: 2-series prostaglandins and 4-series leukotrienes. AA and DHA compete for the same desaturation and elongation enzymes in their synthesis pathways. Thus, AA is the antagonist of DHA and modifying the concentration of one will inversely impact the other [240]. It is believed that the elevated levels of AA in CF patients contribute to the inflammation and pathology of the disease [119]. In 1999, Freedman and colleagues described a mouse model with a lipid membrane imbalance in CF related organs. The observed imbalance in these *Cftr* knock-out mice (CF mice) could be corrected by oral administration of DHA, which also normalized the pathology of the lung and the pancreas [70]. As mentioned in Chapter 1, clinical trials have attempted to correct the PUFA imbalance with oral supplementation of DHA in the forms of fish or algal oils. While DHA levels increased in CF patients in these studies, no improvements in lung function tests were observed (see also Table 1.1) [241].

We have previously reported the correction of AA and DHA imbalances following a twice-weekly 4-week treatment of CF mice with fenretinide, a semi-synthetic retinoid. This treatment was able to prevent osteoporosis in CF mice [242]. This protocol however did not improve lung infection eradication (unpublished data). A more frequent treatment protocol of 28 consecutive days led to a significant improvement in the clearance of lung infection in CF

mice following instillation of *Pseudomonas aeruginosa* (PA) laden beads. These studies also discovered a deficit in ceramide, a sphingolipid, in CF mice and 10 CF patients [180]. In our mouse model, the twice-weekly 4-week treatment protocol could partially correct the defect in ceramide [242], however the daily 28-day protocol was able to normalize ceramide levels in CF mice to those of wild-type (WT) mice [180]. We believe that the complete correction of ceramide is a major factor in the ability of CF mice to fight infection as ceramide was found to be involved in the formation of lipid membrane rafts which are essential in internalizing PA [177].

In this chapter, we examine the relationship between ceramide, AA and DHA using a relatively large cohort of healthy controls and CF patients, and our CF mouse model. To evaluate the importance of the defects in lipids in disease severity, we grouped our patient cohort according to age of diagnosis, the idea being that late diagnosis is linked to milder disease pathology [243]. We also examine the effects of a 28-day treatment with fenretinide on the concentrations of AA, DHA and the AA/DHA ratio in various CF affected organs using CF mice and littermate controls, and we describe the relationship between ceramide and the fatty acids in mice. Additionally, we confirm that fenretinide also affects the expression of inflammatory genes by reducing IL-1β and S100A8 in the lungs of CF mice.

METHODS

Human study

CF patients were recruited from the Adult Cystic Fibrosis Clinic at the Montreal Chest Institute (Montreal, Canada). All CF patients were diagnosed by evidence of CFTR dysfunction (elevated sweat chloride) and/or identification of two pathological *CFTR* mutations resulting in

symptoms characteristic of CF disease (Tag-It^{TM} Cystic Fibrosis 40+4 Test, Tm Bioscience Corp., Toronto, ON). CF patients were following standard CF therapy [244,245]. Clinical data were obtained at the time of visit for blood sampling. These included age of the patient, age of diagnosis, height and weight while dressed in indoor clothing without shoes. BMI was calculated as kg/m^2. Pancreatic status was also extracted from the medical records. Spirometry was obtained at the time of visit for blood sampling or within 2 - 6 weeks from the last visit when the patient was considered clinically stable. It included forced expiratory volume in one second percent predicted (FEV_1%) and forced vital capacity percent predicted (FVC%). It was performed according to standards established by the American Thoracic Society [246]. The better of two reproducible efforts was recorded. They were each expressed as percentage of the predicted normal value for the patient's age, sex, and height, calculated from regression equations derived from a healthy reference population [247]. Since CF patients have lower height-for-age values compared to normal subjects and these values are used to classify lung function as normal or abnormal, the comparison of CF subjects to a healthy reference population is an important limitation. Kulich and colleagues developed CF disease-specific reference equations for lung function based on FEV_1 observations from over 21,000 subjects with CF. We calculated FEV_1 percentiles using these CF-specific reference equations (CF-specific FEV_1 perc.) for our patient cohort. Each CF patient (age 18-39) was compared to a CF reference population of the same sex, age and height [248]. Patients over 39 years of age were attributed the highest percentile. This allowed for a better classification of disease severity.

Seventy-two healthy control subjects were recruited from McGill University Health Centre Hospitals. The study was approved by the Research Ethics Board of the McGill University Health Centre. All participants gave written consent to participate in the study.

Mouse study

C57BL/6-*Cftr*^{tm1unc}–/– (CF) mice, aged 15-16 weeks, were obtained by breeding of heterozygous mice C57BL/6-*Cftr*+/– (HZ). As controls, we used age- and gender-matched C57BL/6-*Cftr*+/+ (WT) mice. All mice were maintained in a pathogen free environment in sterile cages with corn bedding. CF mice are fed a liquid diet, Peptamen (Nestle Canada, Brampton, Canada), throughout their lives. This diet enables CF mice to thrive, maintain healthy weight and it increases their survival into adulthood. WT mice were placed on the liquid diet only throughout the experiment. Fenretinide, N-4-hydroxyphenyl retinamide, powder (generously provided by Dr. R. Smith, National Institutes of Health, Bethesda, USA) was resuspended in 95% ethanol to a concentration of 2 mg/mL and subsequently incorporated into 12.5 mL of the liquid diet at a dose of 5 mg/kg/day. Mice were treated for 28 consecutive days. The treatment solution was protected from light and changed daily. Vehicle-treated controls were fed Peptamen with 95% ethanol corresponding to the equivalent volume used for drug treated mice. Experimental procedures were conducted in accordance with the Canadian Council on Animal Care Guidelines and with the approval of the Facility Animal Care Committee of the Montreal General Hospital Research Institute (Montreal, Canada).

Intratracheal lung infection

Preparation of inoculum. To prepare the inoculum for infection, *Pseudomonas aeruginosa* (PA, strain 508) was grown from an overnight culture for 3 hours at 37°C in a shaking incubator. The 3 hour culture was concentrated and resuspended in 5 mL of phosphate buffered saline (PBS). This mixture was combined with 5 mL of warm agar (3% trypticase soy broth, 1.5% agar; Difco, Detroit, USA) and dispensed into a flask of stirring heavy mineral oil at 52°C. The solution was left to stir for 6 min at room temperature and an additional 10 min cooling with ice. The mixture was centrifuged to precipitate the agar beads and washed several times with PBS to eliminate the residual oil. An aliquot of the final bead mixture was homogenized in PBS and used to plate for quantification of colony forming units (CFU). The PA agar beads were used to infect mice at a dose of 1×10^6 CFU in a volume of 50 µL.

Infection procedure. Prior to the infection procedure, mice were anesthetized using a combination of ketamine (7.5 mg/mL) and xylazine (0.5 mg/mL) administered intraperitoneally at a dose of 20 mL/kg of body weight. Mice were then placed on a support under a microscope (Microscope M650; Wild Leitz, Willowdale, Canada) with their top incisors restrained to ensure the mouth remained opened. To have the larynx in view, the tongue was pushed to the side and held using forceps. A 26-gauge gavage needle was used to deliver the inoculum via the trachea. Immediately following the infection, mice were placed on heating pads until they recovered from anaesthesia. Mice were monitored twice a day until euthanasia, which occurred three days after infection [249].

Lipid analysis

Sample collection. Blood samples, both human and murine, were collected in EDTA coated tubes. Following centrifugation for 10 min at 3000 rpm, 100 µL of plasma was collected from the samples and preserved in 1 mL of 1 mM butylated hydroxyanisole (BHA) in a chloroform/methanol solution (2:1 vol/vol)[250,251]. Approximately 30 mg tissue samples from mouse organs (lung, pancreas, ileum and liver) were collected, minced and preserved in 1 mL of BHA solution.

Lipid extraction. One volume of cold water was added to the mixture and samples were mixed for 90 min at 4°C. The organic phase was collected to which diethyl ether was added to remove any leftover protein contamination.

Ceramide analysis. An aliquot of the organic fraction was dried and resuspended in 100 µL of chloroform. Lipids were further separated by TLC and were detected by iodine [251]. The phospholipid fraction corresponding to ceramide was compared to known standards and collected from the dry silica. The ceramide fraction was resuspended in ethanol and used to coat Nunc plates specific for lipid binding. The plates were washed, incubated with blocking buffer (PBS, 0.1% Tween 20, and 1% bovine serum albumin, Sigma-Aldrich, Oakville, Canada) for 1 h at 37°C followed by incubation with murine anti-ceramide IgM (Sigma-Aldrich) antibody for 1 h at 37°C. The plates were washed again and incubated with peroxidase-conjugated anti-mouse IgM antibody for 1 h at 37°C. Finally, the plates were incubated with the peroxidase substrate (TMB; Roche, Laval, Canada). The intensity of the colorimetric reaction was determined by spectrophotometry at 405 nm. The amount of total ceramide was calculated with reference to a standard curve (Sigma-Aldrich)[180,252,253]. Phosphate levels were

assessed by the PiBlue Phosphate assay (Boehringer Ingelheim, Caracas, Venezuela), according to the manufacturer's instructions, and used to normalize ceramide concentrations. Ceramide levels are expressed as ng/μg phosphate.

PUFA analysis. Following lipid extraction procedure, fatty acids in the organic fraction were esterified and hydrolyzed with diazomethane and methanol as described by Schlenk and Gellerman [254] and the esters were identified by gas chromatography/mass spectrometry (Hewlett Packard 5880A, WCOT capillary column (Supelco-10, 35 m × 0.5 mm, 1 μm thick) using commercial standards (Sigma-Aldrich, Oakville, Canada). In addition, the protein content of the aqueous phase was analysed using the bicinchoninic assay (Pierce Biotechnology, Rockford, USA). The concentrations of AA and DHA are expressed as nmol/μg protein. The AA/DHA ratio represents the amount of AA to DHA in each sample.

RNA extraction and mRNA expression analysis

RNA was extracted from frozen lung tissue using TRIzol (Invitrogen, Burlington, Canada). Following extraction, cDNA was made from the samples using QuantiTect Reverse Transcription Kit (Qiagen, Mississauga, Canada). Gene expression was quantified using real-time PCR. The cDNA amplification was performed using the Brilliant SYBR Green QPCR Kit (Stratagene, Cedar Creek, USA) in the MX-4000 system (Stratagene) according to the manufacturer's instruction. The following primer sequences, forward and reverse, were used for mRNA analysis: Interleukin-1beta (IL-1β, 5'GCTTCAGGCAGGCAGTATCACT 3', 5' CACGGGAAAGACACAGGTAGCT 3'), S100 calcium binding protein A8 (S100A8, calprotectin, 5' CCATGCCCTCTACAAGAATGAC 3', 5' CTACTCCTTGTGGCTGTCTTG 3'), glyceraldehypde 3-phosphate dehydrogenase (GAPDH, 5' ATGTGTCCGTCGTGGATCTGA 3', 5'

TTGAAGTCGCAGGAGACAACCT 3') (IDT, Coralville, USA). Gene expression in relation to WT levels was calculated based on the threshold cycle (C_T) value and standardized by the amount of the house-keeping gene using the $2^{-\Delta\Delta C}{}_T$ method:

$$\Delta\Delta C_T = (C_{T,Target} - C_{T,GADPH})_{KO} - (C_{T,Target} - C_{T,GADPH})_{WT}$$

where the "target" represents the gene of interest tested. Each gene expression was standardized by the expression of the house-keeping gene GAPDH.

Statistical analysis

Data were analyzed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, USA) and SigmaStat 9.01 (Systat Software Inc., Chicago, USA). For comparisons between two groups only, the Student's t-test was used. For comparisons between multiple groups, two-way ANOVAs were used with the Holm-Sidak post-test to evaluate the effects of genotype and treatment on PUFA levels. The Spearman test was used for correlations between data sets. For the incidence of pancreatic insufficiency in Table 2.3, Chi-Square test was used. Significance was set at a two-tailed p value of ≤ 0.05.

RESULTS

Correlation between ceramide and fatty acids in CF patients

Plasma lipid levels from CF patients were evaluated and compared to control subjects (detailed characteristics of the participants shown in Table 2.1 and S2.1A). We observed significant differences in plasma lipid levels of CF patients with low DHA and ceramide levels, and high AA and AA/DHA ratios compared to healthy controls (Table 2.2). We determined the relationship between abnormally low ceramide levels, elevated AA levels and reduced DHA

levels in plasma from CF patients. In CF patients, ceramide concentrations were inversely correlated with AA concentrations (Figure 2.1C) and were directly correlated with DHA levels (Figure 2.1D). AA/DHA ratios are also correlated with ceramide levels in CF patients ($r = -0.78$, $p < 0.0001$), but not in healthy controls ($r = -0.10$, $p = 0.5938$). No correlations between ceramide and AA (Figure 2.1A) or DHA concentrations (Figure 2.1B) were observed in control subjects.

PUFA imbalance is correlated with disease severity

To establish the degree of disease severity in CF patients, we divided our CF patient cohort by age of CF diagnosis and compared lipid levels between the groups (Table 2.3, genotype frequencies by group are listed in Table S2.1B). Thirteen of 58 adult patients were diagnosed after the age of 18 (Late Diagnosis; LD) and 45 of 58 patients were diagnosed before the age of 18 (Early Diagnosis; ED). The LD subjects were clinically milder, as determined by a higher average BMI, their higher CF-specific FEV_1 percentile compared to the ED subjects, and by a lower prevalence of pancreatic insufficiency (Table 2.3). The LD group had significantly lower AA concentrations, 1.7-fold higher concentrations of DHA and 1.9-fold higher ceramide levels compared to ED group. Overall, higher concentrations of AA were observed along with parameters characterizing severe CF disease such as low CF-specific FEV_1 and pancreatic insufficiency in ED patients.

Fenretinide treatment corrects the AA/DHA imbalance

CF mice have imbalances in PUFA compared to WT mice in plasma, lungs, ileum, pancreas and liver. As shown in Table 2.4, CF mice have reduced levels of DHA and increased

levels of AA. The imbalance can be represented as a ratio of AA/DHA which is significantly higher in CF mice than in WT mice in plasma and all CF-related organs.

We evaluated the effect of fenretinide on PUFA in the context of our PA lung infection model as it is more representative of chronically infected CF patients. Following a 28-day treatment protocol of fenretinide, PUFA levels were analyzed in plasma, lung, ileum, pancreas and liver of CF and WT mice. CF mice had higher basal levels of AA compared to WT controls in plasma, lung and liver (Figure 2.2A, and Table 2.5A). AA significantly decreased in fenretinide treated mice compared to vehicle treatments in plasma (Figure 2.2A), lungs and liver (Table 2.5A). AA levels in WT mice did not change after treatment with fenretinide.

There were significant differences in DHA levels between untreated WT and CF mice. DHA concentrations increased in plasma following fenretinide treatment in both WT and CF mice (Figure 2.2B). We found significant increases in DHA concentrations in fenretinide treated CF mice compared to vehicle controls in the lung and liver (Table 2.5B). DHA levels were also increased following drug treatment in WT mice in lungs, ileum, pancreas and liver (Table 2.5B). Decreased AA/DHA ratios in CF mice treated with fenretinide compared to CF vehicle controls were found in plasma (Figure 2.2C) and all tested organs (Table 2.5C). In WT mice, the treatment with fenretinide did not significantly reduce the AA/DHA ratio however decreases were still observed in plasma (Figure 2.2C) and all organs (Table 2.5C).

Correlations between ceramide and PUFAs in CF mice

We assessed the correlations between ceramide and AA concentrations, and between ceramide and DHA in plasma of WT and CF mice which were untreated or fenretinide treated animals (Figure 2.3). In CF mice, ceramide concentrations were inversely correlated with AA

concentrations (Figure 2.3C) and were directly correlated with DHA levels (Figure 2.3D). We also found a correlation between the AA/DHA ratio and ceramide levels in CF mice ($r = - 0.56$, $p = 0.0012$). No correlation between ceramide and AA was found in WT mice (Figure 2.3A). Ceramide levels were directly correlated with DHA levels also in WT mice (Figure 2.3B).The AA/DHA ratio correlated with ceramide levels in WT mice ($r = - 0.30$, $p = 0.0376$). Our results showed links between DHA and ceramide levels in the WT mice which were treated with fenretinide demonstrating that both DHA and ceramide increased with fenretinide treatment within the same mouse.

Expression of inflammatory markers

We evaluated the expression in lung tissue of IL-1β and S100A8 to determine whether fenretinide treatment had an effect on genes involved in inflammation. Our group previously indentified these genes as over-expressed in CF mice [65]. We found a significant difference between WT and CF untreated mice, confirming our previous report (Figure 2.4). Treatment with fenretinide diminished expression of both inflammatory genes tested. We found a statistically significant difference between fenretinide treated CF mice and their untreated controls (IL-1β, Figure 2.5A and S100A8, Figure 2.5B). The mRNA expression of IL-1β and S100A8 levels was also reduced in WT mice following fenretinide treatment, albeit to a much lower extent.

DISCUSSION

The importance of PUFAs and their derivatives is studied in a variety of diseases such as asthma [255], rheumatoid arthritis [255], cardiovascular disease [256], neurodegeneration

[257] and cancer [258]. Such diseases have been linked to imbalances in AA and DHA. Freedman and colleagues described the AA/DHA imbalance in CF patients [119] and their CF mouse model [70]. Ceramide has been studied in the context of cancer [259] and neurodegenerative diseases [260]. Previously, we reported deficiencies in ceramide levels in 10 CF patients and our CF mouse model displaying spontaneous lung disease [180]. Here we confirm the defect in ceramide in a larger CF patient cohort and we describe its relationship between the imbalance in PUFAs. To our knowledge, this is the first study to correlate a deficiency in ceramide to an imbalance in PUFA.

In our human study, we found correlations between low levels of ceramide, high levels of AA and low levels of DHA in the plasma of CF patients, whereas no correlations were found between these biochemical parameters in healthy controls. It is quite interesting that the CF patient cohort contains patients with severe lipid imbalances and others with more normal lipid levels even if they share the same genotype at the *CFTR* locus, e.g. F508del/F508del (26 CF patients in our cohort shared this particular genotype). This variation may be due to polymorphisms in gene modifying genes such as in cyclooxygenases, the enzymes responsible for converting PUFA into prostaglandins [139].

We hypothesized that patients with normal lipid levels might display a milder form of CF. Multiple factors affect disease severity such as pulmonary function, lung colonization with PA and other pathogens, pancreatic status, *CFTR* genotype and the patient's age at the time of CF diagnosis. In a large cohort of older CF patients, Rodman and colleagues showed that patients with CF diagnosed in adulthood differed from individuals diagnosed as children. Those diagnosed later in life have a significantly lower prevalence of pancreatic insufficiency, better

lung function, less frequent PA colonization and CF-related diabetes[243]. Thus, we divided our cohort into patients that were diagnosed before the age of 18 (early diagnosis, ED) and those diagnosed in adulthood (late diagnosis, LD). Our results showed that the ED group had lower BMI, CF-specific FEV_1 percent and higher incidences of pancreatic insufficiency. In terms of lipid concentrations, overall the ED group showed trends towards lower levels of DHA and ceramide, and displayed statistically significantly higher levels of AA compared to LD patients. As AA metabolites are pro-inflammatory, reducing AA in CF patients might enable an amelioration of clinical parameters or at least a deceleration of CF morbidity therefore an early intervention may prevent a severe form of CF disease.

The results obtained in the mouse study demonstrated that the 28-day treatment with fenretinide was able to normalize levels of AA in CF mice to the levels of WT mice in plasma, lungs and liver. DHA levels were improved in CF mice in plasma, lung and liver after treatment however the concentrations did not reach those seen in WT mice. The AA/DHA ratios were reduced in plasma and all CF related organs in CF mice. This suggests that even though AA and DHA levels were not significantly different in the ileum and pancreas following treatment, ultimately, the changes in fatty acid levels in each mouse led to an overall improvement of the AA/DHA ratio even in these organs. Fenretinide treatment had the greatest impact on AA concentrations out of the PUFAs studied causing a complete normalization of AA in CF mice. Interestingly, we saw that the difference in lipid deficiencies between ED and LD patients lays mainly in their plasma AA concentrations. This suggests that potential therapies should attempt to reduce concentrations of AA, and not only looking to increase DHA.

A study by Teichgräber and colleagues described increased levels of ceramide in other mouse models of CF and patients with CF [261]. In their study, ceramide was quantified with immunochemistry, while we have used a TLC-ELISA technique and confirmed our initial results with mass spectrometry [180]. Discrepancies in these two studies may be due to the method of analysis, the use of different antibodies (monoclonal versus polyclonal) and the ceramide species detected by each of the antibodies. All these parameters must be taken into account to reconcile the differences between the two studies. Their study suggested that our results showing lower ceramide levels were due to the use of a liquid diet, Peptamen. However, our data that strongly indicate this is not the case. We have never observed changes in ceramide in mice due to the diet, including Peptamen. We have illustrated that prolonged ingestion of Peptamen in CF mice is not associated with a decrease in ceramide levels in plasma (Figure S2.1A) and lungs (Figure S2.1B). We also tested plasma lipid levels in CF and WT mice on solid mouse chow, without ever having Peptamen throughout their lives. The defects in ceramide as well as PUFA were present in CF mice (Table S2.2). Moreover, none of our CF patients consume Peptamen, yet they have lower ceramide levels.

In addition to improving the lipid defects in CF, a 28-day treatment with fenretinide reduced the over-expression of inflammatory genes. As mentioned in the Chapter 1, IL-1β is abnormally increased in CF tissues. S100A8, also known as calprotectin, was also discovered to be increased in CF tissues, and was called the “CF antigen” prior to the discovery of the CF gene defect [262]. S100A8 is a neutrophil derived marker and has been found to be an indicator of high levels of inflammation [263]. We showed that fenretinide can decrease the expression of pro-inflammatory genes in the airways of our CF mouse model (Figure 2.4). We have identified

these two genes among others to be over-expressed at a basal level in CF mice developing spontaneous lung disease compared to their littermate WT controls [65]. It is unclear from our results whether correcting the lipid defects resets the proper function of the inflammatory response or whether fenretinide is acting independently of lipid pathways to regulate the expression of inflammatory genes.

CONCLUSION

Overall, the results of this study suggest that fenretinide should be considered as a potential therapy to correct the lipid imbalances in CF patients and reduce the over activated inflammatory response. Fenretinide's safety and efficacy have already been investigated in clinical trials for various cancers. The side effects consisted of reversible dermatological, gastrointestinal and ocular symptoms [264]. Regular, three-day drug-free intervals incorporated into treatment protocols prevent the development of these side effects. In a study with 2972 cancer patients, minimal side effects were observed over a 5-year period of daily 200 mg fenretinide [220]. Fenretinide doses up to 4000 mg/m^2 (approximately 33-fold higher than the 5 mg/kg fenretinide dose used in our study) were given during pharmacokinetic and toxicity studies in 54 children with neuroblastoma and resulted in manageable toxicity [265]. As these clinical trials have already included children, fenretinide offers the possibility to prevent severe pathology of CF disease and to reduce the lipid defects early in life. Due to the tolerable side-effects, convenient administration protocols, and its efficacy in a mouse model of CF, fenretinide offers a new potential treatment for CF.

ACKNOWLEDGEMENTS

We would like to thank the patients and volunteers for participating in this study. We are indebted to Chantal Savard and Christine Baichoo for collecting the blood samples from the CF patients and healthy volunteers. Gabriella Wojewodka received funding from the McGill University Health Center Research Institute studentship and a Provost's Graduate Fellowship from McGill University Graduate Training Committee and Dept. of Human Genetics. This study was funded by the Canadian Cystic Fibrosis Foundation.

Figure 2.1. Ceramide levels correlate with polyunsaturated fatty acid levels in plasma from CF patients. AA (panels A and C), DHA (panels B and D) and ceramide were analyzed in human plasma samples. A) In healthy controls (*upper panels, empty circles,* n = 72), no correlation was found between ceramide and AA ($p = 0.5750$) nor B) ceramide and DHA ($p = 0.2128$). C) In CF patients (*lower panels, empty triangles,* n = 58), ceramide levels inversely correlated with AA ($r = - 0.57$, $p < 0.0001$) and D) directly correlated with DHA ($r = 0.75$, $p < 0.0001$).

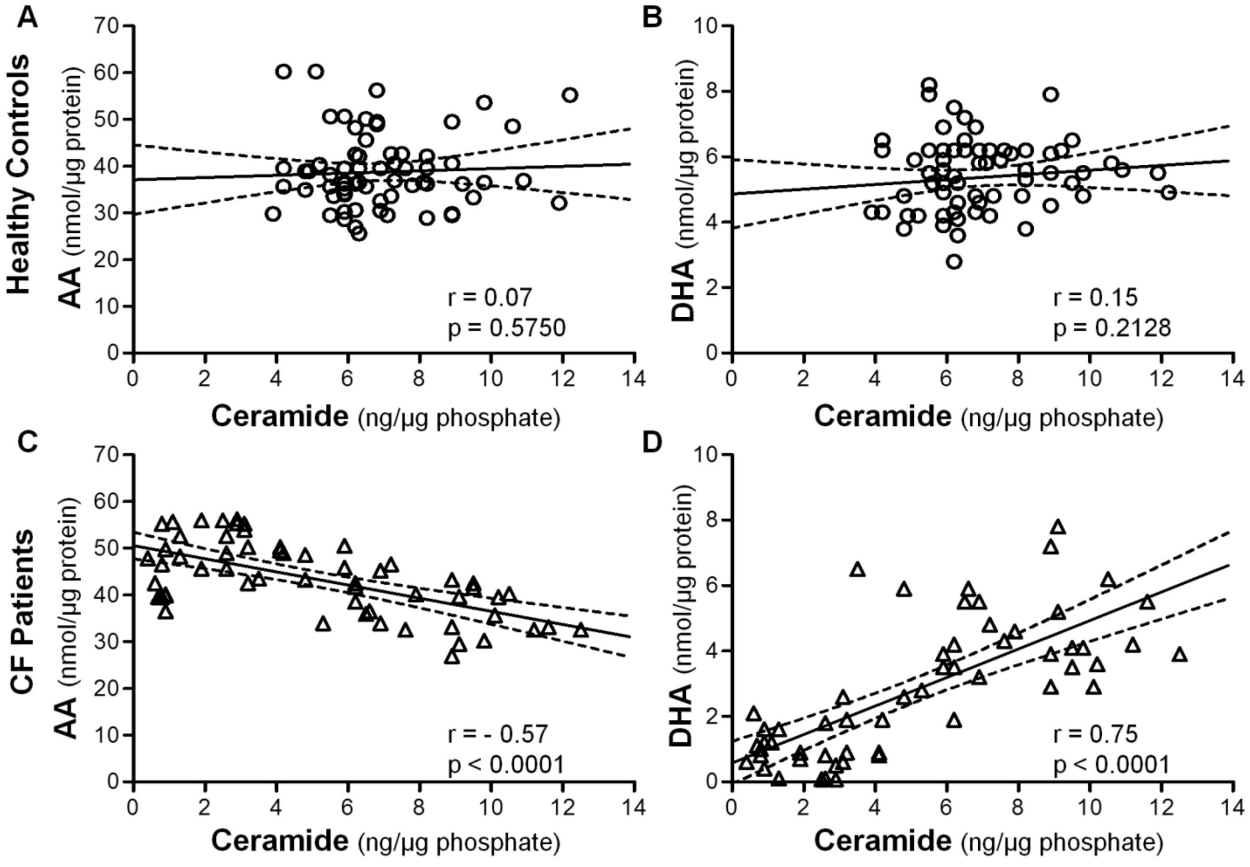

A
Healthy Controls
AA (nmol/µg protein)
r = 0.07
p = 0.5750
Ceramide (ng/µg phosphate)
B
DHA (nmol/µg protein)
r = 0.15
p = 0.2128
Ceramide (ng/µg phosphate)
C
CF Patients
AA (nmol/µg protein)
r = - 0.57
p < 0.0001
Ceramide (ng/µg phosphate)
D
DHA (nmol/µg protein)
r = 0.75
p < 0.0001
Ceramide (ng/µg phosphate)

Figure 2.2. Fenretinide improves polyunsaturated fatty acid defects in CF mice. PUFA concentrations in plasma were evaluated at 3 days post-infection in WT and CF mice infected with PA. A) AA concentrations, B) DHA concentrations and C) AA/DHA ratio were assessed in vehicle-treated (WT, *empty circles*, n = 11; CF, *empty triangles*, n = 11) or fenretinide-treated (WT-FEN, *filled circles*, n = 15; CF-FEN, *filled triangles*, n = 11) mice. The PUFA levels in plasma showed differences between the groups of mice tested (two-way ANOVA). The effect of the genotype was $p < 0.001$ and the effect of the treatment was $p < 0.001$ for AA, DHA and AA/DHA. *indicates a significant difference between groups according to the post-test.

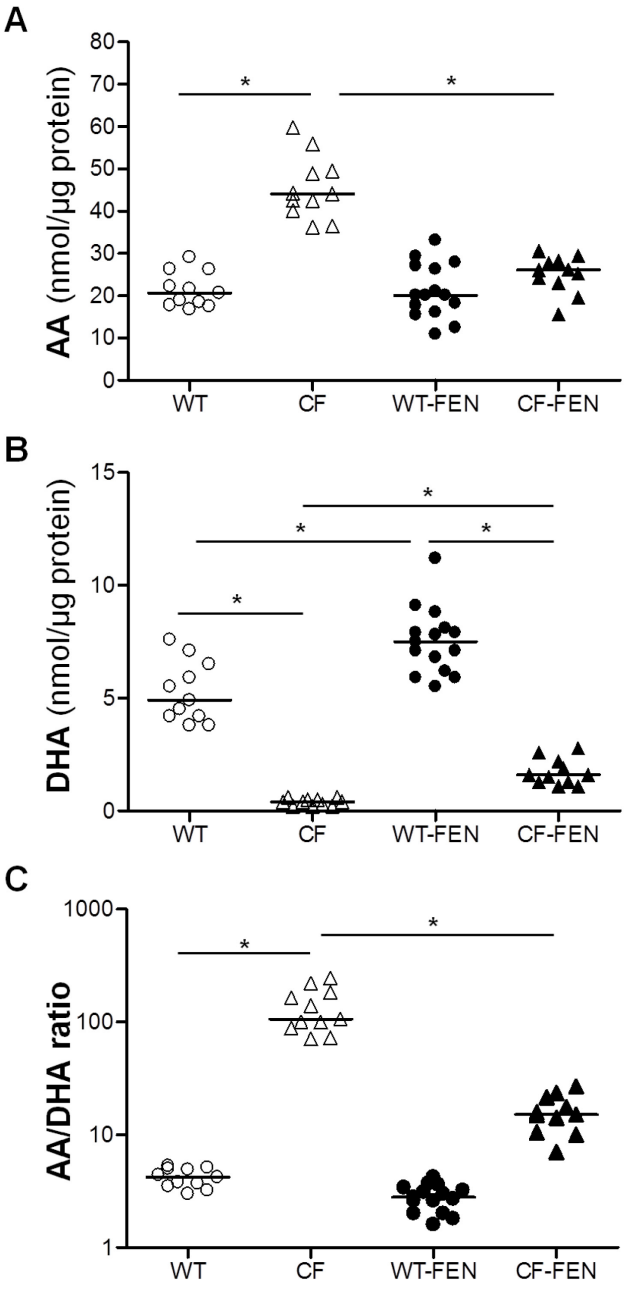

A
AA (nmol/µg protein)
80
70
60
50
40
30
20
10
0
*
*
WT
CF
WT-FEN
CF-FEN
B
DHA (nmol/µg protein)
15
10
5
0
*
*
*
*
WT
CF
WT-FEN
CF-FEN
C
AA/DHA ratio
1000
100
10
1
*
*
WT
CF
WT-FEN
CF-FEN

Figure 2.3. Ceramide levels correlate with polyunsaturated fatty acid levels in plasma from CF mice. AA (panels A and C), DHA (panels B and D) and ceramide were analyzed in mouse plasma samples from vehicle-treated mice (*empty symbols*) and fenretinide-treated (*filled symbols*) mice. A) In WT mice (*upper panels, circles*, n = 48), no correlation was found between concentrations of ceramide and AA ($p = 0.6461$). B) A correlation was observed between ceramide and DHA in WT mice ($r = 0.40$, $p = 0.0053$). C) In CF mice (*lower panels, triangles*, n = 30), ceramide levels inversely correlated with AA ($r = -0.70$, $p < 0.0001$) and D) directly correlated with DHA ($r = 0.54$, $p = 0.0020$).

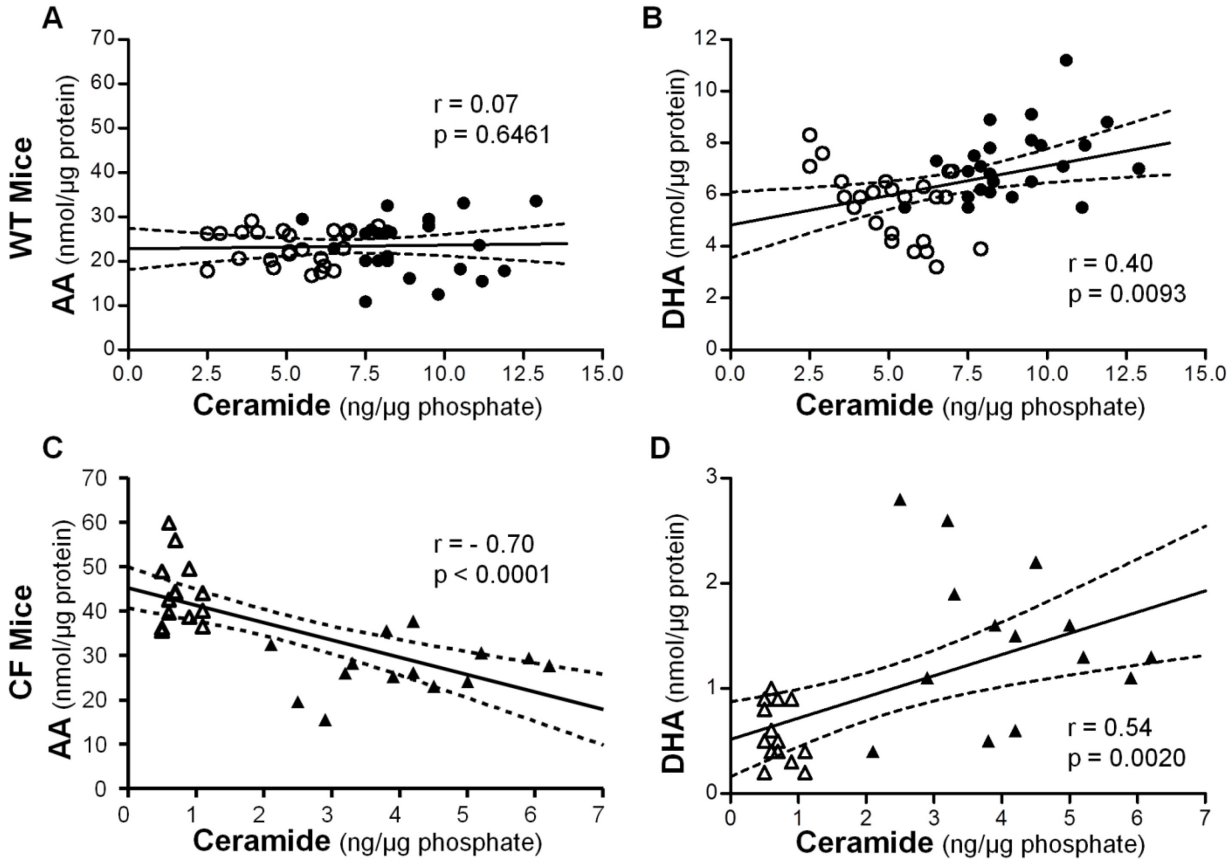

A
WT Mice
AA (nmol/µg protein)
r = 0.07
p = 0.6461
Ceramide (ng/µg phosphate)
B
DHA (nmol/µg protein)
r = 0.40
p = 0.0093
Ceramide (ng/µg phosphate)
C
CF Mice
AA (nmol/µg protein)
r = - 0.70
p < 0.0001
Ceramide (ng/µg phosphate)
D
DHA (nmol/µg protein)
r = 0.54
p = 0.0020
Ceramide (ng/µg phosphate)

Figure 2.4. Effects of fenretinide on mRNA expression of inflammatory genes in WT and CF mice. Gene expression was analyzed in lung tissue from fenretinide treated (FEN) mice (WT, *grey*, n = 3; CF, *white*, n = 3) and age-matched control (CTRL) mice (WT, *grey*, n = 5; CF, *white*, n = 5). A) Relative mRNA expression of IL-1β was statistically different between groups (p = 0.0021): WT and CF CTRL (*), CF CTRL and WT FEN (†) and CF CTRL and CF FEN (‡) B) Relative mRNA expression of S100A8 was statistically different between groups (p = 0.0021). * indicates significant difference between WT and CF CTRL, † CF CTRL and WT FEN, and ‡ CF CTRL and CF FEN. C) Analysis of GAPDH shows no statistically significant difference between groups (p = 0.21). GAPDH mRNA expression was used to standardize amounts of cDNA during the real-time PCR reaction.

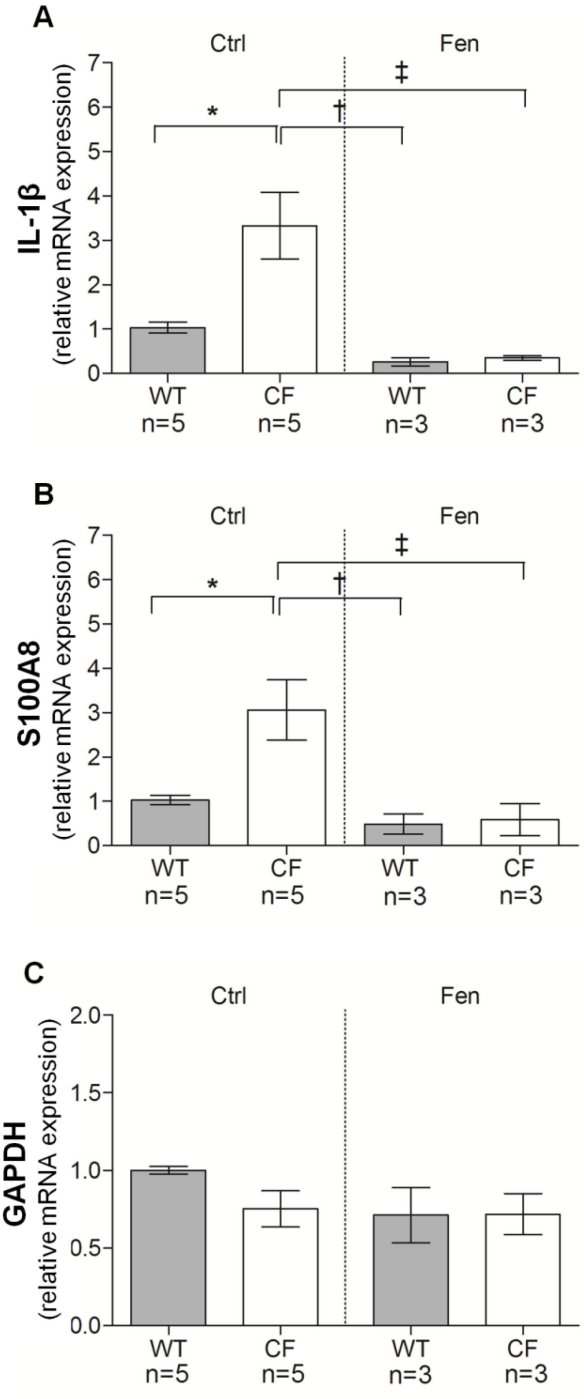

A
Ctrl
Fen
IL-1β
(relative mRNA expression)
0
1
2
3
4
5
6
7
*
†
‡
WT
n=5
CF
n=5
WT
n=3
CF
n=3
B
Ctrl
Fen
S100A8
(relative mRNA expression)
0
1
2
3
4
5
6
7
*
†
‡
WT
n=5
CF
n=5
WT
n=3
CF
n=3
C
Ctrl
Fen
GAPDH
(relative mRNA expression)
0.0
0.5
1.0
1.5
2.0
WT
n=5
CF
n=5
WT
n=3
CF
n=3

Table 2.1. Characteristics of healthy controls and CF patients

	Healthy controls (HC) n = 72	**Range** (min - max)	**CF patients (CF)** n = 58	**Range** (min - max)
Sex M/F	28/44	N/A	33/25	N/A
Age (yr)	38.3 (11.6)	23.0 – 69.4	32.3 (11.3)	18.0- 59.0
Weight (kg)	69.4 (15.7)	50.8 – 136.2	60.8 (14.7)	36.7- 120.0
Height (cm)	168.0 (10.5)	147.0 – 200.0	167.6 (9.1)	151.5.0- 188.0
BMI	24.7 (4.9)	15.5 – 48.2	21.5 (4.2)	14.7- 39.2

Data presented as mean (standard deviation, SD). N/A - Not Applicable.

Table 2.2. Lipid concentrations in healthy controls and CF patients

	Healthy controls (HC) n = 72	**CF patients (CF)** n = 58	**p-value**
AA (nmol/µg protein)	38.7 (7.8)	43.4 (7.8)	0.0004*
DHA (nmol/µg protein)	5.4 (1.1)	2.8 (2.1)	≤ 0.0010*
AA/DHA ratio	7.5 (1.1)	68.4 (152.8)	0.0007*
Ceramide (ng/µg phosphate)	6.9 (1.8)	5.1 (3.5)	0.0003*

Data presented as mean (SD). * Statistically significant difference between HC and CF.

Table 2.3. Comparison between early diagnosed and late diagnosed CF patients

	Early Diagnosed (ED: diagnosed < 18) n = 45	**Late Diagnosed** (LD: diagnosed ≥ 18) n = 13	**p-value**
Age at the time of diagnosis (Years)	2.3 (3.8)	34.4 (11.9)	≤ 0.0001*
BMI	20.9 (3.5)	23.5 (5.6)	0.0495*
CF-specific FEV_1 perc†	62.6 (28.7)	88.8 (14.3)	0.0025*
Pancreatic status‡ Sufficient Insufficient	11.1% 88.9%	38.5% 61.5%	0.0214*
AA (nmol/μg protein)	44.7 (7.7)	39.2 (6.7)	0.0237*
DHA (nmol/μg protein)	2.6 (2.1)	3.5 (2.0)	0.1792
AA/DHA ratio	82.2 (171.0)	20.4 (20.7)	0.2017
Ceramide (ng/μg phosphate)	4.6 (3.3)	6.7 (3.7)	0.0623

Data presented as mean (SD). * Statistically significant difference between the groups. †See Material and Methods, Human Study and Kulich et al., 2005 [248] ‡ p-value calculated using Chi-Square test

Table 2.4. Basal levels of lipids in WT and CF mice

		AA (nmol/µg protein)	**DHA** (nmol/µg protein)	**AA/DHA**
Plasma	**WT**	24.5 (3.2)	6.0 (1.3)	4.4 (1.7)
	CF	41.3 (5.2)	0.9 (0.1)	46 (5.7)
	p-value	< 0.0001*	<0.0001*	<0.0001*
Lung	**WT**	29.9 (6.8)	5.1 (1.3)	6.0 (1.4)
	CF	42.9 (4.0)	0.8 (0.2)	57.3 (14.2)
	p-value	0.0007*	<0.0001*	<0.0001*
Ileum	**WT**	22.5 (5.6)	2.7 (0.8)	9.1 (3.4)
	CF	34.5 (4.6)	0.5 (0.2)	72.1 (22.7)
	p-value	0.0003*	<0.0001*	<0.0001*
Pancreas	**WT**	26.7 (4.5)	4.5 (1.2)	6.4 (2.2)
	CF	34.8 (6.3)	0.5 (0.2)	85.8 (50.4)
	p-value	0.0059*	<0.0001*	<0.0001*
Liver	**WT**	22.6 (6.9)	14.8 (2.8)	1.6 (0.7)
	CF	36.9 (8.1)	2.9 (0.8)	13.3 (3.2)
	p-value	0.0009*	<0.0001*	<0.0001*

Data presented as means ± SD. WT: n = 11-13; KO: n = 5-6. * indicates statistically significant difference between WT and CF values.

Table 2.5A. Fenretinide-induced changes in AA concentrations in organs of CF mice

	Lung			Ileum			Pancreas			Liver		
	Ctrl	**Fen**	Treat. Sig. Diff.	**Ctrl**	**Fen**	Treat. Sig. Diff.	**Ctrl**	**Fen**	Treat. Sig. Diff.	**Ctrl**	**Fen**	Treat. Sig. Diff.
WT	19.6 (4.9)	20.1 (3.9)	NS	19.8 (4.5)	19.4 (5.5)	NS	23.7 (4.9)	22.6 (4.9)	NS	16.6 (3.7)	16.2 (3.2)	NS
CF	39.8 (6.3)	22.4 (4.9)	*	22.9 (6.3)	21.2 (5.3)	NS	32.4 (6.2)	23.8 (5.3)	NS	26.9 (3.4)	18.0 (3.4)	*
Geno. Sig. Diff.	†	NS		NS	NS		NS	NS		†	NS	
overall genotype effect p-value‡	**<0.001**			**0.504**			**0.452**			**<0.001**		
overall treatment effect p-value‡	**<0.001**			**0.805**			**0.484**			**<0.001**		

AA levels shown in nmol/µg protein. Data represented as mean (SD). WT: n = 10 - 15, KO: n = 10 – 12. NS: no statistically significant difference. * indicates a statistically significant difference between treatments (Treat. Sig. Diff.) within the same genotype (i.e. WT CTRL vs. WT FEN, CF CTRL vs. CF FEN). † indicates a statistically significant difference between genotypes (Geno. Sig. Diff.) within the same treatment (i.e. WT CTRL vs. CF CTRL, WT FEN vs. CF FEN). ‡ this row indicates the p-value from the two-way ANOVA for the effect of genotype (WT vs. CF) or the effect of treatment (CTRL vs. FEN) on AA levels.

Table 2.5B. Fenretinide-induced changes in DHA concentrations in organs of CF mice

	Lung			Ileum			Pancreas			Liver		
	Ctrl	**Fen**	Treat. Sig. Diff.	**Ctrl**	**Fen**	Treat. Sig. Diff.	**Ctrl**	**Fen**	Treat. Sig. Diff.	**Ctrl**	**Fen**	Treat. Sig. Diff.
WT	4.4 (1.5)	7.1 (1.3)	*	3.3 (1.1)	6.0 (2.0)	*	4.5 (1.9)	6.6 (1.1)	*	11.7 (4.0)	17.9 (4.9)	*
CF	0.3 (0.2)	1.4 (0.4)	*	0.3 (0.1)	1.4 (0.6)	NS	0.5 (0.2)	1.5 (0.7)	NS	1.0 (0.7)	6.5 (1.0)	*
Geno. Sig. Diff.	†	†		†	†		†	†		†	†	
overall genotype effect p-value‡	**<0.001**			**<0.001**			**<0.001**			**<0.001**		
overall treatment effect p-value‡	**<0.001**			**<0.001**			**<0.001**			**<0.001**		

DHA levels shown in nmol/µg protein. Data represented as mean (SD). WT: n = 10 - 15, KO: n = 10 – 12. NS: no statistically significant difference. * indicates a statistically significant difference between treatments (Treat. Sig. Diff.) within the same genotype (i.e. WT CTRL vs. WT FEN, CF CTRL vs. CF FEN). † indicates a statistically significant difference between genotypes (Geno. Sig. Diff.) within the same treatment (i.e. WT CTRL vs. CF CTRL, WT FEN vs. CF FEN). ‡ indicates the p-value from the two-way ANOVA for the effect of genotype (WT vs. CF) or the effect of treatment (CTRL vs. FEN) on DHA levels.

Table 2.5C. Fenretinide-induced changes in AA/DHA concentrations in organs of CF mice

	Lung			**Ileum**			**Pancreas**			**Liver**		
	Ctrl	**Fen**	Treat. Sig. Diff.	**Ctrl**	**Fen**	Treat. Sig. Diff.	**Ctrl**	**Fen**	Treat. Sig. Diff.	**Ctrl**	**Fen**	Treat. Sig. Diff.
WT	4.8 (1.6)	2.9 (1.0)	NS	6.4 (2.0)	3.5 (1.3)	NS	6.0 (2.5)	3.5 (0.9)	NS	2.0 (2.2)	0.9 (0.2)	NS
CF	221.2 (178.4)	21.1 (20.3)	*	111.0 (80.6)	16.4 (4.7)	*	50.3 (24.4)	18.0 (8.2)	*	34.4 (15.6)	2.8 (0.7)	*
Geno. Sig. Diff.	†	NS		†	NS		†	†		†	NS	
overall genotype effect p-value‡	**<0.001**			**<0.001**			**<0.001**			**<0.001**		
overall treatment effect p-value‡	**<0.001**			**<0.001**			**<0.001**			**<0.001**		

Data represented as mean (SD). WT: n = 10 - 15, KO: n = 10 – 12. NS: no statistically significant difference.
* indicates a statistically significant difference between treatments (Treat. Sig. Diff.) within the same genotype (i.e. WT CTRL vs. WT FEN, CF CTRL vs. CF FEN). † indicates a statistically significant difference between genotypes (Geno. Sig. Diff.) within the same treatment (i.e. WT CTRL vs. CF CTRL, WT FEN vs. CF FEN). ‡ indicates the p-value from the two-way ANOVA for the effect of genotype (WT vs. CF) or the effect of treatment (CTRL vs. FEN) on AA/DHA ratios.

ADDENDUM

Human study

Supplemental Table S2.1A. *CFTR* Genotypes of patients

CFTR genotypes	Number of CF patients
F508del/F508del	26
F508del/A455E	1
F508del/G85e	1
F508del/L206W	1
F508del/L558S	1
F508del/R334W	2
F508del/W1282X	1
F508del/Y1092X	1
F508del/3849+10kb C-T	1
F508del/621+1G=T	1
F508del/711+1G-T	1
F508del/ unidentified *	10
G85e/ G85e	1
R334W/ unidentified	1
W1282X/W1282X	1
621+1G=T/L206W	2
unidentified / unidentified	5
not tested/not tested **	1

* Unidentified mutation means that it could not be identified by the specific PCR kit (Tag-It™ Cystic Fibrosis 40+4 Test) that detects 44 mutations. ** not tested indicates a CF diagnosis based on sweat test results.

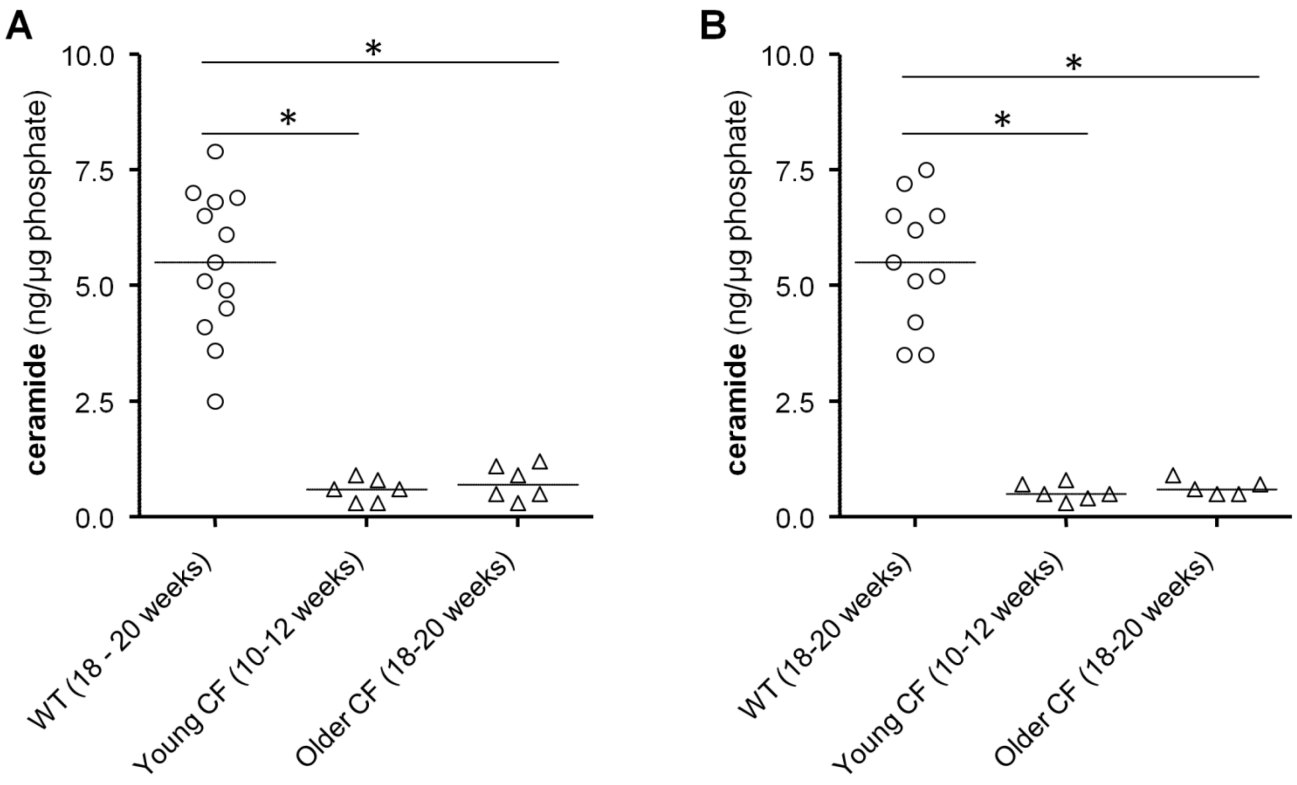
Supplemental Table S2.1B. *CFTR* Genotypes of patients based on age of diagnosis

Genotype	number of patients ED	number of patients LD
F508del/F508del	24	2
F508del/A455E	0	1
F508del/G85e	1	0
F508del/L206W	0	1
F508del/L558S	1	0
F508del/R334W	1	1
F508del/W1282X	1	0
F508del/Y1092X	1	0
F508del/3849+10kb C->T	0	1
F508del/621+1G=T	1	0
F508del/711+1G--T	1	0
F508del/unidentified*	6	4
G85e/G85e	0	1
R334w/unidentified	1	0
W1282X/W1282X	1	0
621+1G>T/ L206W	1	1
unidentified/unidentified	4	1
not tested/not tested**	1	0
total	45	13

* Unidentified mutation means that it could not be identified by the specific PCR kit (Tag-It™ Cystic Fibrosis 40+4 Test) that detects 44 mutations. ** not tested indicates a CF diagnosis based on sweat test results.

Mouse study

Supplemental Figure S2.1. Analysis of ceramide concentrations in young and old CF mice. Ceramide levels in young CF mice (young CF) ages from 10 to 12 weeks and of older CF mice (older CF) ages from 18 to 20 weeks were analyzed. We found no differences between ceramide levels in A) plasma and B) lung of young CF mice and older CF mice. Both groups were significantly different than WT mice (A. plasma ANOVA $p < 0.0001$; B. lung ANOVA $p < 0.0001$). * indicates differences of $p < 0.001$ as per Bonferroni post-test.



Supplemental Table S2.2. Peptamen does not induce lipid defects in CF mice. Peptamen has a different nutritional breakdown than regular chow with more fat (1.43-fold higher) and less protein (0.69-fold lower). We wanted to verify that Peptamen was not causing the lipid defects seen in CF mice. CF mice were not given the liquid diet at any point in their lives. These mice were closely monitored for any signs of discomfort. Despite not being fed Peptamen, the fatty acid defects in blood plasma were still observed in CF mice. AA levels were significantly higher in CF mice than in WT mice, DHA was significantly reduced and the AA/DHA ratio was reduced.

	WT mice n = 4	**CF mice n = 3**	**p-value**
AA (nmol/µg protein)	40.18 ± 1.67	45.70 ± 3.20	0.0293*
DHA (nmol/µg protein)	4.00 ± 0.27	2.00 ± 0.20	0.0001*
AA/DHA	10.08 ± 0.74	23.03 ± 2.69	0.0002*
Ceramide (ng/µg phosphate)	9.05 ± 1.21	4.63 ± 0.76	0.0027*

* Indicates statistically significant difference between WT and CF groups as determined by the Student's t-test.

In Chapter 2, we presented our results regarding fenretinide's effects on improving lipid defects and reducing inflammation [71]. However, many questions remained about the mechanism of fenretinide. In particular, how does fenretinide affect lipid levels? And, would its effects differ from one species to another? In the following chapter, we establish how one dose of fenretinide has prolonged effects on polyunsaturated fatty acids in mice. We also confirm the drug effects on cells from CF patients and we demonstrate that fenretinide reduces high levels of lipid peroxidation and nitrosative stress in mice and in patient tissues. We present data on determining an appropriate formulation for fenretinide treatment in patients by first testing it in monkeys. These experiments were necessary to improve our understanding of fenretinide and the timing of its impact on lipids to present a dosing strategy for clinical trials. Additionally, we find that fenretinide can address other CF phenotypes: high levels of lipid peroxidation and nitrosative stress.

CHAPTER 3: FENRETINIDE REDUCES LIPID PEROXIDATION AND NITROSATIVE STRESS IN CYSTIC FIBROSIS

Based on the manuscript
Wojewodka G., De Sanctis J. B., Hajduch M., Radzioch D. “Fenretinide reduces lipid peroxidation and nitrosative stress in cystic fibrosis” Manuscript in preparation.

ABSTRACT

Cystic fibrosis (CF) patients experience high levels of oxidative and nitrosative stress caused by the over-activation of inflammatory cells, unabated inflammatory responses, chronic infections and low antioxidant defenses. The generation of reactive radicals impact the functioning of proteins, induce lipid peroxidation and cause tissue damage. We hypothesized that fenretinide improves the polyunsaturated fatty acid (PUFA) defects in CF in part by reducing the levels of lipid peroxidation and nitrosative stress.

We assessed the impact of one 5 mg/kg dose of fenretinide in mice to determine how rapidly the drug affects lipid defects. We monitored the levels of AA, DHA and ceramide at specific time points after drug treatment. Additionally, we measured the levels of malondialdehyde (MDA) and 3-nitrotyrosine (NT3) in the same samples. We also confirmed the effects of the drug in leukocytes isolated from 10 CF patients and 11 healthy controls. We tested two formulations of fenretinide in monkeys to determine a potential formulation for patients.

We found that one dose of fenretinide caused rapid and prolonged effects on PUFA and ceramide in mice. The treatment effects on AA lasted up to 90 h while DHA levels began to worsen after 36 h. Leukocytes from CF and healthy controls both responded to treatment with reductions in AA and increases in DHA and ceramide. Fenretinide treatment reduced MDA and NT3 levels in both mice and cells. While testing two fenretinide formulations in monkeys, we found the drug treatment also decreased the AA to DHA ratio.

Fenretinide impacts PUFA and ceramide long after treatment. We found that fenretinide exerts its effects on PUFA in two animal species and in CF patient cells. The drug

also reduces the levels of lipid peroxidation and nitrosative stress which are important phenotypes of CF currently unaddressed by routine CF treatments. These results add to the benefits of fenretinide as a treatment for CF patients.

INTRODUCTION

Oxidation is a major contributor to CF disease causing pathological damage when left unabated. CF patients have high levels of oxidative stress due to the combination of high levels of inflammation, chronic bacterial infections, activated immune cells and reduced antioxidant mechanisms [97]. Studies have shown that superoxide dismutase activity was reduced in plasma of CF patients compared to controls and reactive oxygen species (ROS) levels in immune cells were elevated [266]. Lipid peroxidation occurs when free radicals attack the back bone of polyunsaturated fatty acids (PUFA) [267] which results in the production of malondialdehyde (MDA), the omega-6 oxidation product 4-hydroxynonenal and the omega-3 product 4-hydroxyhexenal [268]. Lipid peroxidation is an important contributor to disease as it disrupts the cell membrane which leads to tissue damage [269]. MDA is elevated in CF patients [97,270,271] and can cross-link to proteins modifying their function [268]. Other by-products of lipid oxidation cause autoactivation of tyrosine kinases [272]. 4-hydroxyhexenal was found to cause the abnormal activation of NF-κB and increased ERK1/2 phosphorylation [273]. Thus it is vital that an increase in omega-3 PUFA in CF is accompanied with decreased lipid peroxidation since the oxidation products of PUFA have detrimental effects.

Higher levels of nitrosative stress were documented in CF with elevated nitrotyrosine (NT3), myeloperoxidase and nitrate [274-276]. NT3 occurs as a result of the interaction between a reactive nitrogen species with a tyrosine residue on proteins. There are many ways

this can occur such as the direct action of NO_2 on tyrosine, or NO with a tyrosine radical. Additionally, NT3 can be formed by the interaction of peroxynitrite with proteins [277]. Peroxynitrite is formed by the reaction between superoxide and NO. Incidentally, NO levels in exhaled breath of CF patients are low compared to healthy subjects [275,276]. This may be explained by the high levels of superoxide produced in CF patients by activated neutrophils and macrophages, which interact with NO to produce more peroxynitrite than normal leading to less exhaled NO. In turn, more peroxynitrite results in a higher production of NT3 [278]. The post-transcriptional modifications on tyrosine leading to the formation of NT3 cause structural and functional changes such as protein inhibition, gain-of-function or increased degradation. For example, the modification of tyrosine in cytochrome c induces peroxidase activity of the protein, while inhibiting its capacity for electron transfer [279].

There are currently no therapies directed at reducing oxidative and nitrosative stress in CF. Retinoids have known antioxidant capacities [280,281] thus we hypothesized that fenretinide would also have this effect. We also postulated that by reducing the oxidation status in CF tissues, the defects in PUFA may be corrected especially DHA which is particularly susceptible to peroxidation.

We thus tested the effects of one dose of fenretinide in mice to determine the kinetics of the changes in lipids, and whether they would coincide with improvements in lipid peroxidation and nitrosative stress. We also evaluated the effects of the drug on leukocytes isolated from CF patients and controls to confirm that fenretinide affects human tissues in similar ways. We then tested different formulations of fenretinide in monkeys and mice to better evaluate how this drug could be administered in CF patients. In this chapter, we will

demonstrate that fenretinide has prolonged effects on lipids in both animal models and human cells, and that markers of lipid peroxidation and nitrosative stress are also reduced.

METHODS

Fenretinide formulations

We used two forms of fenretinide, [N-4-hydroxyphenyl retinamide, 4-HPR], in these studies. We used fenretinide as a powder (provided by Dr. R. Smith, NIH). Depending on the animal model, the powder was either dissolved in 95% ethanol to a stock solution of 20 mg/mL or used without dissolving.

We also tested the capsule formulation of fenretinide (generously provided by the NIH, Bethesda, USA) which was previously used in clinical trials for cancer. It contains 100 mg of fenretinide powder suspended in 1 mL of corn oil/polysorbate 80 (known as McNeil corn oil capsules). For use in experiments, we extracted the contents of the capsules via syringe and analysed the concentration of fenretinide in the solution obtained which was approximately 70 mg/mL.

Mouse study

Mouse maintenance. *Cftr* wild-type (WT), heterozygote (HZ) and knockout (CF) mice were maintained in ventilated cages on corn-bedding. As CF mice are infertile, HZ mice were used for breeding. Due to their intestinal phenotype, CF mice were on a liquid diet (Peptamen, Nestle, Brampton, Canada) for better nutrient absorption and prevention of intestinal blockage. WT and HZ mice were maintained on regular chow and placed on the liquid diet when used in experiments with CF mice.

Fenretinide treatment. In all experiments in this chapter, mice received one dose of fenretinide by gavage. For a dose of 5 mg/kg, the fenretinide/ethanol stock solution was diluted 1:40 in Peptamen. We also used a dose of 20 mg/kg which allowed for a better detection of the bioavailability of the drug in blood. For this dose, the fenretinide/ethanol solution was diluted 1:10 in Peptamen for gavage. The fenretinide solution extracted from the McNeil capsule was given at a dose of 20 mg/kg in our mouse experiment. The McNeil capsule extract solution was diluted 1:35 in Peptamen. In all cases, the final volume of the fenretinide solutions administered to each mouse was determined by weight. For kinetic experiments, mice were euthanized at 4, 9, 18, 24, 36, 48, 72 and 90 h post-treatment (n = 4 per time point). For the assessment of fenretinide bioavailability using different formulations, mice were euthanized 4 h after gavage. All procedures were approved by the Facility Animal Care Committee of the Montreal General Hospital Research Institute (Montreal, Canada).

Human study

We obtained 10 mL of blood in EDTA coated tubes from 10 CF patients and 11 healthy controls from the CF Clinic at the Department of Pediatrics (Palacky University in Olomouc, Olomouc, Czech Republic). Patients were between 18 and 21 years old. Blood samples were centrifuged at 3000 rpm for 10 min. Plasma was collected and 100 µL was stored in 1 mL of 1 mM butylated hydroxyanisole in a chloroform/methanol solution (2:1 vol/vol) for lipid analysis. Leukocytes were obtained by isolation of the buffy coat. Any leftover erythrocytes were lysed. Leukocytes (1×10^6 cells per treatment group) were treated for 24 h after which they were collected into 1 mL of 1 mM butylated hydroxyanisole in a chloroform/methanol solution (2:1 vol/vol) for lipid analysis. Cells from healthy controls were treated with either 1 µM (n = 10) or

2.5 µM (n = 11) of fenretinide dissolved in 95% ethanol. All CF cells were treated with 2.5 µM of the drug and cells from 8 patients with 1 µM. Control cells were treated with 95% ethanol corresponding to the 2.5 µM dose. All procedures involving human subjects were approved by the Palacky University Research Ethics Board.

Monkey study

Monkey maintenance. Four healthy *Macaca fascicularis* monkeys were used for the kinetic experiments using both the fenretinide powder and McNeil capsule extract. They were housed and maintained according to the guidelines of the McIyntre Animal Facility at McGill University (Montreal, Canada).

Fenretinide treatment. The appropriate amount of fenretinide powder corresponding to a 5 mg/kg dose was weighed out for each animal and placed into a 10 mL syringe (one per animal). The syringes were then filled with 10 mL of Peptamen and vigorously shaken. For the experiments testing the capsule formulation of fenretinide, the fenretinide capsule extract was diluted 1:20 in Peptamen. The appropriate volume of this solution was calculated based on the weight of each animal for a dose of 5 mg/kg. Extra Peptamen was added to the dose to make up a final volume of 10 mL. For both treatment types, each monkey received an additional 10 mL Peptamen as a chaser after the drug gavage. Following fenretinide treatment, blood samples were collected at 1, 3, 4 and 6 h post treatment and plasma was isolated from the samples. All procedures were approved by the Facility Animal Care Committee of the McIntyre Animal Facility at McGill University (Montreal, Canada).

Fenretinide analysis

To prepare the samples for fenretinide analysis, acetonitrile was added to blood plasma samples. The acetonitrile contained 4-EPR, a synthetic compound similar in structure to fenretinide used as an internal control. Two methods were used to detect fenretinide: high pressure liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS). The HPLC used was a Waters Alliance 2690 Separation Module (Waters, Milford, MA, USA) [224,282]. The LC-MS analysis was performed using the API-4000 MS (Life Technologies, Burlington, Canada). The method used will be specified in the results section for each experiment. Results are presented as µM.

Lipid analysis

PUFA and ceramide analysis. Sample preparation and lipid analyses are described in detail in Chapter 2. Briefly, 100 µL of plasma (from mouse, human and monkey) was added to 1 mL of 1 mM butylated hydroxianisole solution containing 2:1 vol/vol chloroform/methanol. Lipids were extracted from the samples using the Folch method [251]. The fatty acids in the organic fraction were esterified and hydrolyzed using diazomethane and methanol as described by Schlenk and Gellerman [254] and the esters were identified by gas chromatography - MS (Hewlett Packard 5880A, WCOT capillary column (Supelco-10, 35 m×0.5 mm, 1 µm thick)) using commercial standards (Sigma-Aldrich, Oakville, ON, Canada) [283]. Protein levels were measured and used to normalize PUFA levels. Arachidonic acid (AA) and docosahexaenoic acid (DHA) concentrations are expressed as nmol/µg protein. Ceramide levels were measured by ELISA as described in Chapter 2 and normalized by the levels of phosphate. Ceramide levels are expressed as ng/µg phosphate.

Lipid peroxidation analysis.

The assessment of lipid peroxidation was measured indirectly using the thiobarbituric acid reactive species (TBARS) assay where MDA reacts with thiobarbituric acid to form a compound which can be measured fluorometrically. A 20 µL aliquot of the lipid extract was added to equal volumes of 8.1% sodium dodecyl sulfate, 20% acetic acid and 0.8% thiobarbituric acid. The samples were vortexed and incubated for 1 h at 95°C. Subsequently, 15:1 butanol/pyridine (vol/vol) was added and the mixture was shaken for 10 min and then centrifuged. The fluorescence of the butanol-pyridine layer was measured at 552 nm (515 nm excitation) in a fluorimeter (Shimadzu, Japan). The values of TBARS were normalized per amount of protein and are expressed as nmol of TBARS/mg of protein.

Nitrotyrosine analysis

The total amount of NT3 was determined by ELISA. NT3 concentrations were assessed using a standard curve of known amounts of NT3 from chemically modified bovine serum albumin. The sensitivity of the assay was 50 pg/mL. The antibodies used were polyclonal anti-NT3, monoclonal mouse IgG and polyclonal goat anti-rabbit IgG-peroxidase (Upstate Biotechnology, Millipore, Mississauga, Canada). The levels of NT3 were normalized by the amount of protein and are expressed as ng/mg of protein.

Statistical analysis

Statistics were analyzed using Prism 4 (GraphPad, Lajolla, USA). The one-way ANOVA test with Bonferroni post-test was used to determine differences between three or more groups. The Pearson test was used to determine correlations. Significance was set at $p < 0.05$.

RESULTS

The severity of lipid defects is related to the number of mutated *Cftr* alleles in mice

Freedman and colleagues discovered mild defects in fatty acids in carriers of *CFTR* mutations [119]. We hypothesized that our HZ mice may also have intermediate defects in PUFA, in between CF and WT levels. If so, these mice would be a good model to assess the effects of fenretinide since they represent about 50% of our animals resulting from breeding. We assessed the levels of AA and DHA in blood plasma and lung tissue from WT, HZ and CF mice. Indeed, HZ mice had significantly higher AA levels in blood plasma than WT mice but lower levels than CF mice (Figure 3.1A). DHA levels in HZ mice were also between WT and CF values (Figure 3.1B). Overall, the ratio was lower in HZ than CF mice and tended to be higher than in WT mice however the difference between the two was not statistically significant (Figure 3.1C). Similarly in lung tissue, HZ mice had intermediate levels of PUFA, between WT and CF concentrations (Figure 3.1A and B). HZ mice also had intermediate defects in ceramide levels in plasma and lung with lower levels than WT but higher than CF mice (Figure 3.1D). We concluded that HZ mice would be good models to study fenretinide's effects since we would be able to observe improvements in lipid defects.

A single dose of fenretinide has prolonged effects on lipids

In our previous studies on fenretinide and lipids, we treated the mice for 28 consecutive days (see Chapter 2). To better evaluate the dosing strategy for future studies, we assessed the impact one dose of fenretinide would have on lipids using HZ mice. A single dose of 5 mg/kg of fenretinide was given via gavage. Mice were euthanized at specific time points post-treatment (4, 9, 18, 24, 36, 48, 72 and 90 h). Lipid levels were analyzed in both blood

plasma and lung tissue (Figure 3.2). Fenretinide concentrations were measured in blood plasma by HLPC (Figure 3.2A). Drug levels were highest at 4 h (0.73 μM ± 0.28) and were reduced by approximately 30% at the 9 h time point (0.50 μM ± 0.16). Although fenretinide levels were being eliminated from plasma over time, the effects of fenretinide on lipids were longer lasting. AA levels in plasma were significantly decreased by 9 h post-treatment. After 36 h, a plateau was reached where AA levels at this and subsequent time points were no longer statistically different from each other (Figure 3.2B). The changes in AA occurred with similar kinetics in plasma and lungs and remained at their improved levels up to 90 h.

DHA levels responded to treatment by 9 h in both tissues (Figure 3.2C). However, fenretinide induced a more pronounced increase in DHA in plasma, albeit the levels in the lungs eventually reached plasma equilibrium by 36 h. From this time point, DHA levels in plasma began to decline while they peaked in the lung at 72 h post-treatment. The AA/DHA ratio decreased significantly by 9 h and stabilized by 18 h (Figure 3.2D). As with the DHA kinetic, the improvements in the AA/DHA ratio in the lung occurred later than in plasma and eventually stabilized at 24 h post-treatment.

The impact of fenretinide on ceramide was delayed compared to PUFA (Figure 3.2E). Noticeable increases in ceramide occurred 18 h after drug treatment. Plasma ceramide levels rose faster than in the lungs, yet equilibrated by 48 h. At this time point, ceramide levels began to decline in both tissues.

Lipid peroxidation and nitrosative stress decrease with fenretinide treatment

It is well described that CF patients experience high levels of oxidative stress [97,284] and nitrosative stress [275,276,285]. PUFAs, in particular DHA, are quite susceptible to lipid

peroxidation [286,287] and could explain in part the lipid imbalance observed in CF. In the same kinetic experiment as above, we measured MDA, a marker of lipid peroxidation, and NT3, a marker of nitrosative stress, to determine whether fenretinide exerts effects on these markers. Fenretinide decreased the levels of MDA in both plasma and lung samples (Figure 3.2F). These changes occurred by the 9 h time point, similar to the changes observed in PUFA. The reductions in MDA stabilized by 24 h post-treatment in plasma and 18 h in the lung. Plasma NT3 levels decreased at the 9 h time point and stabilized by 36 h in plasma (Figure 3.2G). In the lung, NT3 levels were also significantly decreased by 9 h and remained relatively stable after 24 h.

MDA and NT3 levels in plasma and lung were significantly correlated with each other and with AA, DHA and the AA/DHA ratio (Table 3.1) indicating that when the imbalance between AA and DHA is large, there is also a high level of lipid peroxidation and nitrosative stress.

Fenretinide improves lipid defects in leukocytes from CF patients

To further examine the effects of fenretinide in CF, we treated leukocytes isolated from CF patients and healthy controls. We first assessed the defects in lipids in plasma from CF patients, from the Palacky University CF clinic (Olomouc, Czech Republic) (Figure 3.3). We also observed elevated levels of MDA and NT3 in plasma from CF patients compared to controls (Figure 3.3E and F). This analysis allowed us to confirm the lipid defects in another CF patient population, complementing our results from Chapter 2.

We tested two doses of fenretinide based on the levels reported in patient clinical trials. Using a dose of 3 mg/kg, plasma levels of fenretinide corresponded to an average of 0.99 μM ± 0.06 in cancer patients [288]. Since we envision treating patients with a slightly higher

dose (5 mg/kg), we treated cells with two doses: 1 μM and 2.5 μM. Using two doses allowed us to determine whether the effects on lipids is dose dependent and whether the higher dose would have detrimental effects. Interestingly, although we did not observe significant defects in AA between untreated CF and control cells (Figure 3.4A), DHA was significantly lower in untreated CF cells compared to controls (Figure 3.4B). Overall, the AA/DHA was significantly higher in untreated CF cells than control cells (Figure 3.4C). Ceramide levels were also reduced in untreated CF cells compared to control cells (Figure 3.4D).

In general, fenretinide improved the lipid defects in CF cells, as seen in the mouse model. The decreases in AA levels in CF cells were dose dependent (Figure 3.4A). DHA levels increased in CF cells with both treatments but only the higher dose was significantly higher than untreated cells (Figure 3.4B). We observed an increase in DHA increase in control cells as well. The AA/DHA ratio was significantly reduced with both doses, but the 2.5 μM dose caused a greater reduction in CF cells (Figure 3.4C). Ceramide levels increased in both cell types with ceramide levels in CF cells reaching normal values with the 2.5 μM dose of fenretinide (Figure 3.4D). No cytotoxicity caused by the drug was observed with either of the two tested concentrations.

Fenretinide reduces lipid peroxidation in CF leukocytes

We assessed MDA and NT3 in CF and control leukocytes to evaluate whether fenretinide has antioxidant properties in human cells, as we have seen in our mouse experiments. MDA levels were significantly higher in untreated CF cells than control cells (Figure 3.4E). The reductions in MDA were dose dependent with 2.5 μM of fenretinide inducing a normalization of lipid peroxidation levels in CF cells. NT3 was also significantly higher in

untreated CF cells compared to healthy controls (Figure 3.4F). Interestingly, both doses of fenretinide reduced NT3 in control cells. The NT3 levels in CF cells also had a tendency to drop with the higher dose but this decrease did not reach statistical significance. However the final concentrations of NT3 using the 2.5 μM dose reached control levels.

Fenretinide improves PUFA levels in monkeys

For our mouse studies, we used a stock solution of fenretinide dissolved in 95%, ethanol, which was then diluted to final concentration containing 2.4% ethanol (1:40 dilution). The exposure to even such a small concentration of ethanol given on a daily basis would not be considered a suitable drug formulation for humans or primates. Thus we performed a kinetic experiment in monkeys to test different formulations of the drug and their impact on lipids (Table 3.2). We used the extracted contents from the McNeil fenretinide capsule previously used in cancer clinical trials and fenretinide powder not dissolved in ethanol. Each formulation was added to Peptamen as described in the Methods section. Since these monkeys were healthy with no diseases or abnormalities, we considered their lipid concentrations to be normal. The McNeil capsule formulation seemed to have greater effects on plasma lipids than the powder. The McNeil capsule formulation induced significant decreases in AA levels from baseline values at 6 h post-treatment, while the powder formulation induced only a slight effect on AA. The McNeil capsule formulation caused a greater increase in DHA than the powder with a significant increase from baseline values at 6 h post-treatment. Overall, the McNeil capsule formulation had a greater impact on reducing the AA/DHA ratio than the powder, with significant decreases observed as early as 4 h post-treatment. There were no effects on ceramide with either formulation at these early time points. In addition, we measured the

concentration of fenretinide by LC-MS in plasma samples from monkeys treated with the McNeil capsule formulation at a dose of 5 mg/kg. And we found that at 1 hour there was 0.01 μM ± 0.02 and at 4 hours there was 1.44 μM ± 0.51 of the drug in plasma.

Treatment with dissolved fenretinide has the highest bioavailability

Our results assessing bioavailability in monkeys demonstrated that the McNeil capsule extract in Peptamen had a greater effect on PUFA levels than the formulation which consisted of fenretinide powder directly suspended in Peptamen. Some cancer trials have reported poor bioavailability of fenretinide using the capsules [224], thus we proceeded to compare the bioavailability of fenretinide in HZ mice using three formulations: fenretinide/ethanol, McNeil capsule extract and powder suspension. The drug concentrations were measured by LC-MS in plasma obtained from HZ mice 4 h after one treatment (Table 3.3). We first compared the effects of two different doses of the fenretinide/ethanol formulation on drug concentrations in the blood of treated animals. We found the concentration of fenretinide after a 20 mg/kg dose was 5.41-fold higher than with the 5 mg/kg dose. Then we compared the McNeil capsule formulation to fenretinide/ethanol at a dose of 20 mg/kg. The McNeil capsule had a lower bioavailability at 1.63 μM ± 0.31 which represents approximately half of the fenretinide/ethanol formulation. The worst formulation was the powder suspension in Peptamen, which explains the little impact on PUFA in the monkey study (Table 3.2).

DISCUSSION

The experiments presented in this chapter were conducted to better understand the effects of fenretinide on PUFAs. Our major hurdle was the limited number of CF mice available

to run large kinetic experiments. It was previously shown that carriers of *CFTR* mutations also have abnormal PUFA levels, intermediate between healthy and CF values [119]. Thus we hypothesized and showed here, that our HZ mice also have moderate defects in PUFA, which facilitated the study of the effects of fenretinide.

One dose of fenretinide and lipid kinetics

Our kinetic experiments were important in determining dosage and sample collection strategies for clinical trials. We found that fenretinide plasma concentrations were at their highest at the 4 h time point and decreased rapidly from this time. These results are similar to previous pharmacokinetic studies. In rats, the concentration of fenretinide in plasma peaked at 4 h post-treatment using a 1:5 ethanol/corn-oil vehicle [289]. Various concentrations of fenretinide (100 to 4000 mg/m^2) peaked at a median of 4 h in plasma of neuroblastoma patients following a single dose using the fenretinide capsule, ranging from 3 to 16 h depending on the dose. However, the timing of the peaks was not statistically different between the doses [265,290].

Fenretinide has been studied in the context of many diseases particularly cancer where high doses were used *in vitro* or *in vivo*, including clinical trials. These high doses were used as chemotherapy to achieve cell death. Compared to cancer studies, our dose is relatively low as we seek to improve the lipid imbalance as opposed to inducing cell death. While pharmacokinetic studies have been performed with various doses, none have reported the kinetics of lipids following fenretinide treatment. Our mouse kinetic experiment showed that 18 h following treatment, fenretinide was no longer detected blood samples from mice. However, there were prolonged effects on lipids with improvements lasting 90 h. The

improvements in AA, DHA, MDA and NT3 began at the 9 h time point. The normalization of AA levels persisted until 90 h following treatment, however after 36 h DHA levels in plasma began to drop again. Ceramide levels also began to decrease 48 h after treatment. These results suggest that fenretinide should be administered at least every 36 h, if not more frequently, to maintain the improvements in both DHA and ceramide.

Fenretinide was found to rapidly equilibrate to plasma levels in certain organs in particular the liver, pancreas and lungs [289] which are CF-affected tissues. Interestingly, changes in PUFA were noticeable in both plasma and lung tissues at similar time points, although fenretinide treatment had a more pronounced impact on DHA in plasma at the earlier time points (Figure 3.2). Eventually similar peak concentrations of DHA were reached in both tissues, although at different time points. We recognize that we did not sample very many early time points thus fenretinide may be affecting lipids much faster in these tissues than we have concluded based on the data collected.

Fenretinide and lipid peroxidation

As we have shown in this chapter, lipid peroxidation levels in CF patients were found to be elevated with high levels of MDA in plasma from CF patients [271,291]. High levels of lipid peroxidation were observed in CF patients with normal levels of antioxidants such as the vitamins A, C and E [291]. The supplementation of β-carotene reduced the levels of MDA in CF children, although they still remained higher than normal levels in non-CF children [271].

Fenretinide decreased lipid peroxidation in HZ mice and CF leukocytes, suggesting a potential mechanism by which fenretinide improves DHA levels. Our human study using leukocytes revealed significant impacts of 24 h treatment with fenretinide on MDA levels, but

not NT3 (Figure 3.4). Despite no significant change in NT3, the PUFA imbalance still improved suggesting a strong link with lipid peroxidation in these cells. NT3 levels did tend to decrease and seemed to reach normal levels using the higher dose of fenretinide.

The mechanism of action of fenretinide has not been clearly established and it may vary depending on the dose of the drug used and on the expression of receptors specific to tissue type which are likely to be engaged in drug response [234]. The effects of fenretinide have been shown to vary even *in vitro* based on cell type. For example, apoptosis was generated after 10 μM of fenretinide in epidermal carcinoma cells while no apoptosis was observed in normal fibroblasts [292]. There have been few published studies reporting effects of fenretinide on oxidation and the effects seemed to depend on the treatment dose, frequency of treatment and the cell type. Many studies have demonstrated an increase in ROS in cancer cells lines using apoptosis-inducing doses of fenretinide [293]. ROS generation was dose dependent in cervical carcinoma cells, however no ROS generation was observed in normal cervical epithelial cells, even at a high 10 μM dose [294]. A study by Takahashi described the dose-dependent inhibitory effect of fenretinide on lipid peroxidation and generation of MDA. In his assay, concentrations of fenretinide between 0 – 5 μM steadily decreased MDA generation in a dose-dependent manner. The inhibition of MDA generation by the drug was reduced at concentrations over 5 μM, with higher doses achieving less inhibition. Our dose of fenretinide used in animal pharmacokinetic experiments ensured a bioavailability of about 1 μM in plasma, which reduced MDA levels. In our CF leukocyte experiment, MDA further decreased when a dose of 2.5 μM of fenretinide was used. The study by Takahashi demonstrated that fenretinide functions as an antioxidant capable of quenching free radicals at

doses ranging from 0 – 20 μM. Fenretinide may be affecting the AA/DHA ratio by reducing overall oxidation, thereby protecting PUFAs. In particular, DHA is more sensible to peroxidation than AA due to the high number of double bonds in its structure [287]. The dose of fenretinide for CF patient studies should be carefully chosen to achieve antioxidant effects and higher doses should be avoided since necrosis of cells might actually lead to an increase in inflammation and increased in oxidation.

One dose of fenretinide in mice induced decreases in NT3 and, to our knowledge, this is the first study to demonstrate a drug effect on reducing NT3 levels in CF. Few studies have looked at the effects of vitamin A on nitrosative stress. A study on rats treated with varying doses of vitamin A showed an approximately 30% increase in NT3 levels in brain tissue with the highest dose used (500 IU/kg) and a small increase in MDA of hippocampal tissue [295]. By contrast, another study found vitamin A pre-treatment of guinea pigs before LPS exposure reduced the formation of NT3 [296]. Fenretinide may be exerting altogether different effects than vitamin A, as is the case for apoptosis of cancer cells [237], thus further studies are warranted to explain the exact molecular mechanism responsible for the NT3 reduction.

Fenretinide bioavailability and inter-species variation

We tested the same 5 mg/kg dose of fenretinide in two animal models: mouse and monkey. Modeling drug bioavailability in different species can be quite challenging due to the differences in metabolic rates. Smaller animals, such as rodents, might have faster metabolisms of some drugs than larger animals such as monkeys and humans [297]. The proportion of the weight of the liver to total body weight in small animals is greater compared to large animals therefore liver enzymes are produced in higher concentrations in smaller animals [298].

Fenretinide is metabolized in humans by cytochrome P450 (CYP) enzymes: CYP3A4, CYP3A5, CYP2C8 [299]. These enzymes are also produced in mice and monkeys but have varying degrees of homology. There are two isoforms of CYP2C in monkeys, CYP2C20 and CYP2C43, the latter having 80% homology with the human counterpart CYP2C8. The monkey CYP3A8 enzyme has 93% homology to CYP3A, however it is expressed 3-4 folds higher than the human counterpart. The mouse CYP3A11 is 76% homologous to the human CYP3A [298]. These differences in enzymatic activities between rodents and human should be taken into consideration when determining drug dosage.

Our studies in monkeys were aimed at assessing the impact of fenretinide on lipids following treatment with a low 5 mg/kg dose of the drug using formulations which did not contain any traces of ethanol. It is unlikely that a tincture containing the large amounts of ethanol needed to achieve at least a 5 mg/kg dosage of fenretinide in humans would ever be permitted to be used. Thus we resorted to using two methods to prepare an ethanol-free solution of fenretinide for treatment of monkeys: one was prepared by homogenizing the fenretinide solution extracted from McNeil capsules with Peptamen and the second by homogenizing the fenretinide powder directly in the liquid diet Peptamen. We used the liquid diet in both cases since the absorption of the drug was found to increase when it was administered with a meal high in fat [300]. The capsule extract in Peptamen was found to affect lipids more efficiently than the same amount of active substance homogenized with Peptamen.

We then used HZ mice to compare these two formulations to our standard protocol of dissolving fenretinide in ethanol. This comparison demonstrated that dissolving fenretinide in ethanol ensures the best absorption. However, the capsule extract was next to the best

regarding its blood concentration. The concentration of fenretinide in plasma after treatment with the drug powder suspended directly in Peptamen was low which explains the lack of effect of this particular preparation on lipid profiles in monkeys (Table 3.2). While the capsule extract was also not dissolved, the added fat from the corn oil along with the detergents present in the formulation may have aided in the absorption of the drug.

Using the McNeil capsule extract, a dose of 20 mg/kg in mice resulted in similar concentrations of fenretinide as in monkeys treated with a 5 mg/kg dose (mice: 1.63 µM ± 0.31; monkeys: 1.44 µM ± 0.51) suggesting a much faster metabolism in mice. In this chapter, we have demonstrated that 1 µM to 2.5 µM of fenretinide, *in vitro* and *in vivo*, have effects on lipid defects and this should be the suggested target drug concentration in CF patients. Based on our results comparing inter species variation, the concentration of fenretinide in CF patients during a clinical trial would have to be closely monitored to ensure adequate dosing.

CONCLUSION

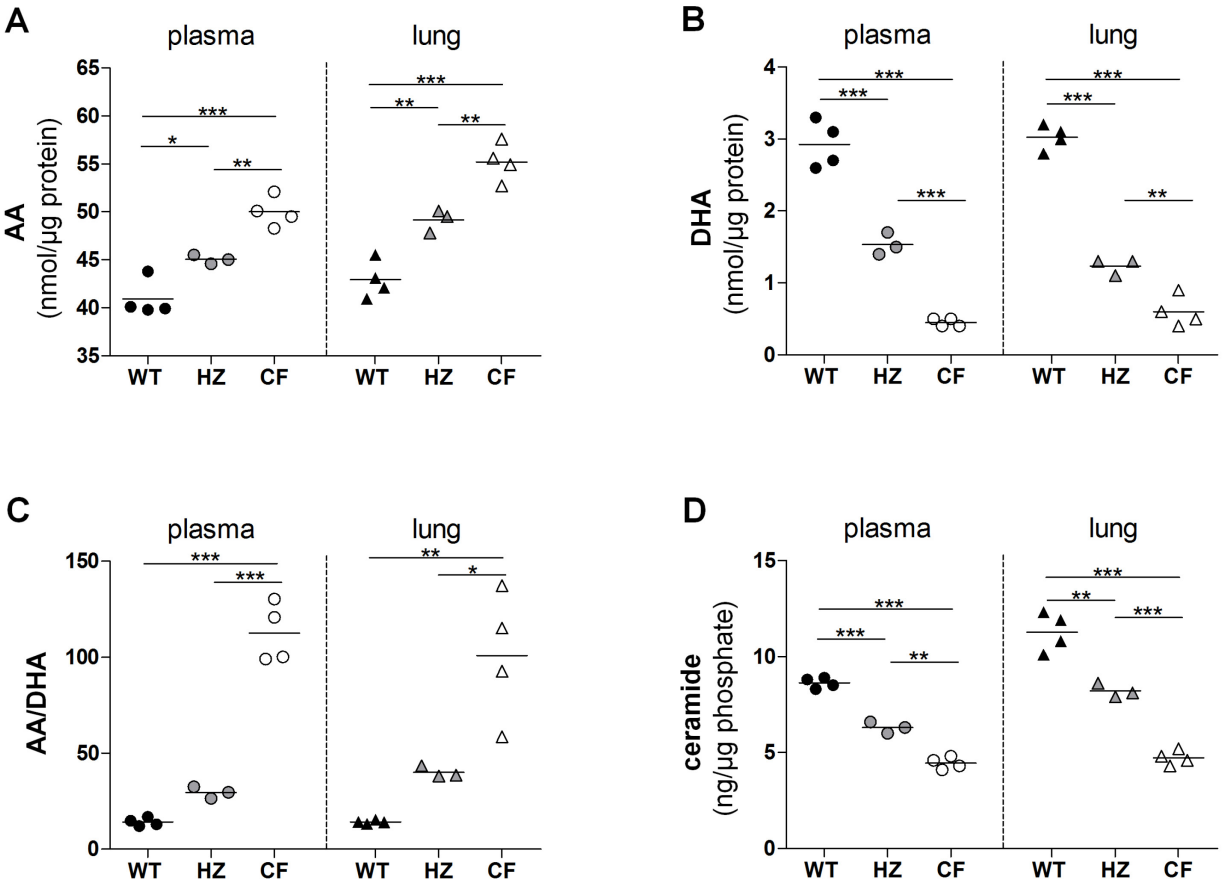
The results presented in this chapter demonstrate that one dose of fenretinide has prolonged effects on AA, DHA and ceramide. We show that the drug treatment reduced the levels of lipid peroxidation. We also demonstrate for the first time a reduction in NT3 following fenretinide treatment. We tested the bioavailability of fenretinide and its effect on lipids in mice, monkeys and cells from CF patients. Fenretinide affects lipids similarly in different species although the dosage needs to be adjusted depending on the species used. Fenretinide can correct other CF phenotypes such as the PUFA imbalance, ceramide defects and inflammation (as presented in Chapter 2) and in this chapter we demonstrate that it is a suitable candidate for improving more CF phenotypes: high lipid peroxidation and high nitrosative stress. There

are currently no medications which could be administered to CF patients to reduce their levels of lipid peroxidation or high levels of nitrosative stress, and thus fenretinide may be able to fill this important gap in CF therapy.

ACKNOWLEDGEMENTS

We would like to thank Steve Nuara for technical assistance for the monkey study. G.W. received a Frederick Banting and Charles Best Canada Graduate Scholarships Doctoral Award from the Canadian Institutes of Health Research and from Cystic Fibrosis Canada. D.R. received funding from Cystic Fibrosis Canada.

Figure 3.1. Number of mutated Cftr alleles associated with severity of lipid defects. Concentrations of PUFA and ceramide were analyzed in plasma (*circles*) and lung tissue (*triangles*) in WT (*black*), HZ (*grey*) and CF (*white*). A) HZ mice had significantly higher AA levels than WT mice but lower than CF mice in both plasma and lung. B) DHA levels in HZ mice were lower that WT mice but higher than CF mice in both plasma and lung. C) Although the AA/DHA ratio was between the WT and CF values, the values were not significantly different from WT. D) Ceramide levels in HZ tissues were moderately affected with lower levels than WT values and higher than CF. Statistical analysis was done using ANOVA with Bonferroni post-tests, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Horizontal lines are set at the means of the groups.


A
plasma
lung
AA
(nmol/µg protein)
65
60
55
50
45
40
35
*
**
***
**
**
***
WT
HZ
CF
WT
HZ
CF
B
plasma
lung
DHA
(nmol/µg protein)
4
3
2
1
0
***
***
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**
WT
HZ
CF
WT
HZ
CF
C
plasma
lung
AA/DHA
150
100
50
0
***
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**
*
WT
HZ
CF
WT
HZ
CF
D
plasma
lung
ceramide
(ng/µg phosphate)
15
10
5
0
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WT
HZ
CF
WT
HZ
CF

Figure 3.2. One treatment with 5 mg/kg of fenretinide induces improvements in lipids in HZ mice. HZ mice were treated with one 5 mg/kg dose of fenretinide by gavage. Lipid levels were measured in plasma (*white bars*) and lung tissue (*grey bars*) obtained at specific time points. A) Analysis of concentrations of fenretinide in the plasma of HZ (*black bars*) shows peak concentrations at 4 h. B) AA levels were rapidly reduced in plasma and lungs with prolonged improvements after one dose of fenretinide. B) DHA levels were improved with a single dose of fenretinide. However this effect was transient and DHA diminished in plasma 48 h after drug treatment and 72 h post-treatment in the lungs. C) The AA/DHA ratio rapidly improved and remained low for 90 h post treatment. D) The increases in ceramide levels were delayed compared to PUFA and fenretinide's effects were observed by 18 h. F) MDA levels, as measured by the surrogate TBARS, are reduced by fenretinide treatment with prolonged improvements 90 h post gavage. G) NT3 levels decreased with fenretinide treatment and these improvements are maintained 90 h post gavage. * represents a statistically significant difference between the 4 h time point vs. subsequent time points of the same sample type (plasma or lung). † represents a statistically significant difference between plasma and lung samples of the same time point. Groups were compared using ANOVA and the Bonferroni post-test. Significance was set to $p < 0.05$. Data presented as mean ± SD, n = 4 per time point.

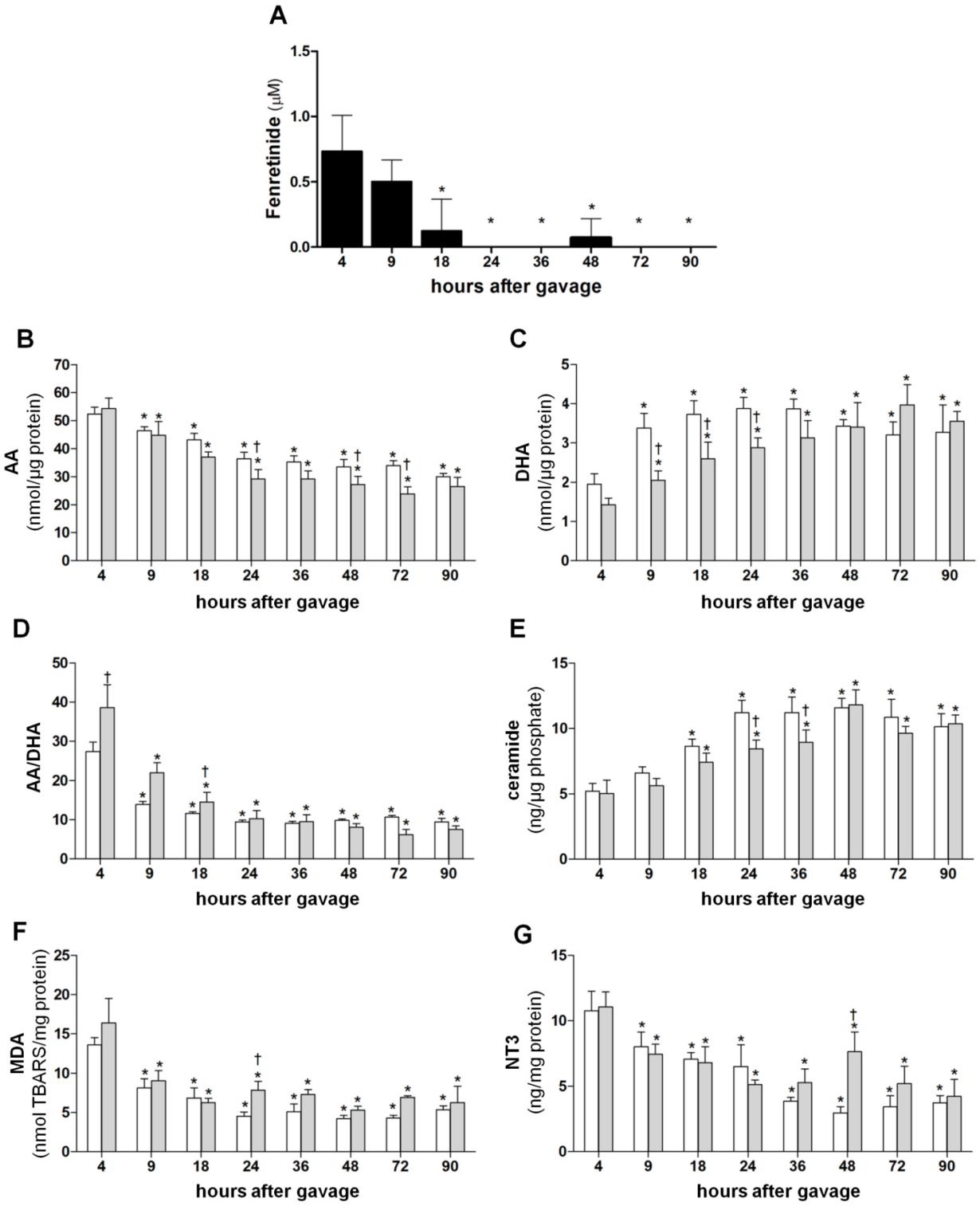

A
Fenretinide (μM)
1.5
1.0
0.5
0.0
4 9 18 24 36 48 72 90
hours after gavage
B
AA
(nmol/μg protein)
70 60 50 40 30 20 10 0
4 9 18 24 36 48 72 90
hours after gavage
C
DHA
(nmol/μg protein)
5 4 3 2 1 0
4 9 18 24 36 48 72 90
hours after gavage
D
AA/DHA
50 40 30 20 10 0
4 9 18 24 36 48 72 90
hours after gavage
E
ceramide
(ng/μg phosphate)
15 10 5 0
4 9 18 24 36 48 72 90
hours after gavage
F
MDA
(nmol TBARS/mg protein)
25 20 15 10 5 0
4 9 18 24 36 48 72 90
hours after gavage
G
NT3
(ng/mg protein)
15 10 5 0
4 9 18 24 36 48 72 90
hours after gavage

Figure 3.3. Lipid defects, high levels of lipid peroxidation and nitrosative stress in CF patients. PUFA, ceramide, lipid peroxidation and nitrosative stress markers were quantified in plasma from CF patients (CF, *white bars*, n = 10) and healthy controls (HC, *black bars*, n = 11). A) AA levels were increased in CF patients ($p = 0.0171$). B) DHA levels were decreased in CF patients ($p < 0.0001$). C) The AA/DHA ratio was higher in CF patients ($p < 0.0001$). D) Ceramide levels were reduced in CF patients ($p < 0.0001$). E) MDA levels, markers of lipid peroxidation measured by the surrogate TBARS, were higher in CF patients ($p < 0.0001$). F) Nitrosative stress, measured by NT3 levels, was also increased in CF patients ($p < 0.0001$). * represents significant difference between the two groups as determined by the Student's t-test, significance was set at $p < 0.05$.

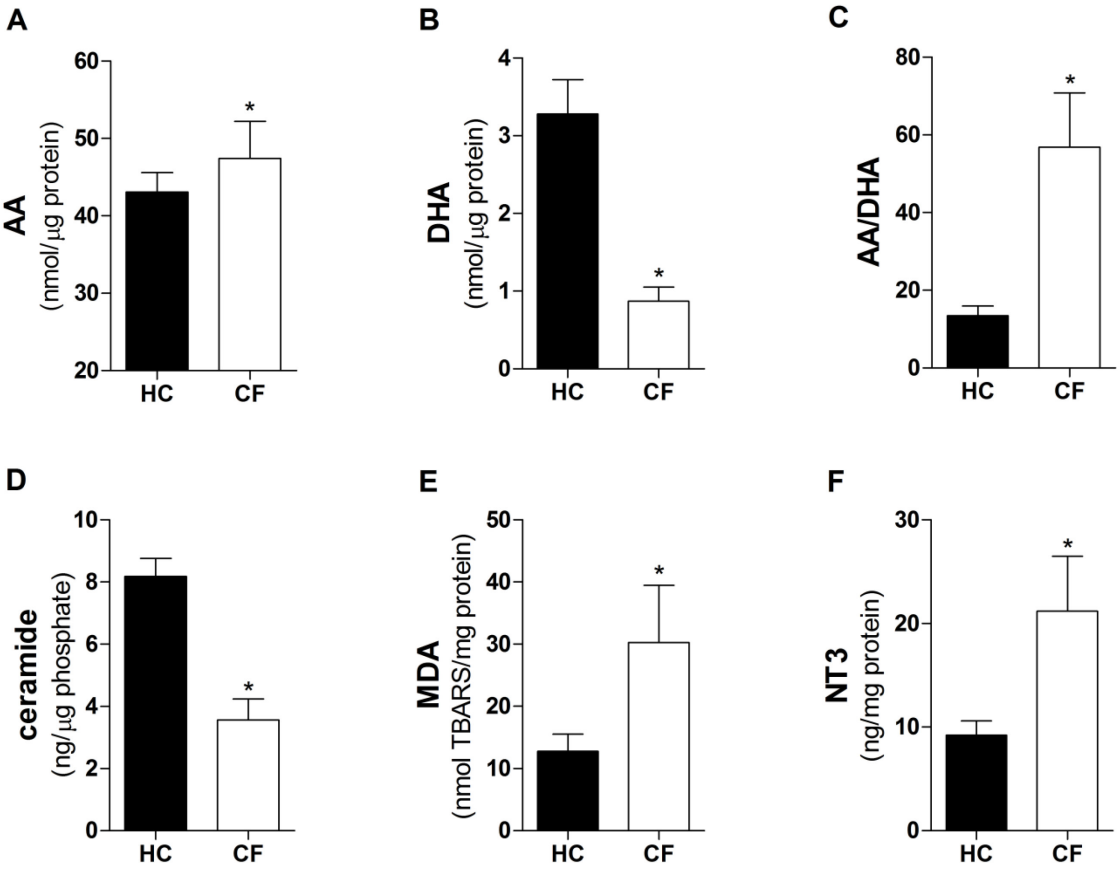
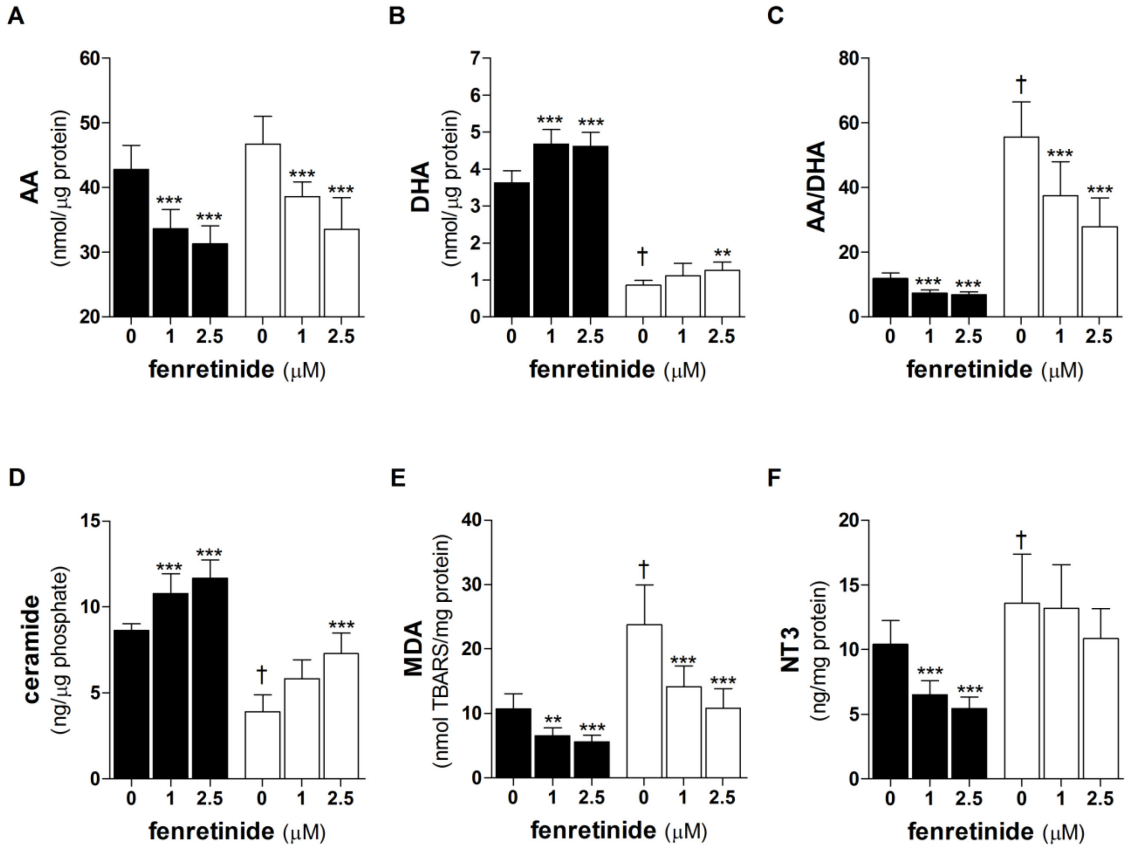

A
AA
(nmol/μg protein)
60
50
40
30
20
HC
CF
*
B
DHA
(nmol/μg protein)
4
3
2
1
0
HC
CF
*
C
AA/DHA
80
60
40
20
0
HC
CF
*
D
ceramide
(ng/μg phosphate)
10
8
6
4
2
0
HC
CF
*
E
MDA
(nmol TBARS/mg protein)
50
40
30
20
10
0
HC
CF
*
F
NT3
(ng/mg protein)
30
20
10
0
HC
CF
*

Figure 3.4. Fenretinide corrects lipid defects and abnormal lipid peroxidation in CF leukocytes. Leukocytes were isolated from blood samples of CF patients (CF, white bars, n = 8 - 10) and healthy controls (HC, black bars, n = 10 – 11). Cells were treated for 24 hours with two concentrations of fenretinide: 1 µM and 2.5 µM. A) AA levels were decreased following fenretinide treatment. B) DHA levels increased in CF cells at the highest dose. C) The AA/DHA ratio decreased in CF cells following their treatment with fenretinide in a dose dependent manner. D) Ceramide levels were significantly lower in CF cells compared to HC and increased with fenretinide treatment. E) Fenretinide treatment reduced MDA levels in both CF and HC cells with greater impact on CF cells. F) NT3 levels were higher in CF than HC cells and tended to decrease with drug treatment. ** represents a statistically significant difference of $p < 0.01$, and *** $p < 0.001$ between treated and untreated cells as determined by ANOVA with Bonferroni post-test. † represents a statistically significant difference between untreated CF and HC cells ($p < 0.05$) as determined by ANOVA with Bonferroni post-test.


A
AA
(nmol/μg protein)
60
50
40
30
20
0 1 2.5 0 1 2.5
fenretinide (μM)
B
DHA
(nmol/μg protein)
7
6
5
4
3
2
1
0
0 1 2.5 0 1 2.5
fenretinide (μM)
C
AA/DHA
80
60
40
20
0
0 1 2.5 0 1 2.5
fenretinide (μM)
D
ceramide
(ng/μg phosphate)
15
10
5
0
0 1 2.5 0 1 2.5
fenretinide (μM)
E
MDA
(nmol TBARS/mg protein)
40
30
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10
0
0 1 2.5 0 1 2.5
fenretinide (μM)
F
NT3
(ng/mg protein)
20
15
10
5
0
0 1 2.5 0 1 2.5
fenretinide (μM)

Table 3.1. Correlations between MDA, NT3 and PUFA levels in mice.

			AA	DHA	AA/DHA	MDA	NT3
Plasma	**MDA**	**r**	0.8524	-0.6766	0.8829	---	0.8273
		p-value	**<0.0001**	**<0.0001**	**<0.0001**	**---**	**<0.0001**
	NT3	**r**	0.8783	-0.4673	0.7568	0.8273	---
		p-value	**<0.0001**	**0.0070**	**<0.0001**	**<0.0001**	---
Lung	**MDA**	**r**	0.8179	-0.6698	0.8841	---	0.6313
		p-value	**<0.0001**	**<0.0001**	**<0.0001**	**---**	**0.0001**
	NT3	**r**	0.7511	0.6487	0.8006	0.6313	---
		p-value	**<0.0001**	**<0.0001**	**<0.0001**	**0.0001**	---

Table 3.2. Differences between the effects of two fenretinide formulations on lipids in monkeys.

	Time point	McNeil Capsule Extract	Powder
AA (nmol/µg protein)	0 h	37.8 (1.0)	37.8 (1.0)
	1 h	37.5 (1.4)	39.9 (2.5)
	3 h	35.0 (0.5)	35.5 (1.3)
	4 h	34.5 (2.3)	35.5 (3.3)
	6 h	32.2 (1.7)†	34.3 (3.3)
DHA (nmol/µg protein)	0 h	3.4 (0.1)	3.4 (0.1)
	1 h	3.5 (0.1)	3.3 (0.2)
	3 h	3.6 (0.1)	3.5 (0.1)
	4 h	3.7 (0.1)	3.6 (0.4)
	6 h	4.0 (0.3)†	3.5 (0.4)*
AA/DHA	0 h	11.1 (0.3)	11.1 (0.3)
	1 h	10.8 (0.8)	12.3 (0.9)
	3 h	10.0 (0.3)	10.1 (0.5)
	4 h	9.4 (0.3)†	10.0 (0.2)
	6 h	8.3 (0.1)†	10.0 (0.9)*
Ceramide (ng/µg phosphate)	0 h	9.2 (0.2)	9.2 (0.2)
	1 h	9.2 (0.5)	8.8 (0.5)
	3 h	9.9 (0.6)	9.6 (0.3)
	4 h	10.0 (0.8)	9.4 (0.5)
	6 h	10.3 (0.3)	10.0 (0.6)

Data represented as mean (SD). * represents statistical difference between "McNeil capsule extract" and "Powder". † represents statistical difference between time point and baseline values as determined by ANOVA with Bonferroni post-test. Significance was set at $p < 0.05$.

Table 3.3. Fenretinide concentrations in mouse plasma using different drug formulations

	fenretinide powder dissolved in EtOH n = 7	**fenretinide powder dissolved in EtOH n = 7**	**McNeil capsule extract (corn oil emulsion) n = 3**	**Fenretinide powder not dissolved n = 4**
Dose (mg/kg)	5	20	20	20
Fenretinide concentration (μM)	0.67 (0.35)	3.63 (1.53)	1.63 (0.31)	0.40 (0.32)

Blood samples were taken 4 h after treatment with fenretinide. Data represented as mean (SD).

In the previous two chapters, we demonstrated that fenretinide corrects phenotypes of CF and that it may have therapeutic potential for patients. This chapter describes a clinical study of CF patients and pulmonary exacerbations. As will be described in more detail, pulmonary exacerbations are important events which progress CF disease. We hypothesized that clinical and molecular markers might help clinicians to discern which patients are at risk of pulmonary exacerbations. In addition to clinical assessments, we looked at markers of inflammation, lipids, lipid peroxidation and nitrosative stress which are affected by fenretinide treatment, as shown in Chapters 2 and 3. We observed transient improvements in polyunsaturated fatty acids following aggressive antibiotic therapy. AA and DHA levels improved, but ceramide levels remained static. Lipid oxidation and nitrosative stress were also reduced with treatment. These improvements occurred in conjunction with reduced levels of inflammation and overall, better clinical status of patients. This study demonstrated that polyunsaturated fatty acids can be modulated in CF patients, without omega-3 supplementation, possibly due to a reduction in lipid peroxidation. Interestingly, ceramide levels were not affected by aggressive treatment for pulmonary exacerbations, adding to the benefits of fenretinide use. Additionally, we showed that patients with high levels of inflammation at the end of treatment re-exacerbated rapidly and thus these patients may benefit from anti-inflammatory therapy such as fenretinide. This study also served as a model for patient assessment, sample collection and analysis which can be used for future clinical trials.

CHAPTER 4: CANDIDATE MARKERS ASSOCIATED WITH THE PROBABILITY OF FUTURE PULMONARY EXACERBATIONS IN CYSTIC FIBROSIS PATIENTS

Adapted from the manuscript: Wojewodka G., De Sanctis J.B., Bernier J., Bérubé J., Ahlgren H.G., Gruber J., Landry J., Lands L.C., Nguyen D., Rousseau S., Benedetti A., Matouk E., Radzioch D. Candidate markers associated with the probability of future pulmonary exacerbations in cystic fibrosis patients. PLoS One. 2014 Feb 12;9(2):e88567.

ABSTRACT

Pulmonary exacerbations (PEx) cause significant morbidity and can severely impact disease progression in cystic fibrosis (CF) lung disease, especially in patients who suffer from recurrent PEx. Assessments able to predict a future PEx or a recurrent PEx are limited. We hypothesized that combining clinical, molecular and patient reported data could identify patients who are at risk of PEx.

We prospectively followed a cohort of 53 adult CF patients for 24 months. Baseline values for spirometry, clinical status using the Matouk Disease Score, quality of life (QOL), inflammatory markers (C-reactive protein (CRP), Interleukin (IL)-1β, -6, -8, -10, Macrophage Inflammatory Protein (MIP)-1β, Tumor Necrosis Factor (TNF) and Vascular Endothelial Growth Factor (VEGF)), lipids, lipid peroxidation and nitrosative stress markers in blood plasma were collected for all patients during periods of stable disease, and patients were monitored for PEx requiring oral or IV antibiotic treatment. In addition, we closely followed 13 patients during PEx collecting longitudinal data on changes in markers from baseline values. We assessed whether any markers were predictors of future PEx at baseline and after antibiotic treatment.

Out of 53 patients, 37 experienced PEx during our study period. At baseline, we found that low values for lung function, clinical scoring and QOL were associated with increased risk of PEx events. PEx were associated with increased inflammatory markers at Day 1 and these biomarkers improved with treatment. The imbalance in arachidonic acid and docosahexaenoic acid levels improved with treatment which coincided with reductions in lipid peroxidation. High levels of inflammatory markers CRP and IL-8 were associated with an early re-exacerbation.

Our results demonstrate that low clinical and QOL assessments during stable disease are associated with a higher risk of future PEx, while higher levels of inflammatory markers at the end of antibiotic treatment may be associated with early re-exacerbation.

INTRODUCTION

Cystic fibrosis (CF) patients often suffer acute exacerbations of their pulmonary symptoms, necessitating more aggressive treatment. Pulmonary exacerbations (PEx) are major events contributing to the morbidity and progression of CF lung disease. The recovery from PEx is largely based on the reversal of symptoms and improvement in lung function. However, pre-PEx lung function levels are not recovered in 15% to 25% of CF patients [301,302]. Even when pulmonary function tests return to the values observed prior to PEx, experiencing each PEx contributes to incremental long-term decline in lung function with similar impacts on survival as would a 12% point reduction in forced expiratory volume in 1 second, expressed as percent of the predicted value (FEV_1%) [303,304]. Higher frequencies of PEx were associated with greater rates of decline in FEV_1%, especially having more than two PEx per year could increase the need for transplant and the risk of death [305]. The survival model characterized by Liou and colleagues also predicted that the impact on 5-year mortality of four PEx events in a single year was as detrimental as *Bulkholderia cepacia* infection or a 48% reduction in FEV_1% [303]. Given the substantial morbidity and mortality associated with PEx, there is an urgent need to identify patients at risk of PEx, particularly recurrent PEx. Improving the clinicians' ability to stratify patients based on their risk to develop PEx will allow for more effective prevention (e.g. treatment of CF related diabetes or allergic bronchopulmonary aspergillosis) and early intervention to prevent irreversible lung damage. Although standards of care that include

inhaled antibiotics, recombinant human DNase and hypertonic saline contribute in reducing the frequency of exacerbations, there is currently no effective and reproducible diagnostic marker for the identification of early stages of PEx which could aid in prevention. The standard criteria used to monitor PEx are mainly focused on lung function indicators such as FEV_1% which are mostly reflective of disease severity and not necessarily disease activity [306]. Patient reported symptoms are important complements to physicians-documented clinical signs in the diagnosis of PEx. In fact, newly developed diaries are being validated for the purpose of early intervention to quickly reduce the development of a full and vigorous inflammatory response and to reduce the severity of PEx with the hope of preventing irreversible lung damage [307].

The events that trigger PEx are still poorly understood and may include respiratory viral infections [308,309] and air pollution [310]. Bacterial pathogens already present in the patients' lungs may be causing PEx by adapting their virulence or colonizing new areas of the lung [311]. Retrospective studies have found risk factors for developing PEx which include the female sex, nutritional status, CF co-morbidities such as CF related diabetes, pancreatic insufficiency, and lung microbiology (e.g. *Aspergillus fumigatus, Bulkholderia cepacia*) [302,303,305,312,313]. Due to the nature of data available to perform retrospective analyses, these risk factors are based on clinical information which identifies a population of CF patients more likely to have PEx. However on an individual basis, it remains difficult to assess which patients will experience a PEx.

There have been few prospective studies looking at factors associated with risk of PEx which included molecular markers as predictors. A study by Sequeiros and colleagues found that the time to the next PEx was shorter in patients with allergic bronchopulmonary

aspergillosis and CF related diabetes. The authors also described that high CRP and low FEV_1 values at the end of antibiotic PEx treatment were associated with shorter intervals until the next PEx. They did not find any other markers of significance [314]. Gray and colleagues demonstrated that serum calprotectin levels at the end of PEx treatment rather than CRP were linked to the time until next PEx [315]. These two studies show the potential of inflammatory markers in predicting risk of future PEx.

For this study, we asked two questions: 1) in a period of stable disease, who is more likely to have a PEx in the future? And 2) After PEx treatment, who is at risk of an early re-exacerbation? We hypothesized that combining clinical and patient reported data with inflammatory markers and lipids may result in a better evaluation of patient disease activity. More specifically, we describe that worse disease-specific patient reported quality of life (QOL) and clinical assessments during stable disease indicated risk of PEx, while higher levels of inflammation at the end of PEx treatment were associated with early re-exacerbation.

METHODS

Study design

Primary study: Markers at stable disease associated with risk of future PEx. CF patients from the Adult Cystic Fibrosis Clinic at the Montreal Chest Institute (Montreal, Qc, Canada) were approached during their regular clinic visit to participate in the study. Fifty-three patients were enrolled in our prospective cohort study and were followed for 12 months after enrollment. The study duration was 24 months in total. Baseline data was recorded during a period of stable disease defined by the absence of any PEx requiring IV or oral antibiotic therapy in the preceding month. One patient was excluded from the study after baseline data

was collected due to lung transplantation, thus we had 52 patients in our patient group. Inhaled antibiotics (tobramycin or aztreonam) were prescribed as maintenance therapy to 39 patients enrolled in the study (75% of the cohort: 32 patients that had PEx and 7 that had no PEx). A PEx was defined as any change in patients' symptoms (increased cough, sputum production and breathlessness, and decreases in lung function, weight, appetite and energy) requiring additional oral or IV antibiotic therapy [305]. The decision to treat was at the physicians' discretion and was not influenced by this study.

Secondary study: Markers at the end of PEx treatment associated with early re-exacerbation. Among patients that experienced PEx (n = 37), we collected serial longitudinal clinical data and blood samples throughout the PEx of 13 randomly selected patients. Data and samples were collected at Day 1 (within 24 hours prior to PEx treatment, n = 13), and on follow-up assessments on Days 7 (n = 12), 14 (n = 11), 21 (n = 9) and 42 (n = 8). The prescribed treatments were based on antibiotic *in vitro* resistance tests and patient history. Patients received oral or IV antibiotics. Treatment length was determined by the treating physician. Four patients received antibiotic treatment for 14 days and nine for 21 days. All patients were treated with β-lactams and/or fluoroquinolones in addition to tobramycin. Some received additional antibiotics such as doxycycline, clindamycin or trimethoprim. Nine patients were given corticosteroids during their first PEx of the study based on previous response and/or severe bronchospasm. Patient compliance limited the sampling of all patients for Days 7, 14 and 21 time points. By Day 42, four patients had already re-exacerbated and were not included in this follow-up time point.

Students and employees at the McGill University Health Centre were recruited as healthy controls. CF patients and healthy controls gave written consent to participate in the study which was approved by the Institutional Review Board of the McGill University Health Centre.

Clinical data collection and clinical scoring

Patient Characteristics. At enrollment, patients' information on age, sex, weight and body mass index (BMI) was recorded. At baseline (n = 52) and at all defined time points during PEx (n = 8 - 13), the patients' weight, BMI, complete blood cell counts and clinical information for the Matouk Disease Score were assessed.

Clinical scoring. The Matouk Modified N. Huang Disease Score (Matouk Disease Score), previously described and validated [316,317], was used to quantify disease activity of CF patients. Briefly, the total score comprises of four subscores: Clinical (weight, weight change, dyspnoea, cough, sputum, physical exam, respiratory rate/breathing pattern/cardiac frequency, bacterial culture, appetite and general condition), Pulmonary Function (PFT, forced vital capacity percent predicted (FVC%), FEV_1% predicted, FEV_1/FVC, forced expiratory flow $(FEF)_{25-75\%}$ predicted, $FEF_{50\%}$ predicted, RV/TLC), Chest Radiography (CXR) score (based on the Bradsfield method for radiography scoring, comprised of scores for air trapping, linear markings, nodular cystic lesions, parenchymal lesions, general impression) and Complications score (number of previous PEx, pneumothorax, haemoptysis, respiratory failure, cardiac enlargement, pulmonary surgery). Healthier patients have higher scores for Clinical, PFT and CXR categories and lower values for the Complications score. To calculate the total score: Total score = Clinical + PFT + CXR – Complications.

Measures of lung function. At baseline (n = 52) and at all defined time points during PEx (n = 8 - 13), lung function was assessed by spirometry, and included FEV_1% and forced vital capacity percent predicted (FVC%). Spirometry was performed according to the American Thoracic Society standards as described in Chapter 2 [246,247].

Patient reported quality of life (QOL). The QOL evaluation was recorded at baseline (n = 52) and at defined time points during PEx (n = 8 – 13) by a self-administered questionnaire using the CF Questionnaire-Revised (CFQ-R), a validated tool for the assessment of QOL in CF patients. It is comprised of 50 items associated with 3 symptom scales (Weight, Respiratory Function and Digestion) and 9 QOL domains (Physical, Vitality, Emotional, Eating Disturbances, Treatment Burden, Health Perceptions, Body Image, Social Functioning and Role/School Functioning) [318]. Higher scores reflect healthier disease status. The total score is the sum of scores for all items in the questionnaire.

Plasma inflammatory biomarker analysis

At baseline (n = 52) and each defined time point during PEx (n = 8 – 13), blood samples were collected in EDTA coated tubes and spun at 3000 rpm for 10 min at 4°C for plasma isolation. Plasma CRP levels were quantified in the hospital's clinical laboratory using ELISA. Cytokines were measured in plasma with the MILLIPLEX® Map multiplex assay kit (Millipore, Mississauga, ON, Canada) and the MAGPIX® multiplex system (Millipore) according to the manufacturer's instructions. The data were assessed for IL-1β, IL-6, IL-8, IL-10, MIP-1β (CCL4), TNF and VEGF using the MILLIPLEX® Analyst software, version 4.2 (Millipore). The concentrations of cytokines are expressed as pg/mL.

Plasma lipid analysis

PUFA analysis. After plasma isolation as described above, 100 µL of plasma was added to 1 mL 2:1 chloroform/methanol solution with added 1 mM of butylated hydroxyanisole (BHA) to prevent oxidation of fatty acids. Samples were stored in -80°C until analysis. Lipids were isolated using the method described by Folch [251] and analyzed as described in Chapter 2. Briefly, the fatty acids in this fraction were esterified and hydrolyzed as described by Schlenk and Gellerman [254] and the esters were identified by GC/MS using commercial standards [283]. In addition, the total protein content of the aqueous phase was analysed using the bicinchoninic assay (Pierce Biotechnology). The concentrations of arachidonic acid (AA) and docosahexaenoic acid (DHA) are expressed as nmol/µg protein. The AA/DHA ratio represents the amount of AA to DHA in each sample.

Ceramide analysis. An aliquot of the organic fraction was dried and resuspended in 100 µL of chloroform. Lipids were further separated by TLC and were detected by iodine [251]. On these separated lipid samples, the concentration of ceramide was determined by ELISA as described in Chapter 2 [180,252,253]. Ceramide concentrations are expressed in ng/µg phosphate.

Analysis of lipid peroxidation and nitrosative stress markers

As described in Chapter 3, lipid peroxidation was assessed by indirectly measuring malondialdehyde (MDA) with the thiobarbituric acid reactive species (TBARS) assay [283] and is expressed as nmol of TBARS/mg protein. The total amount of NT3 was determined by ELISA as explained in Chapter 3. The levels of NT3 were normalized by the amount of protein and are expressed as ng/mg protein.

Statistical analysis

The SAS software version 9.2 (Toronto, Canada) was used to assess the relationship between the markers at baseline and PEx events. First, we used the Cox proportional hazards model adjusted for age and sex using time to first PEx as an outcome. To illustrate the survival analysis data, we used the Kaplan-Meier survival model with log rank test with continuous covariates dichotomized at the median to illustrate the survival analysis data. All other statistical analyses were performed with Prism 5 (GraphPad Software, LaJolla, USA). We used the D'Agostino and Pearson omnibus normality test to assess normality, the Student's t-test or the Mann-Whitney test to compare two groups, and Chi-square test for categorical values. For changes from baseline during PEx, statistical analysis was performed using the percentage change with the one sample t-test or Wilcoxon signed-rank test (Figures 4.2 and 4.3, Table 4.4). The percentage change was calculated as:

$$[(\text{Value}_{\text{PEx time point}} - \text{Value}_{\text{baseline}})/ \text{Value}_{\text{baseline}}] \times 100.$$

Positive values indicate increases and negative values indicate decreases from baseline. The ANOVA test with Bonferroni post-tests were used to evaluate differences between the PUFA levels at all PEx time points (Figure 4.4) and Pearson correlations were estimated for this data. Significance was set at $p < 0.05$.

RESULTS

Patient demographics, baseline clinical and biomarker characteristics.

We prospectively followed our adult CF cohort for a total of 24 months where 37 out of 52 patients experienced at least one PEx during the follow-up period. The patient demographics and baseline values of the cohort are described in Table 4.1 and 4.2. At baseline,

CF patients who experienced at least one PEx ("PEx group") had lower lung function, weight, BMI and Matouk Disease Score compared to the patients who did not have a PEx ("no PEx group") (Table 4.1). The baseline levels of white blood cells and blood neutrophils, although still within the normal range, were higher in the "PEx group", suggesting higher levels of systemic inflammation even during a stable disease state. These patients also had lower QOL assessments for total score, Weight, Physical, Emotion, Health Perceptions, Body Image and Role items (Table 4.1). IL-6 and IL-10 levels were higher in the "PEx group" compared to the "no PEx group" (Table 4.2). Consistent with previously reported data from our group and other investigators [71,117,119] there was a lipid imbalance in PUFA with high AA levels and low DHA levels CF patients compared to controls. Overall the AA/DHA ratio was higher in CF patients than in controls. However, there were no differences in PUFA between the "no PEx group" and "PEx group" during stable disease. Ceramide levels were lower in CF patients than in controls but were not different between "no PEx group" and "PEx group". Overall, these characteristics suggest that the "PEx group" has more severe and active disease, and a lower QOL at baseline, compared the "no PEx group".

Worse clinical disease severity and activity, and QOL are associated with PEx events

Using the Cox proportional hazards model adjusting for age and sex, we tested the association between our markers and the risk of PEx (Table 4.3). Better lung function (FEV_1% and FVC%) and higher Clinical, PFT, CXR subscores and total Matouk Disease Score were associated with a lower risk of PEx. A lower Complications subscore was associated with lower risk of PEx. The Matouk Disease Score incorporates patients' symptoms as well as physician-recorded clinical parameters and other complications adding to disease activity beyond

spirometric evaluations. Thus a high degree of disease severity as measured by lung spirometry was associated with high risk of PEx as was high disease activity assessed by the Matouk Disease Score. The analysis also showed that low CFQ-R symptom scores (Weight and Respiratory) and low QOL domains (Physical, Vitality, Health Perceptions and Role) indicated higher risk of PEx. No inflammatory markers or PUFA were found to be associated with risk of PEx using the Cox proportional hazard model.

To illustrate these relationships, we used the Kaplan-Meier survival model, with patients dichotomized based on median values (Figure 4.1). We obtained similar results to the Cox proportional hazards model. Lower lung function (FEV_1%, Figure 4.1A and FVC%, Figure 4.1B), worse subscores of the Matouk Disease Score (low Clinical subscore, Figure 4.1C and high Complications subscore Figure 4.1D) and low scores for the CFQ-R QOL domains Physical and Health Perceptions were also related to a higher risk of a future PEx (Figures 4.1E and 1F, respectively). No baseline inflammatory markers or PUFA were associated with increased risk of PEx events, although there was a trend with higher CRP levels (median 5.3 mg/L, p = 0.054, Figure 4.1G).

Changes in clinical parameters and QOL during PEx

We performed a second study with 13 patients to evaluate whether any of our markers at the end of PEx treatment would be predictive of an early re-exacerbation. First, we calculated for each patient the percentage change at every time point from their baseline values obtained during a period of stable disease (Figure 4.2 and full data set is presented in Table 4.4). As expected, lung function was most reduced at Day 1 of a PEx (-16.8% FEV_1% and -16.1% FVC% compared to baseline) and steadily improved over the course of PEx treatment

lasting either 14 or 21 days (Figure 4.2A). FEV_1% and FVC% approached pre-PEx baseline values at Day 14. However, both values decreased again by Day 21 and 42 where FVC% values were 10.5% lower than baseline values ($p = 0.028$) (Figure 4.2A). At the onset of PEx (Day 1), all components of the Matouk Disease Score worsened compared to baseline (Clinical score illustrated in Figure 4.2A). The Clinical subscore steadily improved with PEx treatment where it was 3.4% above baseline on Day 21. The CXR subscore did not decrease at PEx onset however showed a trend toward improvement on Day 21, attesting to the limited sensitivity of the Bradsfield radiologic scoring to capture small CXR changes (Table 4.4). The QOL evaluation revealed that patients recognized a decline of their health on Day 1 with improvements on Days 7, 14, 21 and 42 (Respiratory, Physical and Health Perceptions illustrated in Figure 4.2B).

Changes in inflammatory markers during PEx

In previous studies, inflammatory markers such as CRP, IL-1β, IL-6, IL-8 and VEGF were found to increase with PEx onset and to respond to antibiotic treatment for PEx [314,315,319-324]. To examine this in our study, we calculated for each patient the percentage change in the concentrations of inflammatory markers at each time point from baseline values (Figure 4.3 and full data set in Table 4.5). In general, we also found PEx onset induced an inflammatory response which returned to baseline values toward the end of treatment (Days 14 and 21). Once off aggressive treatment for PEx, inflammatory markers tended to increase again in some patients at Day 42. More specifically, we found the elevated CRP levels from baseline values on Day 1 decreased during treatment and significantly worsened once again post-treatment by Day 42 (Figure 4.3A). Similarly, IL-6, IL-8, MIP-1β and VEGF also increased on Day 1 and improved over the course of treatment (Figure 4.3B, C, E, and F, respectively). IL-10 increased

on Days 7 and 14 from baseline values, suggesting that anti-inflammatory mechanisms were activated (Figure 4.3D). Interestingly, MIP-1β levels significantly increased again post-treatment on Day 42 (Figure 4.3F). All other inflammatory markers tended to increase at Day 42, although with a large variation between patients. Neither levels of IL-1β nor TNF were significantly changed throughout PEx (Table 4.5).

Improvements in PUFA, lipid peroxidation and nitrotyrosine during PEx treatment

A hallmark of CF disease is the imbalance in PUFAs with high levels of AA and low levels of DHA [71,117,119,161,325]. AA is pro-inflammatory and its metabolites include prostaglandins and eicosanoids which are also increased in CF [144,146]. DHA is anti-inflammatory and its metabolites include resolvins and protectins. This imbalance in PUFAs may contribute to the inflammatory status observed in CF [110,326]. In fact, as described in Chapter 2, our previous studies in a CF mouse model showed improvements in AA and DHA after treatment with fenretinide which were concurrent with reductions in inflammatory markers and better clearance of lung infections [71,180]. Fenretinide increased ceramide levels which might have important roles in bacterial infections and membrane composition [180].

We found improvements on Day 21 in the PUFA imbalance compared to baseline for both AA (Figure 4.4A) and DHA (Figure 4.4B). At this time point, AA levels dropped to control concentrations, the AA/DHA ratio improved (Figure 4.4C), and both were no longer significantly different from control values. It is important to note that these improvements were transient as AA levels and AA/DHA ratios increased again by Day 42. Ceramide levels remained unchanged throughout PEx treatment (Figure 4.4D). Lipid peroxidation decreased with treatment for PEx and MDA levels were no longer different from controls at Day 21 (Figure 4.4D). We found a

significant positive correlation between MDA and AA in plasma at the end of treatment (r = 0.692, p = 0.013) and an inverse correlation between MDA and DHA, however this trend did not reach significance (r = -0.339, p = 0.290). NT3 levels did not significantly decrease from baseline levels however did approach healthy control levels. At baseline, Days 1, 7 and 21, NT3 levels were significantly higher in CF patients compared to controls. However, at Days 14 and 42, NT3 levels in CF patients were no longer statistically different from controls levels (Figure 4.4E). At the end of treatment, NT3 levels were positively correlated with AA (r = 0.618, p = 0.043) and negatively correlated to DHA levels (r = -0.618, p = 0.043). The AA/DHA ratio was also positively correlated with NT3 levels at the end of treatment (r = 0.660, p = 0.0272).

Candidate markers of early re-exacerbation

To determine whether any of our markers assessed on the last day of treatment (either Day 14, n = 4, or Day 21, n = 8) could indicate an early re-exacerbation, the patient cohort was divided based on whether their next PEx was under or over 42.5 days from the last day of treatment, which was the median number of days for the group (Table 4.6). “Early PEx” refers to the patient group which had their next PEx less than 42.5 days after the last day of treatment, while the group which developed no subsequent PEx or a second PEx more than 42.5 days after the last day of treatment was called “Late PEx”. One patient was excluded from the analysis since information on his (or her) next PEx was not available, thus 12 patients were included. The “Early PEx” group had a trend towards lower lung spirometry values, total Matouk Disease Score and QOL total score at end of treatment compared to the “Late PEx” group, but this did not result in a statistical significance. More importantly, we found increased levels of inflammatory markers in “Early PEx” patients with significantly higher CRP and IL-8,

indicating that these markers may contribute to assessing which patients could rapidly re-exacerbate.

DISCUSSION

The importance of PEx in CF disease progression is well established however the triggers of these events are not fully understood which limits the means for prevention. Retrospective studies have identified patient groups at risk of PEx such as those with liver disease, CF related diabetes, low FEV_1 values [305,312]. However, the difficulty remains in determining on an individual basis which patient has a high risk of a future PEx.

Our prospective study is unique for two main reasons: 1) the large panel of data that were collected at stable disease and 2) the extensive sampling throughout PEx. With these results, we assessed markers predicting future PEx in two distinct cases: from stable disease and at the last day of treatment for PEx.

Assessing risk from stable disease

Patients who developed PEx had worse baseline disease severity (based on lung spirometry and BMI), greater baseline disease activity (based on the Matouk Disease Score) and worse self-reported QOL at stable disease. Interestingly, this was associated with higher inflammatory markers (Table 4.1 and 4.2). Using survival models, the clinical (lung spirometry and Matouk Disease score) and QOL assessments were predictive of future PEx with better scores associated with lower risk of PEx (Figure 4.1 and Table 4.3). The inflammatory markers we assessed were not associated with future PEx when measured at stable disease. Noteworthy, there was a trend with low CRP associated with low risk of PEx, which merits

further investigation with a larger cohort of patients. Monitoring changes in these markers may lead to an early recognition of PEx which would allow for earlier intervention. This, in turn, would reduce the impact of heightened and prolonged inflammation on lung tissue. Previous studies have shown that CF patients who recorded their lung spirometry using a daily diary had lower rates of lung function decline than those who did not [327]. A new study by Lechtzin and colleagues looking at twice weekly electronic symptom recording by CF patients may help determine the usefulness of a more frequent symptom and lung function monitoring for early recognition of PEx [307].

Prediction from end of treatment

Each PEx significantly decreases lung function in CF patients, even more with PEx in rapid succession [304]. In our study, we found that higher inflammation at the end of treatment may be a better indication of early re-exacerbation rather than clinical or patient QOL assessments (Table 4.6). CRP was previously shown to be correlated to the number of days until the next PEx [314], a finding not replicated in some studies [315]. Regardless of the discrepancies, the few prospective studies on predicting the time to next PEx showed the usefulness of inflammatory markers such as CRP and calprotectin in assessing risk of future PEx [314,315]. Sequeiros and colleagues also showed that early re-exacerbation was correlated with more symptoms after 14 days of treatment such as cough, sputum production, breathlessness and fatigue [314].

We found that FEV_1% values tended to be lower in patients that quickly re-exacerbated, and due to our small patient group, we cannot disregard this as a marker of recurring PEx. Thus, inflammatory markers such as CRP and IL-8, in conjunction with patient

reported symptoms, clinical evaluations and spirometry, could be additional indicators of a recurring PEx. And, although symptoms and clinical picture (lung function and Matouk Disease score) have returned to baseline at the end of treatment, patients may still be experiencing some level of unresolved inflammation indicating the PEx has not been cleared completely. There has been evidence that extending the course of antibiotics may only offer a small improvement in symptoms but not lung function or inflammation [328]. In these cases, perhaps a change in antibiotics and/or the use of anti-inflammatory agents to aid in the resolution of inflammation would benefit the patient and prevent early re-exacerbation. However, based on our study, we cannot determine whether the unresolved inflammation itself triggers a new PEx or whether it is a response to other underlying active processes such as a poorly controlled infection. To note, we did not look at causes of PEx such as viral infections which have been reported to impact recovery from PEx [309].

Kinetics of inflammation and lipids throughout PEx

We found in general inflammatory markers increased at PEx onset such as CRP, IL-6, IL-8, MIP-1β and VEGF compared to values at stable disease periods, and responded to treatment as soon as Day 7 (Figure 4.3 and Table 4.5). This corroborates previous findings regarding resolution of inflammation with antibiotic treatment [314,319,324,329]. MIP-1β, a chemoattractant for monocytes, has rarely been assessed in CF. To our knowledge, this is the first time reported to respond to treatment in the context of PEx in CF.

Our study design allowed us to monitor whether improvements during PEx treatment are maintained after treatment was completed. Five patients out of 13 were not available for Day 42 assessments due to a re-exacerbation (n = 4) or other complications (n = 1, same patient

that was excluded from the second study of markers associated with early re-exacerbation). Even with this small number of patients, we were able to observe decreases in FVC% values and increases in CRP and MIP-1β at Day 42 compared to baseline values. Due to the small sample size and large variation at this time point, we could not conclude whether these changes are indicative of future PEx and further studies are necessary. However, it is important to recognize that improvements at the end of treatment, a time point assessed in many PEx studies, may not be representative of the patient's disease status on the long term.

Few studies have assessed the changes in PUFAs at PEx onset and throughout treatment [124,330]. Similar to our results regarding lipid peroxidation, McGrath and colleagues demonstrated a decrease MDA in CF patients after antibiotic treatment for PEx [331]. The improvements in PUFA during PEx occur during treatment but worsen at Day 42, when patients are no longer treated for PEx. Thus the improvements in the AA/DHA ratio may be due to the effects of the treatment itself in reducing lipid peroxidation, also observed in this study. Antibiotics have been shown to protect lung epithelial cells from oxidative damage [332]. Tobramycin in particular was found to act as a potent reactive oxygen species scavenger [333]. Corticosteroids were associated with decreased oxidation in lupus patients [334] and in particular lipid peroxidation in Crohn's disease [335]. In general, PUFAs are very susceptible to peroxidation due to their high content of double bonds compared to other types of fatty acids causing DHA to be more affected than AA [286]. The increase in anti-inflammatory DHA may contribute to the resolution of inflammation. Additionally, the reduction in lipid peroxidation may itself be a factor. Oxidized fatty acids such as DHA were found to act on TLR4, much like its ligand LPS, activating downstream NF-κB signalling [336]. It is important to note that changes in

PUFA were not permanent and routine treatments for disease maintenance do not normalize PUFA levels since CF patients at stable disease still have defects in AA and DHA levels (Figure 4.4 and Table 4.2). Currently, no routine antibiotic or steroid therapy for CF has proven to be successful in correcting PUFA abnormalities, which could improve disease status of patients. The aggressive PEx treatment had no effect on ceramide levels, which remained lower than normal at baseline and all PEx time points. Modulating this class of lipids could offer additional benefits for fighting bacterial infections and improved inflammatory response [71].

Limitations

Our study had several limitations that are important to consider. Among the 52 patients included in our analysis, we had longitudinal clinical and molecular marker measurements in only 13 patients during their PEx. Due to the extensive testing and the availability of the clinical coordinator, this was the volume of patients we could handle for the study period. We recognize that this small number of patients limits the statistical power of our analysis and thus important relationships may be missed. However, due to the large panel of markers we assessed and the prospective nature of the study design, this study remains an important exploratory analysis generating many hypotheses about predictive markers of PEx.

The median time to next PEx was somewhat low suggesting that our patient group had high disease activity. Parkins and colleagues described that 13% of their patient cohort re-exacerbated in 45 days. They used this time point as a definition of “non-response to treatment” which may be another way to describe the six patients with early re-exacerbations since they had higher levels of inflammation [301]. Our Day 42 time point was also affected when it overlapped with new PEx. Noteworthy, our definition of PEx was more inclusive than

the other definitions used [305,314,319] as it included any event needing additional IV or oral therapy thereby including mild and severe types of exacerbations.

CONCLUSION

This study demonstrates that monitoring changes in clinical and patient reported assessments during stable disease may help in determining which patients are at risk for PEx. Our longitudinal analysis of inflammation throughout PEx suggests that, at the end of antibiotic treatment for PEx, inflammatory markers could contribute to monitoring patients at risk of early recurring PEx. Our analysis reveals that CRP and IL-8 in particular may be important in assessing patients at risk of early recurring PEx, which needs to be confirmed in a larger study. The imbalance in PUFA levels improved after treatment for PEx likely due to a decrease in lipid peroxidation. The data presented here offer more insight into potential markers of PEx which, in conjunction with clinical data, may improve earlier recognition of PEx in CF. Additionally, anti-inflammatory therapies may be of benefit when patients display high levels of inflammation after the completion of PEx treatment.

ACKNOWLEDGEMENTS

We would like to thank all the personnel at the Adult Cystic Fibrosis Clinic at the Montreal Chest Institute, all the patients and healthy volunteers participating in this study. G.W. received a Frederick Banting and Charles Best Canada Graduate Scholarships Doctoral Award from the Canadian Institutes of Health Research. D.R. received funding from the Ministère du Développement Économique, de l'Innovation et de l'Exportation (MDEIE/MESRST) de Québec, MSBi Valorisation and Cystic Fibrosis Canada.

Figure 4.1. Probability of having a PEx is associated with low clinical and QOL assessments. Using the Kaplan-Meier survival analysis, we evaluated whether the markers were associated with the risk of a PEx. Continuous covariates were dichotomized at the median. The parameters illustrated here were all influencing the probability of having a PEx. Higher risks of PEx were associated with A) lower FEV1% predicted ($p = 0.020$), B) lower FVC% predicted ($p = 0.032$), C) lower Clinical subscore of the Matouk Disease Score ($p = 0.004$), D) higher Complications subscore of the Matouk Disease Score ($p < 0.0001$), E) lower assessments of QOL Physical ($p = 0.030$) and F) lower Health Perceptions ($p = 0.006$) domains. G) The association between lower CRP levels and lower probabilities of PEx tended towards significance ($p = 0.054$).

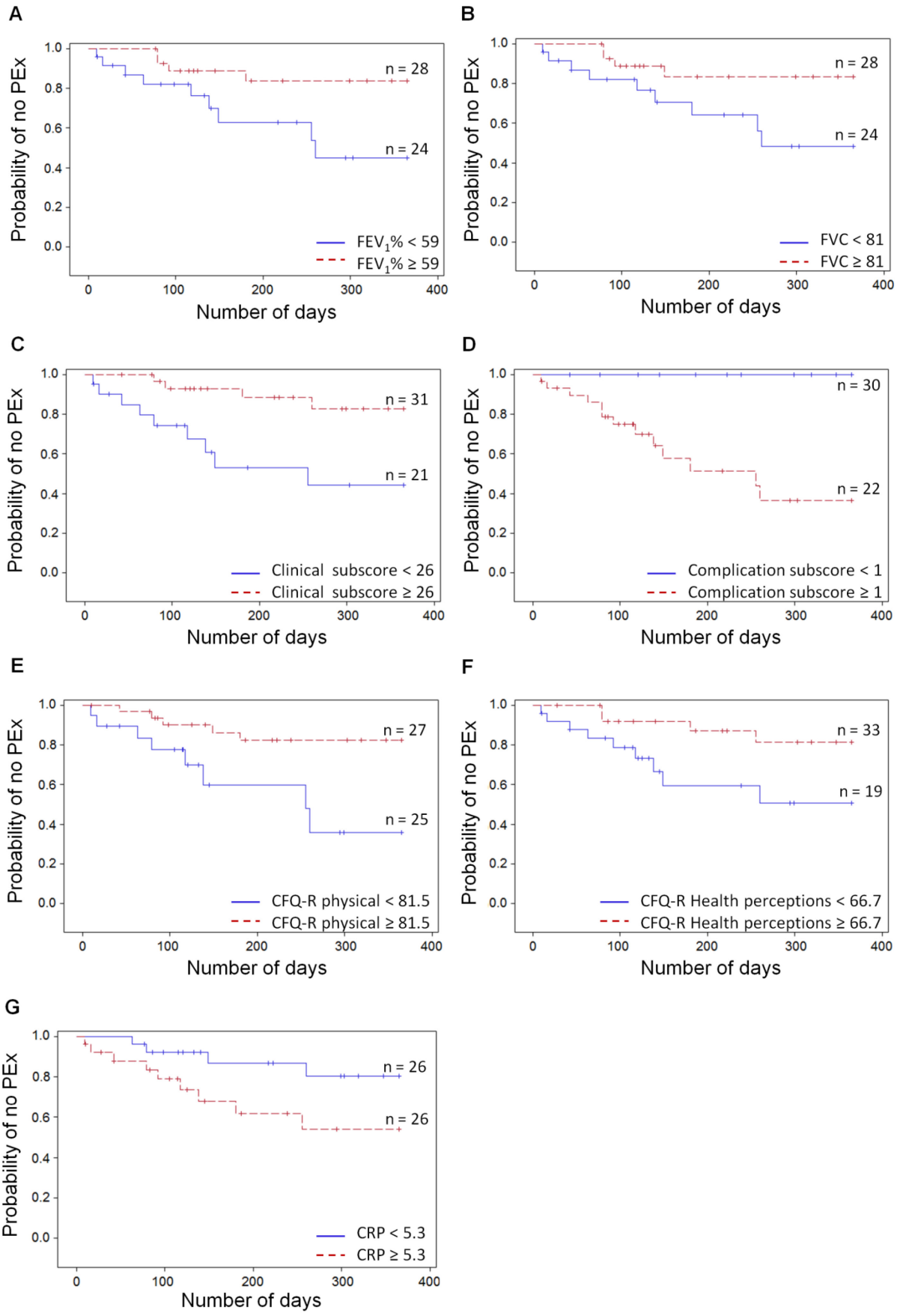
A
Probability of no PEx
1.0
0.8
0.6
0.4
0.2
0.0
n = 28
n = 24
FEV1% < 59
FEV1% ≥ 59
0
100
200
300
400
Number of days
B
Probability of no PEx
n = 28
n = 24
FVC < 81
FVC ≥ 81
Number of days
C
Probability of no PEx
n = 31
n = 21
Clinical subscore < 26
Clinical subscore ≥ 26
Number of days
D
Probability of no PEx
n = 30
n = 22
Complication subscore < 1
Complication subscore ≥ 1
Number of days
E
Probability of no PEx
n = 27
n = 25
CFQ-R physical < 81.5
CFQ-R physical ≥ 81.5
Number of days
F
Probability of no PEx
n = 33
n = 19
CFQ-R Health perceptions < 66.7
CFQ-R Health perceptions ≥ 66.7
Number of days
G
Probability of no PEx
n = 26
n = 26
CRP < 5.3
CRP ≥ 5.3
Number of days

Figure 4.2. Percentage change in clinical and QOL parameters of CF patients throughout PEx. A) FEV1% (*blue, circles*) and FVC% (*red, squares*) were significantly reduced from baseline values at Day 1 of PEx (FEV1% p = 0.001; FVC% p = 0.010). Both parameters subsequently improved with treatment however, FVC% significantly declined at Day 42 (p = 0.028). The Clinical subscore of the Matouk Disease score (*green, triangles*) was significantly decreased from baseline values on Day 1 (p < 0.0001) and Day 7 (p = 0.045). See Table 4.4 for the results for other Matouk Disease subscores. B) QOL items also decreased with PEx onset, Day 1, such as Respiratory (*blue, circles*, p = 0.002), Physical (*red, squares*, p = 0.025) and Health Perceptions domains (*green, triangles*, p < 0.0001). The Physical and Health Perceptions domains remained decreased at Day 7 (p = 0.025 and p = 0.001, respectively). Other QOL domains which decreased at Day 1 include: Vitality, Health Perceptions, Social and Role (Table 4.4). The dotted horizontal line indicates a 0% change or no change from baseline values. * indicates a significant difference from baseline. Day 1, n = 13; Day 7, n = 12, Day 14, n = 11; Day 21, n = 9; Day 42, n = 8. Full table of results can be found in Table 4.4.

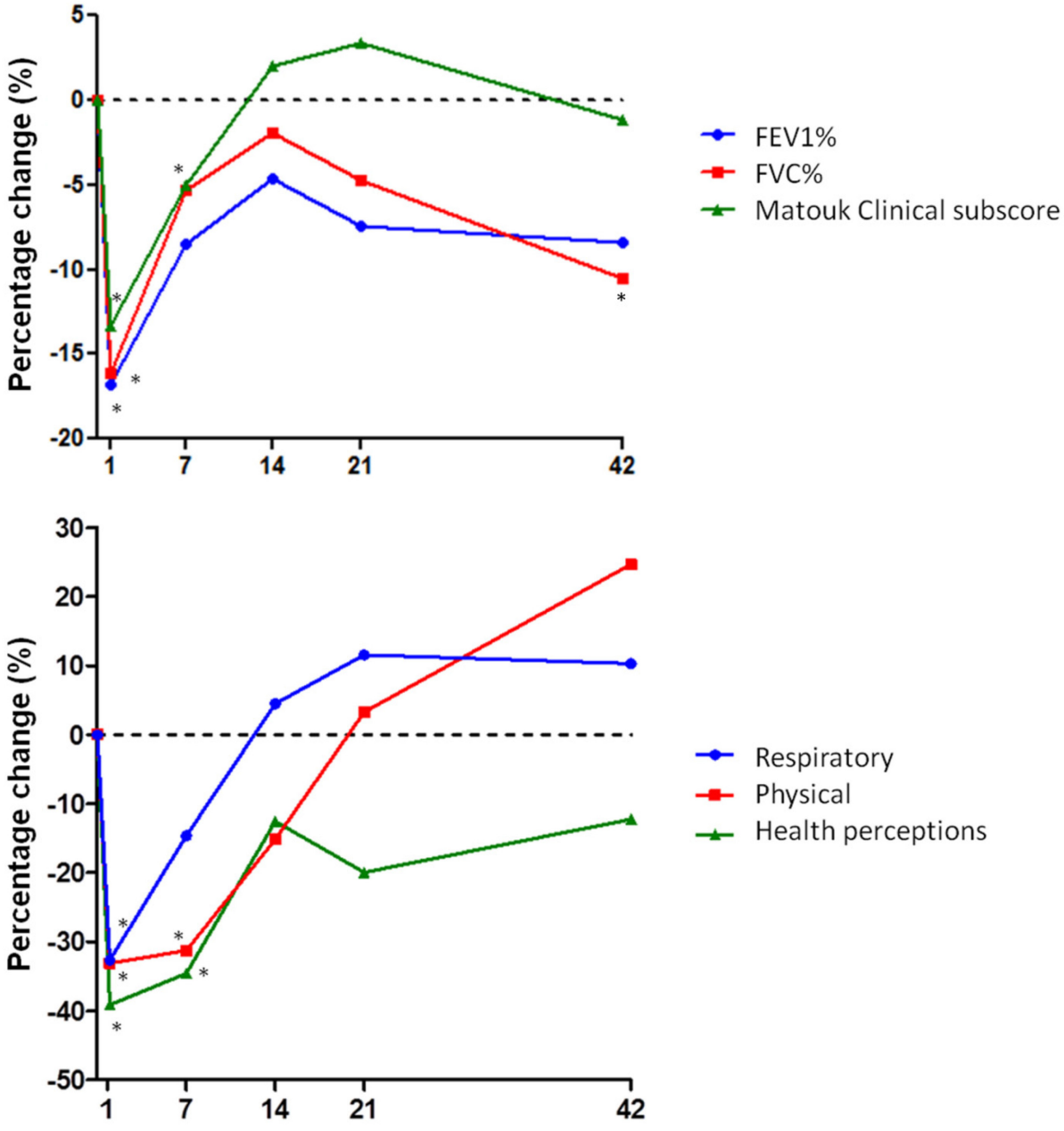
Percentage change (%)
5
0
-5
-10
-15
-20
1
7
14
21
42
FEV1%
FVC%
Matouk Clinical subscore
Percentage change (%)
30
20
10
0
-10
-20
-30
-40
-50
1
7
14
21
42
Respiratory
Physical
Health perceptions

Figure 4.3. Percentage change in inflammatory markers throughout PEx in CF patients. Inflammatory markers were measured in blood plasma from CF patients throughout PEx. The percentage change was calculated at each time point and compared to a 0% change which indicates no change from baseline (represented by dotted horizontal line). A) CRP levels were significantly increased from baseline values on Day 1 of PEx ($p = 0.001$) and Day 42 ($p = 0.039$). B) IL-6 levels were significantly higher on Day 1 of PEx ($p = 0.006$). C) IL-8 concentrations were significantly higher on Day 1 ($p = 0.047$) and significantly lower than baseline values after treatment on Day 21 ($p = 0.022$). D) IL-10 levels rose significantly on Days 7 ($p = 0.021$) and Day 14 ($p = 0.046$). E) MIP-1β increased from baseline on Days 1 ($p = 0.020$) and Day 42 ($p = 0.023$). F) VEGF levels were significantly higher on Day 1 of PEx ($p = 0.043$). Solid horizontal lines are set at the mean. * indicates a significant difference from 0% change from baseline. Day 1, n = 13; Day 7, n = 12, Day 14, n = 11; Day 21, n = 9; Day 42, n = 8. Full data set is found in Table 4.5.

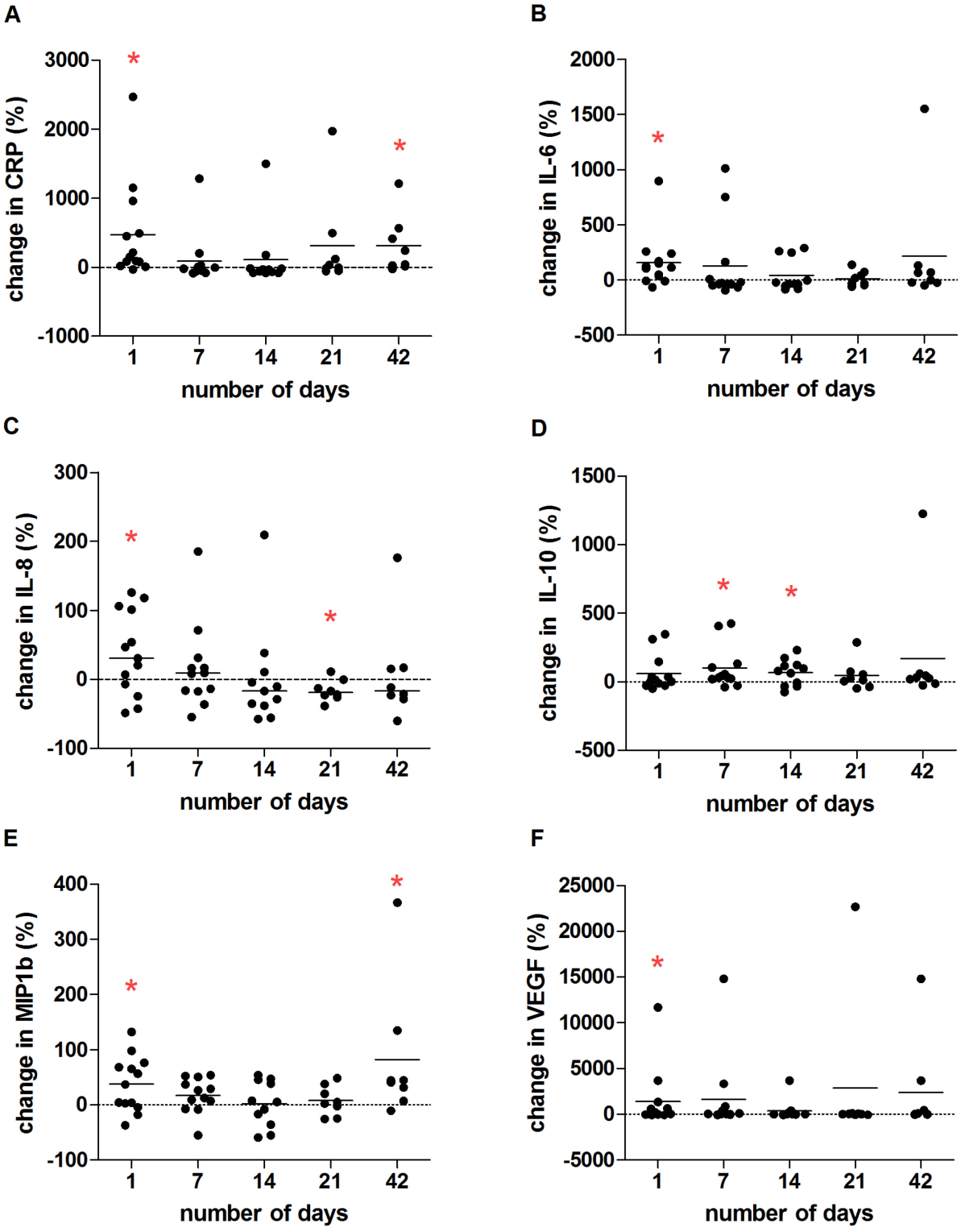
A
change in CRP (%)
3000
2000
1000
0
-1000
1
7
14
21
42
number of days
B
change in IL-6 (%)
2000
1500
1000
500
0
-500
number of days
C
change in IL-8 (%)
300
200
100
0
-100
number of days
D
change in IL-10 (%)
1500
1000
500
0
-500
number of days
E
change in MIP1b (%)
400
300
200
100
0
-100
number of days
F
change in VEGF (%)
25000
20000
15000
10000
5000
0
-5000
number of days

Figure 4.4. The levels of PUFA and lipid peroxidation in CF patients improve with aggressive PEx treatment. PUFA concentrations and lipid peroxidation were measured in blood plasma of healthy controls (HC, *white circles*) and CF patients (*black circles*) at stable disease (Bsl), at PEx onset (Day 1), throughout PEx treatment (Days 7, 14 and 21) and post treatment (Day 42). A) AA levels decreased during PEx treatment and were significantly different from Bsl values at Day 21 ($p < 0.05$). All PEx time points including Bsl were significantly different from HC, except on Day 21 where there was no longer a difference with HC. B) DHA levels improved with PEx treatment and were significantly increased from Bsl values at Day 21 ($p < 0.05$). All PEx time points including Bsl were significantly different from HC. C) Overall, the AA/DHA ratio was significantly decreased from Bsl values on Day 21 ($p < 0.05$). All PEx time points including Bsl were significantly different from HC, except on Day 21 where there was no difference with HC. D) Ceramide levels at Bsl were lower than HC, and remained unchanged throughout treatment. E) MDA levels improved with treatment however no statistical difference was detected from Bsl. All PEx time points including Bsl were significantly different from HC, except on Day 21 where there was no difference with HC. F) NT3 levels were higher than HC at baseline, however were no longer statistically different from HC on Days 14 and 42. Solid lines indicate the means of the groups. Dotted lines indicate the HC mean and grey shadowing illustrates the min-max range for HC values. * represents significant difference between Bsl group and PEx time points using the Bonferroni post-test after ANOVA. † indicates a significant difference between HC and CF samples (including PEx time points) using the Bonferroni post-test after ANOVA. Significance set as $p < 0.05$. HC n = 10, CF n = 8 – 13.

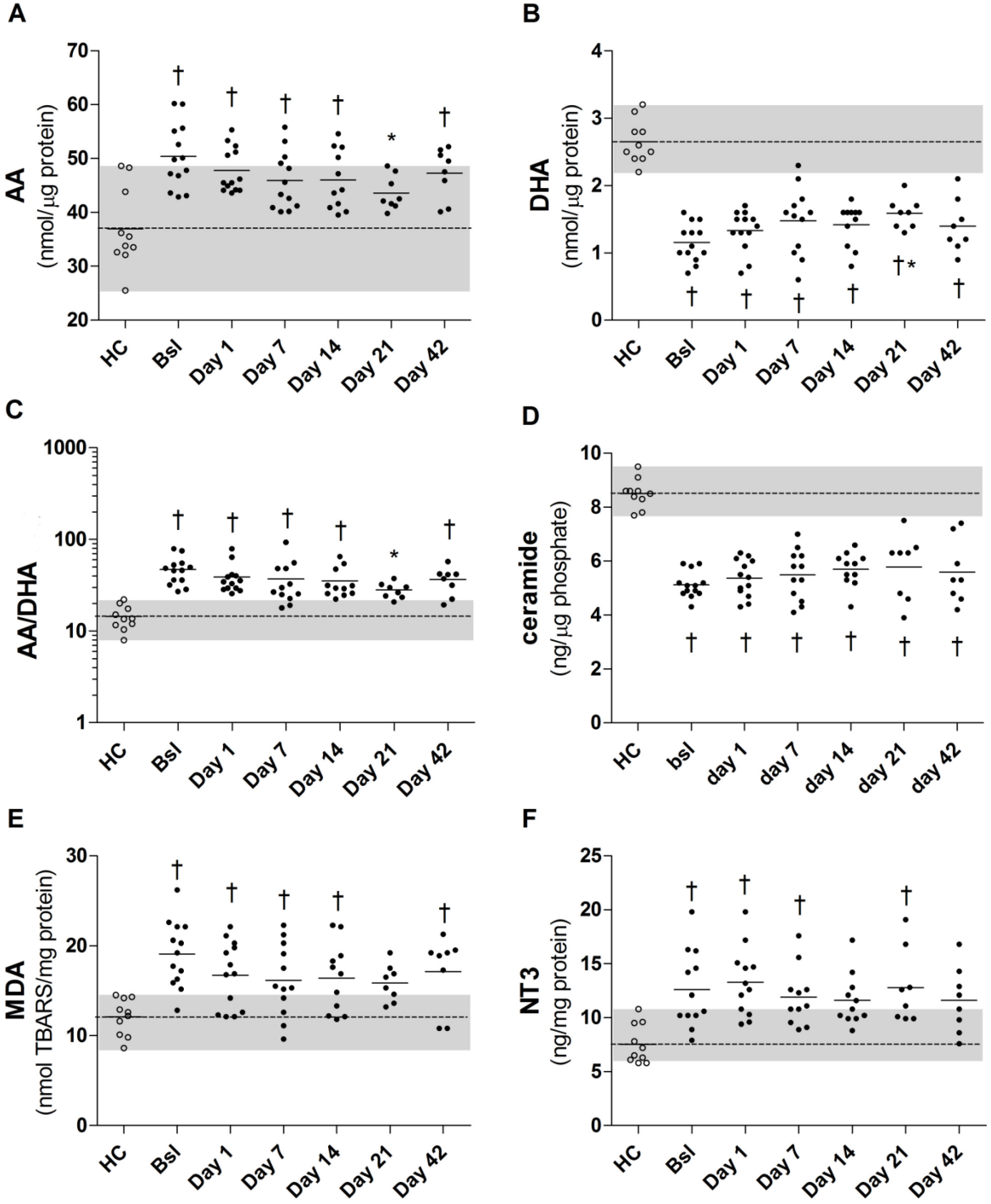
A
AA
(nmol/µg protein)
70
60
50
40
30
20
HC
Bsl
Day 1
Day 7
Day 14
Day 21
Day 42
B
DHA
(nmol/µg protein)
4
3
2
1
0
C
AA/DHA
1000
100
10
1
D
ceramide
(ng/µg phosphate)
10
8
6
4
2
0
bsl
day 1
day 7
day 14
day 21
day 42
E
MDA
(nmol TBARS/mg protein)
30
20
10
0
F
NT3
(ng/mg protein)
25
20
15
10
5
0

Table 4.1. Patient demographics and baseline values of clinical and QOL assessments

		Total CF patient group	Non-exacerbating (no PEx)	Exacerbating (PEx)	p-value no PEx vs. PEx
Demographics	**Number of patients**	52	15	37	
	Sex (F/M)	24/28	5/10	19/18	0.358
	Age	32.8 (1.8)	36.1 (3.4)	31.5 (2.3)	0.112
	Weight (kg)	60.8 (2.1)	**64.9 (2.8)**	**59.1 (2.6)**	**0.031***
	BMI (kg/m^2) (NR: 18.5-24.9)	21.9 (0.6)	**23.4 (0.7)**	**21.3 (0.7)**	**0.009***
Clinical assessments	**FEV1% predicted (%)**	63.0 (3.8)	**78.5 (5.0)**	**56.8 (4.6)**	**0.009***
	FVC% predicted (%)	79.8 (3.7)	**95.1 (5.2)**	**73.6 (4.4)**	**0.008***
	White blood cells (10^9/L) (NR: 4.8 – 10.8)	9.4 (0.4)	**8.1 (0.6)**	**10.0 (0.4)**	**0.018***
	Neutrophils (10^9/L) (NR: 1.6 – 7.7)	6.7 (0.3)	**5.6 (0.5)**	**7.1 (0.4)**	**0.033***
	Eosinophils (10^9/L) (NR: 0.0 – 0.5)	0.2 (0.02)	0.2 (0.07)	0.2 (0.01)	0.562
	Platelets (10^9/L) (NR: 140 – 440)	284.6 (8.4)	**244.0 (14.6)**	**301.6 (9.0)**	**0.001***
Matouk Disease Score	**Clinical** (0 – 50 points)	36.3 (0.6)	**39.3 (1.0)**	**35.1 (0.7)**	**0.002***
	PFT (0 – 25 points)	16.6 (0.9)	**19.9 (1.1)**	**15.2 (1.0)**	**0.018***
	CXR (0 – 25 points)	16.5 (0.4)	**18.2 (0.9)**	**15.8 (0.4)**	**0.008***
	Complication (0 – 37 points)	2.6 (0.5)	**0.3 (0.2)**	**3.5 (0.7)**	**<0.0001***
	Total	66.9 (2.0)	**77.3 (2.3)**	**62.6 (2.4)**	**0.001***
Quality of life (each item is scored 0 – 100 points)	**Weight**	64.1 (5.2)	**84.5 (5.5)**	**55.9 (6.5)**	**0.016***
	Respiratory	68.3 (2.3)	74.8 (4.5)	65.6 (2.6)	0.072
	Digestion	78.0 (2.5)	84.5 (4.0)	75.4 (3.1)	0.064
	Physical	71.8 (3.4)	**83.3 (3.8)**	**67.2 (4.4)**	**0.041***
	Vitality	64.4 (2.2)	67.8 (4.4)	63.1 (2.6)	0.341
	Emotion	81.7 (2.2)	**90.7 (2.6)**	**78.0 (2.7)**	**0.008***
	Eating	90.3 (2.3)	91.1 (4.8)	89.9 (2.7)	0.537
	Treatment burden	66.2 (3.4)	74.8 (6.7)	62.8 (3.9)	0.113
	Health Perceptions	69.5 (2.8)	**80.8 (4.0)**	**54.9 (3.3)**	**0.009***
	Body Image	73.5 (3.5)	**88.9 (3.1)**	**67.3 (4.3)**	**0.004***
	Social	74.6 (2.5)	81.5 (4.4)	71.8 (2.9)	0.074
	Role	82.8 (2.8)	**91.7 (3.0)**	**79.2 (3.6)**	**0.044***
	Total (0 – 1200 points)	885.2 (21.8)	**994.2 (28.9)**	**841.0 (25.1)**	**0.001***

Data represented as mean (SEM). * p-value designates significant statistical difference between non-exacerbating (no PEx) and exacerbating patients (PEx) using Student's t-test or Mann-Whitney t-test when values were not normally distributed. NR indicates normal range for cell counts. BMI normal range from Health Canada (http://www.hc-sc.gc.ca).

Table 4.2. Baseline values of inflammatory markers and lipids

	Healthy controls (HC) n = 3 - 11	Total CF patients group (CF) n = 52	Non-exacerbating CF patients (no PEx) n = 15	Exacerbating CF patients (PEx) n = 37
CRP (mg/L)	2.0 (1.1)	7.4 (1.0)	6.04 (1.7)	8.0 (1.2)
IL-1β (pg/mL)	3.8 (2.7)	0.9 (0.1)	0.79 (0.2)	0.9 (0.1)
IL-6 (pg/mL)	2.7 (1.1)	5.2 (1.2)	**2.93 (0.6)**	**6.0 (1.6)†**
IL-8 (pg/mL)	3.6 (0.9)	5.0 (0.5)	4.08 (0.6)	5.8 (0.6)
IL-10 (pg/mL)	**2.1 (0.9)**	**29.1 (3.2)***	**19.04 (2.6)**	**38.8 (7.2)†**
MIP-1β (pg/mL)	31.8 (6.9)	24.3 (1.6)	24.83 (3.2)	24.2 (1.9)
TNF (pg/mL)	**5.3 (0.5)**	**3.8 (0.3)***	3.45 (0.5)	3.9 (0.4)
VEGF (pg/mL)	129.5 (46.7)	95.7 (16.5)	82.45 (17.3)	101.0 (22.2)
AA (nmol/µg protein)	**37.0 (2.4)**	**49.5 (0.8)***	48.45 (1.5)	49.9 (1.0)
DHA (nmol/µg protein)	**2.7 (0.1)**	**1.2 (0.1)***	1.30 (0.1)	1.2 (0.1)
AA/DHA ratio	**14.4 (1.4)**	**48.8 (4.6)***	40.92 (3.8)	51.8 (6.1)
Ceramide (ng/µg phosphate)	**8.5 (0.2)**	**5.4 (0.1)***	5.7 (0.3)	5.2 (0.1)

Data represented as mean (SEM). * p-value represents statistically significant difference between HC and CF groups . † represents statistically significant difference between "no PEx" and "PEx" groups. Significance was set at $p < 0.05$.

Table 4.3. Markers influencing time to first PEx during stable disease

Marker		Hazard ratio[1]	95% hazard ratio confidence limits	p-value
Clinical assessments	**FEV1% predicted**	0.97	0.94 – 0.99	0.006
	FVC% predicted	0.97	0.95 – 0.99	0.010
Matouk Disease Score	**Clinical**	0.74	0.63 – 0.86	<0.0001
	PFT	0.88	0.80 – 0.98	0.015
	CXR	0.71	0.50 – 0.94	0.017
	Complication	1.33	1.17 – 1.51	<0.0001
	Total	0.92	0.88 – 0.96	<0.0001
Quality of life	**Weight**	0.97	0.95 – 0.99	0.006
	Respiratory	0.96	0.92 – 0.99	0.024
	Physical	0.96	0.93 – 0.99	0.003
	Vitality	0.96	0.93 – 1.00	0.043
	Health perceptions	0.95	0.92 – 0.99	0.005
	Role	0.96	0.94 – 0.99	0.020
	Total	0.99	0.99 – 1.00	0.004

[1]HR < 1 is associated with lower risk of future PEx

Table 4.4. Percentage change in clinical and QOL assessments throughout PEx in CF.

(*on following page*)

Data represented as means of percentage changes (SEM). * p-value indicates significant statistical difference between time point vs. baseline values as determined using one sample t-test or Wilcoxon Signed Rank test when values were not normally distributed.

		Day 1 n = 13	p-value	Day 7 n = 12	p-value	Day 14 n = 11	p-value	Day 21 n = 8	p-value	Day 42 n = 8	p-value
Clinical parameters	FEV1% predicted	-16.8 (3.9)	**0.001***	-8.5 (4.2)	0.068	-4.7 (5.3)	0.397	-7.4 (3.9)	0.141	-8.4 (5.3)	0.159
	FVC% predicted	-16.1 (5.3)	**0.010***	-5.3 (4.3)	0.240	-1.9 (5.2)	0.719	-4.7 (4.3)	0.150	-10.5 (3.8)	**0.028***
	White blood cells	28.4 (12.4)	**0.002***	30.9 (11.7)	**0.023***	35.5 (21.4)	0.175	11.4 (15.5)	0.547	0.9 (8.2)	0.920
	Neutrophils (%)	45.3 (19.3)	**0.005***	36.1 (12.9)	**0.018***	43.8 (28.1)	0.150	17.5 (25.0)	0.742	6.3 (12.2)	0.621
	Eosinophils (%)	37.0 (16.2)	**0.041***	79.8 (26.2)	**0.011***	73.1 (26.3)	**0.020***	177.8 (62.0)	**0.016***	26.3 (24.5)	0.483
	Platelets (%)	4.5 (5.9)	0.461	5.7 (5.6)	0.328	4.9 (5.6)	0.403	-0.02 (4.6)	0.996	3.1 (9.2)	0.743
Matouk Disease Score	Clinical	-13.3 (2.5)	**<0.0001***	-5.0 (1.9)	**0.045***	2.0 (1.8)	0.273	3.4 (4.2)	0.446	-1.1 (6.0)	0.856
	PFT	-11.0 (3.5)	**0.009***	-6.3 (4.0)	0.150	-7.4 (4.7)	0.148	-6.8 (6.7)	0.176	-5.6 (6.3)	0.400
	CXR	-1.9 (2.0)	0.375	0.2 (2.4)	0.949	-0.5 (2.4)	0.853	3.5 (1.8)	0.099	-1.5 (2.9)	0.615
	Compli-cations	169.9 (67.1)	**0.006***	171.6 (73.0)	**0.004***	197.0 (76.9)	**0.009***	203.9 (104.2)	**0.036***	216.6 (103.5)	**0.022***
	Total	-19.0 (1.8)	**<0.0001***	-11.2 (2.3)	**0.001***	-7.6 (1.8)	**0.002***	-5.6 (2.1)	**0.016***	-10.2 (4.1)	**0.042***
Quality of life	Weight	-6.4 (9.4)	0.505	7.0 (16.6)	0.683	3.0 (17.0)	0.862	6.2 (6.2)	1.000	37.5 (24.6)	0.174
	Respiratory	-32.7 (5.6)	**0.002***	-14.6 (9.5)	0.240	4.5 (10.0)	0.844	11.6 (9.9)	0.280	10.3 (9.6)	0.438
	Digestion	9.5 (7.7)	0.362	3.6 (6.1)	0.352	9.3 (7.0)	0.214	23.9 (10.4)	0.055	18.1 (11.7)	0.166
	Physical	-33.1 (15.4)	**0.025***	-31.3 (10.1)	**0.010***	-15.2 (6.3)	0.146	14.1 (21.4)	0.531	24.7 (48.2)	0.195
	Vitality	-34.7 (5.2)	**0.002***	-34.9 (7.9)	**0.001***	-18.0 (5.5)	**0.009***	-17.9 (13.1)	0.213	-17.1 (12.1)	0.200
	Emotion	-9.8 (8.8)	0.288	-4.0 (6.2)	0.531	1.5 (6.5)	0.826	-3.5 (7.8)	0.672	6.9 (7.2)	0.469
	Eating	-11.7 (7.8)	0.123	-21.1 (5.9)	**0.004***	-11.1 (6.4)	0.115	-1.6 (4.6)	0.745	-8.3 (10.0)	0.434
	Tx burden	-3.9 (5.8)	0.291	-18.5 (7.1)	**0.024***	-11.7 (5.6)	0.064	-26.6 (11.5)	**0.036***	6.9 (10.6)	1.000
	Health Perceptions	-39.1 (7.3)	**<0.0001***	-34.6 (7.5)	**0.001***	-12.5 (6.4)	0.079	-18.3 (11.6)	0.160	-12.3 (12.4)	0.357
	Body image	2.7 (20.5)	0.844	11.4 (13.8)	0.624	-3.6 (11.6)	0.762	11.1 (22.1)	0.633	1.3 (17.5)	0.943
	Social	-14.3 (4.0)	**0.004***	-16.7 (5.5)	**0.011***	-14.3 (8.9)	0.203	-14.2 (11.7)	0.266	-7.4 (7.9)	0.375
	Role	-11.4 (8.4)	0.196	-22.3 (8.9)	**0.019***	-2.2 (15.3)	0.641	-9.8 (6.6)	0.279	-5.2 (10.5)	0.638
	Total	-17.4 (4.2)	**0.001***	-24.2 (7.8)	**0.001***	-7.7 (3.1)	**0.035***	-2.6 (4.6)	0.585	1.0 (7.3)	0.844

Table 4.5. Percentage change in inflammatory markers during PEx

	Day 1 n = 13	p-value	Day 7 n = 12	p-value	Day 14 n = 11	p-value	Day 21 n = 8	p-value	Day 42 n = 8	p-value
CRP (%)	470.9 (196.2)	**0.001***	85.7 (111.5)	0.380	107.6 (141.0)	0.320	309.8 (245.8)	0.461	310.1 (149.4)	**0.039***
IL-1β (%)	120.3 (83.4)	0.839	154.5 (102.1)	0.064	204.3 (225.1)	0.638	131.0 (120.1)	0.250	22.0 (29.25)	1.000
IL-6 (%)	157.7 (67.0)	**0.006***	129.6 (104.2)	0.733	40.1 (44.4)	0.831	11.6 (24.3)	0.648	214.9 (192.0)	0.313
IL-8 (%)	37.4 (16.9)	**0.047***	16.6 (18.0)	0.733	1.0 (22.5)	0.365	-16.1 (5.5)	**0.022***	8.0 (25.6)	0.547
IL-10 (%)	58.6 (35.8)	0.376	99.4 (44.7)	**0.021***	65.7 (28.8)	**0.046***	45.6 (37.3)	0.250	169.7 (150.8)	0.078
MIP-1β (%)	37.2 (13.9)	**0.020***	17.1 (9.1)	0.088	1.9 (12.4)	0.882	7.5 (9.4)	0.456	82.2 (43.3)	**0.023***
TNF (%)	101.7 (89.8)	0.685	51.2 (23.6)	0.053	43.3 (39.1)	0.294	-13.4 (12.3)	0.311	37.2 (32.6)	0.461
VEGF (%)	1381.0 (902.8)	**0.043***	1610.0 (1232.0)	0.160	366.7 (332.1)	0.469	2839.0 (2833.0)	0.563	2367.0 (1834.0)	0.156

Data represented as means of percentage changes (SEM). *p-value indicates significant statistical difference between time point vs. baseline values as determined using one sample t-test or Wilcoxon Signed Rank test when values were not normally distributed.

Table 4.6. Markers associated with early re-exacerbation at the end of PEx treatment

	Early PEx (n = 6)	Late PEx (n = 6)	p- value
Days until next PEx mean (min – max)	17.3 (1 – 29)	142.5 (56 – 365)	N/A
FEV1% predicted (%)	38.5 (7.2)	50.0 (8.5)	0.328
FVC% predicted (%)	56.5 (9.9)	64.0 (10.0)	0.606
Matouk Total score	46.5 (6.2)	56.8 (3.7)	0.180
QOL Total score	752.0 (86.7)	765.5 (62.8)	0.902
CRP (mg/L)	**42.5 (26.1)**	**5.2 (2.7)**	**0.045***
IL-6 (pg/mL)	4.9 (1.6)	2.6 (0.6)	0.199
IL-8 (pg/mL)	**3.9 (0.4)**	**2.7 (0.3)**	**0.034***
IL-10 (pg/mL)	24.4 (2.5)	77.0 (37.8)	0.195

Data presented as mean (SEM) unless specified. * indicates significant difference between the groups using Student's t-test or Mann-Whitney test if values were not normally distributed.

CHAPTER 5: GENERAL DISCUSSION

Discussion on ceramide adapted from the review:

Wojewodka G., De Sanctis J.B., Radzioch D., Ceramide in cystic fibrosis: a potential new target for therapeutic intervention. *J Lipids*, 2011:674968

1. FENRETINIDE: A THERAPY FOR CF

Table 5.1 summarizes the recommended treatments for CF patients and their effects on CF phenotypes in comparison to fenretinide. Many of the drugs listed can be classified into more than one functional category.

Tobramycin is the primary anti-infective medication used by CF patients. It was also found to have some anti-inflammatory properties by reducing the migration of neutrophils *in vitro*, and preventing the activity of neutrophil elastase via α1-antitrypsin, however its effect on cytokines has not been reported. As mentioned in Chapter 4, it also behaves as a scavenger for reactive oxygen species *in vitro* [333]. We showed that aggressive treatment of CF patients during PEx can improve the PUFA defects, and we hypothesized that IV treatment with tobramycin may be reducing oxidation and thus protecting DHA from peroxidation. However, future studies are required to confirm this finding.

Hypertonic saline is used primarily to increase the volume of the ASL. Studies have shown its effects on reducing IL-8 levels in sputum from CF patients and *in vitro*. In CF lungs, IL-8 binds to glycosaminoglycans through electrostatic interactions which prevent its degradation. By altering the ion concentration of the ASL, this interaction is disturbed, releasing IL-8 which can then be degraded [337]. The effect of hypertonic saline on other inflammatory markers has not been reported.

Recombinant human DNase (rhDNase) is used to breakdown the thick mucosal layer in the airways. Consequently, the environment for pathogens is disrupted and patients have lower bacterial counts after treatment, without additional antibiotic treatment. DNase was also found

to have anti-inflammatory effects by preventing the increase of IL-8 and neutrophil elastase over time in young uninfected CF patients [338].

Azithromycin is another anti-infective treatment used in CF which has been shown to have anti-inflammatory properties. *In vitro* studies showed no effect of azithromycin on inflammation in CF epithelial cells [339]. However, it was found to reduce serum levels of CRP and calprotectin in uninfected CF patients after 28 days of treatment [340]. Azithromycin was also found to decrease the levels of eicosanoids *in vitro* by blocking $cPLA_2$ [341]. Aztreonam has found to be a powerful anti-infective which significantly benefits CF patients by reducing the bacterial burden in lungs [79] however it has not be assessed for other CF phenotypes.

Ibuprofen is a known anti-inflammatory medication which was found to reduce the levels of NF-κB activation in CF epithelial cells [342]. It shows important effects in young CF patients on prevention of lung function decline [88] however the risk of gastrointestinal bleeding remains an important factor in chronic treatment.

Ivacaftor is the newest drug for CF on the market proven to substantially improve the symptoms of CF patients with the G551D mutation by potentiating the ion current through the chloride channel. It was also found to improve insulin secretion in CF patients [343]. The effects of this drug on ASL, mucus, inflammation and oxidation are yet to be fully described. The issue remains that the majority of CF patients cannot benefit from this treatment and for those that can, the cost is quite high.

The evidence presented in this thesis demonstrates the potential therapeutic benefits of fenretinide for CF which requires clinical trials to fully assess its safety and efficacy. Chapter 1 presented the major pathological contributions to CF disease and how current treatments

address these issues. It also introduced areas in CF disease which are currently lacking in viable treatment options such as correction of PUFA and ceramide defects, reducing inflammation and lipid peroxidation.

In Chapter 2, we showed that defects in PUFA in CF are correlated to those in ceramide. Greater defects in AA in particular were associated with a more severe presentation of CF disease. We demonstrated that a 28-day fenretinide treatment protocol, previously found to increase ceramide levels and improve infection clearance, also improved the PUFA imbalance by reducing AA to normal levels and increasing DHA in CF mice. This treatment also reduced inflammation in the lungs of CF mice. This study concluded that fenretinide may help to address two characteristics of CF disease: PUFA defects and high levels of inflammation. Since the publication of the results presented in Chapter 2, we tested the effects of fenretinide in the context of other diseases. It was found to reduce inflammation incurred after acute spinal cord injury in mice by reducing the levels of TNF and iNOS [344]. The results of *in vitro* experiments in macrophages demonstrated that fenretinide decreases the inflammatory response following LPS exposure via the reduction in ERK1/2 phosphorylation [111]. Although there are a few current treatments for CF that also act as anti-inflammatories, there is no medication given routinely for this purpose.

Chapter 3 showed the impressive effects of one dose of fenretinide on PUFA and ceramide. The action of fenretinide in normalizing ceramide levels is most likely due to the direct effects on the ceramide synthesis pathway [217]. The mechanism of fenretinide on PUFA was unknown until we explored markers of lipid peroxidation such as MDA, which is reduced with fenretinide treatment. The improvements in DHA may be explained in part by reduced

levels of lipid peroxidation, which consequently would decrease the levels of AA. We confirmed the high levels of systemic peroxidation in a cohort of CF patients from which we obtained leukocytes. Fenretinide treatment of the cells caused improvements in the PUFA imbalance and a reduction of peroxidation. Additionally, we showed reductions in NT3 levels following fenretinide treatment, a CF phenotype not addressed by any CF treatment available. This chapter concluded that fenretinide can address other issues in CF disease: high levels of lipid peroxidation and nitrosative stress. Currently there are no established therapies to reduce oxidation, although as mentioned in Chapter 1, some are being tested. A recent trial of inhaled glutathione concluded with no benefits of this treatment on improved lung function, no changes in oxidation nor inflammation status and thus does not seem to be beneficial for CF patients [100].

Chapter 4 presented a clinical study on the risk of PEx in CF patients. This study was performed to determine whether a combination of clinical and patient-reported data together with inflammatory markers and lipids could help determine which patient is more likely to have a PEx. Additionally, this study served as a model on which to base our protocol for future clinical trials in terms of sample collection, processing and markers to analyze and a characterization of each individual CF patient. We found that at periods of stable disease, worsening of the clinical picture and quality of life may place the patient at risk of a future PEx. While at the end of treatment, unresolved inflammation may indicate which patients will experience a rapid re-exacerbation. Surprisingly, we found that while being treated aggressively with antibiotics and corticosteroids, patients had systemic improvements in PUFA levels with reduced AA, increased DHA leading to improved AA/DHA ratios. MDA and NT3 levels were

correlated with PUFA levels, demonstrating again an association between lipid peroxidation, nitrosative stress and PUFA defects, as seen in mice in Chapter 3. Ceramide levels remained relatively unchanged throughout PEx. Based on the data included in Chapter 4, we concluded that patients may benefit from anti-inflammatory therapies to regulate unabated inflammation after PEx treatment. Importantly, we presented throughout this thesis clear defects in lipids in CF patients during stable disease compared to healthy controls indicating that routine therapies for disease maintenance in CF do not improve these abnormalities. Inhaled antibiotics are prescribed to patients in our clinic when tested positive for PA infection. In our patient cohort for the study in Chapter 4, 75% were taking inhaled antibiotic therapy yet still had defects in PUFA and ceramide levels compared to healthy individuals. We observed some improvements in lipid levels with aggressive antibiotic treatment but these improved levels were not maintained once treatment was completed. Importantly, these aggressive antibiotic treatments are not feasible as chronic therapies, while fenretinide can be used daily.

2. CERAMIDE IN CF

Following our study regarding the modulation of ceramide with fenretinide in CF, other studies were published with results in conflict with ours. This next section will discuss the various studies published on ceramide in CF.

The most important factor to consider in the studies on ceramide in CF involves the various methodologies used to detect these sphingolipids. Phospholipids constitute approximately 30% of lipids in cells[345]. Ceramide alone represents only 1% of lipids present in the cell [167]. The ceramide species are similar in structure thus their analysis requires high level of specificity to differentiate them from each other.

One of the most common biochemical assays for ceramide detection involves the use of diacylglycerol (DAG) kinase which can convert ceramide to ceramide-1-phosphate. The assay quantifies levels of ceramide by incubating extracted lipids with [P^{32}]ATP and DAG kinase, and assessing the levels of [P^{32}]ceramide-1-phosphate. There are certain caveats with this method [346] and there has been some question regarding the validity of the results generated with this assay [347,348]. The source and amounts of the enzyme and ATP can alter the results obtained. Additionally, an internal standard should ideally be used to normalize the level of enzyme activity [167]. In summary, proper care must be taken into consideration to produce valid results [349] and ideally data should be verified using a more sensitive technique.

Certain studies employ immunodetection for total ceramide quantification. There exist two antibodies which have been employed for these methods. A monoclonal antibody clone 15B4 (Sigma-Aldrich) was employed by our group using TLC-purified lipids in an ELISA assay [180] and others in immunohistochemistry [350]. A polyclonal antibody, IgM-enriched mouse anti-ceramide antiserum (Glycobiotech, Kükels, Germany, formerly identified as MAS 0010 or S58-9, same molecule as MAS 0010 sold without the addition of mouse serum, Glycobiotech) has been used by some CF investigators [261,350].

Both antibodies were evaluated and compared in a study by Cowart and colleagues which found that each antibody recognizes different species of ceramide and both recognize dihydroceramide [351]. Some cross-reactivity was observed to other sphingolipids and cholesterol depending on epitope exposure methods [351,352].

The studies using antibodies in ceramide quantification have used different methods. Our group has used the monoclonal antibody in ELISAs only after the ceramide fraction was

isolated from lipids using TLC [180]. Other studies have used these antibodies for immunohistochemistry detecting the amount of staining in a designated area with computer software [261,350].

The most accurate and sensitive method for an accurate analysis of total ceramide levels as well as the multiple subspecies of ceramide involves refined separation techniques such as liquid and gas chromatography (LC and GC) and mass spectrometry (MS) [345,353-356]. There are technical issues which can affect the quantification of ceramide by MS such as the purification of lipids from biological samples, the use of a non-natural ceramide as an internal control, the generation of accurate standard curves for each of the analytes and the concentration of lipids in the sample prior to analysis. However, MS is the most reliable method for ceramide analysis available to date.

Contradicting findings have been published about ceramide levels in CF [180,238,261,350]. Our laboratory published observations regarding reduced levels of ceramide in patients with CF. We analyzed ceramide levels in plasma samples from 10 CF patients and 10 healthy controls by high pressure LC (HPLC)/MS. Our results showed that specific species of ceramide were lower in CF patients: C14, C22, C22:1, C24, C20:1 and also DHC16. When looking at the total levels, CF patients were found to have an overall deficiency in ceramide [180]. The analysis of ceramide levels in CF affected organs also showed defects in CF mice compared to WT mice in plasma, lung, ileum and pancreas [180,242].

Vilela and colleagues demonstrated an increase in IL-8 levels in human tracheal epithelial cells with and without CFTR expression following TNF exposure. The study showed that fenretinide treatment on cells lacking CFTR expression reduced IL-8 levels after TNF

stimulation. Using an HPLC method of ceramide detection, they also demonstrated increased ceramide levels following fenretinide treatment of CFTR deficient cells [238].

Other groups found no differences in ceramide levels between cells expressing and lacking CFTR. There was however a difference in ceramide response to stressors. *In vitro* studies by Yu and colleagues revealed an differences in the response of aSMase to PA in bronchial epithelial cells where CFTR expressing cells increased ceramide levels following infection, while CFTR-deficient cells did not [357]. A lower incidence of cellular apoptosis was noticed following infection of CFTR deficient cells which was improved with the addition of aSMase. An increase in pulmonary ceramide levels was observed in normal mice (C57BL/6) mice but not in CF gut-corrected mice ($Cftr^{tm1unc}$-$TgN^{(FABPCFTR)}$) following oropharyngeal instillation of PA however, no differences between the strains were seen pre-infection [357]. Further *in vitro* studies by Noe and colleagues in endothelial cells revealed defects in ceramide metabolism. Human microvascular endothelial cells respond to H_2O_2 by increasing intracellular ceramide. Pre-treating the same cells with an inhibitor of CFTR ($CFTR_{inh}$-172) prevented the increase in ceramide levels. The authors linked the defects in ceramide to the defects observed in apoptosis whereby suggesting that the lack of apoptosis seen in CF may be due to the defect in upregulating ceramide in response to oxidative stress [358].

In contrast to our studies, the studies by the Gulbins group found an accumulation of ceramide in CF [261]. First, using two mouse models of CF ($Cftr^{tm1unc}$-$Tg^{(FABPCFTR)}$ and B6.129P2(CF/3)-$Cftr^{TgH(neoim)Hgu}$), ceramide levels were quantified by liquid scintillation counting of ceramide spots ran on TLC plates and immunochemistry was used on paraffin embedded lungs. The results showed elevated levels of ceramide in vesicles of epithelial cells from CF mice

compared to the appropriate controls. Similar results were obtained using epithelial cells obtained from CF patients by nasal scraping and lung tissue obtained post-transplant. Their study revealed that ceramide accumulated in intracellular lysosomes. The authors attributed this accumulation to the impairment in acidification of the vesicles which inhibited acid ceramidase, the enzyme converting ceramide to sphingosine. The views on whether pH regulation in vesicles is altered in CF are still conflicting. Certain studies have shown that pH is abnormal in CF vesicles [359] while others claim there are no differences [360,361]. The authors treated mice with aSMase inhibitors which resulted in the reduction of ceramide levels and improved clearance of bacterial infections [261]. As in the study by Yu and colleagues mentioned previously [357], when aSMase is not active, ceramide levels do not increase. However, Yu and colleagues observed detrimental effects during bacterial infections when aSMase function is defective while the Gulbins study suggested that inhibition of aSMase enables increased survival of mice infected with PA. The Gulbins group studied the effects of PA infection in macrophages from WT mice and from macrophages obtained from their CF mouse models. Using the DAG kinase assay they showed higher basal levels of ceramide in CF cells compared to WT cells. Using an immunofluorescence based method, they reported increased ceramide concentrations in WT cells following bacterial infection but not in CF macrophages. In response to the infection, WT cells had increased clustering of ceramide in the plasma membrane but this response was much reduced in CF cells [362].

An immunofluorescence based method was also used in another study by the same group to evaluate the levels of ceramide in alveoli from explanted lung tissue from CF patients. Concurrent to their previous results obtained with the same methods, they had seen higher

staining with anti-ceramide antibodies in CF tissue compared to donor tissue [363]. No additional methods were used to corroborate their data. In a fourth study, the group utilized three methods of detection of ceramide: immunofluorescence, DAG kinase assay and mass spectrometry. The authors looked at three specific species of ceramide by MS: C16, C18 and C20. The levels of each of the three species were not specified in the study but their sum was higher in lungs from their CF mouse models compared to their controls [364]. The authors tested their hypothesis in CF patients using amitriptyline, an anti-depressant which inhibits aSMase. Following 28 days of treatment, FEV_1% levels increased 3.6% relative to baseline (when patients with high CRP levels were excluded from the analysis). Ceramide levels were evaluated in nasal epithelial cells using immunofluorescence and given a score 1 – 4 for intensity. In 9 out of 14 patients treated with the drug, fluorescence decreased. However, 6 out of 14 placebo treated patients also had decreased staining in their cells. There were no changes in bacteria load, leukocyte levels, cytokine levels or DNA levels in sputum between both groups [365].

Another group tested the modulation of ceramide levels in mice using myriocin, an inhibitor of serine palmitoyl transferase from the *de novo* pathway for ceramide generation (see Figure 1.2). Ceramide levels in CF gut-corrected mice were not different compared to WT mice. They did have a greater inflammatory response to PA infection which was reduced with myriocin treatment. The treatment also reduced the total level of ceramide as measured using LS/MS [366].

The levels of ceramide in CF were assessed by UK investigators using lung tissue from transplant patients with CF and unused donor lungs. The authors used two methods of ceramide quantification: immunochemistry with the two antibodies and HPLC/MS. The

immunochemistry results indicated that CF lung tissues had higher median levels of staining compared to all other lung tissue. The authors also found variability in staining between the two antibodies although one was not systematically staining more than the other. Ceramide levels were additionally measured by HPLC/MS although only four species of ceramide were analyzed. It was found that C16, C18 and C20 were elevated in CF lung tissue while no difference was observed for C22 [350].

Another study has shown a combination of the two hypothesis finding elevated levels of certain species of ceramide while others were reduced. Using a mass spectrometry method, ceramide species were analysed in 16HBE14o(-) cells either expressing *CFTR* or the antisense control gene construct. Four ceramide species were found to be elevated (DHC16, C22, C24, C26) when no *CFTR* was expressed while 2 species were reduced (C18 and C18:1) compared to cells expressing *CFTR* [367].

The question remains why these studies report such different results. The first suggestion is the use of different models either animal or cellular. Different cell types or sources of cells (patient samples vs. immortalized cell lines) may respond differently to stress and ceramide may act differently within each cell type. As for animal models, complete *Cftr* KOs have different phenotypes compared to gut-corrected models which may lead to differences in the ceramide response. The issue of diet has been brought up by the Gulbins group as a point of contention between *in vivo* ceramide studies [261]. It has been suggested that the liquid diet Peptamen is responsible for the reduced levels of ceramide by elevating cholesterol levels which in turn inhibit aSMase activity [261]; however, the authors overlooked the data from CF patients presented in our same study [180]. The CF patients assessed in the study were not

consuming Peptamen in their diet, were monitored for their cholesterol levels and none of the patients were placed on a cholesterol reducing diet. Importantly, these findings were assessed using LC/MS analysis of all detectable species of ceramide which demonstrated that several ceramide species were impaired in CF (both in mice and CF patients) [180]. This deficiency of specific ceramide species was correctable by treatment with fenretinide in mice [180].

A genome-wide association study was performed on 4,400 samples collected from five European populations regarding their levels of circulating sphingolipids. The results concluded that there are genetic variations in genes involved in the sphingolipid metabolism indicating that different populations may have varying basal levels of ceramide [368]. This is especially important clinically in CF as genetic variation can affect the severity of the disease, but also in terms of comparing published studies, differences seen in ceramide levels between studies may be attributed to genetic differences of the populations studied.

Another point of conflict can arise between the various methods of ceramide analysis. Using antibodies in the detection of ceramide can be misleading as they can bind to a selection of species of ceramide and lipids in general. While immunohistochemistry offers an opportunity to visualize staining of tissues, one must be careful to interpret the staining as pure ceramide detection. With this method, ceramide is not purified from other lipids and remnants of degraded ceramide molecules may be present which are likely to cause cross-reactivity, especially when using antibodies recognizing multiple epitopes. Using the antibodies in ELISAs necessitates an initial separation of lipids by TLC from which the ceramide fraction can be isolated. This step also eliminates partly degraded ceramide. Without purification, the cross-reactivity of the antibodies prevents the reliable quantification of ceramide. While this assay

can reduce costs for the quantification of ceramide for many samples, it cannot provide data on the types of ceramide species nor the concentration of each species present in the sample. DAG kinase assays can be an inexpensive method for ceramide analysis however only total levels of ceramide can be assessed and once again, ceramide may not be effectively quantified in the end. MS offers unmistakable identification of various species of ceramide, therefore it represents the best solution for accurate quantification. Many are turning to MS as a form of analysis however it is questionable why certain researchers chose to only look at a very small subset of species (sometimes only 3 species) when it is possible to look at all known and detectable forms of ceramide. This is especially valid when the cost of analysis of 3 species versus a complete set of ceramides is the same, once the standards are purchased. The simple explanation would be that the cost per sample using MS is still much greater than the cost for DAG kinase assays and immunodetection. Also, MS requires specialized equipment and knowledge in the technique which may not be available to all laboratories. Regardless, MS is still the most accurate technique currently available for ceramide quantification.

Whether ceramide in CF is increased, decreased or similar to normal levels remains a controversial point. There are many variables between the different published studies which continue to make comparisons difficult.

3. FENRETINIDE AND CERAMIDE

Initial studies of fenretinide on cancer described its effects on apoptosis to be related to the induction of ceramide levels. Fenretinide was found to increase the levels of serine palmitoyl transferase and ceramide synthase, the enzymes involved in the early steps of ceramide synthesis through the *de novo* pathway (Figure 1.2) [217]. With the advent of more

sensitive detection methods, fenretinide is now thought to target dihydroceramide (DHC) to a greater extent than ceramide [369]. Fenretinide was found to reduce DHC desaturase which converts DHC into ceramide, thus inducing an accumulation of DHC [370]. Ceramide levels are affected but to a lesser extent than their precursors [369].

In our first fenretinide study on ceramide measured by HPLC/MS, we did in fact observe an 8.1 fold increase in DHC16:0, which was greater than each of the affected ceramide species (1.7 fold increase for C22:0, 2.0 fold increase for C24:0 and 3.0 fold increase for C24:1) [180]. Since then, we have not assessed the levels of other DHC species which may show greater increases than ceramide itself. The functions of the various dihydroceramide and ceramide species remains to be elucidated. We do know that these molecules play important roles in cell membrane structure and in the inflammatory response. However, whether each species has a specific role is not currently known. Fenretinide normalizes the levels of ceramide in our studies, however we recognize that DHC might be affected even more. The TLC-ELISA method we used to quantify total ceramide in our experiments does not distinguish DHC from ceramide. We note striking effects of fenretinide on total ceramide levels quantified by TLC-ELISA and it is likely we are assessing a change in DHC as well. To confirm this effect of fenretinide, future studies using MS to quantify the various DHC species are needed.

Table 5.1. Summary of the effects of fenretinide in comparison to current routine treatments for CF patients

	Tobramycin	Hypertonic saline	rhDNase	Azithromycin	Aztreonam	Ibuprofen	Ivacaftor	Fenretinide
Anti-infective	✓ [371]		✓ [372]	✓ [373]	✓ [374]			✓ [180]
Restoring ASL volume		✓ [82]						
Mucus Breakdown			✓ [375]					
Anti-inflammatory	✓ [333]	✓ IL-8 [337]	✓ [338]	✓ [340]		✓ [87,342]		✓ [1]
Antioxidant	✓ [333]							✓ [2]
PUFA correction	? [3]							✓ [1,2]
Ceramide correction								✓ [1,2]
Protein defect							✓ [8]	
Other CF related pathologies							✓ insulin secretion [343]	✓ osteo-porosis [242]

[1] as shown in Chapter 2
[2] as shown in Chapter 3
[3] as shown in Chapter 4 with the aggressive IV treatment for PEx

SUMMARY AND CONCLUSION

The results presented in this thesis demonstrate the effects of fenretinide, a vitamin A derivative, on phenotypes of CF. In Chapter 2, we showed that fenretinide corrected the lipid defects in CF mice. More specifically, the levels of AA were reduced, DHA levels were increased leading to a decreased AA/DHA ratio. Ceramide levels were increased after fenretinide treatment and these improvements were correlated to ameliorated PUFA levels. The drug treatment also reduced the high levels of inflammation in the lungs of CF mice. We also describe more severe lipid imbalances in patients diagnosed after the age of 18, especially with higher AA levels.

In Chapter 3, we evaluated the effects of one dose of fenretinide and found it had prolonged effects on decreasing AA levels, increasing DHA and ceramide levels. The improvements in DHA and ceramide levels were not maintained as long as the effects on AA. We showed a dose-dependent effect of fenretinide on improvements in PUFA in leukocytes from CF patients. In both mice and human cells, fenretinide reduced the levels of MDA and NT3 thus may be capable of affecting more CF phenotypes. We determined the capsule formulation of the drug to be effective in monkeys and that there is a difference between species in metabolizing fenretinide.

In chapter 4, we established markers related to increased risk of PEx in CF patients. At stable disease, worse clinical and patient quality of life evaluations were associated with increased risk of PEx. At the end of treatment, patients experiencing rapid re-exacerbations had higher levels of systemic inflammation. We showed that lung function tests, clinical scores and quality of life scores improved with treatment for PEx, along with inflammatory markers. The

improvements in inflammatory markers are not maintained once treatment is completed. This aggressive treatment for PEx improved the PUFA imbalance by decreasing AA and increasing DHA. Ceramide levels were not significantly affected by this treatment. MDA and NT3 levels were improved with aggressive antibiotic treatment as well. As with inflammatory markers, once treatment was completed, the improvements in PUFA were not maintained. While the aggressive antibiotic treatment combined with use of steroids improved inflammation, PUFA defects, MDA and NT3 levels, it is not a feasible therapy for chronic use due to the high risk of antibiotic and steroid resistance as well as steroid-induced increase in susceptibility to viral, fungal and bacterial infections.

Taken together, our studies presented in this thesis show that fenretinide is a serious and very promising candidate for CF therapy affecting lipid defects, inflammatory markers, lipid peroxidation and nitrosative stress. Based on the results presented in this thesis and those from our previous studies, fenretinide received the Federal Drug Administration's Orphan Drug designation in 2010 for treatment of CF patients. This is a designation given to promising therapies for treating orphan diseases which are rare diseases affecting less than 200,000 people in the USA. We are also very pleased to report that on December 17th 2013, the Phase IB clinical trial studying fenretinide in CF patients infected with PA was approved by Health Canada with patient enrollment set to begin early 2014.

APPENDIX

Significant contributions by the thesis author to other projects:

1) The effects of fenretinide on spinal contusion injuries in mice

Protective Effect of Fenretinide in Spinal Injury.
Radzioch D, Kozak H, DeSanctis J, **Wojewodka G,** Curan L, Tsatas O, Zarruk J, Greenhalgh A, Kroner A, Parent S, Radhakrishna M, Ouellet J, Makriyianni I, David S. Conference Abstract: Presented at the Military Health Research Symposium Florida USA, Aug 2012.

Fenretinide promotes functional recovery and tissue protection after spinal cord contusion injury in mice.
López-Vales R, Redensek A, Skinner TA, Rathore KI, Ghasemlou N, **Wojewodka G,** DeSanctis J, Radzioch D, David S. *J Neurosci*. 2010 Mar 3;30(9):3220-6.

2) Distortion of PUFA and kinetics of inflammation in patients with traumas to the spinal cord

Kinetics of inflammatory markers following traumatic injuries to the spinal cord.
Radzioch D, **Wojewodka G,** David S, Makriyianni I, Radhakrishna M, Parent S, Ouellet J. Conference Abstract: 15th International Congress of Immunology (ICI), Milan, IT, Aug 2013.

Lipid profile analysis in spinal trauma patients shows severe distortion of AA/DHA after injury.
Radzioch D, **Wojewodka G,** David S, Makriyianni I, Radhakrishna M, Parent S, Ouellet J. Conference Abstract: 15th International Congress of Immunology (ICI), Milan, IT, Aug 2013.

Lipid profile analysis in spinal injury patients confirms severe distortion of AA/DHA previously observed in the SCI animal model.
Radzioch D, DeSanctis J, **Wojewodka G,** Parent S, Radhakrishna M, Ouellet J, Makriyianni I, David S. Presented at the Military Health Research Symposium Florida USA, Aug 2012.

3) The effects of fenretinide on inflammation in macrophages

Fenretinide corrects the imbalance between omega-6 to omega-3 polyunsaturated fatty acids and inhibits macrophage inflammatory mediators via the ERK pathway.
Lachance C, **Wojewodka G**, Skinner TA, Guilbault C, De Sanctis JB, Radzioch D. PLoS One. 2013 Sep 12;8(9): e74875.

4) The role of polyunsaturated fatty acids in infertility

Lycopene improves the distorted ratio between AA/DHA in the seminal plasma of infertile males and increases the likelihood of successful pregnancy.
Filipcikova R, Oborna I, Brezinova J, Novotny J**, Wojewodka G,** De Sanctis JB, Radova L, Hajduch M, Radzioch D. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2013 Feb 25.

Increased lipid peroxidation and abnormal fatty acid profiles in seminal and blood plasma of normozoospermic males from infertile couples.
Oborna I, **Wojewodka G,** De Sanctis JB, Fingerova H, Svobodova M, Brezinova J, Hajduch M, Novotny J, Radova L, Radzioch D. *Hum Reprod* 2010, Feb;25(2):308-16.

Other publications:

Gene-metabolite expression in blood can discriminate allergen-induced isolated early from dual asthmatic responses.
Singh A, Yamamoto M, Kam SH, Ruan J, Gauvreau GM, O'Byrne PM, FitzGerald JM, Schellenberg R, Boulet LP, **Wojewodka G**, Kanagaratham C, De Sanctis JB, Radzioch D, Tebbutt SJ. PLoS One. 2013 Jul 2;8(7):e67907.

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