Effects of LAU-7b and Triple therapy combination in a F508del mouse model of Cystic Fibrosis lung disease

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Abstract
Background: Cystic fibrosis (CF) is the most common autosomal recessive genetic disease in Caucasians, affecting more than 100,000 individuals worldwide. It is caused by pathogenic variants in the gene encoding CFTR, an anion channel at the plasma membrane of epithelial and other cells. The most frequent mutation, loss of a phenylalanine at position 508 (F508del), leads to misfolding, retention in the endoplasmic reticulum, and premature degradation of the protein. While there are many therapeutics that treat various facets of the disease, to date, no cure for this life shortening disease has been found.
Methods: We examined the effect of combining LAU-7b oral treatment and Triple therapy (contains all three modulators of TRIKAFTA®) combination on lung function in a F508del^{tm1EUR} mouse model that displays lung abnormalities relevant to human CF. We assessed lung function, lung histopathology, protein oxidation, lipid oxidation, and fatty acid and lipid profiles in F508del^{tm1EUR} mice.
Results: The combined treatment of LAU-7b and Triple therapy resulted in an improvement in airway resistance in the lungs, lung histopathology and a decrease in oxidation markers. Fatty acid and lipid profiles were also corrected following the repeated dose, oral treatment of LAU-7b and Triple therapy.
Conclusion: The combined treatment of LAU-7b and Triple therapy was significantly better and improving multiple markers of CF lung disease, as opposed to Triple therapy alone. The *in vivo* results obtained indicate a potential clinical benefit in persons with CF upon combinatory treatment of LAU-7b and TRIKAFTA®.

Résumé
Contexte: La fibrose kystique (FK) est la maladie génétique autosomique récessive la plus fréquente chez les Caucasiens. Elle touche plus de 100 000 personnes dans le monde. Elle est causée par des variantes pathogènes du gène codant pour le CFTR, un canal anionique situé sur la membrane plasmique des cellules épithéliales et autres. La mutation la plus fréquente, la perte d'une phénylalanine en position 508 (F508del), entraîne un mauvais repliement, une rétention dans le réticulum endoplasmique et une dégradation prématurée de la protéine. Bien qu'il existe de nombreuses thérapies qui traitent les différentes facettes de la maladie, à ce jour, aucun remède pour cette maladie qui raccourcit la vie n'a été trouvé.
Méthodes: Nous avons examiné l'effet de la combinaison du traitement oral LAU-7b et de la trithérapie (contient les trois modulateurs de TRIKAFTA®) sur la fonction pulmonaire dans un modèle de souris F508del^{tm1EUR} qui présente des anomalies pulmonaires pertinentes pour la FK humaine. Nous avons évalué la fonction pulmonaire, l'histopathologie pulmonaire, l'oxydation des protéines, l'oxydation des lipides et les profils des acides gras et des lipides chez les souris F508del^{tm1EUR}.
Résultats: Le traitement combiné de LAU-7b et de la trithérapie a entraîné une amélioration de la résistance des voies aériennes dans les poumons, de l'histopathologie pulmonaire et une diminution des marqueurs d'oxydation. Les profils des acides gras et des lipides ont également été corrigés après l'administration de doses répétées de LAU-7b et de la trithérapie par voie orale.
Conclusion: Le traitement combiné de LAU-7b et de la trithérapie a été significativement meilleur et a amélioré plusieurs marqueurs de la maladie pulmonaire de la FK, par rapport à la trithérapie seule. Les résultats *in vivo* obtenus indiquent un bénéfice clinique potentiel chez les personnes atteintes de FK lors du traitement combiné de LAU-7b et de TRIKAFTA®.

List of Abbreviations

CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Regulator
cAMP	Cyclic adenosine monophosphate
ATP	Adenosine triphosphate
PwCF	Persons with Cystic fibrosis
P. aeruginosa	*Pseudomonas aeruginosa*
GWAS	Genome-wide association study
G551D	Gly55Asp, substitution of glycine for aspartate at position 551
mRNA	Messenger ribonucleic acid
ER	Endoplasmic reticulum
F508del	Deletion of phenylalanine codon at position 508 of *CFTR*
DNA	Deoxyribonucleic acid
A. fumigatus	*Aspergillus fumigatus*
NTB	Non-tuberculosis bacteria
MDR	Multidrug resistance
NETs	Neutrophil extracellular traps
KO	Knock-out
WT	Wild type
rhDNase	Recombinant human deoxyribonuclease
FEV_1	Forced expiratory volume in one second
NaCl	Sodium chloride
SABAs	Short-acting ß-2 agonists
AMP	Adenosine monophosphate
LABAs	Long-acting ß-2 agonists
SAMAs	Short-acting muscarinic antagonists
cGMP	Cyclic guanosine monophosphate
LAMAs	Long-acting muscarinic antagonists
FEN	Fenretinide
IL	Interleukin
ERK1/2	Extracellular signal-regulated protein kinase 1/2

Zn^{2+}	Zinc
VLCCs	Very-long-chain ceramides
LCCs	Long-chain ceramides
AA	Arachidonic acid
DHA	Docosahexaenoic acid
FDA	Food and Drug Administration
MSD	Membrane spanning domains
NBD	Nucleotide-binding domains
AEs	Adverse events
$ppFEV_1$	Percent predicted forced expiratory volume in one second
CFQ-R RD	Cystic Fibrosis Questionnaire-Revised respiratory domain
BMI	Body mass index
TNF	Tumor necrosis factor
PPARγ	Peroxisome proliferator-activated receptor-γ
EPA	eicosapentaenoic acid
MCh	Methacholine
p.o	per os
H&E	Hematoxylin and Eosin
MD	Malondialdehyde
3-NT	3-nitrotyrosine
DD	Homozygous (F508del/F508del) mice
NT	Non-treated
VEH	Vehicle
SPF	Specific pathogen free
RARs	Retinoid receptors

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We thank Dr. Bob Scholte for generously providing us with the *Cftr*^{tm1EUR} mouse model (C57BL/6J), heterozygous for the F508del *Cftr* mutation, was obtained from the Erasmus Medical Center (Rotterdam, Netherlands). We want to acknowledge Laurent Pharmaceuticals Inc. for having generously provided the clinical capsules used for treatment of *Cftr*^{tm1EUR} F508del mice and their littermate controls. We thank the staff at the ARD for their continual care for the mice and to the technicians who helped with my experiments. We also thank Dr. Gergely L. Lukacs for providing us with the CFBE cell lines used for our *in vitro* work.

Contributions

Amanda Centorame for thesis writing, *in vitro* and *in vivo* sample processing, maintenance of F508del colony, animal experimentation, analysis and quantification of histopathology, Western blot, figure preparation. Danuta Radzioch for study concept and design, data analysis, manuscript and thesis corrections/editing and thesis approval. Daciana Catalina Dumut for maintenance of F508del colony, animal experimentation, collection of blood plasma and purification of samples, lung function analysis of F508del mice, corrections and editing for sections included from the manuscript title: Treatment With LAU-7b Complements CFTR Modulator Therapy by Improving Lung Physiology and Normalizing Lipid Imbalance Associated with CF Lung Disease by Centorame et al [1]. Mina Youssef for animal experimentation, analysis of lung function and quantification of histopathology. Martin Ondra for *in vitro* sample processing and tissue culture, Western blot analysis. Juhi Shah for *in vitro* sample processing and Western blot analysis. Radu Alexandru Paun for aiding in animal experimentation and editing of manuscript

[1]. Tomas Ozdian for lipid extraction and ceramide analysis. Juan Bautista De Sanctis for lipidomic analysis, protein and lipid oxidation analysis, normalization, data analyses and manuscript editing. Ekaterina Gusev for genotyping of the mice and help with maintenance of F508del colony and manuscript editing. John W. Hanrahan for manuscript corrections and editing. Basil Petrof for manuscript corrections and editing. Irenej Kianicka for study design and consulting for animal experimentation. Radu Pislariu for study design, consulting for animal experimentation and manuscript editing. Marian Hajduch for analysis of lipid oxidation, discussion on the conceptual data analysis, exploration of molecular mechanism of action of the drug and manuscript editing.

Chapter 1. Introduction

1.1. Cystic fibrosis etiology

Cystic Fibrosis (CF, OMIM #219700) is an autosomal recessive disorder that commonly affects Caucasians. With every individual having two chromosomes, one inherited by the mother and father respectively, this contribution allows for the inheritance of two copies of the gene encoding the cystic fibrosis transmembrane regulator (*CFTR)*. Given CF is a recessive disease, both alleles of *CFTR* must have two loss-of-function mutations, to cause disease [2]. However, it is possible to have CF-like symptoms with a heterozygote genotype which has only one mutant allele.

It is estimated that 70,000 people worldwide are affected by CF, where North America, Europe and Australia see the highest prevalence [2]. The gene was first characterized in 1989 in a series of three papers and was named the cystic fibrosis transmembrane conductance regulator (*CFTR*) [3-6].

CF is the result of genetic mutations in a gene on the long arm of human chromosome 7, regions q31-q32 [7]. This gene codes for an epithelial membrane protein that acts as a cyclic adenosine monophosphate (cAMP) - activated adenosine triphosphate (ATP) - gated chloride and bicarbonate channel and water transport regulator [6]. Mutations affecting CFTR gene expression result in difficulties with the processing, folding, or trafficking of the protein to the membrane which adversely affect its function.

Phenotypically, CF manifestations are extremely variable and can be observed in multiple organs such as the pancreas, the gastrointestinal tract, the reproductive system, and most prominently the lungs [8, 9].

While the mutations play a major role in the severity of the disease and its progression, there are other factors that can be influential such as gene modifiers (i.e., non-CFTR) and environmental factors [9]. Interestingly, analyses from twin and sibling studies have shown that variation in lung function in persons with cystic fibrosis (PwCF) have shown that non-CFTR genetic modifiers account for 50% of that variation, with the remaining variation attributed to environmental exposure and stochastic effects [9-11].

Non-CFTR gene modifiers have been found in many genes that also encode other plasma membrane proteins, which are located near CFTR, which in turn increase risks of meconium ileus in newborns [9, 12-14]. The genes which contain alleles that increase this risk would be SLC26A9, SLC9A3 and SLC6A14, all of which are pleiotropic and can impact CF comorbidities in early childhood [9, 15]. It has also been shown that these non-CFTR modifier genes can play a role in causing lung damage and allow infection with *Pseudomonas aeruginosa* (*P. aeruginosa*) in children at a younger age than expected [9, 15]. While one genome-wide association study (GWAS) showed a link between lung disease severity and loci on chromosomes 11 and 20 [9, 16]. Moreover, another larger GWAS meta-analysis of 6,365 patients showed correlation with loci on chromosomes 3, 5, 6, X and 11, as previously seen in the earlier GWAS [9, 16, 17].

Environmental factors that can play a role in disease progression include exposure to second-hand smoke, pollutants, climate and microorganisms, access to care and adherence to the treatment regime [9]. Given the complexity of the disease, an array of factors can affect the disease and its progression as well as various tissues differently, with variability from one patient to the next.

1. 2 Pathogenic variants in the *CFTR* gene and their effect on CFTR protein expression and function

To date, there have been over 2,000 different genetic variants reported in the *CFTR* gene, however, while some are known to cause CF, others are known to be linked to the disease severity [2]. The mutations can affect the number of CFTR channels, their function, or the combination of the two. Furthermore, different combinations of the variants can affect the severity of the disease via different cellular mechanisms [2].

The mutations in *CFTR* had been originally classified into categories by Lap-Chee Tsui and further improved by Welsh and Smith [18-20]. Over the years, various classification systems have been suggested with six or even seven classes. The complexity with these mutations lies in the fact that they can exhibit more than one defect that can be classified into several classes, making classification complex [9, 18]. Recently, De Boeck and Amaral have suggested a new classification that consists of seven classes [18, 21].

Class I mutations reduce the number of functional CFTR protein which is due to major disruptions of the *CFTR* gene, known as production mutations. This includes non-sense mutations,

insertions, and deletions (e.g., G542X) [9]. For class III mutations, CFTR protein is created and moves to the cellular membrane, but due to disordered gating, ATP binding and subsequent hydrolysis is reduced which hinders the channel's gate opening, leading to a loss of chloride transport [9]. This is referred to as a gating mutation, caused by amino acid substitutions [9, 22]. An example of such a mutation would be Gly551Asp (G551D), a substitution of the amino acid glycine for aspartate at position 551 [22]. Class IV mutations are referred to as conduction mutations. Unlike class III mutations, class IV mutations can open their gates, but the channel is faulty leading to defective chloride conductance (e.g., R117H) [9]. Class V mutations result from abnormalities in the promoter or splicing that adversely affects, by diminishing or eliminating, messenger ribonucleic acid (mRNA) transcripts [9]. Ultimately, while the conformation of the protein is not affected, this class of mutations leads to less functional CFTR protein due to less synthesis, which reduces its abundance at the plasma membrane [23]. An example for this class of mutations would be A455E.

Although class VI mutations have fully functional CFTR mRNA and protein, these mutations affect its surface stability at the plasma membrane and/or in post-endoplasmic reticulum (ER) compartments due to an increase in internalization signals [9, 23]. The result is a faster plasma membrane turnover and less CFTR protein expression at the membrane (e.g. c.120del23) [23].

Class II mutations are caused by abnormal folding of the CFTR protein and ultimately, degradation protein occurs mostly at the proteasome, after ER retention, which prevents its trafficking to the Golgi apparatus [24]. This results in little to no CFTR protein at the plasma membrane. The most common class II mutation is a deletion of the codon for phenylalanine at position 508 (F508del), a missense mutation. There are more than 80% of individuals with CF having at least one mutant copy of F508del CFTR [25].

The seventh class that De Boeck and Amaral suggested comprises variants that contain large deletions, thus complete absence of mRNA transcription, making them non-rescuable [21]. While Marson et al. agreed with the addition of a seventh class, they proposed it be referred to as class IA, given their defect has similar consequences to what is seen in regards to the possibility of rescuing CFTR protein expression as seen in PwCF cases with a *CFTR* gene containing a class I mutations [18, 26].

1.3 CF lung disease manifestations and most prevalent lung infections

The airway and lungs of CF patients plays a major role in the progression of the disease and is the main cause of morbidity and mortality among PwCF [9]. One characteristic in particular causes a snowball effect of problems, which starts with mucus accumulation, leading to obstructive pulmonary disease and development of bronchiectasis, all playing a role in the progression of the disease [9, 27]. The mucus has abnormal properties which makes it particularly adhesive to epithelial surfaces [27]. Increased viscosity of mucins produced by goblet cells is contributed, at least in part, by deoxyribonucleic acid (DNA) released from dying neutrophils [28].

Thus, this impacts mucus clearance due to liquid depletion of the airway surface [27]. The abnormal mucosal defences lead to a predisposition of infection which in turn negatively impacts the inflammatory milieu leading to lung damage and eventually lung failure, the main cause of morbidity and mortality [27, 29, 30]. A variety of airway pathogens such as bacteria and fungi can be seen in the CF lung [27]. The most common bacterial infections seen in CF patients are *P. aeruginosa* and *Staphylococcus aureus*, however with increasing age, infections with other bacterial pathogens become more common, such as *Achromobacter xylosoxidans* and *Burkholderia cepacia complex* [27]. In the US, 305 of patients present with a methicillin-resistant strain of *S. aureus* (MRSA). As for fungi, the most commonly found types of fungal infections are *Aspergillus fumigatus (A. fumigatus)*, *Candida albicans*, *Scedosporium apiospermum*, and *Exophiala dermatitidis* [30]. In recent years, due to increasing age in PwCF, non-tuberculosis mycobacteria (NTB) have become more apparent and pose a new threat due to their complex nature [9, 31]. The most dominant species of NTB fall into two classes that cause human disease: *Mycobacterium avium* complex and *Mycobacterium abscessus* [31].

P. aeruginosa infection has been the focus of researchers for numerous reasons. One being that *P. aeruginosa* infection can be seen in 60-70% of adults with CF and can cause recurrent acute and chronic episodes of infection [32, 33]. Bacterial infections from common pathogens have been shown to result in decreased lung function [30]. Similarly, bacterial infections as well as fungi, which includes Aspergillus species, have both shown to result in much higher rates of pulmonary exacerbations [27]. To control and treat bacterial infections, antibiotics are often the first step. However, due to recurrent infections, chronic antibiotic use often triggers allows for multidrug resistance (MDR) [33]. The dangers that accompany MDR is the limited antibiotic options that

can be used to treat patients [33]. MDR is often seen in CF patients that have a more aggressive disease and is indicative of a decreased diversity of the lung microbiome [33].

One important feature of bacterial-fungal infections and a possible reason as to why they thrive in the CF lung is the ability to produce sticky protein leading to the development of biofilms in the lungs [27, 30]. In *P. aeruginosa* infections, neutrophils are recruited to the lung which form neutrophil extracellular traps (NETs) enhancing biofilm development [27]. The biofilm environment allows for the increased development of antibiotic resistance which can be further altered in the presence of a variety of bacterial-fungal species [30].

1.4 Animal models of CF lung disease

Animal models of CF have become a pillar in the scientific community to help researchers delve further into understanding of diseases and the mechanisms at play. They have also been utilized as a steppingstone in testing safety and efficacy of various treatments related to diseases, before taking the leap to humans [34]. The efficacy and safety data obtained in animal models are essential for regulatory agency approval of clinical protocols.

Although CF mice have played a pivotal role over the years, advancements in technologies pertaining to genetic engineering, have allowed a wider range of animals, which includes large mammals, to be used in CF research [35]. To date, there are various CF models to choose from including, murine models, rats, pigs, ferrets, sheep, rabbits, zebrafish and Drosophila [35] Each of these models have varying percentages of CFTR amino acid identity compared to humans: mouse = 78%, rats = 75.5%, pigs = 93%, ferrets = 92%, sheep = 91%, zebrafish = 55% and Drosophila = 40% [36, 37].

When choosing a model to use, many elements must be considered. One of the first factors would include the housing of the animals, labor, and training for breeding and all the associated costs [35]. Next, the model and species characteristics must be considered so that they suit the desired study goals, this includes the lifespan of the model, the severity of the disease, as well as the size of the species. While larger models may be better suited for shorter studies as well as translational studies, smaller animal models are easier to handle, and can be used for proof-of-concept investigations [35]. Other parameters such as the reproductive cycles and depending on the objectives of the study, immune response can be species-dependent and therefore should be

considered [35]. Lastly, the genotype and phenotype of interest must be known as well as accessibility of the model, to make an informed decision [35].

In comparison to humans, porcine models have similar lungs, the same ion properties, can rapidly develop infection, and elicit strong inflammatory reactions [18, 38, 39]. Ferrets, which have similar pulmonary characteristics, have been of interest for studies of innate immunity and inflammatory responses, however, their lipid metabolism is very different compared to human or rodent metabolisms resulting in high mortality rates, which limits the usefulness of this model when studying chronic CF lung disease [18, 39]. Moreover, other models such as the rat would be useful for studying lung disease, growth failure and bone disease over a longer period. The zebrafish has shown to be helpful to study pancreatic disease, mucosal immunology and also for initial sorting of modulator therapies that can improve specific combinations of mutated CFTR protein trafficking which helps progress towards personalized medicine [18, 39].

The first mouse model was created in 1992 at the University of North Carolina, three years after discovery and cloning of the CFTR gene [40-42]. To achieve this model, the endogenous CFTR gene was disrupted by targeting embryonic stem cells, which now has a stop codon in exon 10. The offspring were mated with mice from various genetic backgrounds, and the heterozygotes from this cross were then crossed to produced homozygous mice, termed $CFTR^{tm1UNC}$ knockout (KO) mice. However, with less than 5% making it to maturity, Kent and colleagues suspected that the mixed genetic background seen in the $CFTR^{tm1UNC}$ mice affected pulmonary pathology [40, 43]. They created a congenic strain by backcrossing a heterozygote $CFTR^{tm1UNC}$ mouse onto a C57BL/6 background, termed B6- $CFTR^{tm1UNC}$/$CFTR^{tm1UNC}$ KO mouse, which has been utilized to study the impact that multiple organs have in relation to CFTR [40, 44]. Meanwhile, Dorin and colleagues generated the $CFTR^{tm1HGU}$ strain, a KO strain on an MF1/129 background, which has an insertional mutation in exon 10 resulting in a mild disease phenotype and 95% of the mice survived to maturity [40, 45, 46]. Ratcliff and colleagues created another strain that closely resembled the $CFTR^{tm1UNC}$ KO strain, however, this new strain, $CFTR^{tm1CAM}$, which has a null mutation exhibited lacrimal gland pathology [40, 47]. Moreover, another strain of mice, $CFTR^{tm1BAY}$, generated in Texas and Iowa in 1993**,** had a duplication of exon 3 [40, 48]. These mice had less than 2% of normal levels of wild-type (WT) mRNA levels, a severe phenotype, and a 40% survival rate past Day 7. Other strains were also created such as the $CFTR^{tm3BAY}$ strain by Hasty and colleagues in 1995, which had a null mutation at exon 2, and the $CFTR^{tm1HSC}$ strain by

Rozmahel and colleagues, which had a disruption at exon 1 of the *CFTR* gene [40, 49, 50]. While the CFTR^{tm3BAY} strain had a 40% survival rate after 30 days, the original CFTR^{tm1HSC} strain only had 30% survival, and following crossing with other inbred strains, these KO mice had varying survival rates and phenotypes.

Models that represent common CF mutations have also been generated, such as those for the most common mutation, F508del, and the class III mutation, G551D. The first F508del mouse model, which introduces the mutation into the endogenous mouse *CFTR* gene, was generated by van Doorninck et al., by a double homologous recombination technique into exon 10, and referred to as CFTR^{tm1EUR} [40, 51]. However, the mice which were generated on an FVB/129 background, did not show severe disease [51]. F508del KO mice on a C57BL/6/129 background were generated by Colledge and colleagues in which exon 10 was replaced, were designated CFTR^{tm2CAM}, while another similar model, CFTR^{tm1KTH} was generated by Zeiher et al [52, 53]. Following the generation of these two models, using the hit-and-run procedure, French et al. created and F508del mouse model that did not affect the intron structure of the gene, allowing for normal mRNA expression levels [54]. This model provides many opportunities to further understand how the defect affects various processes and the opportunity to test new therapeutics in a relevant model [54]. The G551D model, designated as CFTR^{tm1G551D} with replacement of exon 11, was generated by Delaney et al. in CD1/129 mice [40, 55]. In all CF mice models, the lung phenotype can only be observed if the genetically modified mouse is backcrossed to a strain that does not produce significant expression levels of calcium chloride transport in the lungs. The calcium chloride would substitute for the deficient chloride channel function of the CFTR protein in the lungs.

1.5 Current Therapies for CF

1.5.1 Mucoactive agents

The mucus in the CF patients lungs have abnormal properties which make it extra adhesive to epithelial surfaces [27]. Both DNA released from dead neutrophils' and mucins, secreted by hyperplastic goblet cells were shown to be responsible for this increase in viscosity [28]. An increase in mucus viscosity impacts mucus clearance due to liquid depletion of the airway surface from the improper or lack of the CFTR protein function [27, 56]. The abnormal mucosal defences lead to a predisposition of infection which in turn negatively impacts the inflammatory milieu [27].

Mucoactive agents, which consists of two categories: mucolytics and hyperosmolar agents, both aim to improve mucus clearance [56].

1.5.1.1 Mucolytics

To date, the only mucolytic proven to help with CF in patients is called recombinant human deoxyribonuclease (rhDNase), also known as dornase alfa and sold under the brand name Pulmozyme [56]. RhDNase, an enzyme, works by targeting and cleaving the DNA released from the dead neutrophils, a major component of the mucus, which can reduce its viscosity and elasticity [56, 57]. It is administered to patients via jet nebulisers which need to be attached to a compressor [57]. After many studies using rhDNase, researchers confirmed that inflammation can be reduced by the improved clearance of secretions, which in turn also reduces duration of exacerbations [56]. Its efficacy was demonstrated in a Cochrane study over time points of 1 month, 6 months and 2 years, all of which showed improved lung function [56, 58]. Furthermore, in another study with patients whose force expiratory volume in one second (FEV_1), a measurement of lung function, was below 30%, had more than doubled their median survival rate, which suggest that such an improvement was due to the use of rhDNase [56, 59]. The use of dornase alfa has become a common practice for CF patients over the age of 6 [56]. The most common side effects seen are voice alteration and a rash [57].

1.5.1.2 Hyperosmolar agents

To account for the liquid depletion seen in the CF lung, nebulised hypertonic saline can be used. Hypertonic saline is composed of sterile water with sodium chloride ions (NaCl) at concentrations of 3 to 7% [57]. With higher salt concentration on the luminal side, water movement will move outwards, hydrating the mucus, which will enhance mucociliary clearance [57]. In 2014, Michon et al. assessed antimicrobial effect of treatment with hypertonic saline by isolating *P. aeruginosa* from patients' respiratory tracts. *P. aeruginosa* is a bacterium that infects 60-70% of adults with CF and can cause recurrent acute and chronic episodes of infection [32, 33]. They found that 6% NaCl solution inhibited growth of all isolates and 3% or more could inhibit biofilm growth, a problem that occurs with recurring infections, for 69% of isolates [60]. Hypertonic saline's use has also been recommended for CF patients 6 years and up, by the Cystic Fibrosis Pulmonary Clinical Practice Guidelines Committee established by the CF Foundation [61].

Mannitol, sold under the brand name of Bronchitol, is another hyperosmolar agent. It creates an osmotic gradient by drawing water into the airway which has also been shown to facilitate mucus clearance in CF [56]. It is a licensed medication in Europe and Australia due to its effects on FEV_1 improvements and its ability to reduce the likelihood of exacerbations [56, 62]. Its advantage lies in the fact that it is portable and can be dispensed as a dry powder inhaler [56]. The mucoactive agents can be taken in combination, such that one cleaves the DNA, reducing mucus viscosity, while the hyperosmolar agent can improve the surface liquid of the airway [56].

1.5.2 Bronchodilators

Although there is still much debate as to whether or not bronchodilators are an effective treatment for symptoms of pulmonary obstruction seen in cystic fibrosis, bronchodilators are very commonly prescribed to patients to alleviate some symptoms [63, 64]. Common pulmonary symptoms seen in children and adults which can be treated with bronchodilators would be coughing, wheezing, and hyperinflation [65]. Bronchodilators can be either short-acting or long-acting, with the goal of opening the airways by relaxing the smooth muscles in the bronchial wall such that clearance is facilitated [63, 65]. The main difference between the short and long-acting displays lies in the duration at which their effects can be seen following use. Short-acting bronchodilators are most often used to treat acute symptoms and before therapy for airway clearance. On the other hand, long-acting bronchodilators are most often used when the desired effect is long-lasting and for maintenance and prevention [65]. The medication is often delivered as a dry powder device, a dose inhaler or as an aerosol with a nebuliser [65].

1.5.2.1 ß-2 agonists (SABAs and LABAs)

Short-acting ß-2 agonists (SABAs) can interact with the ß-2 receptors on smooth muscles which activates the cyclic adenosine monophosphate (AMP) intracellular cascade allowing for airway relaxation through phosphorylation and a change in calcium concentrations [63]. Examples of SABAs would be salbutamol or albuterol, terbutaline and fenoterol. Their effects can begin within 15 minutes and last for up to four hours [63]. Long-acting ß-2 agonists (LABAs), such as salmeterol and formoterol, act similarly to SABAs. Their difference lies in the presence of a lipophilic tails, allowing it to stay bound to the ß-2 receptor longer [65]. Both salmeterol and formoterol allow for bronchodilatation with effects lasting at least 12 hours [65].

1.5.2.2 Muscarinic antagonists (SAMAs and LAMAs)

Short-acting muscarinic antagonists (SAMAs) allow for better airflow by blocking cholinergic activity and acetylcholine binding to the M3 muscarinic receptors via cyclic guanosine monophosphate (cGMP) to prevent "smooth muscle contraction and mucus secretion" [63]. The most common muscarinic antagonist would be ipratropium bromide that can produce effects within the first hour and can last for four to six hours post administration [63]. Long-acting muscarinic antagonists (LAMAs), which act in the same way as SAMAs, only need to be taken once a day, with effects occurring as quickly as 20 minutes and lasting for 24 hours [65]. Examples of long-acting muscarinic antagonists include tiotropium and aclidinium [65].

1.5.3 Antibiotics

Antibiotics target the acute, and chronic pulmonary infections seen in PwCF, with the goal to improve, or at least stabilize, the infection present and overall lung function [66]. Repeated and prolonged antibiotic cycles due to recurrent pulmonary exacerbations and the combination of more than one infection presiding in the airway microbiota, can create resistant bacteria, making eradication challenging [66, 67]. One of the main triggers of antibiotic resistance, is thought to be due to sub-optimal concentrations in treatment doses [67]. Nonetheless, antibiotic therapy has become a pivotal player in prolonging the durations of patient lives and overall management of chronic and acute bacterial infections occurring in PwCF [67].

Given *P. aeruginosa* infection is seen in many PwCF, especially as they get older, the standard of care of administration of antibiotics in chronic infections is via inhalation [66]. The most used aerosolized antibiotics for this bacterium are tobramycin, aztreonam lysine, colistin and levofloxacin [66, 68].

Tobramycin is an aminoglycoside, that works by interfering with mRNA translation, creating an accumulation of truncated or non-functional proteins [67]. It is administered via nebulizer, with the benefit of administering higher concentration of antibiotic to the lungs [67, 68]. Its aerosolized form has been shown to be well-tolerated for long periods of time with no unexpected effects [67].

Aztreonam lysine, part of the monobactams, target the cell wall of the bacteria by inhibiting its biosynthesis via bactericidal activity and has been shown to work on various gram-negative

bacteria, including *P. aeruginosa* [66, 67]. It has been shown to improve respiratory symptoms and lung function, reduce sputum density and reduce the number of exacerbations [66, 69-71].

Colistimethate sodium, or colistin, part of the polymyxins antibiotic class, can disrupt the bacterial cell membrane [66]. It has been extensively used in Europe, and in its inhaled form, had a similar safety profile as other anti-pseudomonal antibiotics [66, 72].

Levofloxacin, a fluoroquinolone, is a broad-spectrum antibiotic that blocks DNA gyrase and topoisomerase IV activity, inhibiting DNA synthesis [67]. Many studies have shown its safety and effectiveness, by demonstrating a minimal risk of systemic toxicity and reduced *P. aeruginosa* levels in sputum, improved pulmonary function and reduced the need to use other anti-pseudomonal medications [66, 73, 74]. Furthermore, levofloxacin was shown the be superior to tobramycin when looking at mean FEV_1 and reduce pulmonary exacerbation risk [66, 75].

1.5.4 Anti-inflammatories

While the role of inflammation in CF has been shown to play a key role, there are few anti-inflammatory treatments available. Oral corticosteroids have been studied in the past, however, long-term use is no longer advocated due to adverse effects, such as glucose intolerance, growth deficits, osteopenia, osteoporosis, and skeletal muscle weakness [76-78]. Inhaled corticosteroids were then investigated, however, their use in CF has not been substantiated with efficient evidence and is therefore not advised for long-term use [78, 79].

Non-steroidal anti-inflammatory therapies, such as Ibuprofen, have also been investigated for use in PwCF. While like corticosteroids in terms of their properties, they tend to have fewer adverse effects, and their ability to act against neutrophils [78]. However, Ibuprofen use has been debated over the years, with some reports proving its benefits, while others report no benefits [78, 80, 81]. Moreover, it has not been widely adopted due to the need to monitor blood levels to establish the correct dose in each patient [9, 78, 80].

In 2006, Fenretinide (FEN), a synthetic retinoid, was reported to effectively inhibit interleukin (IL)-8 release from CFTR-deficient lung epithelial cells [82]. Subsequently, FEN has been studied in the context of inflammation in CF disease and its effects have been attributed to its overall ability to trigger the resolution phase of inflammation, as demonstrated by the modulation of macrophage-secreted inflammatory cytokines, to the correction of omega-3/omega-6 fatty acid imbalances impacting phosphorylation of extracellular signal-regulated protein kinase

1/2 (ERK1/2), to the correction of the ceramide deficiency in PwCF, and to the resolution of lung mucus plugging under infection with *P. aeruginosa* [28, 83, 84]. Furthermore, Garić et al. found a partial synergistic relationship between FEN and Zinc (Zn^{2+}), whose deficiency has been reported in PwCF and is associated with severity of CF lung disease [85, 86].

Preclinical studies with FEN have shown to normalize the aberrant ratio between very-long-chain ceramides (VLCCs) and long-chain ceramides (LCCs) as well as arachidonic acid (AA)/docosahexaenoic acid (DHA) ratio *in vitro* F508del-CFTR expressing cell lines and *in vivo* (LAU-7b formulation) in mouse models of CF [28, 83, 84]. In 2015, a novel oral formulation of Fenretinide, LAU-7b, was used in a Phase 1, double-blinded, randomized, (3:1, active:placebo), placebo-controlled clinical study, involving 15 adult PwCF (clinicaltrials.gov, NCT02141958). The results from this trial indicated that LAU-7b was safe and well tolerated, while also normalizing lipid imbalances and reducing oxidative stress, as measured in pre-selected plasma biomarkers. It is hypothesized that the cumulated benefits of FEN, both during and after the exacerbation episodes, will translate into clinical benefits such as preservation of pulmonary function, reduced duration of hospital stay and severity of exacerbation episodes, and ultimately a better quality of life for CF patients. A Phase II clinical trial with LAU-7b in adult patients with CF (clinicaltrials.gov, NCT03265288) treated and untreated with TRIKAFTA® provided further support for this hypothesis and results are to be presented in the upcoming NACF conference that will be held in November 2022 (abstract in-press for NACFC by Konstan et al.).

1.5.5 CFTR modulator and corrector therapies

1.5.5.1 Ivacaftor

Ivacaftor (VX-770), also known as KALYDECO®, was developed by Vertex Pharmaceuticals as a novel gene therapy for CF patients. Ivacaftor was originally approved by the Food and Drug Administration (FDA) in 2012 for patients aged 6 and older with CF with at least one G551D mutation [87]. Ivacaftor targets the CFTR protein's ability to open the channel, ultimately, improving ion flow at the surface [88]. This increases the opening probability, which classifies Ivacaftor as a CFTR potentiator. By improving ion transport, Ivacaftor should allow for better air-surface fluidity and increase ciliary beating [88].

Three trials were conducted with the goal of examining ivacaftor's efficacy and safety [89-91]. Each study demonstrated an improvement in FEV_1 and a reduction in mean sweat chloride

levels when compared to placebo [88]. One notable adverse effect seen in some patients was elevated levels of hepatic enzymes. Therefore, during the first year of treatment, liver function had to be monitored every three months, and then yearly after that.

It is available for use as an oral agent in tablets of 150 mg. Dosages can be adjusted if necessary, so that CYP3A inhibitors, moderate or strong, can be administered at the same time as ivacaftor. Ivacaftor therapy comes with a hefty price tag of approximately 294,000$ per year [88]. Nonetheless, it brings to light a new set of therapies which target the defects themselves with the hopes of improving overall quality of life in patients.

1.5.5.2 Orkambi

Shortly after the approval of Ivacaftor, a new CFTR corrector and potentiator mix was brought into clinical trials. This CFTR corrector was referred to as Lumacaftor (VX-809), also developed by Vertex Pharmaceuticals.

CFTR's structure is extremely important to understand how potentiators and correctors work. The CFTR protein consists of 1480-amino acids, that contains two membrane spanning domains (MSD1 and MSD2), two nucleotide-binding domains (NBD1 and NBD2) and a regulatory domain [92-94].

Ren et al. had investigated the mechanism of action of Lumacaftor. In their *in vitro* studies, the CFTR corrector, Lumacaftor, can help in stabilizing the MSD1 region of the CFTR protein, which suppresses folding defects, making it less likely to be degraded in the ER [92]. Lumacaftor was used to help improve processing of the CFTR protein with a F508del mutation, a class II mutation. However, results from a clinical trial with patients homozygous for F508del, showed only slight respiratory benefit [95, 96].

In 2015, Vertex Pharmaceuticals combined Lumacaftor, a corrector, and Ivacaftor, a potentiator, into a fixed-dose tablet, which received FDA approval and was referred to as ORKAMBI®. This was the first combination therapy for CF patients homozygous for F508del, aged 12 years and up, that could treat the underlying defect, rather than the CF symptoms [95].

As for its efficacy, Lumacaftor/Ivacaftor was used in two phase 3 clinical trials, with patients homozygous for F508del CFTR, ages 12 years and older (TRAFFIC: NCT01807923 and TRANSPORT: NCT01807949) [97]. Although the combination treatment did not improve

respiratory symptoms, it was associated with lung function improvement of 3% compared to placebo and reduced pulmonary exacerbations [95].

Adverse events (AEs) were either respiratory or gastrointestinal and more severe AEs were 11.3% greater in the lumacaftor/ivacaftor group, such as pneumonia, hemoptysis, cough, increased blood creatine and phosphokinase and transaminase levels [95].

1.5.5.3 Symdeko

In 2018, a more effective combination, Symdeko® (Tezacaftor/Ivacaftor) addressing a larger number of CF patients (30%) was released by Vertex Pharmaceuticals. Tezacaftor (VX-661) was found to affect the CYP3A4 system since CYP3A4 inhibitors would increase effect of Tezacaftor [98]. While Lumacaftor was associated with a decrease in efficacy of oral contraceptives, the Tezacaftor/Ivacaftor combination had not shown any significant effects [98, 99].

This new combination was tested in a randomized phase 2 placebo controlled clinical trial to assess both safety and efficacy of the combination (tezacaftor/ivacaftor), tezacaftor alone, or placebo (NCT01531673) [100]. The patient cohort was composed of F508del homozygotes or heterozygote patients with the F508del/G551D variants. Results from the trial saw greatest improvement in absolute precent predicted forced expiratory volume in 1 second ($ppFEV_1$) by 3.75% in the tezacaftor (100mg daily)/ivacaftor (150 mg twice daily) group while sweat chloride levels were reduced by 6.04 mmol/L for F508del homozygotes [98, 100]. As for the F508del/G551D subjects, addition of 100 mg tezacaftor or placebo to ivacaftor (150 mg twice daily) that had previously been prescribed, saw an absolute increase of 4.60% in $ppFEV_1$ and a reduction by 7.02 mmol/L was found in sweat chloride levels [98, 100]. AEs although present, were similar across the study with the majority, 81.4%, being mild to moderate. While more serious events occurred in 6.1% of the pooled tezacaftor group, 7.5% in the pooled tezacaftor/ivacaftor group and 15.2% in the pooled placebo groups [98, 100].

Following the phase 2 trial, two subsequent phase 3 trials were conducted to test the efficacy of the combination treatment, tezacaftor/ivacaftor, in F508del homozygotes (EVOLVE, NCT02347657) and in patients with one F508del mutation and the other being a mutation with residual function (EXPAND, NCT02392234) [101, 102].

The EVOLVE trial, which included patients homozygous for F508del, compared the combination of tezacaftor (100 mg daily)/ivacaftor (150 mg twice daily) to placebo, in a double-blind, randomized trial [101]. The combination treatment group showed a 4.0% difference in $ppFEV_1$in the combination treatment group, compared to a 3.4% improvement in the placebo group at 24 weeks [98, 101]. Moreover, tezacaftor/ivacaftor treatment group demonstrated lower pulmonary exacerbation rate, better cystic fibrosis questionnaire-revised (CFQ-R) scores (mean difference of 5.1 points) and a 10.1 mmol/L improvement in sweat chloride levels compared to placebo [98, 101]. The EXPAND trial, while similar to the EVOLVE trial, was comprised of CF patients with one F508del allele and the other CFTR allele had a residual function mutation, also contained an extra arm to the study, that looked at ivacaftor monotherapy (150 mg twice daily) [98, 102]. The primary endpoint results showed improvement in absolute $ppFEV_1$ for the tezacaftor/ivacaftor group (6.8%) and the ivacaftor monotherapy group (4.7%) compared to placebo, with a statistically significant difference between the combination (tezacaftor/ivacaftor) and the ivacaftor monotherapy [98, 102]. CFQ-R scores favored the combination group by 11.1 points and the monotherapy group by 9.7 points, compared to placebo [98, 102].

Another phase 3 clinical trial (NCT02516410) with patients having on F508del mutation and the other being a minimal function mutation, was terminated early due to pre-determined futility criteria, and after reporting no difference in $ppFEV_1$ after 12 weeks with the combination treatment [98]. A fourth phase 3 trial (NCT02412111) in patients with CF with one copy of F508del and a gating mutation (e.g., G551D) was performed [103]. This trial did not meet their primary endpoint, but an absolute $ppFEV_1$ at 8 weeks showed a 0.5% increase for the combination treatment compared to a 0.2% increase for placebo ($p = 0.5846$), and an improvement of 5.8 mmol/L in sweat chloride for the tezacaftor/ivacaftor group [103].

The EXTEND trial (NCT02565914) was a 96-week open-label extension study, and the results from this study were recently conveyed [98, 104]. Overall, the study demonstrated that the tezacaftor/ivacaftor combination treatment was safe, well tolerated, and efficacious during a 120-week period [104]. Benefits could be seen at 48 weeks or more in absolute change in $ppFEV_1$ and CFQ-R score. Furthermore, in F508del homozygotes, the rate of lung function decline was significantly reduced [104].

1.5.5.5 Trikafta

In October 2019, the Triple-combination therapy TRIKAFTA® was approved in the US for PwCF over the age of 12 with at least one copy of F508del-CFTR and recently has been expanded to include an additional 171 CFTR mutations (Table 3) [105]. TRIKAFTA® is composed of three drugs: Elexacaftor (VX-445), Tezacaftor (VX-661) and Ivacaftor (VX-770) which when used *in vitro* are referred to as a Triple therapy. Elexacaftor and Tezacaftor are CFTR correctors, which work to increase the amount F508del-CFTR protein that reaches the plasma membrane by aiding its processing and trafficking, while Ivacaftor is a CFTR potentiator that works at the cell surface to increase chloride channel activity [106].

Early *in vitro* work on HBE cells from CF patients, solidified the findings that correctors, Elexacaftor and Tezacaftor, in the presence of Ivacaftor or not, does in fact improve mature CFTR protein and chloride transport [107, 108]. This then led to a multinational phase 2, double-blind, proof of concept study in CF patients ≥ 18 years old, homozygous for F508del or with one F508del allele and one with minimal function (NCT03227471) [108]. Primary endpoint of increasing $ppFEV_1$ in patients was obtained for both groups during the 4-week trial, seen as quickly as 2-weeks into treatment and was maintained during the study period. Other improvements included reduction in sweat chloride concentrations and an increase in CFQ-R scores [107, 108].

The AURORA program included three, phase 3 clinical trials: NCT03525444, NCT03525548 and NCT03525574 [25, 107, 109, 110]. The trial conducted by Heijerman et al. (NCT03525548), followed CF patients 12 years and older, homozygous for F508del and was a multicenter, double-blind study, over a 4-week period [109]. Improvements were seen in the triple combination group for $ppFEV_1$, CFQ-R scores, body mass index (BMI) and a reduction in sweat chloride levels [109]. Middleton et al. conducted a 24-week, phase 3 trial (NCT03525444) that took place at multiple treatment centres, in patients heterozygous for F508del and a minimal function mutation, 12 years and older [25]. Similar results seen in Heijermen at al. were also seen for this trial. Overall, the combination therapy was deemed safe and effective [25]. Patients included in the Heijermen et al. and the Middleton et al. trials were given the option to continue treatment in an open-label 96-week extension study (NCT03525574) [110].

There have been several other phase 3 trials following the ones previously discussed. A few examples would be: NCT04058353, NCT04105972, NCT03691779, NCT03525574,

NCT04043806, NCT04058366 [111-113]. These trials have seen significant improvements and confirmed its safety in patients.

A new case-control study done in Palermo, Italy, followed 26 patients over the course of a year, who had at least one F508del mutation [114]. The triple therapy combination showed improvements in $ppFEV_1$, sweat chloride levels, CFQ-R scores, BMI as well as a reduction in pulmonary damage and bronchial destruction after computed tomography imaging [114]. Interestingly, in the treated patients, pulmonary colonization showed a significant decrease after one-year of treatment, where 45.3% of collected sputum samples became negative for pathogenic microorganisms [114].

1.6 Inflammation in CF

1.6.1 Cytokine dysregulation

CF is characterized by an abnormally activated inflammatory response in the lung, which overreacts in the presence of pathogens and leads to irreversible lung damage (Balough, McCubbin et al. 1995, Khan, Wagener et al. 1995). More recent evidence suggests that the excessive and persistent inflammation in the human CF airways is indicative of an inflammatory response that begins early in life, is of greater magnitude than is observed in patients without CF and persists beyond apparent eradication of infectious stimuli (Rottner, Freyssinet et al. 2009).

Neutrophils form the first line of defense against pathogens and represent 50-70% of white blood cells in human blood, making them the most abundant circulating immune cell [115, 116]. Their recruitment in the airways of PwCF plays an important role on pathophysiology, due to inflammatory mediators or in response to pathogens [115]. In response to persistent inflammation, tumor necrosis factor (TNF)-α and IL-17 cytokines, further promote the recruitment of neutrophils to the airways, creating a vicious cycle, which is detrimental to the lungs [78, 117]. Moreover, other cytokines, such as IL-1β and GM-CSF are also up-regulated [78]. It has also been shown that CF cells produce disproportionate amounts of IL-8 and IL-6, which are pro-inflammatory cytokines [118]. Moreover, Weber and colleagues demonstrated a link between defective CFTR protein and its implications in the activation of the NF-κB pathway, associated with the drastic increase of IL-8 [118, 119]. Other transcription factors, namely AP-1 and MAPK, are also responsible for the increase in cytokine levels [78]. Moreover, while the proinflammatory response

is dysregulated, anti-inflammatory cytokines, such as IL-10, and nitric oxide, a signaling molecule, are downregulated [78].

1.6.2 Fatty acid imbalance

The impaired inflammatory response in CF reflects an imbalance between the initiation and the resolution of the inflammatory response, with the overexpression of pro-inflammatory cytokines and mediators, while anti-inflammatory and pro-resolving mediators are suppressed. When counter-regulatory controls are abnormal, an imbalance occurs, resulting in prolonged and excessive inflammatory mediator production, fuelling the destructive inflammatory cascade and difficulty in maintaining of homeostatic functions. Many lipid mediators, including prostaglandins, are formed when AA, a 20-carbon unsaturated fatty acid, is metabolized by sequential synthases after its release from the plasma membrane phospholipids due to an increased activity of phospholipase A2 and metabolized by sequential synthases [120]. AA, an omega-6 fatty acid, is metabolized into proinflammatory eicosanoids: 2-series prostaglandins and 4-series leukotrienes [40]. On the other hand, DHA, a 22-carbon unsaturated omega-3 fatty acid, is metabolized to yield anti-inflammatory resolvins and protectins, which can orchestrate the timely resolution of inflammation [121, 122]. Several studies show that functional impairment in the CFTR protein is associated with elevation of AA in the lung epithelial cells and a reciprocal decrease of DHA and eicosapentaenoic acid (EPA) levels [86, 123-129]. Therefore, the elevated AA levels in PwCF directly contribute to an increased production of pro-inflammatory mediators, meanwhile, a decrease in DHA and EPA play important roles in reducing the production of anti-inflammatory mediators [129]

1.6.3 Lipid metabolism abnormalities and their consequences

It has been well documented that sphingolipid metabolism is dysregulated in CF [40, 129-131]. Ceramides are produced through various pathways and the relative composition of specific species of ceramides depends on the expression of specific combination of ceramide synthases in the organs. In the lungs of healthy mice, VLCCs (C24:0 CER and C26:0 CER) were shown to represent more than 70% of the total ceramide pool, whereas LCCs (C14:0 and C16:0) constitute less than 20% of the total ceramide pool [132]. The relative ratios between VLCCs and LCCs change dramatically during infection.

Upon bacterial infection, ceramides arrange into ceramide-enriched membrane platforms vital for host defenses [133-135]. Ceramides present in lipid rafts were shown to play an important role in the regulation and resolution of the inflammatory response mounted in *P. aeruginosa* infected mice [133, 136]. Inhibition of ceramide platform formation creates excess ceramides which can lead to pulmonary inflammation and hinder anion secretion [137-139]. However, the exact composition of ceramide platforms and changes in their composition occurring during infection and leading to deterioration of lung function have not been elucidated thus far. Disturbance in their synthesis and recycling were shown to be associated with several lung diseases and an increased susceptibility to lung infection [132, 140].

1.6.4 Resolution of inflammation in Cystic fibrosis

In recent years, the advancement in CFTR modulator and corrector therapies have allowed for improvements to the specific defect that underlies this disease, however, their ability to help resolve inflammation is still being studied. In 2006, FEN was reported to effectively inhibit IL-8 release from CFTR-deficient lung epithelial cells [82]. Subsequently, FEN has been studied in the context of inflammation in CF disease and its effects have been attributed to its overall ability to trigger the resolution phase of inflammation, as demonstrated by the modulation of macrophage-secreted inflammatory cytokines, to the correction of omega-3/omega-6 fatty acid imbalances impacting phosphorylation of ERK1/2, to the correction of the ceramide deficiency in PwCF, and to the resolution of lung mucus plugging under infection with *P. aeruginosa* [28, 83, 84]. Furthermore, Garić et al. found a partial synergistic relationship between FEN and Zn^{2+}, whose deficiency has been reported in PwCF and is associated with severity of CF lung disease [85, 86].

1.7 Study Rationale and Objectives

Given that TRIKAFTA has become standard of care for PwCF with class II mutation in the *CFTR* gene, and that LAU-7b has shown to be extremely beneficial in clinical trials thus far, we decided to study the effects of the combination of Triple therapy drugs and LAU-7b in a homozygous F508del transgenic mouse model of CF lung disease. Our primary goal was to test if the combination of the two approaches would be more efficacious at improving the disease than Triple therapy alone.

We **hypothesized** that the combination of Triple therapy, which works to improve processing of the mutated CFTR protein to the surface and therapy using fenretinide, which normalizes aberrant fatty acid and lipid imbalance, activates peroxisome proliferator-activated receptor-γ (PPARγ), and controls the inflammatory mediator's expression will facilitate the corrective effect of triple therapy in our *in vivo* F508del-CFTR mouse model (DD mice).

Objective 1: Test the effect of combined therapy consisting of triple therapy and fenretinide on VLCC/LCCs *in vitro* on CFBE41o-(P) and CFBE41o-(F508del) cell lines.

Objective 2: Assess total CFTR protein levels in the CFBE41o-(F508del) cell line following various treatment combinations.

Objective 3: Test the effect of combined therapy consisting of triple and fenretinide on oxidation markers, fatty acids and VLCC/LCCs in lungs, liver, and plasma of DD mice.

Objective 4: Test the effect of combined triple therapy and fenretinide on lung physiology (airway resistance and hyperplasia) in a F508del transgenic mouse model.

Chapter 2. Materials and Methods

Methods are from the published manuscript in *Frontiers in Pharmacology* by Centorame et al. titled:

Treatment with LAU-7b complements CFTR modulator therapy by improving lung physiology and normalizing lipid imbalance associated with CF lung disease [1**].**

2.1 F508del^{tm1EUR} Mice

The *Cftr^{tm1EUR}* mouse model (C57BL/6J), heterozygous for the F508del *CFTR* mutation, was obtained from the Erasmus Medical Center (Rotterdam, Netherlands) [141, 142]. Mice were fed with standard diet and water *ad libitum*. Mice of 8-20 months were randomly assigned to 5 groups: 1) Non-treated, 2) Vehicle-treated, 3) LAU-7b-treated, 4) Triple drug-treated, and 5) combined Triple Drug and LAU-7b-treated. The treated mice received daily doses of Vehicle, LAU-7b (25 mg/kg) representing 10mg/kg fenretinide content, Triple drugs (32 mg/kg Ivacaftor, 21 mg/kg Tezacaftor, 42 mg/kg Elexacaftor) or a combination of LAU-7b with Triple drug therapy by daily gavage *per os* (p.o) over the course of 14 days. The daily doses of Ivacaftor, Tezacaftor and Elexacaftor used in the mouse study were derived by allometric scaling from the daily dose of each individual modulator contained in TRIKAFTA® that was approved for treatment in adult CF patients. Based on the preclinical data available in the NDA/BLA Multi-disciplinary Review and Evaluation (NDA 212273) for TRIKAFTA®, the bioavailability of the three CFTR modulators are similar in rodents and humans. Furthermore, all the derived doses used in the mouse study were below the NOAEL (no observed adverse event level) determined in the rodent toxicity studies with a duration of 28 days up to 3 months.

All animals were harvested 24 hours after the last treatment. All experimental procedures were in accordance with Facility Animal Care Committee of the McGill University Health Center, Montreal, QC, Canada.

2.2 Lung Resistance Analysis

Airway resistance was measured using a Buxco plethysmograph system (Buxco Research System, Wilmington, NC, USA), ventilators, and nebulizers (Harvard Apparatus, Holliston, MA,

USA). Mice were anesthetized using a cocktail of ketamine, acepromazine and xylazine and were connected to a ventilator through tracheotomy as previously described [143]. Standard invasive lung resistance measurement was done just prior to mouse harvest. A nebulizer was used to administer a saline dose followed by ascending doses (50 mg/mL to 100 mg/mL) of methacholine (MCh, Acetyl β-methyl choline, Cat: A2251, Sigma Aldrich, Saint Louis, MO, USA). The maximal resistance at each dose of MCh was determined for each mouse.

2.3 Lung Histology Analysis

The left lung lobe of mice in each treatment group was inflated in 10% PBS-buffered formalin and kept in the solution for 48 hours. The lung section was then processed, paraffinized, sectioned at 4 μm thickness, then deparaffinized, hydrated, and stained with hematoxylin and eosin (H&E). Infiltrating cells were quantified in 4 airways/mice lung at 20X magnification, that of which were averaged and then normalized with the perimeter squares of the airway basement membrane as previously described [143].

2.4 CFBE41o- Epithelial cells overexpressing wt-CFTR or F508del-CFTR

The human bronchial epithelial cell line CFBE41o- homozygous for the F508del mutation was shown to retain several characteristics of human CF bronchial epithelial cells [144]. Parental CFBE41o- (F508del/F508del) cells are referred to as CFBE41o-(P) and have negligible expression of CFTR. CFBE41o-(P) cells overexpressing F508del/F508del are referred to as CFBE41o-(F508del). CFBE41o-(P) cells overexpressing wt-CFTR are referred to as CFBE41o-(WT). The CFBE41o-(P) cells originally generated by Prof. Dr. D. C. Gruenert [145], with a stable expression of wt-CFTR, CFBE41o-(WT), and the CFBE41o-(F508del) [146] were generously provided from Dr. John W. Hanrahan (McGill University at Montreal, Quebec, Canada). Cells were grown in Eagle's Minimum Essential Medium (Wisent Bioproducts) supplemented with 10% fetal bovine serum (Wisent Bioproducts), 5% penicillin- streptomycin (Wisent Bioproducts) in a 5% CO_2 - 95% air incubator at 37°C.

2.5 Treatment of CFBE41o-(P), CFBE41o-(WT) and (F508del) cell lines for lipid and protein analysis

For lipid analysis, cell lines were seeded in 100 mm plates with 1,000,000 cells in 12 mL of media and grown overnight to 80% confluence and treated the next day. They were treated with fresh drugs every 24 hours, for a total time of 72 hours with 1.25 μM LAU-7b (Laurent Pharmaceuticals) and 12.5 μM zinc-sulphate (Sigma-Aldrich), as well as Triple therapy composed of 3 μM Elexacaftor (VX-445), 3 μM Tezacaftor (VX-661) and 10 nM Ivacaftor (VX-770). After completion of treatment, the cell monolayer was washed twice with warmed D-PBS (Wisent Bioproducts), gently scraped with a cell scraper (Sarstedt), and pipetted into a 1.5 ml screw cap tube filled with 1 ml of 1 mM butylated hydroxyanisole (BHA) in a chloroform/methanol solution (2:1 vol/vol) for mass spectrometry lipid analysis. For protein analysis, CFBE41o-(F508del) cell line was seeded in 60 mm plates with 500,000 cells in 6 mL of media and grown overnight to 80% confluence and treated the next day. The cells were also treated for 72 hours, with the same drug concentrations as indicated above for the various treatment combinations.

2.6 Fatty acid analysis

Lipid analysis was done using CFBE41o-(P), CFBE41o-(WT) and CFBE41o-(F508del) cells, 25 mg of macerated lung and liver mouse tissue as well as 50 μl of plasma from each mouse, preserved in BHA and stored at -80°C. Classical purification of lipids was done as previously described by Folch et al. [147] and phospholipids were identified by thin layer chromatography extraction.

In parallel, from the chloroform fraction of the extracted lipids, the fatty acids were methylated under standard conditions and the esters were identified by gas chromatography. An Agilent Technologies 6890 N gas chromatograph (Germany) was used, equipped with a flame ionization detector and capillary column (30m, 0.53mm) (Agilent Technologies 6890 N, Germany), silica was used as stationary phase. The chromatographic conditions: detector temperature 280°C; injector temperature 250°C; initial column temperature 120°C for 1 min, and programmed to escalate at a rate of 10°C per minute up to 200°C and then at 4°C per minute up to the final temperature of 220°C. Nitrogen and hydrogen were used as carrier and auxiliary gas, respectively, with a flow rate of 1.3 mL/min. To perform the determination, 1 μL of the derived sample was injected, alternatively with a sample volume/internal standard ratio of 80/20. Fatty

acids were identified by comparing the retention times and relative retention times of the standards with those of the samples purchased from Sigma Aldrich. The results obtained in mg/100 g of the sample were calculated according to AOCS methodology (AOCS 2017). Output signals were monitored using Agilent Chem Station for GC systems, data analysis and A/D converter 35900E. The data were estimated by automated integration of the area under the resolved chromatographic profile.

2.7 Ceramide Analysis

Tubes were mixed vigorously and centrifuged at 4°C for 5 min at 3,000 rpm. The organic phase was recovered and evaporated using a Speedvac. The extracted lipids were separated as previously described [86, 129-131]. Total ceramides were measured by ELISA after TLC purification, whereas quantification of the specific ceramides' species among purified ceramide pool were quantified using mass spectrometry. LC-MS/MS was carried out using a TripleTOF 5600+ mass spectrometer (AB sciex) coupled to a Dionex UltiMate 3000 LC-system. The separation column was Kinetex 2.1 × 50 mm C18, guarded with a SecurityGuard 4 × 2.0 mm C18 guard pre-column (Phenomenex). The mobile phases were MilliQ water with 50 mM ammonium acetate and 0.1% formic acid (A) and isopropanol/acetonitrile (4:3) with 50 mM ammonium acetate and 0.1% formic acid (B), The flow rate was 300 µl/min and the gradient was set up to 15 min run time with first 1.5 min running 15% B, then increasing to 85% B in 4.5 minute, further increase to 100% B in 12 minute and decrease to 15% B in 15 minute. The injection volume was 10 µl using µl pick-up option and 15% B as loading buffer using 20 µl sample loop. The sampler solvent was pure isopropanol to prevent sample carry over between runs. The MS was run in positive mode using AB Sciex DuoSpray ion source. The ion source was set up to ion source gas flow 1 to 45, gas flow 2 to 40, curtain gas to 30, temperature to 200 and ion source voltage to 4500 V. The instrument was run in product ion mode with eleven separate experiments, one per each monitored analyte. Lipid standards were purchased from Sigma Aldrich and Avanti Polar Lipids

2.8 Analysis of Lipid & Protein Oxidation

Lipid peroxidation was measured fluorometrically using 2-thiobarbituric acid-reactive substances (TBARs species) as the end product of lipid peroxidation [148, 149]. Briefly, the samples of cells or macerated lung tissue were mixed with 8.1% sodium dodecyl sulfate, 20%

acetic acid, and 0.8% 2-thiobarbituric acid. After vortexing, the samples were incubated for 1 hour at 95 °C after which butanol-pyridine was added at a 15:1 (*v/v*) ratio. The mixture was shaken for 10 minutes and then centrifuged. The butanol-pyridine layer was measured fluorometrically at 552 nm after excitation at 515 nm (OptiPlate Perkin-Elmer USA). The results are expressed in nmoles of malondialdehyde (MD) (TBARs species) per mg of protein in the samples reflecting all thiobarbituric acid reactive substances [150]. Oxidative damage of proteins was assessed using 3-nitrotyrosine as a surrogate marker. 3-nitrotyrosine (3-NT) was determined by ELISA as previously described using well-characterized antibodies [151, 152]. The antibodies (mouse IgG monoclonal, polyclonal against 3-nitrotyrosine and polyclonal goat anti-rabbit IgG-peroxidase) were obtained from Upstate Biotechnology (Lake Placid, NY). The quantification of 3-NT was performed using a standard curve with known concentrations of 3-NT from chemically modified bovine serum albumin. The sensitivity of the assay was 50 pg/ml.

2.9 Western Blot analysis

CFBE41o-(F508del) cells were lysed in homemade RIPA buffer (50 mM Tris at pH=7.4, 150 mM NaCl, 50 mM NaF, 0.2 mM Na_3VO_4, 0.1% SDS, 2 mM EDTA, 1% Triton-X, 0.5% Na-deoxycholate) with freshly dissolved protease inhibitor tablet (Sigma # 4693132001). The protein concentration was determined using BCA Protein Assay Kit (Thermo Scientific #23227) and proteins were denatured using 4x Laemmli Sample Buffer (Bio-Rad, #1610747) with 10% (v/v) *2*-mercaptoethanol (1x final) and RIPA. 20 µg of total protein was loaded on a gel and proteins were separated on a precast 4-15% polyacrylamide gradient gel (Bio-Rad, #4561085) by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane using semi-dry transfer and the fast semi-dry transfer buffer (1X, final) containing 48mM Tris, 15 mM HEPPS with freshly added sodium bisulfate (1 mM final), EDTA (1.0 mM final) and 4 N, N-dimethylformamide (1.3 mM final) as previously described [153]. Membranes were incubated with primary antibodies: against the R domain of CFTR (23C5, provided by Dr. John Hanrahan lab) and against β-Actin (Santa Cruz, #sc-1616). Membranes were then incubated with secondary antibodies: goat anti-mouse (IgG-HRP, sc-2005). Lastly, membranes were developed using chemiluminescent kit (Bio-Rad, #170-5060).

2.10 Statistical Analyses

All statistical analyses were performed using GraphPad Prism 9 (GraphPad, San Diego, CA, USA). For lung function, a two-way ANOVA with Sidak. For infiltrating cells in the airways of the mice, a two-way ANOVA with Bonferroni correction was performed. As for the analysis of the oxidation markers, fatty acids, VLCCs and LCCs, a Brown-Forsythe and Welch one-way ANOVA with Dunnett T3 correction was used. For the analysis of the VLCC/LCC ratio, a Kruskal-Wallis one-way ANOVA with Dunn's correction was performed. Lastly, for Western Blot quantification, a two-way ANOVA with Sidak correction was done.

Chapter 3. Results

Results presented in this section have been published in *Frontiers in Pharmacology* by Centorame et al. entitled:

Treatment with LAU-7b complements CFTR modulator therapy by improving lung physiology and normalizing lipid imbalance associated with CF lung disease [1**].**

3.1 Triple therapy and LAU-7b combination normalizes lung function in homozygous F508del/F508del mice

Lung function of F508del/F508del (DD) mice and WT mice treated with LAU-7b, Triple therapy, the combination of the two, or vehicle, was assessed using a classical lung resistance evaluation following aerosolised challenge of the lung with increasing concentrations of methacholine. Lung resistance of 14-20-month-old DD mice compared to their age-matched WT controls (red) is shown in Figure 1**A**. While treatment with Triple therapy (green) decreases the airway resistance in DD mice, treatment with LAU-7b (grey) shows lower airway hyperresponsiveness than all other treatment groups, comparable to WT control mice. Triple therapy alone, LAU-7b alone and their combination (black) shows protective effects at the level of lung function. Lung function was improved to a higher extent by combinatory treatment with Triple and LAU-7b than Triple therapy alone, as demonstrated by a lower airway resistance in this group of mice compared to Triple alone. In Figure 1**B**, resistance values recorded for the saline and 100 mg/ml MCh doses are shown to better illustrate statistical significance observed between the treatment groups. While DD NT & VEH mice compared to Triple have a *p*-value of less than 0.05, when compared to Triple + LAU-7b combination, the *p*-value is less than 0.0005. The complete results of statistical analysis are provided in Table 1.

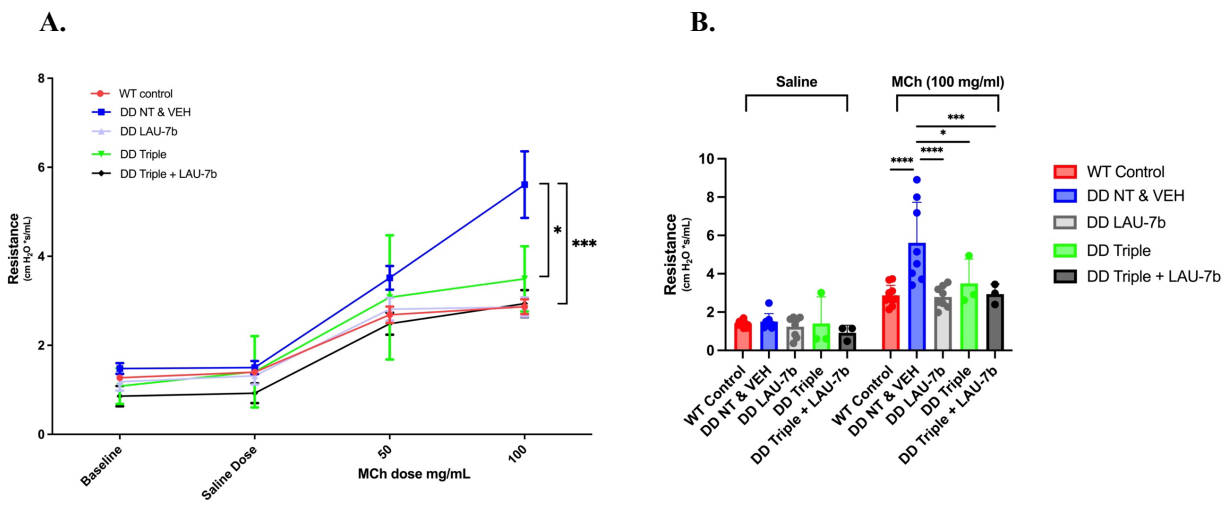


Figure 1. Airway function measure following MCh challenge, is significantly improved in F508del/F508del (DD) mice upon treatment with Triple therapy, and further improved by addition of LAU-7b. Treatment groups include Wild-type (WT) control (Non-treated & Vehicle, NT & VEH) (Red), DD NT & VEH (Blue), DD LAU-7b (Grey), DD Triple therapy (Green) and DD Triple + LAU-7b (Black). **(A)** Comparisons shown between DD NT & VEH and DD Triple (p= 0.0118) and between DD NT & VEH and FF Triple + LAU-7b (p= 0.0003). **(B)** Detailed analysis of the saline and 100 mg/mL MCh dose among the treatment groups. n = 3-12 mice for each group. Two-way ANOVA with Sidak correction where *p<0.05, **p<0.005, ***p<0.0005 and ****p<0.0001

Dunnett's T3 multiple comparisons test	Mean Diff.	95.00% CI of diff.	Summary	Adjusted P Value
S - WT control vs. M - WT control	-1.472	-2.195 to -0.7493	***	0.0001
S - WT control vs. M - DD VEH & NT	-4.213	-7.734 to -0.6923	*	0.0193
S - WT control vs. M - DD LAU-7b	-1.392	-2.324 to -0.4597	**	0.0043
S - WT control vs. M - DD Triple + LAU-7b	-1.540	-2.841 to -0.2389	*	0.0166
S - DD VEH & NT vs. M - WT control	-1.367	-2.223 to -0.5111	***	0.0006
S - DD VEH & NT vs. M - DD VEH & NT	-4.108	-7.535 to -0.6808	*	0.0176
S - DD VEH & NT vs. M - DD LAU-7b	-1.287	-2.280 to -0.2940	**	0.0066
S - DD VEH & NT vs. M - DD Triple + LAU-7b	-1.435	-2.780 to -0.08981	*	0.0316
S - DD LAU-7b vs. M - WT control	-1.633	-2.615 to -0.6509	***	0.0004
S - DD LAU-7b vs. M - DD VEH & NT	-4.374	-7.843 to -0.9049	*	0.0129
S - DD LAU-7b vs. M - DD LAU-7b	-1.553	-2.639 to -0.4666	**	0.0024
S - DD LAU-7b vs. M - DD Triple + LAU-7b	-1.701	-3.092 to -0.3104	**	0.0097
S - DD Triple + LAU-7b vs. M - WT control	-1.945	-3.442 to -0.4475	*	0.0165
S - DD Triple + LAU-7b vs. M - DD VEH & NT	-4.686	-8.194 to -1.178	**	0.0089
S - DD Triple + LAU-7b vs. M - DD LAU-7b	-1.865	-3.354 to -0.3756	*	0.0169
S - DD Triple + LAU-7b vs. M - DD Triple + LAU-7b	-2.013	-3.647 to -0.3790	*	0.0136

Table 1. Table of One-Way ANOVA analysis of airway resistance comparing saline (S) and 100 mg/ml MCh (M).

As shown in Figure 2**A** and **B**, the comparative analysis of weights for DD mice across all 5 groups does not show any statistical significance prior to the experiment (Day 0) and 24-hours after final gavage (Day 15), respectively. In fact, the DD mice did not display any statistical differences in weights between treatment groups over the entire course of the oral gavage **(Figure 3)**. When comparing controls (non-treated, NT and vehicle treated, VEH) WT and DD mice **(Figure 2C, D)**, as expected there was a statistically significant difference between the weights of WT and DD mice, but there was no statistically significant weight difference among the experimental groups within the same genotype.

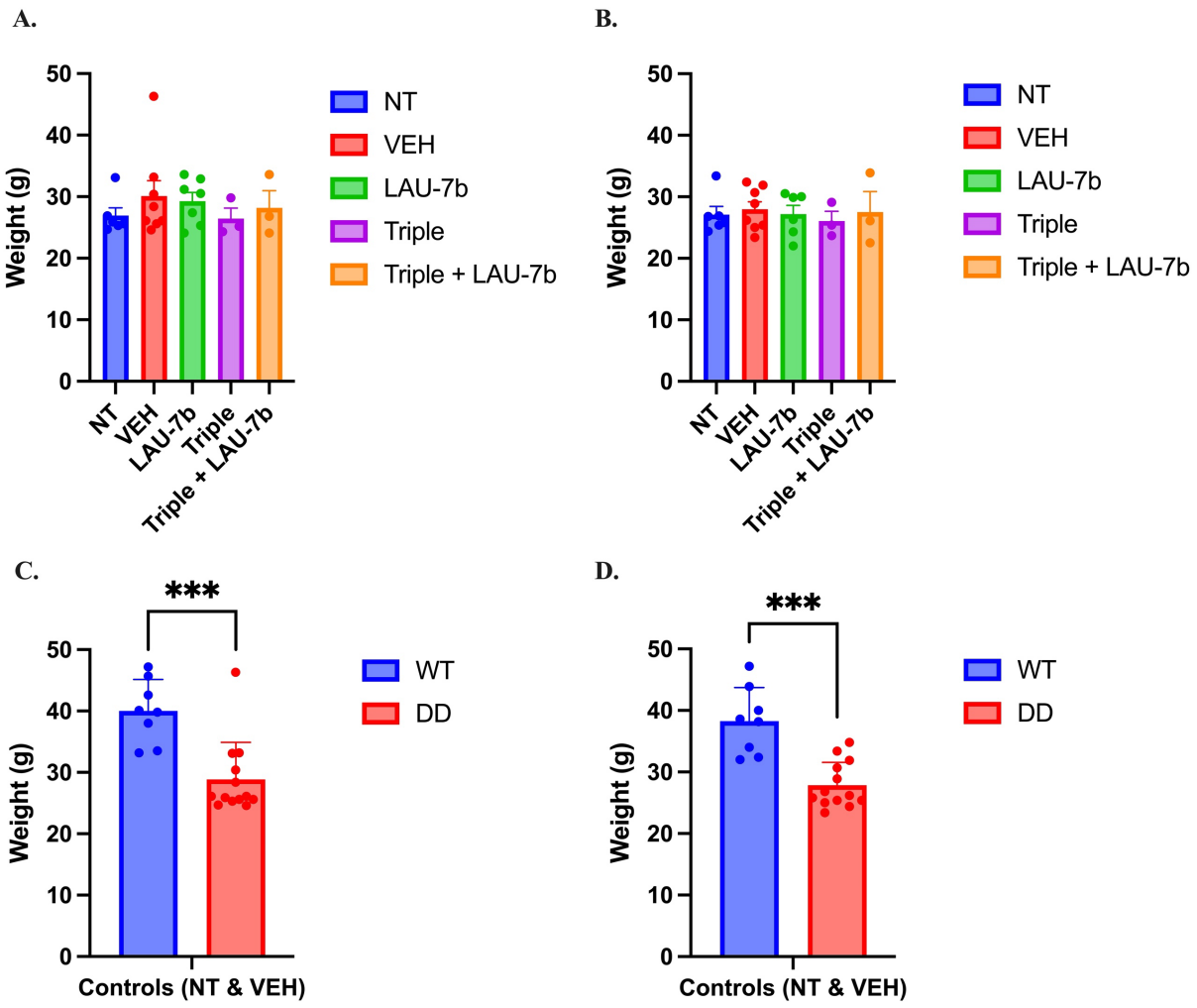


Figure 2. Mice weights prior to (Day 0) and following 14-day treatment (Day 15) in Wild type (WT) and F508del/F508del (DD) mice. (A, B) DD mice weights (g) before treatment (Day 0) and 24 hours following final treatment (Day 15) among treatment groups. There was no statistical significance between any of these groups. n = 3-12 mice for each group. **(C, D)** Comparison between WT and DD mice controls which include non-treated (NT), or vehicle treated (VEH) mice. n = 8-13 mice for each group. Welch's t test was performed where p = 0.0003.

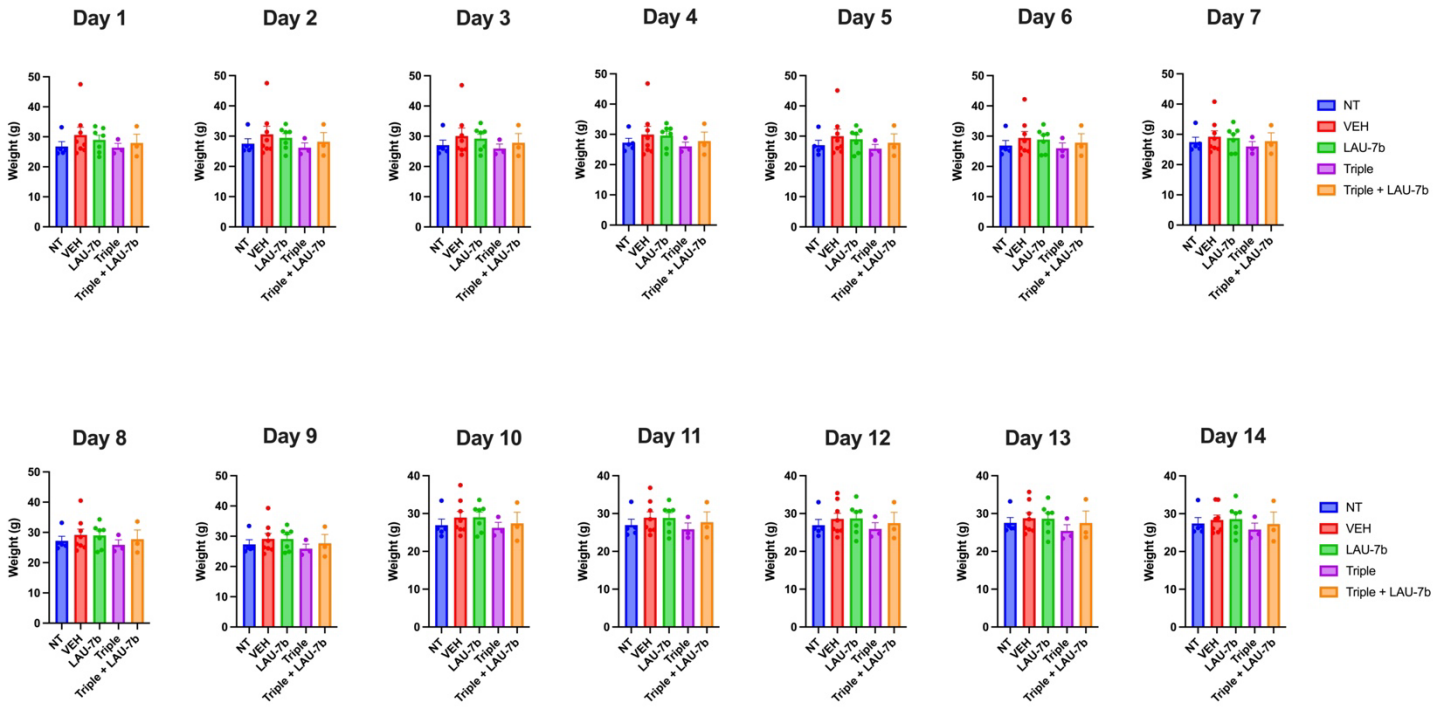


Figure 3. F508del/F508del (DD) mice weights during 14-day treatment. Mice were weighed every day during their 14-day treatment. No statistical significance was seen among treatment groups. n=3-8 mice per group.

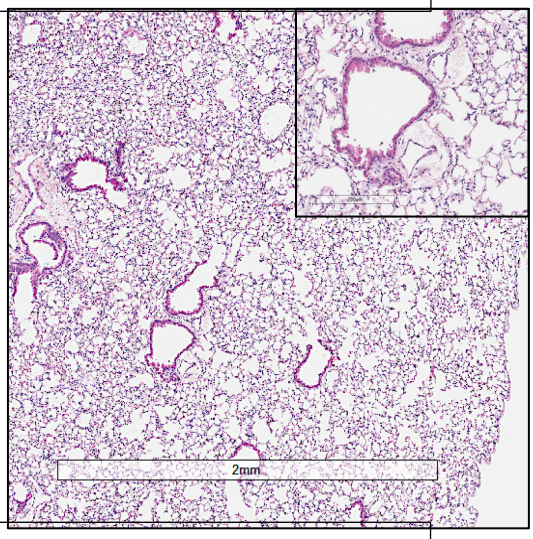
3.2 Triple therapy and LAU-7b combination reduces airway hyperplasia and immune cell infiltration of the airways in the lungs of DD mice

After observing an improvement in airway hyperresponsiveness following combinatory treatment, we assessed lung histopathology using H&E staining **(Figure 4A)**. DD mice were split into 5 treatment groups: non-treated, vehicle treated, LAU-7b, Triple therapy and LAU-7b combined with Triple therapy **(Figure 4B-F)**. DD non-treated and vehicle treated mice with deteriorating lung function show significantly higher airway hyperplasia **(Figure 4B, C)**. Triple therapy, as well as LAU-7b treatments alone, reduced elevated airway hyperplasia in DD mice **(Figure 4D, E)**. The combination of the two treatments, given daily in a 14-day therapeutic cycle, resulted in further improvement and homogenous looking lungs which show no regions of hyperplasia in any of the multiple lung sections analyzed **(Figure 4F)**. However, although a significant improvement in airway resistance following Triple therapy treatment was found, some lung sections still contain pathologically unchanged lung tissue **(Figure 4E)**. DD NT mice develop thickening of the airway due to airway hyperplasia at 8-months old, without further worsening in mice 14-20 months old **(Figure 5A-D)**. As for the WT NT males, there is minimal hyperplasia seen in the airways upon comparison between 9-month-old and 14-month-old WT mice **(Figure 5E, F)**. We also investigated the impacts of sex and age using our DD and WT NT mice **(Figure 5)**. WT and DD mice between the ages of 8-9 months and 14-20 months, both male and female, were analyzed by H&E staining of the lungs **(Figure 5)**. There was no difference in hyperplasia observed when comparing males and females within each experimental group. Furthermore, based on the histological assessment of Figure 4 **A-F**, the treatments have beneficial effects on cellular infiltration in the lung parenchyma in DD mice treated with LAU-7b **(Figure 4D)**, Triple **(Figure 4E)** and Triple + LAU-7b **(Figure 4F)** compared to DD NT and VEH treated mice **(Figure 4B, C)**. In fact, DD mice treated with Triple and LAU-7b had lungs that look very similar to WT control mice, except for hyperplasia that was still observed in some airways examined.

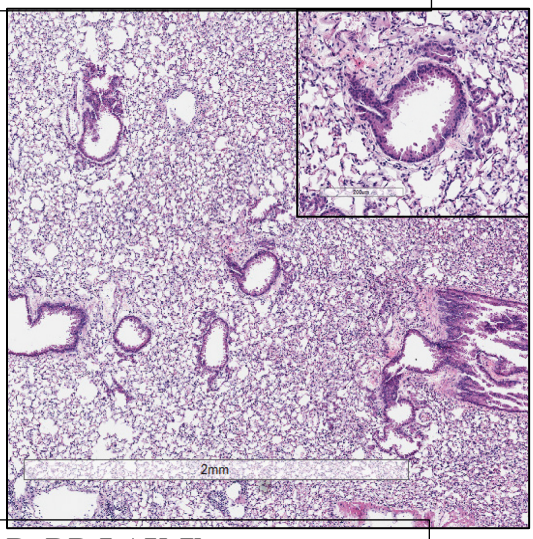
In addition, we evaluated airway thickening caused by cell infiltration through quantification of these cells from H&E-stained lungs **(Figure 4G)**. A significant increase is observed between the WT control group (NT & VEH, mean value 151.08, n=6, SD 30.31) and both the DD non-treated (mean value 277.5, n=6, SD 87.98) and vehicle (mean value 321.6, n=6,

SD 30.37) treated groups. A significant decrease can be seen between vehicle treated mice compared to LAU-7b treated DD mice (mean value 158.7, n=11, SD 62.18). A greater improvement was observed between vehicle treated mice (mean value 321.6, n=6, SD 30.37) and Triple therapy (mean value 158.9, n=3, SD 12.21), as well as with their combination (mean value 136, n=3, SD 40.06). The results indicate a potential benefit with combinatory treatment of LAU-7b and Triple therapy, reducing cellular infiltration in the airways of DD mice.

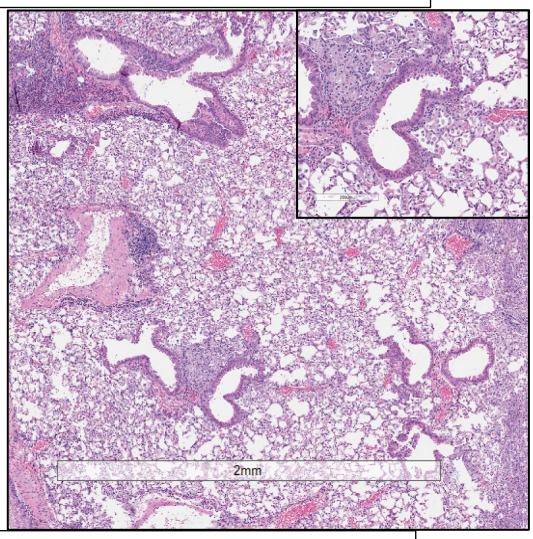
A. WT Control



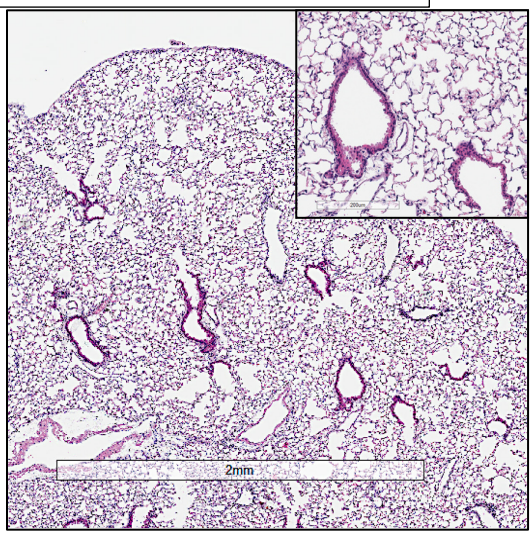
B. DD NT



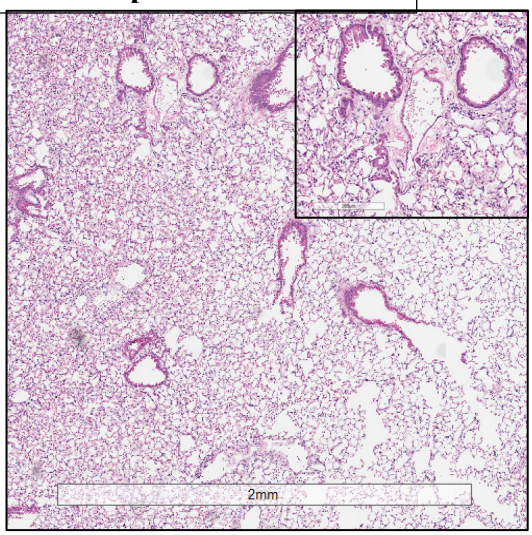
C. DD VEH



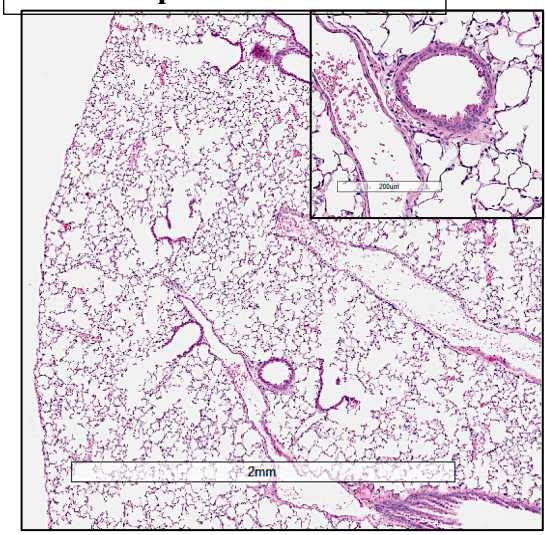
D. DD LAU-7b



E. DD Triple



F. DD Triple + LAU-7b



G.

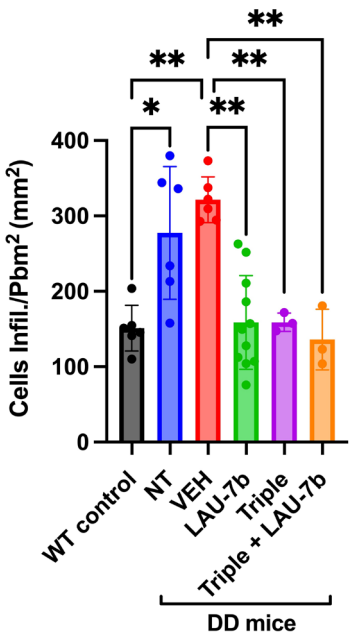
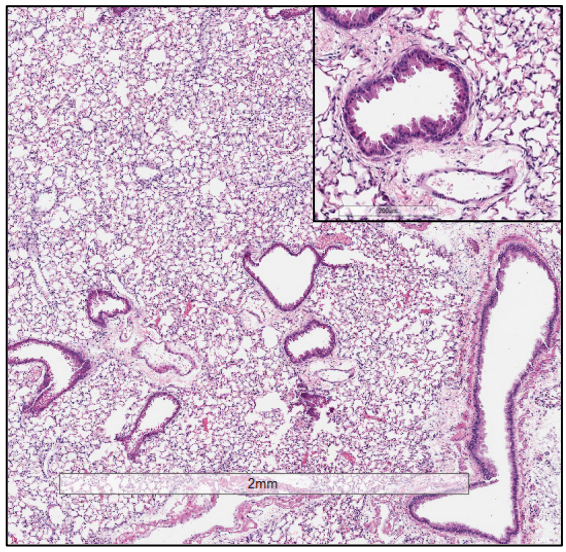


Figure 4. Airway hyperplasia and cellular infiltration in the lungs of Wild-type (WT) and F508del/F508del (DD) mice; Hematoxylin and eosin (H&E) staining. (A) WT control mice (non-treated, NT & vehicle, VEH) **(B-F)** DD NT, VEH, LAU-7b, Triple and Triple + LAU-7b, respectively. **(G)** LAU-7b and Triple therapy treated DD mice have significantly lower lung cell infiltration compared to placebo treated DD mice. For each mouse, measurements were done with at least 4 different airways per lung. Quantification was done by counting the number of infiltrating cells around each of the 4 airways per lung and normalized by dividing the square of the perimeter "in millimeter" of the airway basement membrane. n equal 3-11 mice for each group, Two-way ANOVA with Bonferroni correction where *p<0.05, **p<0.01.

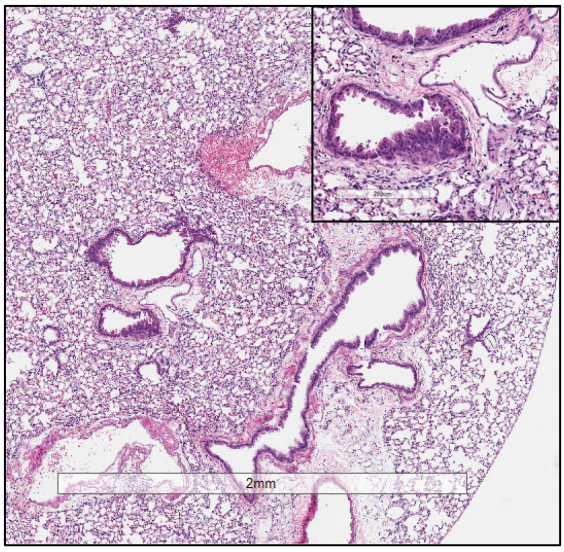
DD NT

8–10-month-old mice

A. Male

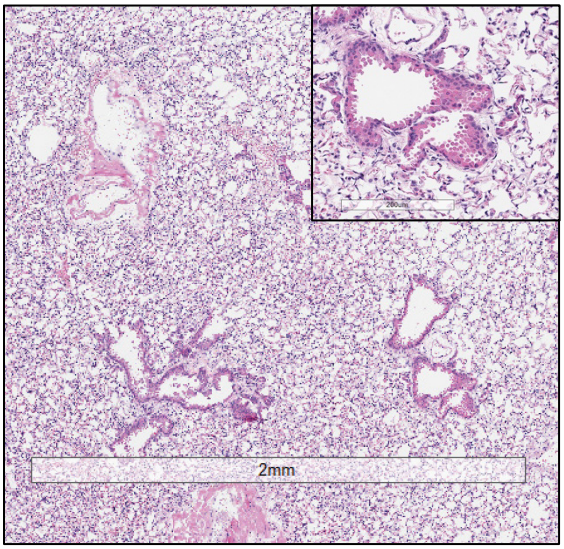


B. Female

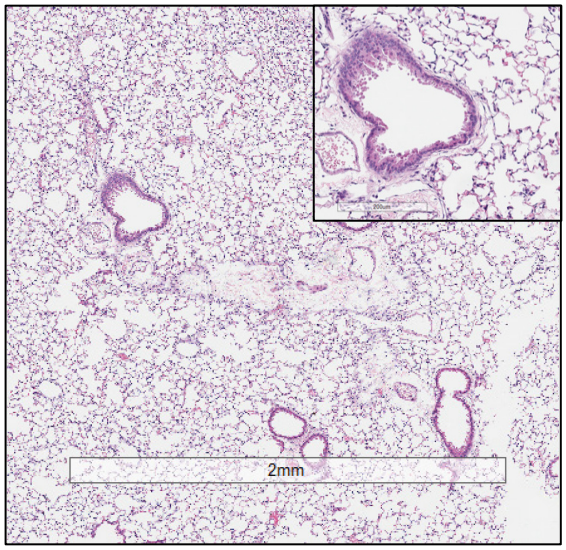


14-20-month-old mice

C. Male



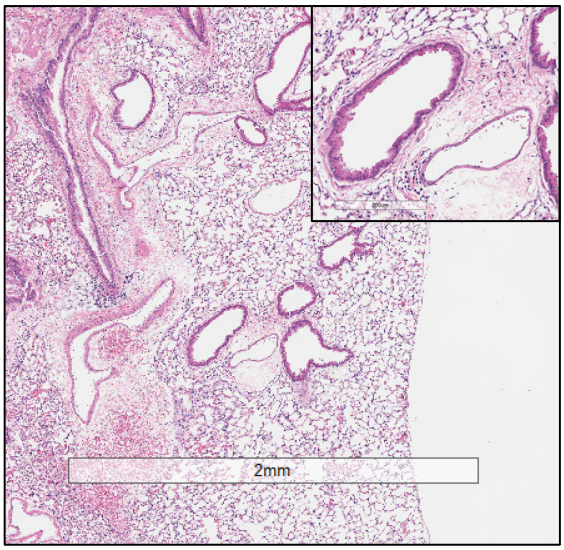
D. Female



WT NT

9-month-old mouse

E. Male



14-month-old mouse

F. Male

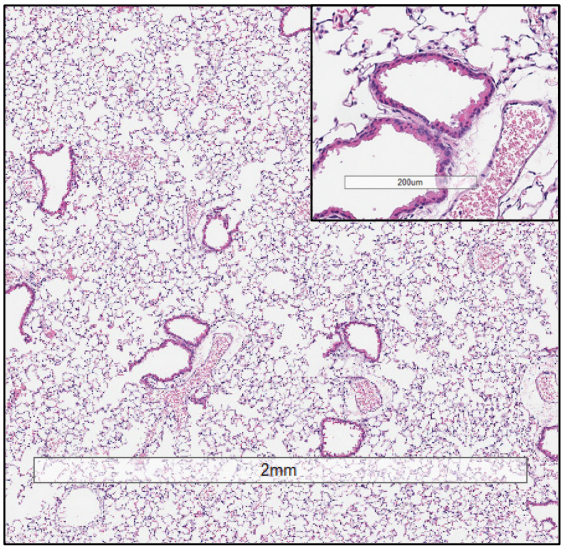


Figure 5. Airway hyperplasia in males and females of different ages seen in F508del/F508del (DD) and Wild-type (WT) Non-treated mice; Hematoxylin and eosin (H&E) staining. (A, B) DD NT mice, 8–10-month-old, male and female respectively. **(C, D)** DD NT mice, 14–20-month-old, male and female respectively. **(E, F)** WT NT mice, 9 month and 14-month-old males, respectively. DD NT mice develop airway hyperplasia by 8-months-old, which remains in older mice, aged 14-20-months-old without further decline. WT NT male mice have little hyperplasia at both 9 and 14-months old.

3.3 Correction of oxidation markers, fatty acids and VLCC/LCCs in the lungs, liver and plasma of DD mice treated with Triple therapy and LAU-7b combination

3.3.1 Oxidation markers

Non-treated and vehicle treated DD mice show increased baseline levels of Malondialdehyde (MD), marker of lipid oxidation, and 3-nitrotyrosine (3-NT), marker of protein oxidation, compared to WT control mice (dotted green line) in lungs, liver, and plasma **(Figure 6)**. Triple therapy, and LAU-7b treatment significantly decrease levels of MD and 3-NT, in lungs, liver, and plasma when compared to vehicle treated DD mice. Across all samples shown in Figure 6 **(A-F)**, combinatory treatment of Triple with LAU-7b significantly decrease both MD and 3-NT to comparable levels with WT mice (dotted green line) or improves these levels below WT, accentuating the benefit of the combinatorial treatment in slowing CF disease progression.

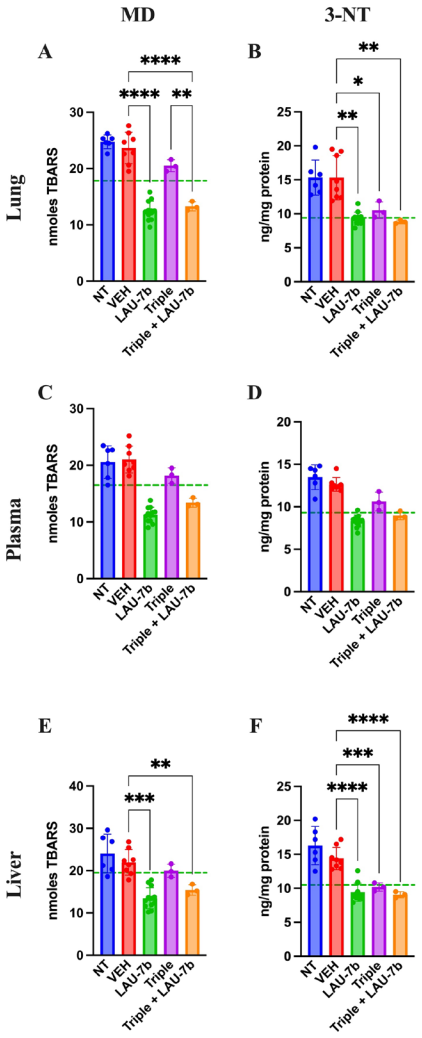


Figure 6. Combination of LAU-7b with Triple therapy restores the imbalances of oxidative stress markers in the lungs, plasma, and liver of F508del/F508del (DD) mice. ELISA analysis of mouse lungs **(A, B)**, plasma **(C, D)** and liver **(E, F)**. Levels for both 3-nitrotyrosine (3-NT) and malondialdehyde (MD) are significantly elevated in Non-treated (NT, n=6) and vehicle treated (VEH, n=8) DD mice as compared to mean levels seen in WT mice (dotted green line). LAU-7b treatment (n=12) significantly decreases levels of MD and 3-NT in all organs and plasma. Triple therapy (n=3) treatment on its own has minimal effect. However, combinatory treatment with LAU-7b and Triple therapy (n=3) restores the decrease in 3-NT and MD seen in the LAU-7b treated mice, when compared to VEH treated DD mice. Brown-Forsythe and Welch One-Way ANOVA with Dunnett T3 correction, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

3.3.2 Fatty acids

In Figure 7, levels of omega-3 (AA) and omega-6 (EPA and DHA) fatty acids were investigated in DD mice lungs, liver, and plasma. EPA and DHA levels are significantly increased with LAU-7b treatment p.o. when compared to placebo treated DD mice. Combinatory treatment of LAU-7b with Triple therapy also significantly increases EPA and DHA comparably, if not higher, than what is seen in WT controls. On the other hand, AA levels are significantly decreased by LAU-7b treatment, and this decrease is further enhanced with Triple and LAU-7b combination. Overall, combination treatment drastically improves the imbalance observed in AA/DHA and AA/EPA ratios in different organs and plasma of DD mice.

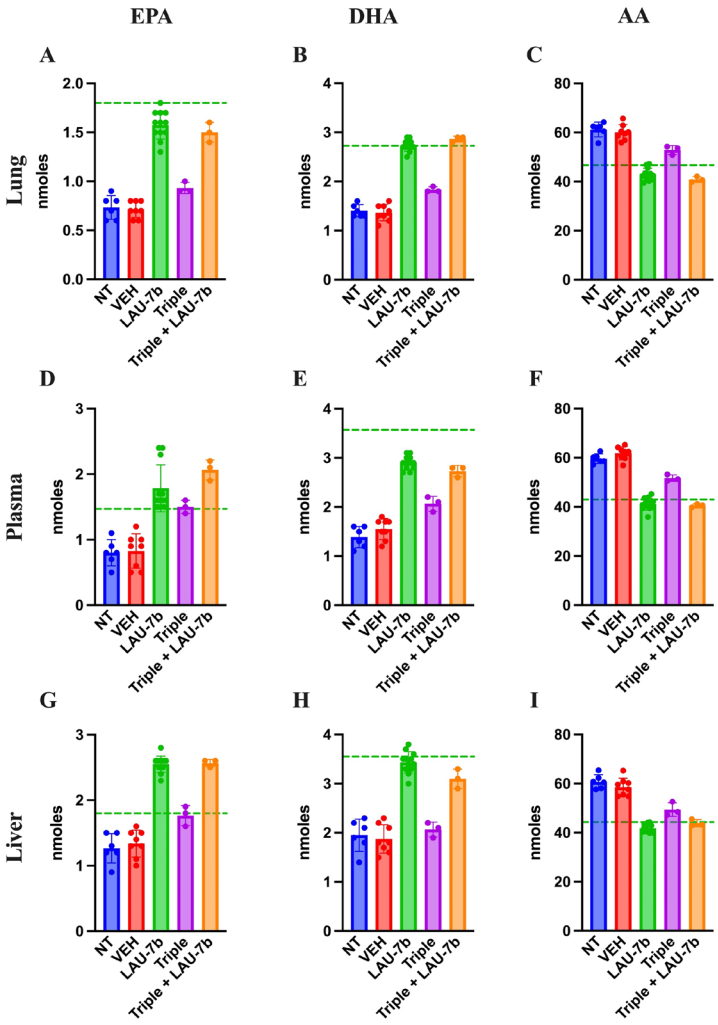


Figure 7. Correction of fatty acids in the lungs, plasma and liver of F508del/F508del (DD) mice is enhanced upon combination of Triple therapy and LAU-7b. Gas chromatography analysis of mouse lungs **(A-C)**, plasma **(D-F)** and liver **(G-I)** fatty acids. LAU-7b significantly increases omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in all three samples comparably to mean levels seen in WT mice (dotted green line). While Triple therapy alone only shows limited level of correction in lungs and plasma, the combination of the two significantly increases EPA and DHA in all samples when compared to vehicle (VEH) treated DD mice. Meanwhile, omega-6 fatty acid, arachidonic acid (AA) levels are significantly decreased in both LAU-7b and Triple therapy treated DD mice seen in all samples. Further improvement is seen upon combination of the two treatments. (n = 6, 8, 12, 3, 3, respectively for DD-NT, DD-VEH, DD-LAU-7b, DD-Triple, DD-Triple + LAU-7b). Brown-Forsythe and Welch One-Way ANOVA with Dunnett T3 correction, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

3.3.3 LCCs

Next, long-chain ceramides (LCCs), C14:0 and C16:0, which are typically elevated in DD mice as compared to WT controls (dotted green line), show improvements upon treatment with Triple therapy, or LAU-7b, both of which bring these levels below those of vehicle treated DD mice **(Figure 8)**. Interestingly, the combination of Triple therapy with LAU-7b further decreases LCC levels below the threshold seen in WT mice across all samples.

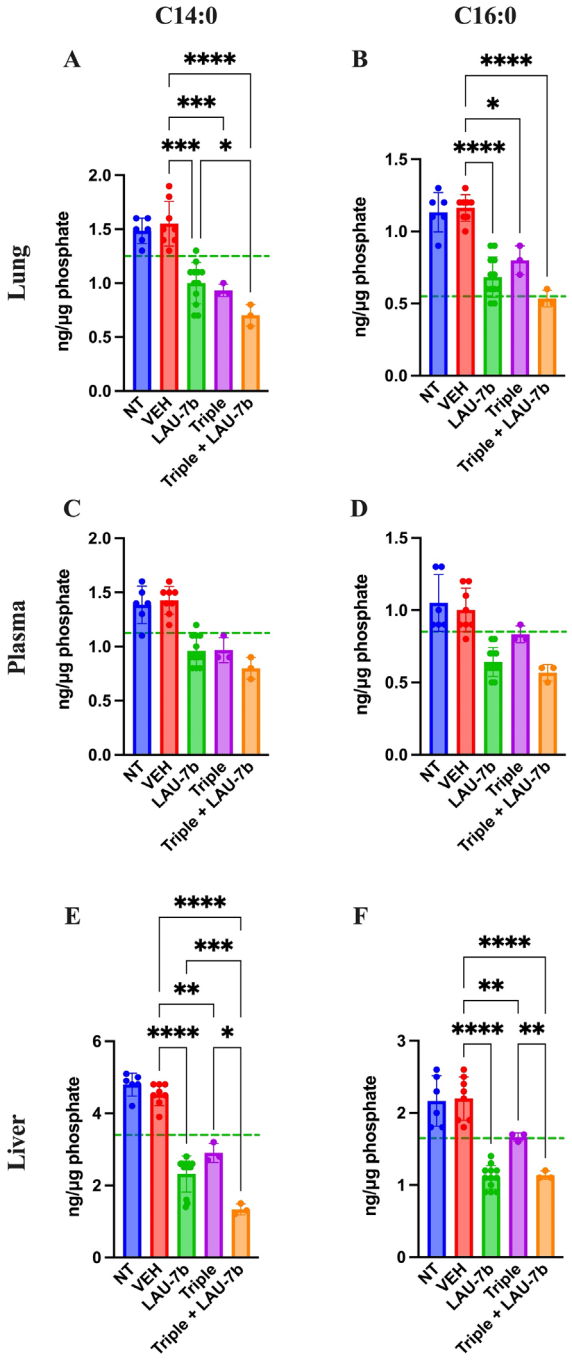


Figure 8. Decrease of long-chain ceramides (LCCs) is enhanced upon Triple therapy and LAU-7b combination in the lungs, plasma, and liver of F508del/F508del (DD) mice. Mass spectrometry analysis of mouse lungs **(A, B)**, plasma **(C, D)**, and liver **(E, F)**. LAU-7b treatment significantly decreases LCCs C14:0 and C16:0 when compared to vehicle (VEH) treated DD mice in all three samples below mean levels seen in WT mice (dotted green line). Similar decrease is also seen upon treatment with Triple therapy. The combination of the two treatments yields a stronger decrease in LCCs than with Triple alone in all samples. (n = 6, 8, 12, 3, 3, respectively for DD-NT, DD-VEH, DD-LAU-7b, DD-Triple, DD-Triple + LAU-7b). Brown-Forsythe and Welch One-Way ANOVA with Dunnett T3 correction, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

3.3.4 VLCCs

As for the very-long chain ceramides (VLCCs), Figure 9 shows a baseline increase in C22:0 and a decrease in C24:0, C24:1 and C26:0 levels in both DD non-treated mice and vehicle treated mice. These levels are normalized to the levels seen in WT mice (dotted green line) upon treatment with LAU-7b, and Triple therapy. Again, combinatory treatment significantly increases in C24:0 and C24:1 compared to WT mice while also improving C26:0 levels. Combination treatment also significantly decreases levels of C22:0, below WT levels.

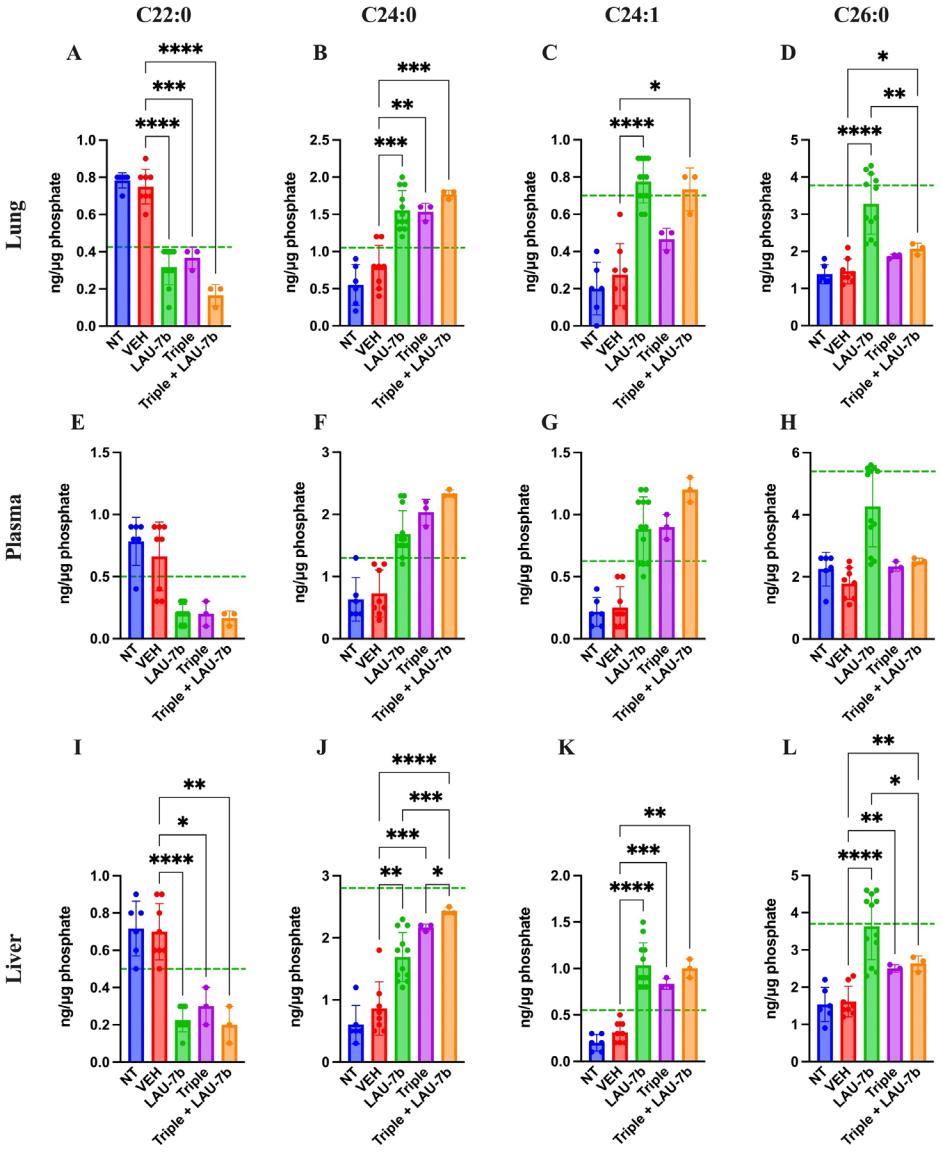


Figure 9. Combination of Triple therapy and LAU-7b exhibits stronger effects in correcting levels of very long-chain ceramides (VLCCs) than Triple therapy treatment alone in the lungs, plasma, and liver of F508del/F508del (DD) mice. Mass spectrometry analysis of mouse lungs **(A-D)**, plasma **(E-H)** and liver **(I-L)**. Upon treatment of LAU-7b and Triple therapy on their own, VLCC C22:0 shows significant decrease while C24:0 and C24:1 show a significant increase when compared to vehicle treated DD mice in all samples. Moreover, levels are corrected towards mean levels seen in WT mice (dotted green line). Meanwhile, only LAU-7b significantly increase C26:0 levels. Combination treatment of LAU-7b and Triple therapy show stronger effects for VLCCs C22:0, C24:0 and C24:1 than with Triple alone. However, VLCC C26:0 is somewhat increased by Triple and combination therapy but is not as strong as with LAU-7b alone. (n = 6, 8, 12, 3, 3, respectively for DD-NT, DD-VEH, DD-LAU-7b, DD-Triple, DD-Triple + LAU-7b). Brown-Forsythe and Welch One-Way ANOVA with Dunnett T3 correction, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

3.3.5 VLCC/LCC ratio

Lastly, the ratios of VLCCs/LLCs for the lungs, plasma and liver of DD mice were studied **(Figure 10)**. Treatment with LAU-7b (green) drastically improves the ratio of VLCCs/LCCs in lungs, plasma and liver of DD mice. While Triple therapy treatment may not show a statistically significant improvement, it does show a trend of correction of the ratio (purple). Moreover, the combination of LAU-7b and Triple (orange) can restore and bring the VLCC/LCC ratio above the levels seen in WT mice (dotted green line). Such correction is like that of LAU-7b's effect on its own.

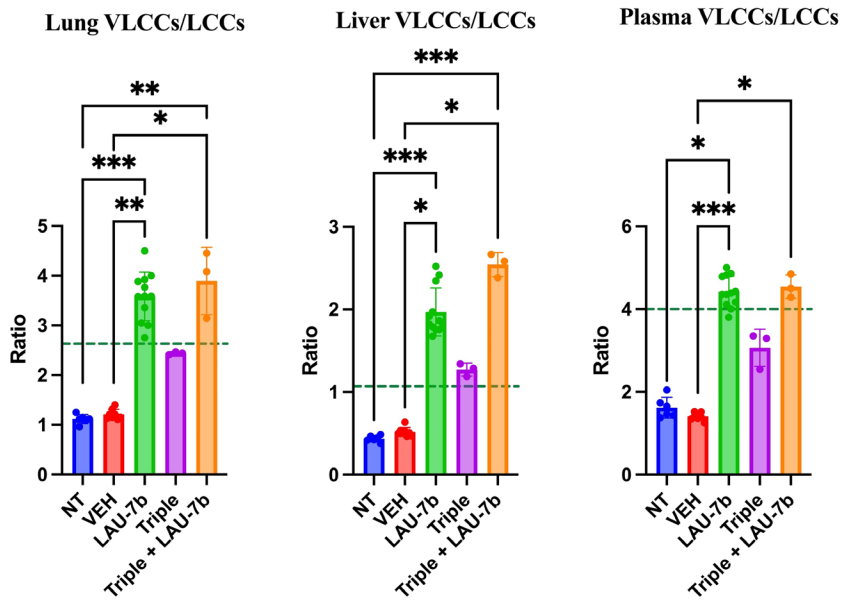


Figure 10. Combination of Triple therapy and LAU-7b improves VLCCs/LCCs ratios in lungs, plasma and liver of F508del/F508del (DD) mice. Treatment of LAU-7b improves the ratio of VLCC/LCC in all samples of DD mice which is higher than mean levels seen in WT mice (dotted green line). Meanwhile, the same effect is not seen in DD mice treated with Triple therapy alone. However, the combination of LAU-7b and Triple can restore and bring the VLCC/LCC ratio in lungs, plasma and liver of DD mice above the levels seen in WT mice. (n = 6, 8, 12, 3, 3, respectively for DD-NT, DD-VEH, DD-LAU-7b, DD-Triple, DD-Triple + LAU-7b). Kruskal-Wallis One-Way ANOVA with Dunn's correction, where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

3.4 Improvement of VLCC/LCC ratio in CFBE cell lines

To assess causality of the combination treatment we also studied VLCC/LCC ratio improvements *in vitro*, in CFBE41o-(P), CFBE41o-(WT) and CFBE41o-(F508del) cell lines. Levels of LCCs (C14:0, C16:0), as well as VLCCs (C22:0, C24:0, C24:1, C26:0) were measured, and their ratios were obtained **(Figure 11)**. Similar trends are observed for CFBE41o-(P) and CFBE41o-(F508del) cell lines when it comes to their VLCC/LCC ratios. At baseline, CFBE41o-(P) and CFBE41o-(F508del) display an 8% and 19% decrease in the VLCC/LCC ratio, when compared to CFBE41o-(WT) cells (dotted line). Fenretinide and physiological levels of zinc improve VLCC/LCC ratio by approximately 2-fold when compared to vehicle, in both cell lines. Triple therapy treatment does not show improvements in VLCC/LCC ratio. However, Triple therapy in combination with fenretinide and physiological concentration of zinc restores the VLCC/LCC ratio to a similar fold increase as seen with fenretinide and zinc treatment alone, with both treatments elevating the ratio above the baseline seen in CFBE41o-(WT).

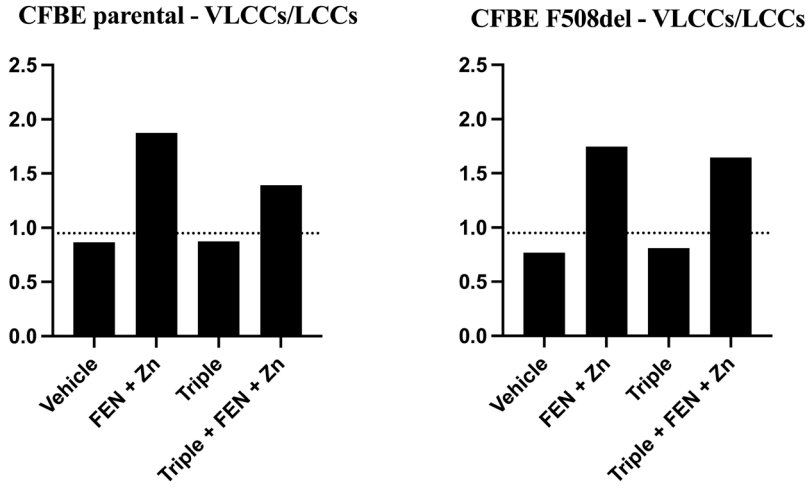


Figure 11. Combination of Triple therapy, LAU-7b and Zinc improves VLCCs/LCCs ratios in CFBE41o-(P) and (F508del) cell lines compared to CFBE41o-(WT) baseline (dotted line).

3.5 CFTR glycosylation status improvement upon Triple therapy and LAU-7b combination at physiological Zinc levels

Given that CFBE41o-(P) does not have any detectable CFTR protein, the total levels of CFTR protein were assessed in CFBE41o-(F508del) cells treated with Triple therapy, fenretinide or the combination of both treatments, by Western blotting (Figure 12**A**). The B band for CFTR is ~131 kDa, which represents the core glycosylated CFTR processed in the endoplasmic reticulum [154-156]. The C band for CFTR, which is ~ 160 kDa, represents the fully glycosylated mature CFTR and would indicate processing of the protein in the Golgi apparatus [155, 156]. As expected, the CFBE41o-(F508del) cell line does not express the fully glycosylated and mature CFTR. Only the B band can be observed on the immunoblot. Figure 12**B** illustrates the quantification data normalized to actin expression levels and shows the differences that were statistically significant. Following 3-day treatment with FEN, the B band was enhanced by 1.5-fold (50%), and upon combination of FEN with Zn^{2+}, the B band is further enhanced to 1.85-fold. This indicates an increased amount of core glycosylated CFTR protein in the cells, while the fully glycosylated mature form represented by the C band remains almost undetectable. While treatment of 1-day or 3-day Triple therapy does not enhance the B band for CFTR, a substantial improvement can be seen upon 3-day treatment with FEN and Triple therapy by 2.1-fold, and upon addition of Zn^{2+}, is further ameliorated to 2.6-fold increase. As for the C band, enhancement is seen for 3-day FEN treatment, Triple therapy treatment for 1 and 3-days, as well as the addition of Zn^{2+}. Of all the treatments, combinatorial treatment with 3-day FEN and 3-day Triple therapy in the presence of physiological concentration of Zn^{2+}, yields the highest intensity for the C band, with a 7.5-fold increase, compared to vehicle. These results demonstrate that treatment with combination of both FEN and Triple augments the total CFTR protein levels further than with Triple therapy alone. These effects can be attributed to increased protein synthesis, stabilization, and/or decreased degradation, ultimately enhancing the efficacy of the modulators. The mechanism of additive effect of this combinatory treatment should be further investigated.

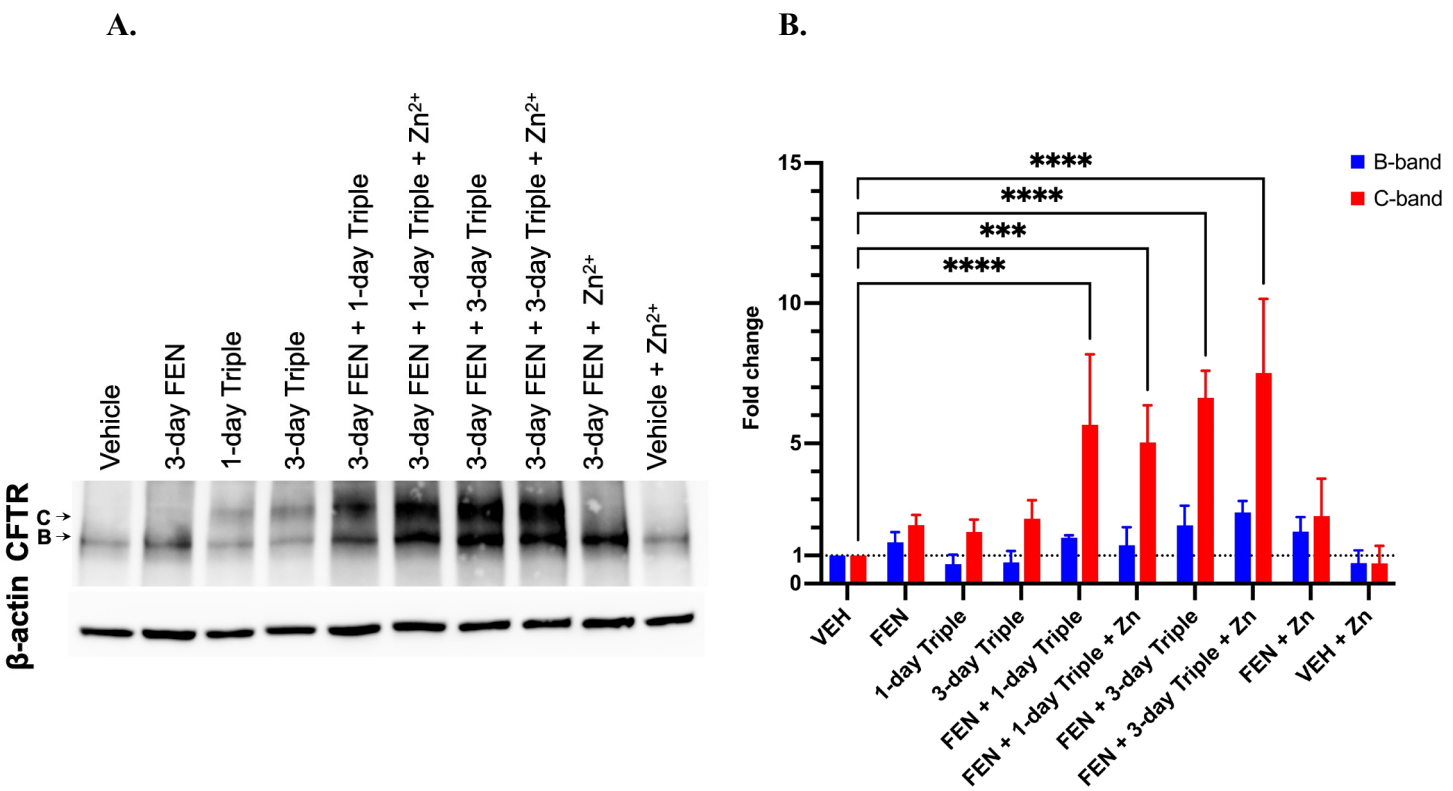


Figure 12. Total CFTR protein level in CFBE41o-(F508del) cell line. Western blot analysis (A) and quantification of three replicates (B). The CFTR protein has two glycosylated forms, the B band which is ~ 131 kDa, or the C band at ~ 160 kDa. For both B and C bands, the greatest fold change can be seen upon combinatory treatment of FEN, Triple therapy, and Zinc. Two-way ANOVA with Sidak correction, where ***p<0.001, ****p<0.0001

Chapter 4. Discussion

Discussion presented in this section has been published in *Frontiers in Pharmacology* by Centorame et al. entitled:

Treatment with LAU-7b complements CFTR modulator therapy by improving lung physiology and normalizing lipid imbalance associated with CF lung disease [1**].**

CF impacts more than 70,000 individuals worldwide [157]. To date, there is no cure for CF and available treatments target its symptoms. However, CFTR modulator and potentiator therapies which target the genetic defect of the disease, have brought much hope to the CF community. Recently, two randomized, double-blind, phase 3 clinical trials involving TRIKAFTA®, for CF patients 12 years and older, heterozygous, or homozygous for the F508del mutation (NCT03525444 and NCT03525548) [109, 158, 159] were successfully completed and led to the approval of TRIKAFTA® treatment in 2019. More recently, TRIKAFTA® was approved in younger patients, 6 to 11 years of age, heterozygous or homozygous for the F508del mutation, following a confirmatory Phase 3 trial in this CF population (NCT03691779) [113]. Across all three studies, improvement in $ppFEV_1$ indicated better lung function, lower sweat chloride concentrations and a higher CFQ-R RD score indicating less respiratory symptoms and a better overall quality of life [159]. Furthermore, an observational study in PwCF homozygous for the F508del mutation ranging from 20.8-48.3 years old (median age =31.1 years) taking TRIKAFTA® over the course of 48 weeks was done [160]. Although inflammation was not evaluated, an improvement in FEV_1, body mass index and sweat chloride was observed. On the other hand, a decrease in exacerbation and a need for intravenous antibiotics was reported. However, it is important to note that due to COVID-19, CF clinics have seen less bacterial exacerbations since patients were isolating and wearing masks. This is relevant given it is a retrospective study (i.e., without adequate control group comparisons) [160]. For the same reason during 2020-2022 period, according to reports from the Centers for Disease Control, the frequency of typical respiratory infections with RSV, Rotavirus, and seasonal influenza infections, decreased worldwide.

While the CF disease triggers occurrence of many symptoms, lung deterioration caused by repeated bacterial, viral infections, and exacerbations are the main cause of mortality and morbidity [9]. To treat these infections, a combination of anti-bacterial, antifungal, and antiviral

medications is needed, but cause complications after repeated and extended periods of use [161]. Therefore, novel treatment options that can mitigate the genetic defects of the CFTR protein while also addressing the inflammatory storm seen in PwCF, still need to be explored. Interestingly, a study by Gentzsch et al. found that the rescue of mutant CFTR was improved upon CFTR modulator therapies *in vitro* undergoing airway epithelial inflammation [162]. This could prove to be very intriguing and would be interesting to see in an *in vivo* model.

An interesting strategy, which has gained much ground across various fields in the last decade, is the repurposing and combination of drugs, in the hope of achieving additive and even synergistic effects in treating diseases. In CF disease, small molecule combination has been the strategy employed by the pharmaceutical industries and academic laboratories, which yielded the most successful results [163-165].

It was therefore important to investigate whether the inflammation-controlling (pro-resolution) treatment, LAU-7b, would be complementary to the recently approved TRIKAFTA® therapy and not interfere with the efficacy of the combination in mice with CF lung disease. With the eventual aim of improving the quality of life for PwCF and to address a broader range of the pathology observed in CF lung disease, we have assessed the efficacy of the combination of pro-resolution LAU-7b, with modulator and corrector therapies.

This study examined the impacts that combinatory treatment of LAU-7b (oral fenretinide) and a Triple therapy similar to TRIKAFTA® have *in vivo* on DD mice, and also *in vitro,* using a CFBE overexpressing F508del cell line. While each treatment on its own has demonstrated ameliorative effects in the studies conducted thus far, their combination is studied here for the first time. TRIKAFTA®'s mechanism of action remains to be fully elucidated. However, it is thought that correctors, Elexacaftor and Tezacaftor, directly interact with the mutant CFTR protein, facilitating its movement and trafficking to the Golgi, while the potentiator Ivacaftor, increases channel activity at the membrane level [159]. LAU-7b's mechanism of action was previously discussed; functioning as a membrane lipid modulator and exerting inflammation-controlling (pro-resolution) in multiple *in vitro* and *in vivo* systems [86, 129].

Before studying the effect of various infections on the efficacy of TRIKAFTA®, it was important to first study mice that develop CF lung disease even under pathogen-free conditions. This occurs when CF mice reach an age corresponding to that of PwCF. PwCF with class 1 and 2 mutations in the *CFTR* gene have a decline in FEV_1. Given our understanding of the two treatments

and the benefits they each have, we studied their combination *in vivo* in our older, 14 to 20-month-old DD and WT mice that show advanced deterioration of their lung histopathology and physiology.

After diagnosis of CF lung disease, a standard measure of disease progression and airway obstruction is FEV_1 tested by spirometry [166]. Declining pulmonary function among PwCF is age-dependent, however, the most dramatic drop in FEV_1 occurs between puberty and the age of 30, although it is dependent on the CFTR gene mutation. The decline in lung function over time in CF patients results from chronic airway inflammation and mucus dehydration, which are triggered by infection-induced increases in pro-inflammatory lipid mediators, which are regulated by AA release [140, 167]. In mice, an invasive measure of airway resistance is a gold standard assay performed to monitor pulmonary function. In Figure 1 **(A)**, our WT control group showed an expected slight increase upon MCh addition. However, a significant impairment in airway function is seen for the DD NT and VEH treated mice, most notably at the 100 mg/ml MCh dose, upon comparison to WT control (Figure 1**B**). Moreover, a significant improvement is seen upon treatment with LAU-7b, which shows airway resistance levels comparable to healthy WT controls. While Triple therapy treatment improves airway function, its effects are less marked than those seen in LAU-7b treated animals. The mice treated with the combination of Triple therapy and LAU-7b, displayed a significant improvement in lung function. The normalization of lung function upon combinatory treatment denotes a lower airway hyperresponsiveness, like that of WT control mice. This indicates that mice treated with LAU-7b, or LAU-7b + Triple therapy, have significantly better pulmonary function. Mice were monitored closely and weighed prior to (Day 0) and following (Day 15) the 14-day treatment period as seen in Figure 2. There was no statistically significant difference among our DD mice (Figure 2**A**, **B**), but the weights of the DD mice compared to WT mice were significantly lower (Figure 2**C**, **D**). Figure 3, which depicts the weights of the DD treated mice throughout the 14-day treatment period, showed no statistically significant difference among treatment groups on any of the days.

In PwCF, the abnormal mucosal defences facilitate recurrent chronic infection, most often with *P. aeruginosa* and sometimes with *Aspergillus fumigatus*, which negatively impacts the inflammatory milieu leading to lung damage and eventually lung failure, the main cause of morbidity and mortality in PwCF [9, 157, 168]. It is well documented that MUC5AC overexpression plays a key role in airway plugging, and both MUC5AC and MUC5B are not only

upregulated in chronically infected PwCF but are further augmented during lung exacerbations that happen in a large percentage of PwCF 3-4 times a year [28, 94, 169]. To improve mucus clearance, mucoactive agents, consisting of mucolytics and hyperosmolar agents, can be used [170]. Bronchodilators work to improve airway opening by relaxing the smooth muscles in the bronchial wall facilitating mucus clearance [171, 172]. Despite various treatments, delayed resolution of inflammation that frequently occurs in PwCF following exacerbation, often results in permanent lung damage. To assess lung deterioration in our F508del mice that were kept in a specific pathogen free condition (SPF), histological analysis of lung sections stained with H&E was done (Figure 4). Histology revealed overall ameliorated pathological parameters in mice treated with LAU-7b and LAU-7b in combination with Triple therapy as compared to vehicle treated controls, further supporting the results obtained for the physiological assessment. Hyperplasia of airways with significantly thickened airway lining is a pathological feature of cystic fibrosis and other respiratory diseases [173]. In Figure 4, a major difference can be seen between the airways of the WT control mouse and the DD-NT or DD-VEH mouse. Both DD-NT and DD-VEH mice have elevated levels of hyperplasia compared to WT control. These results corroborate the findings of a lower airway resistance in WT mice, and higher resistance in DD NT & VEH treated mice, highlighting the drastic difference in overall pulmonary health for these two groups. While treatment with LAU-7b or Triple therapy reduce airway cell hyperplasia, the combination of LAU-7b and Triple therapy yields superior results compared to Triple therapy alone, with uniform looking lungs, comparable to those of WT controls. The histology images are further supported by Figure 4 (**G**), displaying the lowest number of infiltrating cells for the combinatory treatment group.

In response to allergic reactions or infections, goblet cells present in the airways start production of mucus, and an increase in the reproduction rate of these cells leads to mucus hypersecretion, leading to abnormal mucus accumulation and airway plugging. This results in decreased antimicrobial functions and impaired mucociliary clearance, which can further worsen the CF condition [174]. Histological assessment of lung sections demonstrates that treatment with LAU-7b and Triple therapy dramatically diminish the thickening of the airway lining. As the inflammatory response in the lungs of PwCF is self-perpetuating and can be worsened by abnormal immune activity, it is important that any developing CF treatment considers the likely possibility of goblet cell hyperplasia and its consequences. Mucin expression that is inducible upon allergic

response to Aspergillus antigens and infections with Pseudomonas species, *Staphylcoccus aureus* and other bacterial and fungal induced lung pathology was not evaluated in the current study but is one of the important aspects of our ongoing studies.

Since oxidative markers, fatty acids, and overall lipid profiles, are known to be dysregulated in both CF mice and PwCF from birth, prior to their development of CF lung disease [126, 175, 176], further analysis was done using a portion of the liver, lung and plasma of WT and DD mice under the various treatments to obtain the lipidomic profile of those mice, first, oxidative stress markers were analyzed. Being a universal biological response, oxidative stress plays a major role in a variety of inflammatory disease conditions. In CF, neutrophils are continuously recruited to the airways and liberate their toxic products such as oxidants, in an uncontrolled fashion [177]. While antioxidants shield the lung from free oxidative damage in healthy patients, the amount and duration of neutrophilic inflammation overwhelms these defence systems in CF subjects, leading to increased protein and lipid oxidation in the lungs. To improve the defense system in PwCF, it is important to introduce a treatment which reduces levels of lipid and protein oxidation as marked by MD and 3-NT, respectively. In Figure 6, LAU-7b treatment in DD mice significantly reduced levels of MD and 3-NT in the lungs, plasma, and liver, to a greater extent than Triple therapy treatment. Upon combination with Triple therapy, improvements are comparable to that of LAU-7b alone, lowering the levels of those oxidative markers below the WT threshold. Thus, the combination of Triple therapy with LAU-7b, is significantly better than Triple therapy alone, given LAU-7b's antioxidant properties counteracting the overwhelming recruitment of neutrophils seen in lung inflammation.

Moreover, fatty acids abnormalities, in AA, DHA, and EPA, are consistently reported in CF [178]. These abnormalities create an imbalance in the DHA/AA ratio in favour of AA, contributing to the increase in pulmonary inflammation and mucus dehydration, resulting in deterioration of PwCF's condition. However, the causal connection between the expression of CFTR protein and this phenomenon has rarely been examined. Some evidence that suggests a role for CFTR in fatty acid metabolism as it was shown in cell culture models, where CFTR dysfunction results in defective fatty acid composition [179]. Furthermore, similar polyunsaturated fatty acid changes in CF affected organs such as the lung, pancreas and ileum have been reported in CFTR knock-out mice, further suggesting a causal link between CFTR and fatty acid metabolism [180]. Further studies also report that modulation of saturated fatty acids correlates with the modulation

of LCCs and VLCCs [28]. In Figure 7, EPA and DHA levels are shown in the lungs, plasma and liver of DD mice. Upon treatment with LAU-7b, a significant increase in those omega-3 fatty acids was observed. While treatment with Triple therapy alone did show significant improvement, it is only after its combination with LAU-7b that we see an increase in EPA and DHA comparable and even exceeding levels of EPA and DHA seen in WT mice. Combinatory treatment with Triple therapy and LAU-7b shows a significant decrease in AA levels, which is not seen with Triple therapy treatment alone. Figures 8 and 9 show LCCs and VLCCs levels in lung, liver and plasma, respectively. In 2020, Liessi et al. performed an untargeted lipidomic analysis on CFBE41o- cells upon various treatment groups, which included the Triple combination therapy (VX-661/VX-445/VX-770) [181]. Our results, as seen in Figures 8 and 9 are consistent with their findings (Figure 4), of a downregulation in C14:0 and C16:0 and a concomitant upregulation in VLCCs [181]. Recently, an interesting study was published by Westholter and colleagues reporting ceramide levels obtained from plasma analysis of 25 PwCF (age 35.56 +/-12.75; 20 out of 25 with intermittent or chronic Pseudomonas infection) treated for 4 weeks with TRIKAFTA® [182]. A very modest improvement (decrease) in C16:0 ceramide levels (0.218 +/- 0.09 before treatment and 0.178 +/- 0.06 (p = 0.0051) after 4 weeks of treatment) with TRIKAFTA® was reached. Furthermore, a very modest improvement in the levels of C24:0 (1.354 +/- 0.47 before and 1.674 +/- 0.65 after 4 weeks of treatment (p = 0.0048)) was also achieved. However, only 3 PwCF out of 25 reached levels for C24:0 of 2.5 after treatment, with none of the patients reaching a level of 4, which is typical for healthy individuals [86]. No improvement in C22:0 or C24:1 ceramide was reported following the treatment with TRIKAFTA® and no analysis of C26:0 ceramides was done. The ratio between C16Cer/C24Cer has improved from 0.171 +/- 0.06 before treatment to 0.112 +/- 0.03 after 4 weeks of TRIKAFTA® treatment (p-value = <0.001) [182].

In Figure 10 the VLCC/LCC ratio, which is known to be altered in CF disease, is emphasized. While Triple therapy can partially improve this ratio by increasing VLCC levels and decreasing LCC levels, the greatest correction occurs upon combinatory treatment with LAU-7b, surpassing the WT threshold in lungs, liver, and plasma of WT mice. Overall, Triple therapy alone seems to be beneficial in terms of correcting the pool of fatty acids, and ceramides, however, the combination of Triple and LAU-7b is demonstrated to be superior in further ameliorating these levels to similarity with what is seen in WT mice. This cooperative effect might be particularly

important for PwCF since most of them are chronically infected with bacteria such as *P. aeruginosa*, and/or fungi.

Results obtained *in vitro* corroborate the *in vivo* findings, once again demonstrating that the combination of Triple with LAU-7b is more efficacious in improving the VLCC/LCC ratio of the CFBE41o-(F508del) cell line (Figure 11), than Triple therapy alone. Interestingly, our data demonstrated that fenretinide treatment increases total CFTR protein levels (Figure 12), in the same cell line, thereby providing more protein that can be subsequently processed to the cell membrane with the help of modulators and the potentiator, enabling functional recovery of CFTR channel.

While the study presented above can have a lasting impact for PwCF, there are limitations. The cost of the Triple treatment was a limiting factor in our decision for the number of mice to include in each treatment group. Therefore, we chose to include 3 mice in each Triple and Triple + LAU-7b groups, given it is the minimum number of mice required to achieve statistical significance. Furthermore, kinetics of inflammatory mediators and mucin induction, which is usually following exposure to lung pathogens or their filtrates, could not be evaluated in this study since animals were maintained in a SPF condition. The airway thickening quantification in Figure 4 **(G)** is based on a semi-quantitative assessment of 4 airways per mouse lung at 20X magnification and establishment of a fully quantitative approach would be desirable in the future.

Chapter 5. Conclusion and Summary

While therapies for PwCF have evolved immensely over the years and helped in improving the lives of many, there is a constant need for new and better therapies that could help PwCF who have mutations that have yet to be approved for triple therapy use, discontinued use off triple therapy due to side effects or for patients where triple therapy is not working efficiently due to difficulties in eradicating infections. Ultimately, we found that using a F508del mouse model for CF lung disease, we observed an improvement in their lung histopathology and physiology as seen for PwCF from TRIKAFTA®. The combination of LAU-7b with Triple therapy, showed enhanced protective effects compared to Triple therapy alone. We successfully completed all four objectives.

Our results demonstrated that the treatment with LAU-7b triggers an increase of total CFTR protein levels, which to date is the only drug to do so. Therefore, with the help of modulator and potentiator therapies, which help process the pool of CFTR protein to the membrane, CFTR can preside in the membrane and perform is function. Moreover, our results demonstrated that the combined therapy of triple and fenretinide leads to enhanced expression of the glycosylated form of the CFTR protein. Our *in vitro* results have also been validated *in vivo* using our F508del mouse model of CF lung disease demonstrating that treatment with a combination of triple and fenretinide shows superior effect at the level of lung histopathology and lung physiology than triple therapy alone, confirming our hypothesis. Our results also demonstrated that LAU-7b treatment results in a significantly better improvement in VLCC/LCCs ratio as compared to the effect of Triple therapy alone *in vitro*. The addition of LAU-7b to Triple therapy resulted in an increase in VLCC/LCC ratio, enhancing the effect of Triple therapy treatment. In our *in vivo* model, following 14-day oral treatment (gavage), combined treatment with LAU-7b and Triple therapy combination not only improved airway resistance and hyperplasia, but also had positive effects in lungs, liver, and plasma on oxidation markers and fatty acids.

It is well known that neutrophils play a major role in the airways of PwCF, given they are the most abundant immune cell with the ultimate goal of killing bacteria and other microbes via phagocytosis, produce various oxygen species in phagolysosomes and granular enzymes and proteins [183]. While CFTR expression in neutrophils is important for chloride transport into phagolysosome, impaired CFTR expression affects neutrophils' ability to clear bacteria from the airway [184]. It was previously reported, that Ivacaftor (VX-770), a CFTR potentiator and one of

the three drugs that comprise TRIKAFTA®, was able to restore bacterial function in neutrophils, in patients with class II and III mutations [184, 185].

Although leukocytes show expression of CFTR mRNA, the membrane CFTR protein levels nor its function have yet to be convincingly demonstrated. The activity of phagocytes is altered in PwCF and CF animal models [186]. One reason for this could be that CF patients have systemically elevated activity of PLA_2, leading to aberrant levels of lipid metabolites and excessive oxidation status [187].

Moreover, alveolar macrophages, play an equally as important role in the CF lung by regulating the immune response. The CF airway is comprised of excessive amounts of cytokines and chemokines [183]. Recently, a study conducted by Gabillard-Lefort et al. looked at the purine P2X7 receptor (P2X7R) expressed in macrophages, and its link in activation of inflammasomes via K+ efflux, which subsequently releases IL-1β [188]. Given that P2X7R expression is regulated by CFTR function and subsequently Cl^- levels, it's overexpression in CF macrophages can have damaging effects in the CF airway [188]. They found that TRIKAFTA® improved CFTR expression and reduced P2X7R levels in CF macrophages, reducing inflammasome activation and IL-1β release [188].

Moving forward, it is imperative to study whether the effect of TRIKAFTA® is long-lasting and whether lung infection and acute inflammation resulting from recurrent lung infections would not diminish its efficacy over time, as previously seen with Kalydeco®, a similarly effective *in vitro* CFTR modulator [189].

TRIKAFTA® is composed of two correctors, Elexacaftor and Tezacaftor, and one potentiator, Ivacaftor. While the correctors help to allow better processing and trafficking of the CFTR protein, the potentiator increasing the channels opening-probability, ultimately allowing more and better functioning CFTR protein on the membrane. Elexacaftor (VX-445), one of the two correctors, was found to interact with NBD1 of CFTR and suppress misfolding of F508del cells *in vitro,* making it a class III corrector [190]. Furthermore, VX-445 was shown to be synergistic with other class I and II correctors, such as Tezacaftor (VX-661), allowing increased amounts of F508del-CFTR to localize in the plasma membrane [190]. While this new combination has been helpful for the F508del-CFTR mutation, there is much promise that given its ability to correct the whole confirmation, it could also benefit many other CF mutations that affect the MSDs

and NBDs [190]. However, further details regarding its mechanisms of action remain to be discovered.

As for Fenretinide, while perhaps not every pathway in which it plays a role in has been elucidated, more is known regarding its mechanism of action. In the family of nuclear receptors, the retinoid receptors (RARs) are where Fenretinide interacts, and upon binding can form several isoforms of RARs [129]. These isoforms, namely RAR-γ and -β, can bind to specific retinoic acid receptor elements on the DNA sequence, which then allows for regulation of transcription for many genes [129]. Another major pathway that Fenretinide plays a role in is the activation of transcription by PPARγ, in which the receptor is known to be reduced in PwCF. Moreover, Fenretinide can prevent NF-κB pathway activation, ultimately reducing pro-inflammatory cytokine production [129]. Lastly, given the role the NF-κB pathway plays in the expression of mucin genes, Fenretinide was able to prevent MUC5AC overexpression, which is the major cause of airway plugging and obstruction, without disturbing MUC5B expression, required for mucociliary clearance [28].

Interestingly, a new cell type found in the airways, referred to as pulmonary ionocytes, have shown extremely high levels of CFTR protein [191]. In fact, of all the airway epithelial cells, ionocytes express the highest levels of CFTR protein [191]. However, little is known about how this cell type responds to treatments such as TRIKAFTA® or LAU-7b.

Taken together, the results obtained in this study strongly support a potential clinical benefit from using TRIKAFTA® in combination with LAU-7b, with the aim of slowing the lung degradation and further improving overall quality of life in PwCF.

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