Protective Effect of Treatment with Fenretinide against Allergic Asthma and Progressive Cystic Fibrosis Lung Disease

By

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A thesis submitted to the Faculty of Graduate and Postdoctoral Studies at McGill University in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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To:

The Coptic Orthodox Church of Alexandria; my root …

Egypt; my beloved country …

Canada; my new home …

Abstract

It has been well documented that treatment with Fenretinide (FEN) leads to a decrease in the inflammatory cytokine and chemokine production in several diseases including Cystic Fibrosis (CF). Previously, our laboratory demonstrated that Cystic fibrosis transmembrane regulator knockout (*Cftr* KO) mice treated with FEN following infection with *P. aeruginosa* show a 10-fold decrease in bacterial load in the infected lungs. These biologically important outcomes in *Cftr* KO mice were also associated with normalization of fatty acid imbalance and ceramide imbalance in the drug-treated mice.

FEN, [N-(4-hydroxyphenyl)retinamide, 4-HPR], a vitamin A semi-synthetic derivative, is lipid soluble and is insoluble in water, making the generation of suitable formulation very challenging. The development of LAU-7b, a dry formulation of FEN, by Laurent Pharmaceuticals Inc., which displays great bioavailability, has opened new possibilities for basic and clinical research assessing its efficacy. Subsequently, our laboratory investigated both the safety and efficacy of LAU-7b in treating CF disease in mice and in patients (Phase I, Phase II trial APPLAUD in progress in 26 US CF Centers and 6 Canadian CF centers).

Since FEN treatment improves the resolution of lung infection and inflammation in *Cftr* KO mice, we hypothesized that it might also be very effective in controlling the lung inflammation that is caused by allergic asthma. The results of our studies demonstrated that the lungs of allergic mice display a regulatory imbalance in fatty acids and ceramides. Treatment with a very low dose of LAU-7b (10 mg/kg) for a relatively short time (9 days) protects against allergic asthma induced lung inflammation in atopic A/J mice which are genetically predisposed to airway

hyperresponsiveness (AHR). We have developed the protocol of treatment with LAU-7b which normalizes the distribution of ceramide composition in the lungs of our atopic and genetically prone hyperresponsive A/J mice and improves their inflammatory markers.

One of the candidate regions identified in the genome wide association studies (GWAS) as associated with the regulation of allergic asthma predisposition contained both *ZPBP2* and *ORMDL3* genes. *ORMDL3* has been shown to be involved in modulating the *de novo* ceramide biosynthesis. Therefore, to validate the protective effect of this region on house dust mite (HDM)-induced asthma we used atopic A/J mice with a deletion of the region containing the *Zpbp2* gene located upstream from *Ormdl3* gene resulting in a decrease in its expression. We also demonstrated that the normalizing effect of treatment with LAU-7b on the fatty acid imbalance, lipid oxidation and imbalance of long chain ceramides (LCCs) and very long chain ceramides (VLCCs) in mice is associated with LAU-7b's ability to inhibit expression of *Ormdl3* gene expression.

Lung physiology deteriorates with age even in healthy people. Since we have seen a significant improvement in the lung physiology parameters of allergic asthma and of *Cftr* KO mice following treatment with LAU-7b, we wondered if the age-dependent deterioration in the lung physiology could be prevented in LAU-7b treated mice. We also studied the histopathology of two, four and seven months old *Cftr* KO mice compared to their littermate controls. Our data demonstrated that *Cftr* KO mice compared to their littermate controls show significantly worse lung physiology parameters due to chronic inflammation-induced changes in their lungs. We demonstrated that the *Cftr* gene deletion-associated difference in lung physiology is more pronounced in older mice than younger mice. Finally, our results demonstrated that LAU-7b treatment prevents age-related deterioration of lung physiology parameters in *Cftr* KO mice and normalizes the fatty acid and lipid imbalance that is typically observed in the *Cftr* KO mice.

Interestingly, even healthy WT littermate controls profited from LAU-7b treatment as 7-month-old mice, following a 3-week treatment with LAU-7b, showed very similar histology results compared to the lungs of 2-month-old mice, suggesting that the treatment was able to prevent age dependent deterioration in their lung physiology and histopathology.

Overall, the results presented in this thesis validated the protective role of two genes (*Zpbp2* and *Ormdl3*) that are postulated to be associated with allergic asthma and demonstrated the outstanding efficacy of LAU-7b therapy against allergic asthma symptoms and age-dependent deterioration in lung functions.

Résumé de la thèse

Il a été établi que le traitement au Fenretinide (FEN) entraîne une diminution de la production de cytokines et de chimiokines inflammatoires. Les souris traitées par FEN à la suite d'une infection avec P. *aeruginosa* présentent une diminution de 10 fois la charge bactérienne dans les poumons des souris infectées.

Le FEN, [N-(4-hydroxyphényl)rétinamide,4-HPR], un dérivé semi-synthétique de la vitamine A, est liposoluble et insoluble dans l'eau, ce qui rend très difficile la création d'une formulation appropriée. Le développement de LAU-7b, une formulation sèche de FEN, par Laurent Pharmaceuticals Inc., qui permet maintenant une grande biodisponibilité, a ouvert de nouvelles possibilités pour la recherche fondamentale et clinique évaluant son efficacité. Conséquemment, notre laboratoire a étudié à la fois l'innocuité et l'efficacité de LAU-7b dans le traitement de la fibrose kystique (FK) chez la souris et chez les patients (essai de phase I, phase II APPLAUD en cours dans 26 centres américains et 6 centres canadiens).

Étant donné que le traitement par FEN améliore la résolution de l'infection et de l'inflammation pulmonaire chez les souris *Cftr* KO, nous avons émis l'hypothèse qu'il pourrait également être très efficace pour contrôler l'inflammation pulmonaire provoquée par l'asthme allergique. Les résultats de nos études ont montré que les poumons des souris allergiques présentaient un déséquilibre réglementaire entre les acides gras et les céramides. Le traitement avec une très faible dose de LAU-7b (10 mg / kg) pendant une période relativement courte (9 jours) protège contre l'inflammation pulmonaire induite par l'asthme allergique chez les souris atopiques A / J génétiquement prédisposées à l'hypersensibilité des voies respiratoires. Nous avons

développé le protocole de traitement avec LAU-7b qui normalise la répartition de la composition en céramides dans les poumons des souris A/J hypersensibles, atopiques, et génétiquement prédisposées et améliore leurs marqueurs inflammatoires.

L'une des régions candidates identifiée dans les études d'association à l'échelle du génome (GWAS) comme étant associée à la régulation de la prédisposition à l'asthme allergique contenait à la fois les gènes *ZPBP2* et *ORMDL3*. *ORMDL3* s'est avéré impliqué dans la modulation de la biosynthèse de céramides de novo. Par conséquent, nous avons utilisé des souris avec une délétion de la région contenant le gène *Zpbp2* et entraînant une diminution de l'expression du gène *Ormdl3* sur le fond génétique atopique A/J afin de valider l'effet protecteur de cette région sur l'asthme induit par l'allergène HDM. Nous avons également démontré que l'effet normalisant du traitement au LAU-7b sur le déséquilibre en acides gras, l'oxydation des lipides et le déséquilibre entre les céramides à longue chaîne (LCC) et les céramides à très longue chaîne (VLCCs) chez la souris est associé à la capacité de LAU-7b à inhiber l'expression de l'expression du gène *Ormdl3*.

La physiologie pulmonaire se détériore avec l'âge, même chez les personnes en bonne santé. Depuis que nous avons constaté une amélioration significative des paramètres physiologiques pulmonaires de l'asthme allergique et des souris *Cftr* KO à la suite du traitement par LAU-7b, nous nous sommes demandé si la détérioration de la physiologie pulmonaire liée à l'âge pouvait être prévenue chez les souris traitées par LAU-7b. Nous avons également étudié l'histopathologie des souris *Cftr* KO âgées de deux, quatre et sept mois par rapport à leurs contrôles WT. Nos données ont démontré que les souris *Cftr* KO comparées à leurs contrôles affichaient des paramètres de physiologie pulmonaire nettement détériorés en raison de modifications induites par leur inflammation pulmonaire. Nous avons démontré que la différence de physiologie pulmonaire

associée à la délétion du gène *Cftr* est plus prononcée chez les souris âgées que chez les souris plus jeunes.

Enfin, nos résultats ont démontré que le traitement par LAU-7b prévient la détérioration liée à l'âge des paramètres physiologiques du poumon chez les souris *Cftr* KO et normalise le déséquilibre en acide gras et lipidique généralement observé chez les souris *Cftr* KO. Fait intéressant, même les contrôles de litière en bonne santé WT ont bénéficié du traitement par LAU-7b lorsque des souris âgées de 7 mois, après un traitement de 3 semaines avec LAU-7b, ont montré des résultats histologiques très similaires à ceux des souris de 2 mois, suggérant que le traitement a permis d'éviter une détérioration de la physiologie et de l'histopathologie pulmonaires liée à l'âge.

Globalement, nos résultats présentés dans la thèse ont validé le rôle protecteur de deux gènes supposés être associés à l'asthme allergique et ont démontré l'efficacité remarquable du traitement par LAU-7b contre les symptômes de l'asthme allergique et la détérioration de la fonction pulmonaire en fonction de l'âge.

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List of Abbreviations

µg	Microgram
^{o}C	Degrees Celsius
5-LOX	5-lipoxygenase
AA	Arachidonic acid
ADAM33	Disintegrin and metalloproteinase domain-containing protein 33
AHR	Airway hyperresponsiveness
ANOVA	Analysis of variance
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
CCL	Chemokine ligand
cDNA	Complementary deoxyribonucleic acid
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
CXCL	Chemokine (C-X-C motif) ligand
cysLTR	Cystenyl leukotriene receptor
cysTL	Cystenyl leukotriene
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay

EPA	Eicosapentaenoic acid
eQTL	Expression quantitative loci
FcεRI	High-affinity receptor for the Fc region of immunoglobulin E (IgE)
FcεRII	Low-affinity receptor for the Fc region of immunoglobulin E (IgE)
FEN	Fenretinide, 4-HPR
FEV1	Forced exhaled volume in one second
FVC	Forced vital capacity
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage
GSDMB	Gasdermin B
GWAS	Genome wide association studies
H&E	Hematoxylin and eosin
HDM	House dust mite
HPLC	High performance liquid chromatography
IFN	Interferon
IFNAR	Interferon alpha and beta receptor
IgE	Immunoglobulin epsilon
IgG	Immunoglobulin gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF-1	Interferon regulatory factor 1
kg	Kilogram
KO	Knockout

LABA	Long acting beta agonist
LC	Liquid chromatography
LPR	Late phase response
LPS	Lipopolysaccharide
LTB	Leukotriene B
LTRA	Leukotriene receptor antagonists
Mbp	Megabase pair
MDA	Malondialdehyde
mg	Milligram
MHC	Major histocompatibility complex
min	Minutes
mL	Milliliters
Mmp2	Matrix metallopeptidase 2
MS	Mass spectrometry
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NPSR	Neuropeptide S receptor 1
nsSNPs	Nonsynonymous single nucleotide polymorphism
ORMDL3	ORMDL sphingolipid biosynthesis regulator 3
OVA	Ovalbumin
PAS	Periodic acid-Schiff stain
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor

Penh	Enhanced pause
PUFA	Polyunsaturated fatty acid
QTL	Quantitative trait locus
RCS	Recombinant congenic strains
RNA	Ribonucleic acid
SABA	Short acting beta agonist
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
STAT6	Signal transducer and activator of transcription 6
TGF	Transforming growth factor
TLR	Toll like receptor
Tnf-α	Tumor necrosis factor alpha
Ttc6	Tetratricopeptide repeat domain 6
U/mL	Units per milliliter
WBP	Whole body plethysmography
WHO	World Health Organization
WT	Wildtype
ZPBP2	Zona pellucida binding protein 2

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Acknowledgments

The completion of this thesis would not have been possible without the support of certain persons. I am deeply grateful to my supervisor Dr. Danuta Radzioch for her invaluable experience and support throughout my PhD research at McGill. Her commitment to research, her strong spirit and her compassionate attitudes have taught me skills for all aspects of life.

A special thanks to our great collaborators Dr. Juan De Sanctis and Dr. Anna Naumova for all their valuable expertise, help and guidance. I would like also to thank my supervisory committee member Dr. Elizabeth Fixman for her council and support throughout my graduate study.

To the members of the Radzioch lab, past and present, and to my friends in Glen hospital; Dr. Cynthia Kanagaratham, Daniel Houle, Dr. Xu YonZhong, Catalina Dumut, Juhi Shah, Dr. Dusan Garic, Halema Haiub, Nancy Kaddour, Dana Sedki, Dr. Mostafa Ghozlan, Ekaterina Gusev and Fazila Chouiali, thank you for all the help, discussions, friendships and good memories. A special thanks to Dr. Cynthia Kanagaratham and Daniel for being great lab mentors and wonderful mates.

My friends have been a precious source of support and encouragement during the journey. My close friends in Canada; Dr. Sherif Soliman, Emad Farag, Michael Geriges, Nader Samaan, and my best friends in Egypt; Morcos Guirguis, Morad Nasry, Maxim Mark and Mark Hany. Your friendships, warmth and support are truly inspiring and the reason for my success.

Last but not least, I am deeply indebted to my parents for their love, support and inspiration, without which I could not have done my PhD. I am fully grateful to my Dad who is in the same field and had always encouraged me to continue the path. I am very grateful to my Mom for believing in me, celebrating my successes, listening to me all the time, and for always cheering me on.

I am thankful to you all … Mina

Claims to Originality

The PhD candidate was the first to demonstrate that:

- In allergic asthma, the levels of very long chain ceramides (VLCCs) are decreased, and the levels of long chain ceramides (LCCs) are increased.

- On A/J background, loss of *Zpbp2* gene renders the mice less sensitive to airway hyper-responsiveness (AHR) as measured by MCh.

- Loss of *Zpbp2* in A/J KO mice results in significantly lower *Ormdl3* gene expression, even after HDM challenge, compared to WT mice. This observation shed light on the fact that these two genes are co-regulated together.

- *Cftr* KO C57BL/6 mouse model displays clear age-dependent progressive deterioration demonstrated by the increase in lung resistance, number of cell infiltration and epithelium hyperplasia of the lung airways at age of 2, 4 and 7 months.

Additionally, the PhD candidate was the first to use Fenretinide, and its novel oral clinical formulation (LAU-7b), to demonstrate that:

- Treatment with Fenretinide restores the aberrant levels of ceramides in allergic asthma by elevating the levels of the VLCCs and reducing the levels of the LCCs in lungs and plasma.

- Downregulation of *Ormdl3* gene expression using Fenretinide therapy is associated with the modulation of the levels of the VLCCs and the LCCs in allergic asthma.

- 10 mg/kg of oral (p.o.) LAU-7b or 5 or 10 mg/kg of intraperitoneal (i.p.) Fenretinide for 9 days is able to correct diverse pro-inflammatory events, improve lung physiology and histology phenotypes in both OVA- and HDM-sensitized and challenged A/J mice.

- LAU-7b treatment in dose of 10 mg/kg for 9 days is able to correct diverse pro-inflammatory events and improve lung physiology in HDM-sensitized and challenged *Zpbp2* KO A/J mice.

- Treatment of *Cftr* KO C57BL/6 mice for 21 days with LAU-7b at dose of 10 mg/kg is able to improve dramatically lung physiology and histopathology when the treatment is administrated at the age of 4 and 7 months.

- LAU-7b treatment reverses the progressive age-related changes in the lung which normally occur with age in WT C57BL/6 mice.

Preface, Thesis Format and Contribution of Authors

This thesis is presented in a manuscript-based format for the Doctoral Thesis, according to the Thesis Preparation Guidelines provided by the Department of Graduate and Postdoctoral Studies. The studies described in all the Chapters were performed under the supervision of Professor Danuta Radzioch, PhD, initially at the Research Institute of the Montreal General Hospital and subsequently at the Research Institute of McGill University Health Center (Glen Hospital, 1001 Decarie Blvd, Montreal, QC H4A 3J1). Each of the Chapters containing experimental results is preceded by a Chapter Preface that links the thesis together.

Chapter 1 is adapted from a book chapter entitled *Genetics of Allergic Asthma and Current Perspectives on Therapeutic Management*, In: *Asthma - From Childhood Asthma to ACOS Phenotypes*, Intechopen, London, United Kingdom (137–184, 2016. doi:10.5772/63651) by Mina Youssef, Cynthia Kanagaratham, Mohamed I. Saad, and Danuta Radzioch.

Mina Youssef (M.Y.) wrote and performed the literature search for Chapter 1. This chapter contains a comprehensive introduction addressing the epidemiology, pathogenesis, available treatments, and mouse models of both diseases investigated throughout this study. Additionally, the last section of Chapter 1 discusses the medication used in this study, putative mechanisms of action, possible applications, and available formulations. Figures 1.1 and 1.5 included in Chapter 1 were produced by the PhD candidate and published in the above-mentioned book chapter. Figure 1.2, 1.3 and 1.4 were reproduced with permission from Elsevier and John Wiley and sons; the publishers of the two journal review articles (refer to the list of copyrighted materials). Chapter 1 was edited and revised by another graduate student in our laboratory, Daciana Catalina Dumut (D.C.D.) and by the supervisor, Danuta Radzioch (D.R.).

Chapter 2 is adapted from the manuscript entitled: *Efficacy of optimized treatment protocol using LAU-7b formulation against ovalbumin (OVA) and house dust mite (HDM)-induced allergic asthma in atopic hypersensitive A/J mice by* Mina Youssef, Juan B. De Sanctis, Cynthia Kanagaratham, Shao Tao, Eisha Ahmed and Danuta Radzioch.

This manuscript was submitted for publication in the journal of *Pharmaceutical Research* and was assessed by the reviewers positively with several comments. Additional experiments were done, and the revised manuscript has been resubmitted in its revised version a few weeks ago and we are waiting for the final decision of the journal regarding its' final acceptance. In terms of the contribution of the authors, M.Y. and D.R. designed all experiments. D.R. supervised the study. The protocols of Fenretinide and LAU-7b treatment, and the allergic asthma models used in this work were developed by the PhD candidate. M.Y. participated in all experiments and wrote the manuscript. Specifically, animal experiments and airway resistance measurements were performed by M.Y and C.K. with the assistance of an animal technician, Danial Houle (D.H.). The histology work presented in this manuscript was done and analyzed by M.Y. Measurements of lipids, ceramides and markers of oxidative stress were done by Dr. J. De Sanctis (J.D.S.). Shao Tao (S.T.) and Eisha Ahmed (E.A.) performed the immunophenotyping experiment of this manuscript. Statistical analyses were performed by the M.Y. All authors participated in the editing of the manuscript.

Chapter 3 is adapted from the manuscript entitled: *Treatment of allergic asthma with Fenretinide downregulates Ormdl3 expression and normalizes ceramides imbalance.* Prepared for submission by Mina Youssef, Juan B. De Sanctis, Juhi Shah, Daciana Catalina Dumut, Marian Hajduch, Anna K. Naumova, and Danuta Radzioch.

M.Y., D.R. and Anna K. Naumova (A.N.) designed the experiments. D.R. supervised the study. M.Y. participated in all the experiments and wrote the manuscript. The animal experiments were performed by M.Y. and these experiments were assisted by D.H. M.Y. performed and analyzed the whole-body plethysmography, the airway resistance measurements and the IgE measurements by ELISA. The RNA expression analysis was performed by M.Y., Juhi Shah (J.S.) and D.C.D. The histology work was performed by M.Y. and the Histopathology Platform, RI-MUHC. The analysis of the histology results was done by M.Y. Measurements of lipids, ceramides and markers of oxidative stress was done by J.D.S. and Marian Hajduch (M.H.). Statistical analyses were performed by M.Y. All the other authors participated in the editing of the manuscript.

Chapter 4 *(Age-related deterioration of Cftr knock-out mice and protective effect of treatment with LAU-7b)* by Mina Youssef, Juan B. De Sanctis, Juhi Shah, Daciana Catalina Dumut, Marian Hajduch, Basil Petrof, and Danuta Radzioch. The manuscript has been submitted for publication in the *Journal of Cystic Fibrosis*.

M.Y. and D.R. designed the experiments. D.R. supervised the study. M.Y. participated in all the experiments and wrote the manuscript. The animal experiments were performed by M.Y., with the help of J.S. and D.C.D. (J.S. and D.C.D. helped in all experiments which involved 2- and 4-month-old mice). These experiments were assisted by the animal technician Ekaterina Gusev (E.G.). *Cftr* KO Mice were bred with the assistance of Dr. Basil Petrof's (B.P.) laboratory. M.Y. performed and analyzed the whole-body plethysmography, the airway resistance measurements and the IgE measurements by ELISA. The histology work was performed by M.Y. and the Histopathology Platform, RI-MUHC. The analysis of the histology results was done by M.Y. Measurements of lipids, ceramides and markers of oxidative stress was done by J.D.S. and M.H.

Statistical analyses were performed by M.Y. All the other authors participated in the editing of the manuscript.

Chapter 5 contains a general discussion of the results presented in the thesis in the context of the existing literature. It was written by M.Y.

Chapter 6 contains a description of possible future research directions. It was written by M.Y.

Chapter 7 contains the master reference list which contains the all the references used in all chapters. It was written by M.Y.

I, Mina Youssef, have read, understood and abided by all norms and regulations of academic integrity of McGill University.

Chapter 1. General Introduction

Adapted from:

- **Youssef, M.**, Kanagaratham, C., Saad, M. I. & Radzioch, D. in *Asthma - From Childhood Asthma to ACOS Phenotypes* 137–184 (InTech, 2016). doi:10.5772/63651
- Regulation of Ceramides Imbalances in Allergic Asthma and Cystic Fibrosis Using Fenretinide: A Clinical Perspective. Manuscript in preparation.

1.1 Allergic Asthma

1.1.1 Worldwide asthma burden

Asthma is an inflammatory chronic condition that has reached globally epidemic levels. Although no cure exists, symptoms are treatable in most patients [1]. Statistically, the number of asthmatic cases have been on the rise over the past ten years and affecting up to 10% of adults and 20% of children worldwide [2]. Globally, more than 300 million people are asthmatics, and this estimate is predicted to become 400 million by 2025 [3]. The worldwide economic burden caused by asthma is predicted to be more than that of both AIDS and tuberculosis combined together. For example, in the United States of America, annual asthma care costs exceeds US$6 billion [4]. Moreover, these numbers are due to the fact that more than 50% of asthmatic cases are poorly controlled by medication, since the response to treatments varies considerably among patients despite having similar clinical features [3,5]. Asthma is characterized by altered and distinct clinical changes in the lung airways obstructing the flow of air into the lungs. The most prominent airway remodeling features include epithelial and sub-epithelial layer thickening, increased airway smooth muscle mass and angiogenesis [6]. Different classes of asthma therapies address one or more of the phenotypes of asthma; however the heterogeneous nature of the disease prevents homogenous clinical outcomes in response to the current treatment guidelines [7].

In the past two decades, the field of human genetics has evolved due to the advancements in the human genome project and high throughput sequencing technologies [8,9]. Currently, the advances in genetic, pharmacodynamic and pharmacokinetic studies, analyzing responsiveness of patients to various therapies, may eventually allow the prescription of personalized treatments and a shift of asthma therapies from classical standards, using mostly corticosteroids and β-adrenergic agonists, to a highly tailored approach [10]. Future genetic profiles of the population would form the

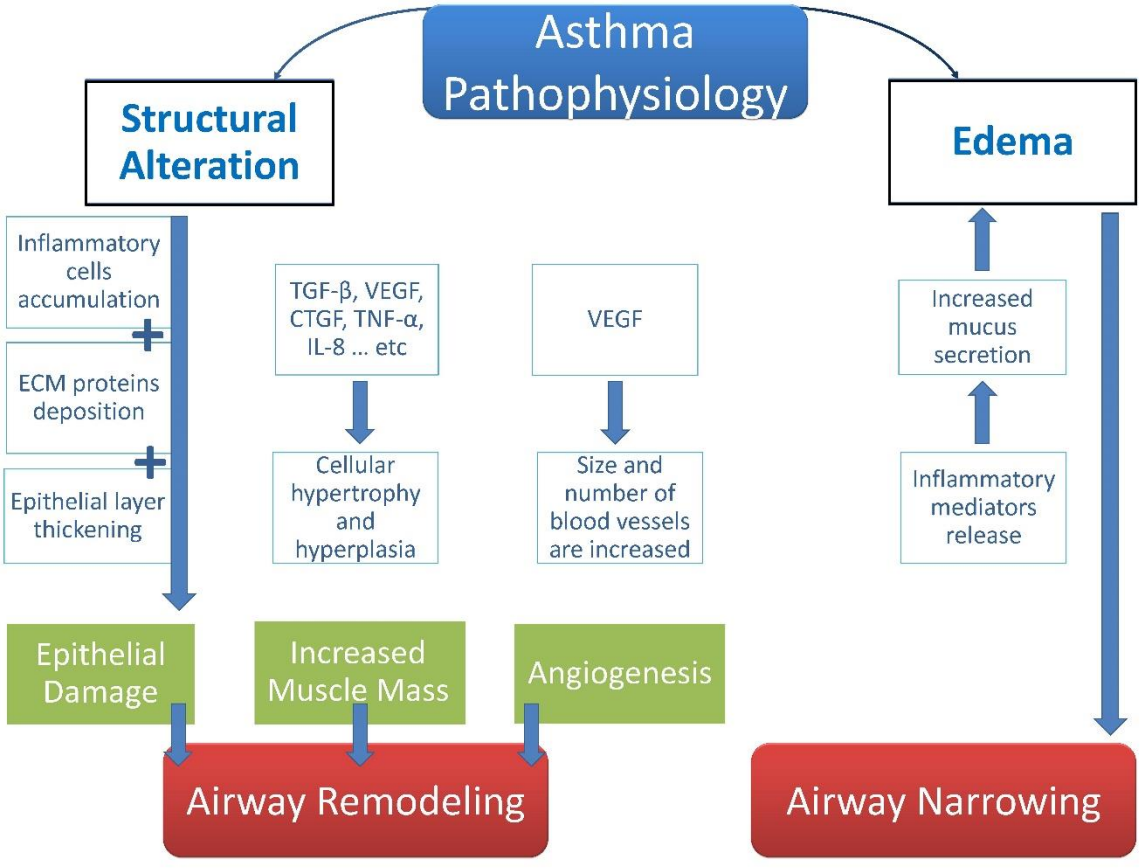
basis of tomorrow's treatments, because the treatments will be highly tailored, in order to potentiate the required therapeutic benefits, and to diminish any possible adverse effect risks. Overall, there remains a great need for comprehensive drug research, paralleled with high throughput genetic profiling, in order to treat asthma in a personalized or stratified manner [11].

1.1.2 Asthma pathophysiology

Scientists have tried to uncover alterations related to asthma for a long time. One of the oldest publications that discussed asthma pathophysiology was in 1873 [12]. Later on, in 1886, F.H. Bosworth concluded a possible relation between asthma and hay fever [13]. Clearly, it is well known that asthmatic patients suffer from reversible airway obstruction resulting from an allergen exposure, consequently releasing multiple broncho-constricting mediators that stimulate airway muscles to contract. Furthermore, airway narrowing results from past and current mucus and edema occlusion [14]. The chronic inflammation and associated repair of lung airways lead to structural changes, referred to as *'airway remodeling'*. Airway remodeling (figure 1.1) usually involves lung epithelial layer injury, and includes features such as sub-epithelial thickening, airway smooth muscle hyperplasia and angiogenesis [6].

Figure 1.1 Asthma pathophysiology

A complex set of inflammatory events leading to airway remodeling and narrowing. Chronic inflammation and associated repair of lung airways leads to airway remodeling, which involves 3 major events: sub-epithelial thickening, airway smooth muscle hyperplasia and angiogenesis. Airways narrowing results from past and current mucus, and edema, occlusion. Figure reproduced from Youssef *et. al.* 2016 [15] (open access article under the terms of the Creative Commons Attribution 3.0 License, no permission required).



Asthma and COPD (chronic obstructive pulmonary disease) are now considered to be discrete respiratory disorders. Although both share several similar underlying mechanisms, driving airway obstruction in COPD and hyper-responsiveness in asthma, core molecular pathology remains to be mostly different for these two diseases [16]. Pauwels *et al.* [17] defined COPD as "a disease state characterized by airflow limitation that is not fully reversible". The airflow limitation is usually progressive and associated with an abnormal inflammatory response of lungs to noxious particles and gases. One important reason of asthma and COPD overlap is the effect of aging. Asthma-COPD overlap syndrome (ACOS) is a medically recognized coexisting syndrome of both asthma and COPD [18]. Some other health conditions may occur more frequently in asthmatic patients. Rhino-sinusitis [19], obstructive sleep apnea [20] or GERD [21] are the most common documented comorbidities. Substantially, the impact of these diseases on asthma is variable and still not fully clear [22].

1.1.3 Structural alterations in asthmatic airway walls

1.1.3.1 Epithelial/Subepithelial layer thickening

Epithelial changes are not unique to asthma, they are also observed, in more or less of similar manner, in lungs of smokers and cancer patients [23]. Epithelial layer damage in asthma includes loss of ciliated cell layer, shedding of the epithelium, goblet cell hyperplasia and growth factors, cytokines and chemokines up-regulation [24].

One important feature of asthma, which has been routinely used as an asthma severity index, is the thickening of the subepithelial airways layer. The epithelial and subepithelial layer thickening is caused by the over deposition of extracellular matrix (ECM) proteins [25]. Roche *et al.* observed that intensive layers of collagen sedimentation contribute to the thickened subepithelial basement membrane. Through immunohistochemistry, they have shown that the commonly

involved collagen types are collagen I, III and V, and fibronectin [26]. Additionally, the cells that are responsible for ECM protein production are myofibroblasts and fibroblasts, as both are embedded in the sophisticated ECM which they secrete [27]. Meanwhile, some inflammatory cells *e.g.*, T cells, mast cells and eosinophils also accumulate in the submucosal layer [28]. Moreover, transforming growth factor-β (TGF-β), and some similar growth factors, are usually secreted by the lung epithelial cells echoing any ongoing lung injury, and consequently, directly impress the matrix proteins' production by fibroblasts/myofibroblasts. By increasing the airways' rigidity, however, Holgate *et al.* suggested that the airway thickening due to the ECM proteins precipitation may in fact have a remodeling protective effect *via* postponing long-term bronchoconstriction events [24]. Collectively, the ECM proteins, the lung structural cells (i.e. epithelial cells and fibroblasts), and the immune system inflammatory cells all interact together and control the overall airways remodeling and fibrosis [29].

1.1.3.2 Hyper proliferation of airway smooth muscle mass (ASM)

Hyper proliferation of airway smooth muscle mass (ASM) is a common event in asthma and has been suggested to be implicated in its pathophysiology. Hyperplasia and hypertrophy of the ASM in the bronchial airways of asthmatics can be observed by 3D morphometric studies [30]. Airway smooth muscle layer thickness is estimated to be increased by 25-55% in non-fatal asthma and up to 50-200% in fatal asthma [31]. Meanwhile, in response to some growth factors like TGF-β, Vascular endothelial growth factor [VEGF], and connective tissue growth factor [CTG+F], ASM cells actively participate in the remodeling process through the process of ECM synthesis [32]. ASM cells also express cellular adhesion molecules (CAMs), receptors for cytokines (e.g. tumor necrosis factor-α [TNF-α]), Toll-like receptors, and chemokines (eotaxin, macrophage inflammatory protein 1α, and interleukin 8 [IL-8]), presenting multiple mechanisms for the inflammatory and remodeling process [33]. Additionally, one characteristic event of the airway remodeling is the ASM

cells migration toward the epithelium [34]. Since ASM cells are crucial in asthma, Zuyderduyn *et al.* suggested that these cells should be targeted, rather than targeting inflammation or dealing with the symptoms [35].

1.1.3.3 Angiogenesis

Accumulating evidence indicates that there is an abnormal elevation in the size and number of blood vessels, as well as micro-vessel vascular leakage within the bronchial tissue in remodeled airways [36]. It is assumed that VEGF strongly affects airways remodeling *via* its angiogenic effects, but the exact molecular mechanism linking the increase in the VEGF expression to remodeling of the airways is not fully understood [37].

Correlation between angiogenesis and asthma severity has been also documented. Dense vascularity occurs in severe asthmatics, followed by moderate, and then finally mild asthmatics, who experience less angiogenesis events [38]. This pattern was also observed in fatal asthmatics compared with non-fatal asthmatics [39]. While current asthma therapeutics are not directly targeting vascular remodeling, recent trials investigate some anti-angiogenic therapies as a new approach for asthma. Yuksel *et al.* showed that Bevucizamab, which significantly neutralizes VEGF, results in a reduced thickening of lung epithelium, a reduced ASM and a reduced basement membrane thickness compared with untreated ovalbumin (OVA) challenged mice [40].

1.1.4 Etiology of allergic asthma

1.1.4.1 Environmental factors contributing to allergic asthma

Parallel to genetic factors, environmental factors are also involved in the development and progression of asthma. The exposure to some environmental factors was shown to contribute not only to asthma, but also to other related respiratory disorders, e.g. emphysema development. By contrast, there are also some other environmental factors that seem to be solely linked to the

development of asthma but not to other inflammatory or/and respiratory disorders [41]. Various studies assessed the risk factors of asthma and found evidence that allergen exposure, respiratory tract infections, gastroesophageal reflux disease (GERD), physical and psychosocial stress might represent individual risk factors. It is important to keep in mind that some other environmental factors are protective; such as maternal diet, breastfeeding, and farming conditions [42].

Allergen exposure is the major factor impacting sensitization and constitutes the most common cause of asthmatic exacerbations in adults and children. A wide variety of inhaled allergens may trigger asthma symptoms *e.g.* house dust mite [43], pollens [44], cockroaches [45], and animal fur [46]. Respiratory tract infections have been implicated in asthma occurrence and exacerbation as well. Examples include infection with viruses [47,48], *Mycoplasma* [49] and *Chlamydia species* [50]. Based on the conclusions from a Japanese study that included 3085 patients, the change in weather followed by smoking were identified as two leading asthma exacerbating factors [51] . Although active and passive smoking are predominant contributing factors for the development of asthma [52], one occupational study [53] has shown that non-smokers might also develop asthma due to occupational air pollutant exposure.

Additionally, a correlation has been observed between the presence of asthma and gastroesophageal reflux-induced disease (GERD), with reports showing one third of asthmatic patients also diagnosed with GERD [54,55]. Although the coexistence of GERD in asthmatic patients did not affect asthma severity, the airway resistance was significantly higher in asthmatic patients with GERD [56]. Some other psychosocial factors like parental stress during childhood [57] and socioeconomic status [58] are reported to influence allergic inflammation severity. It is estimated that psychopathology is six times more common among asthmatics, and accordingly, correlates more closely with the asthmatic quality of life, rather than with lung physiological functions [59,60].

In both directions, psychopathology is supposed to precipitate asthma or vice versa; psychopathology may develop as a consequence of asthma [61].

1.1.4.2 Genetic control of allergic asthma

The heritable nature of asthma has been demonstrated through various types of studies over the past decades. Family and twin studies indicate that 60 to 70% of asthma cases are due to genetic factors. Moreover, it has been proven that the concordance of asthma is greater among monozygotic twins rather than dizygotic ones. Adoption studies have shown a greater disease prevalence within biological relatives of the affected people compared to the adopted family [62].

Higher prevalence of allergic disease phenotypes is observed among relatives of atopic individuals compared to non-atopic individuals. Overall, the heritability estimates remains in between the range of 30-66% for airway hyper-responsiveness, 35-95% for asthma, and 35-84% for total serum IgE levels [63]. It is clear that the inter-genetic individual differences and the degree of allergens exposure both contribute to these variations in heritability. Heritability of asthma is linked to both disease susceptibility and severity. While the main concern of asthma genetic studies has been on disease susceptibility, increasing evidence shows that many genetics variants are important in asthma progression and severity as well [64]. Lung function tests in asthma showed that genes in the T helper lymphocyte 1 (Th1) pathway affect asthma severity, while T helper lymphocyte 2 (Th2) pathway genes relates to susceptibility [64]. Based on these hypotheses, genes associated with asthma susceptibility differ from those related to asthma severity; hence, it is important to define both groups distinctly.

By knowing the genetic signature associated with allergic asthma, geneticists can help to understand better the molecular mechanism of this disease, and the shared and distinct pathways among other allergic diseases. Moreover, the genetic signature of asthma associated genes with altered expression during the peak of asthmatic episodes may help predict the severity and response

to therapy. Unfavorable response might be identified and, consequently, more targeted and personalized treatments can be considered for this complex trait. The human genome project and the ongoing advancements in sequencing technologies have resulted in more successful gene discovery over the last years, even in diseases as complexed as asthma. Since then, dozens of susceptibility genes were identified through a large variety of methods and rationales. *ADAM33* is the first asthma susceptibility gene to be discovered through positional cloning [65]. ADAM33 (also known as Disintegrin and metalloproteinase domain-containing protein 33) is a membrane bound metalloproteinase enzyme that has been involved in several cellular interactions involving cell-cell and cell-matrix events [66]. Variants in this gene have been correlated to asthma susceptibility and bronchial hyper responsiveness, but not atopy. Due to its clinical significance, *ADAM33* studies were conducted among 33 different asthmatic population samples all over the world. Additionally, numerous studies have suggested that altered *ADAM33* DNA methylation patterns could result in diversely unbalanced biological effects in the airways [67]. Studies focused on candidate genes have examined a number of genes involved in asthma and highlighted more than 100 interesting genetic spots; however, the role of those loci in asthma susceptibility remains largely unexplored [68].

Genome-wide association studies (GWAS) extensively investigate the unknown genetic bases of many intricate disorders including asthma [69,70]. In the first reported GWAS study for asthma susceptibility, Moffatt *et al.* [71] identified the 17q21 locus, containing several genes (*e.g.*, *ORMDL3* and *ZPBP2*) as being associated with childhood asthma. Importance of this region was later on replicated in numerous subsequent studies [72–74].

To increases the power of detection of modest alleles, the results of individual GWAS need to be collated through meta-analysis. The scientific literature recognizes two meta-analyses of asthma GWAS. One was done by the GABRIEL Consortium [75] of the European investigators, and

the other was conducted by the EVE Consortium [72] of the US investigators. While the EVE meta-analysis included diverse subjects from different ethnic background (US and Mexico population backgrounds), the GABRIEL meta-analysis included only European subjects. Overall, these two thorough meta-analyses present a comprehensive overview of the genetic associations for asthma. Some associations are shared among different populations; by contrast, others are specific to one race. Grouping GWAS in this way increases the power of genetic detection, contrasts different ethnic groups' genotypes, and highlights the worldwide populations' genetic patterns. Overall independent GWAS have identified large number of candidate loci that deserve further testing. Replication studies helps to prioritize which genes deserve further study, based on their identification in multiple populations.

Additionally more loci were identified to be associated with asthma; these include IL33 (on 9p24), *HLA-DR/DQ* (on 6p21), *IL1RL1/IL18R1* (on 2q12), *TSLP* (on 5q22), and *IL13* (on 5q31) [72,75,76]. Collectively with *ORMDL3*/*ZPBP2*/*GSDMB* (on 17q21), these are the most remarkable and consistent loci which are identified for asthma. Since Moffatt *et al.* published the first GWAS results for asthma, identifying *ORMDL3* as a candidate gene, numerous other studies have been conducted investigating an array of phenotypes observed in allergic diseases; for example, FCER1A, RAD50 and STAT6 have been associated with total serum IgE levels [77].

1.1.4.2.1 ORMDL1-3: Custodians of cellular sphingolipids

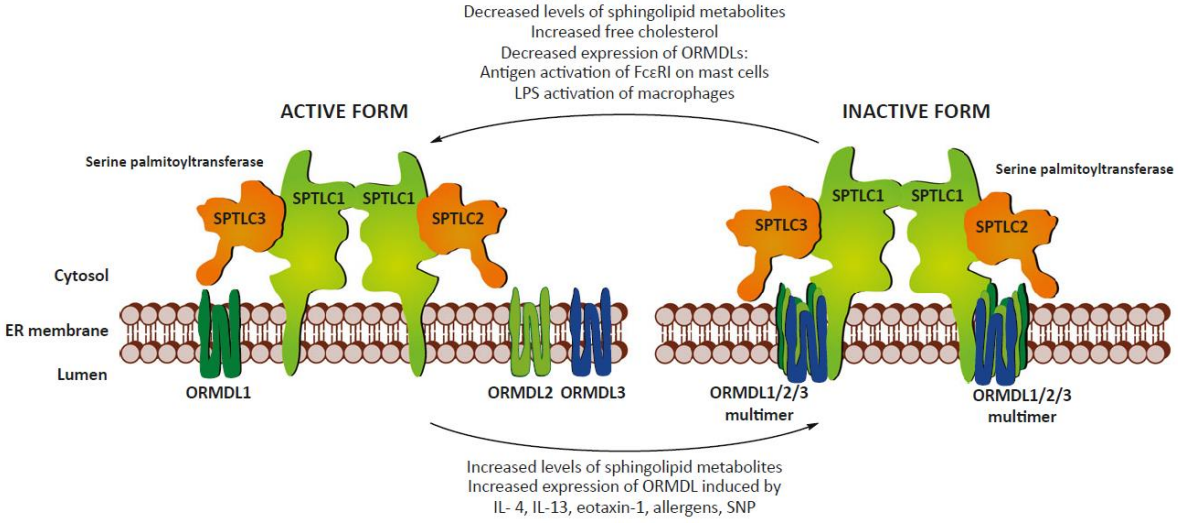
Human *ORMDL1*, *ORMDL2*, and *ORMDL3* are localized on chromosomes 2q32, 12q13.2, and 17q21, respectively, whereas mouse *Ormdl1*, *Ormdl2*, and *Ormdl3* are localized on chromosomes 1, 10, and 11, respectively [78]. Several studies showed that *ORMDL3* locus is not only associated with asthma, but also with other numerous pathologies (e.g. allergic rhinitis [79], type 1 diabetes [80], primary biliary cirrhosis [81], rheumatoid arthritis [82], ulcerative colitis [83], Crohn's

disease [84], and ankylosing spondylitis [85]). By contrast to *ORMDL3*, very few studies have been done on the association of *ORMDL1* or *ORMDL2* with diseases.

The *ORMDL3* gene encodes a protein that acts as an inhibitor of sphingolipid biosynthesis, by directly binding to serine palmitoyl transferase, the first and rate-limiting enzyme in sphingolipid production. In general, Orm family proteins were shown to be implicated in the control of sphingolipid homeostasis (figure 1.2) [86]. The next section will discuss in detail the sphingolipid metabolism and will be followed by reviewing the involvement of *ORMDL3* in allergic asthma.

Figure 1.2 Regulation of serine palmitoyl transferase (SPT) by ORMDL proteins

SPT is an octamer formed by three different subunits: SPTLC1, SPTLC2, and SPTLC3 localized to ER membrane with its catalytic core formed in the cytosol. In active state, SPT is free and able to catalyze condensation of serine with palmitoyl CoA. When the cell senses increased levels of sphingolipid metabolites, or the increased expression of ORMDLs is initiated by cytokines (IL-4, IL-13, eotaxin-1) or allergens, ORMDLs bind SPT, presumably in the form of homo- or hetero-oligomers, and they inhibit its activity and stop *de novo* sphingolipid biosynthesis. When sphingolipid levels in the cell are low, the cell is activated (FceRI antigen, LPS), or the amount of free cholesterol is high, the concentration of ORMDL proteins is decreased and sphingolipid biosynthesis is reactivated. Figure reproduced with permission from Paulenda *et. al.* 2016 [78].



1.1.4.2.2 Metabolism of sphingolipids

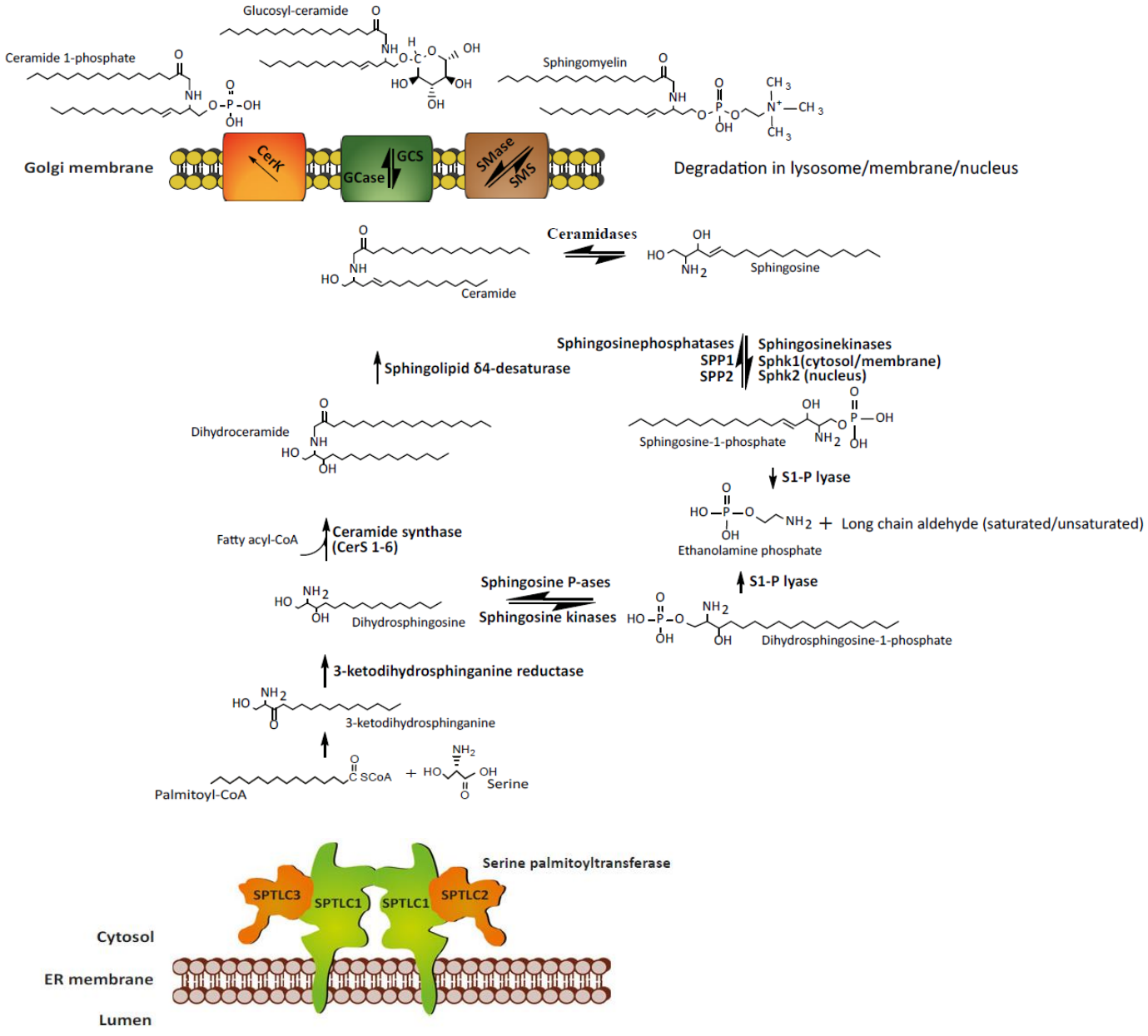
The *de novo* synthesis of all sphingolipids starts by the condensation of palmitoyl-CoA and serine by the serine palmitoyl transferases (Sptlc1-3) in the smooth endoplasmic reticulum. As depicted in figure 1.3, the resulting 3-ketodihydrosphinganine is further reduced to dihydrosphingosine [87].

In vertebrates, one of the six ceramide synthases (Cers1–6) acylates the dihydrosphingosine by using a fatty acid molecule of a specific chain length to produce dihydroceramides having side chains of different lengths. Dihydroceramides are then saturated, by hydrogenation of the double bond *via* the action of sphingolipid desaturase, to produce ceramides. Based on the length of the acyl side chain of the ceramide molecule, ceramides are: long chain (C14:0-C20:0), very long chain (C22:0-C26:0) or ultra-long chain ceramides (> 26 carbons) [87,88]. Subsequently, ceramides are translocated from the endoplasmic reticulum to the Golgi apparatus by the ceramide transfer protein for sphingomyelin synthesis or by vesicular transport for glucosyl ceramide synthesis [89].

Ceramides are degraded by one of four ceramidases (Asah1 and 2, Acer1 and 2) to sphingosine which can be reused as a substrate for ceramide synthases to synthesize new ceramides through a salvage pathway. Alternatively, sphingosine can be phosphorylated into sphingosine-1-phosphate through the action of sphingosine kinases (Sphk1 in the plasma membrane and Sphk2 in the nucleus). The full picture of ceramides synthesis and recycling is quite complex, as beside their *de novo* synthesis pathway, three different salvage pathways are known to recycle ceramides from complex sphingolipids; cerebrosides, sphingomyelins or phosphorylated ceramides [87–89].

Figure 1.3 Sphingolipid biosynthesis in vertebrates

Serine palmitoyl-transferase (SPT) enzyme catalyzes condensation of acyl-CoA with serine leading to production of 3-keto dihydrosphinganine, which is subsequently reduced to dihydrosphingosine (DHS). DHS can be phosphorylated by sphingosine kinase to DHS 1-phosphate, or it is acetylated by ceramide synthase (CerS) to dihydroceramide. Inhuman, six different CerS are known, each of which uses specific acyl chains, typically with saturated or mono-unsaturated fatty acids with 14 to 26 carbons. After that, dihydroceramide is dehydrogenated by sphingosine-4-desaturase to ceramide. Upon trans-port of ceramides from ER to Golgi, ceramides undergo various modifications such as glycosylation resulting in glycosphingolipids phosphorylation resulting in phosphoceramides, and addition of phosphoethanolamine (PEA). Addition of PEA to ceramide produces sphingomyelin. Sphingolipids are molecules undergoing rapid turn-over. Complex sphingolipids can be recycled to ceramide by degradation enzymes, for example, sphingolmyelinase (SMase), glucosylceraminase (GCase), and sphingosine can be recycled to ceramide, which are important steps in sphingolipid homeostasis. Sphingosine can also be further phosphorylated by sphingosinekinase (Sphk1 acting in the cytosol and membrane and SphK2 acting in the nucleus) to sphingosine 1-phosphate. Degradation of sphingosine 1-P is mediated by sphingosine 1-P lyase (S1P lyase) producing phosphoethanolamine and long-chain aldehyde. Figure reproduced with permission from Paulenda *et. al.* 2016 [78].


Glucosyl-ceramide
Ceramide 1-phosphate
Sphingomyelin
Golgi membrane
CerK
GCase
GCS
SMase
SMS
Degradation in lysosome/membrane/nucleus
Ceramidases
Ceramide
Sphingosine
Sphingosinephosphatases
SPP1
SPP2
Sphingosinekinases
Sphk1(cytosol/membrane)
Sphk2 (nucleus)
Sphingolipid δ4-desaturase
Dihydroceramide
Sphingosine-1-phosphate
S1-P lyase
Ethanolamine phosphate
Long chain aldehyde (saturated/unsaturated)
Fatty acyl-CoA
Ceramide synthase
(CerS 1-6)
Sphingosine P-ases
Sphingosine kinases
S1-P lyase
Dihydrosphingosine
Dihydrosphingosine-1-phosphate
3-ketodihydrosphinganine reductase
3-ketodihydrosphinganine
Palmitoyl-CoA
Serine
Serine palmitoyltransferase
SPTLC3
SPTLC1
SPTLC1
SPTLC2
Cytosol
ER membrane
Lumen

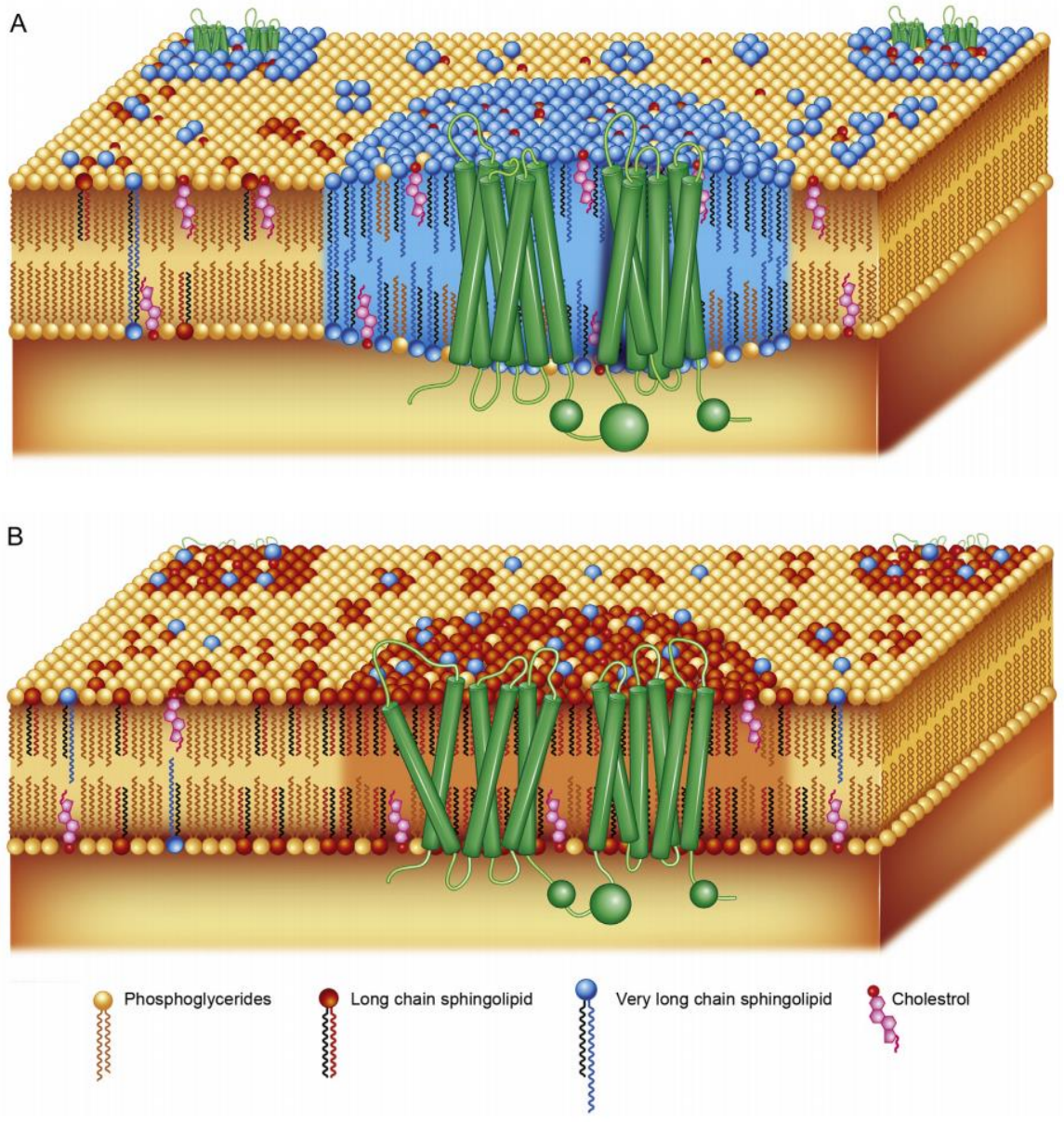
1.1.4.2.3 Metabolic actions of ceramides

Ceramides can act as both building blocks in the cell membranes and as signaling molecules [90]. Therefore, the roles of ceramides can be viewed as structural components of the cellular membranes, maintaining the membrane integrity, and as bioactive signaling molecules produced upon various stimuli e.g. inflammation or stress.

In the cell membrane, lipid rafts are cholesterol- and sphingolipid- rich ordered platforms, carrying different domains or receptors and floating in a liquid disordered matrix (i.e. the rest of cell membrane) [91]. The concentration of cholesterol and sphingomyelins in the lipid rafts is estimated to be twice as high as in non-raft portions of the membrane, resulting in tight packing of the portions of the membrane that contain lipid rafts. An interesting structure model of the lipid rafts under normal and pathological conditions has been hypothesized by Garic *et al.* (figure 1.4) [88]. Nevertheless, the composition and ratios of specific ceramides serving as building blocks of the sphingolipids in those rafts have not been fully established yet. Moreover, the exact role of lipid rafts in maintaining membrane stability and controlling transmembrane signals transduction remains undiscovered [88,91].

Figure 1.4 Structures of lipid rafts under normal and pathological conditions

Under normal conditions (A), when VLCCs are more abundant than LCCs, tight packing of VLCCs whose acyl chain interdigitate from both leaflets of the cell membrane forms a gel-like environment in the lipid rafts that may stabilize the folding of large transmembrane proteins (green). On the other hand, in the membranes of cells with imbalance of ceramides (B), where LCCs are more abundant than VLCCs, lipid rafts are more fluid, because their side chain do not interdigitate from the two leaflets of the cell membrane, and under these conditions the folding of large transmembrane proteins (green) may be compromised. Phosphoglycerides (yellow patterns), Long-chain ceramides (red/black patterns), Very-long-chain ceramides (blue/black patterns), cholesterol (red/pink patterns). Figure reproduced with permission from Garic *et. al.* 2019 [88].


A
B
Phosphoglycerides
Long chain sphingolipid
Very long chain sphingolipid
Cholestrol

Very few studies described the interaction between ceramides and their target proteins [88,90]. Long-chain ceramides indirectly activate protein phosphatase 2A (PP2A) by binding to its inhibitor I2PP2A (Inhibitor-2 of PP2A), thereby stopping its inhibitory effects on PP2A. PP2A are a family of enzymes composed of 3 subunits, having different isoforms, which can assemble in different combinatorial associations to control different cell functions [90]. Additionally, long-chain ceramides form large channels in the outer mitochondrial membrane, which lead to permeabilization, release of cytochrome c and initiation of apoptosis [92]. Very-long chain ceramides counteract the formation of these pores made by long-chain ceramides, thus inhibiting apoptosis [93]. As of 2019, the direct intracellular targets of very-long-chain ceramide remain unknown.

Beside allergic asthma, it has been shown that ceramides play an important role in several other diseases, such as cystic fibrosis (discussed in detail in the next section of this review of literature), Alzheimer's disease [94], coronary artery disease [95], multiple sclerosis [96], neuroblastoma [97], breast cancer [98], atherosclerosis [99], osteoporosis [100], ulcerative colitis [101], Parkinson's disease [102], hypertension [103] and obesity [104]. In addition to their important role as promising biomarker [105], scientists have started to view ceramides as possible therapeutic targets, however, as of 2019, this field of pharmaceutical research is still in infancy [105,106].

1.1.4.2.4 ORMDL3 and allergic asthma

The expression levels of *ORMDL3* gene was found to be upregulated in allergic asthma, and it has been shown that *ORMDL3* transgenic mice have increased airway responsiveness and airway remodeling; the two main landmarks of asthma [107]. Since it was observed that ORMDL3 protein acts as an inhibitor of sphingolipid biosynthesis, the Orm family of proteins, and how they control sphingolipid homeostasis, has been investigated in greater detail. Zhakupova *et al.* have observed that cells from *Ormdl3*$^{-/-}$ KO mice did not show an altered SPT activity compared to wild type mice, however, parallel knockdown of all the three ORMDL isoforms increased the SPT

enzyme activity significantly [108]. This highlights in part the interconnected function of all the three isoforms of the ORMDL proteins. Eventually, dysregulated sphingolipid formation in the respiratory tract, influenced by the alteration of Orm family proteins, instigates airway hyper reactivity [109] although the full picture is still not known.

ORMDL3 protein regulates the sphingolipid biosynthesis, by inhibiting SPT enzyme, in a more complicated manner than originally thought. Moderate ORMDL3 overexpression inhibited SPT enzyme activity, which, in turn, resulted in inhibiting the *de novo* biosynthesis of sphingolipids [110]. In contrast, when strong overexpression of ORMDL3 was induced, the sphingolipids levels remained unchanged [111] or even increased [110]. This observation can be interpreted in terms of increased recycling of complex ceramides, in case of strong overexpression of ORMDL3, which overall results in unchanged or even higher levels of sphingolipids.

In immune cells, ORMDL3 is involved in the production of many proinflammatory cytokines, being widely expressed in macrophages [107], lymphocytes [112], eosinophils [113], and in the epithelial cells of the lungs [114]. Moreover, ORMDL3 has been shown to play an important role in endoplasmic reticulum homeostasis by regulating the unfolded protein response and calcium response [115–117].

In eosinophils, overexpression of ORMDL3 significantly resulted in increased rolling, distinct cytoskeletal rearrangement, extracellular signal-regulated kinase (1/2) phosphorylation and nuclear translocation of nuclear factor kappa B. By contrast, knockdown of ORMDL3 expression inhibited activation-induced cell shape changes and decreased the eosinophils adhesion and recruitment to sites of inflammation [113].

It has been shown that transgenic mice which universally overexpress human ORMDL3 have increased airway smooth muscle thickening, increased subepithelial fibrosis, overproduction of mucus, and overall significant levels of airway remodeling [107]. The observed increase of airway

remodeling in this study [107] was associated with activation of the unfolded protein response pathway transcription factor ATF6. Overexpressing ORMDL3 in lung epithelial cells of transgenic mice activated ATF6 and induced sarco–endoplasmic reticulum Ca^{2+} ATPase, SERCA2b, which is implicated in the airway remodeling in asthma [107,114].

For activation, the T lymphocytes depend on a Ca^{2+} signal; this signal is known as store-operated calcium entry (SOCE). Either the activation of a T-cell receptor or the depletion of endoplasmic reticulum (ER) Ca^{2+} stores triggers this signal. ORMDL3 overexpression inhibits the Ca^{2+} influx resulting in depletion of endoplasmic reticulum (ER) Ca^{2+}, SOCE signaling, and a final activation of T lymphocytes [112].

Dysregulated production of the Th2 cytokines interleukin (IL)-4 and IL-13 has long been associated with the pathogenesis of allergic asthma [118]. It has been shown that IL-4 and IL-13, the two prototypical Th2 cytokines, are induced by ORMDL3 *via* STAT6 dependent pathway [114]. IL-4 has a consistent and primary role in Th2 differentiation and IgE synthesis, however, IL-13 is not involved in these responses. On the other hand, production of mucus, increased proliferation of ASM and collagen deposition are IL-13–, not IL-4–, dependent reactions in the lungs of allergen-challenged mice [118]. Altogether, IL-4 and IL-13, which are induced by ORMDL3, play a pivotal role in the asthma pathogenesis.

1.1.4.2.5 ZPBP2 and allergic asthma

Human *ZPBP2* is located on chromosome 17, while mouse *Zpbp2* is located on chromosome 11. Zona pellucida binding protein 2 (ZPBP2) is a protein that localizes to the sperm acrosome and was confirmed to be highly expressed in the testes in both mice and humans, however, *ZPBP2* gene expression is not restricted to testis. Mouse studies [119,120] pertinent to allergic asthma have shown that knocking down *Zpbp2* reduces AHR, moreover, higher methylation of the *Zpbp2* promoter, which is also associated with lower *Zpbp2* gene transcription,

is protective against asthma [121]. Collectively, these studies confirm the role of *ZPBP2* in predisposition to allergic asthma as previously associated by the GWAS published by Moffatt *et al.* [71]

Our group has observed that the ablation of *Zpbp2* results in lower levels of VLCCs (e.g., C24:0, C24:1, C26:0) and higher levels of LCCs (C14:0, C16:0, and C18:0) in *Zpbp2* KO mice on C57BL/6 genetic background [120]. Also, it has been published that *Zpbp2* KO mice have reduced levels of ORMDL3 in the airway epithelial cells [119]. Overall, these studies suggest that effect of *Zpbp2* in allergic asthma may be mediated through changes in ceramides metabolism and reduction of the levels of *Ormdl3* expression. The third chapter of this thesis further investigates the role of these two genes; *Zpbp2* and *Ormdl3,* in parallel with the ceramides biosignature in a mouse model of allergic asthma (A/J atopic strain).

Ultimately, several studies suggested that the mechanisms of allergic asthma development are linked with genetically determined abnormalities in some patients resulting in their inability to control the imbalance generated by the inflammation cascade upon the encountering of allergens. In addition to genetically determined abnormalities, the mechanisms of allergic asthma development are linked with other environmental factors. Altogether, these alterations seem to explain the differences between people who are susceptible to allergic asthma as opposed to other people who are not.

1.1.5 Mouse models of allergic asthma

Mouse models represent good and practical venues for studying the molecular mechanisms of diseases, improving our understanding of how medications work and shaping out the fine details of treatment protocols. Some questions may be answered using *in vitro* experiments, however,

exploring complex disease traits, like in the case of allergic asthma and medications used for it, usually requires the utilization of animal models [122].

Mice do not spontaneously develop AHR or airway inflammation, unless genetically engineered to do so, as observed in transgenic mice overexpressing the human *ORMDL3* [107]. As of right now, there are many allergic protocols for studying allergic asthma in animal models and each research group usually establishes its own protocol for studying the disease [122]. The fact that there are diverse and different existing allergic asthma models makes it difficult to compare data among research groups.

Due to the complexity of the nature of the disease in human, it is not convenient at all to capture the entire picture in one model. In addition to the effect of the genetic background, humans are usually sensitized by more than one allergen; thus the underlying disease mechanism, the disease severity and the resulting phenotype differ from one person to another [123]. Ultimately, the selection of the appropriate protocol depends mainly on the goal of the project, among many other factors. People usually experience episodes of severe and acute exacerbations in specific situations e.g. acute allergen exposure. however, allergic asthma, as a disease, is considered chronic and progressive over long term. For that, both acute and chronic allergic asthma models were developed to address this point [124].

1.1.5.1 Acute and chronic mouse models

Acute models of allergic asthma in mice have successfully replicated many human features of the disease. For example; AHR, epithelial hypertrophy, airway inflammation, goblet cells hyperplasia, and high levels of serum IgE are easily developed after a series of allergen sensitizations and challenges. On the other hand, since in this model mice are exposed to the allergen fewer times than what normally happens in humans, chronic remodeling and inflammation of the airways might not be observed. In animals, airway inflammation resolves within a few weeks

after the final antigen exposure. Nevertheless, in human, inflammation persists and a new exposure to the allergen tends to induce recurrence of symptoms [125]. Although having some limitations, the acute model has demonstrated many aspects of allergic asthma being a Th2-mediated disease [125].

Chronic murine models of allergic asthma mimic the disease more closely in humans. It involves repeated airway exposure to the allergen for periods up to 12 weeks. Chronic models reproduce airway remodeling which is one of the disease events in human adults. In addition, pulmonary inflammation persists for several weeks after the last allergen provocation in this model, resembling the asthmatic patients. However, the degree of severity and length of symptoms of lung inflammation and AHR vary widely according to the protocol used [126].

1.1.5.2 Allergens used in asthma mouse models

After deciding whether an acute or a chronic animal murine model is the appropriate choice for the study, the next step in the study design would be choosing the allergen(s) and the route of allergen administration (during the sensitizations and challenges). Ovalbumin (OVA), House Dust Mite (HDM) *Dermatophagoides pteronyssinus* (Der p, or European dust mite) or *D. farinae* (Der f, or American dust mite), ragweed, fungi (*Aspergillus fumigatus, Alternaria alternate*), *Ascaris* antigens, cockroach extracts, cotton dust, latex (*Hevea brasiliensis*), and pollen grains are among the common allergens that have been widely used to induce allergic asthma in mouse models [127]. The choice of allergen depends on the condition to be replicated, additionally, allergens can be used separately or in combination.

OVA has been used traditionally to induce allergic asthma in mice. Because OVA is derived from chicken egg and can be produced in large quantities with cheaper costs, OVA-sensitized and challenged mice have been classically used for allergic asthma studies for many years. According to the Asthma and Allergy Foundation of America (aafa.org), egg allergy is considered the second most common ingested food allergen in young children after milk allergy.

Although OVA can produce airway inflammation and induce AHR in mice, it does not induce airway inflammation or AHR in humans. Apparently, OVA can induce food, but not airway, allergies in human. As a result, this model has been commonly questioned whether it could successfully mimic the human "naturally" inhaled allergens [124]. Recently, HDM, the most common allergen which causes allergic asthma in humans, became one of the most routinely-used allergens to induce allergic asthma in rodents.

HDM is the most common inhaled human allergen because up to 85% of all asthmatic patients are sensitized against HDM allergens. Additionally, as compared to OVA which contains 1 main protein allergen (> 98% albumin), 23 allergens (Der p1 - Der p23) have been characterized from the extract of HDM. Consequently, two main advantages are provided when using HDM as an allergen; strong immunogenicity and intrinsic enzymatic activity [128]. One of the limitations of using the HDM allergen is that the purified allergen is much more expensive than OVA allergen.

1.1.5.3 Routes of allergen administration in asthma mouse models

In human beings, allergen sensitization (or challenge) happens by allergen exposure to the airway mucosa, then immunological recognition, and finally Th2 inflammatory response. In animal models, a wide range of allergen administration are used, e.g., subcutaneous (s.c.) injection, intraperitoneal (i.p.) injection, intranasal (i.n.) drops or by aerosol inhalation. It has been described in the literature that sensitizing the mice by inhalation better mimics the way in which humans are sensitized to allergens, however, numerous asthma protocols traditionally use s.c. or i.p. injections for simplicity, consistency, and greater systemic exposure for administrating the allergen by these two routes [124].

If the allergen is administrated s.c. or i.p., coadministration of an adjuvant is routinely, but not always, required. Aluminum hydroxide, which is considered the adjuvant of choice of allergic asthma models, is also known to promote a Th2 type response. Omitting the use of adjuvants in

the model of allergy may be more representative of what occurs in humans, but it may also require longer periods of sensitization to compensate for the rapid clear up of the allergen by the organism following its administration [129].

Any model of allergic asthma has its advantages, disadvantages and applications. Nonetheless, the best model should be selected or developed to answer the research questions. Generally, studying allergic asthma from as many angles as possible helps to gain a complete understanding of this complex disease.

1.1.6 Therapies for allergic asthma

Modern treatments for asthma have been tested and used since the early 20th century [130]. However, the oldest documented drug for asthma dates back to ancient Egypt. Kyphi, an incense mixture drink, was used inside the temples by the priests as a multipurpose lung medicament. There was more than one recipe for Kyphi; each may include as many as ten herbs [131]. Following this, about 4000 years ago, Atropa Belladonna alkaloids, also called "deadly nightshade" because of their poisonous properties ("Natural Medicinal Herbs"), were derived from the leaves of thorn-apple plant and smoked by the Indians as cough suppressant [133]. Until today, natural and synthesized entities related to the tropane alkaloids class are still widely used. This includes anticholinergics (*e.g.* natural atropine, hyoscyamine [the levo-isomer of atropine], acopolamine, and the synthetic Ipratropium Bromide and stimulants (*e.g.* cocaine and hydroxytropacocaine) [134]. In 1872, one of the first papers published on asthma states that rubbing the chest of asthmatics with chloroform liniment can resolve airway constriction [135]. Adrenergic stimulants were in use for asthma over 100 years ago. In 1901, the adrenaline isolated from sheep and oxen adrenal glands was used to treat asthma [136]. The first documented publication of adrenaline as a bronchodilator therapy for asthma was written in 1903 by James Burnett, a physician in Edinburgh [137]. One year

later in 1904, adrenaline was synthesized in the laboratories of Friedrich Stolz and Henry Drysdale Dakin, independently [138].

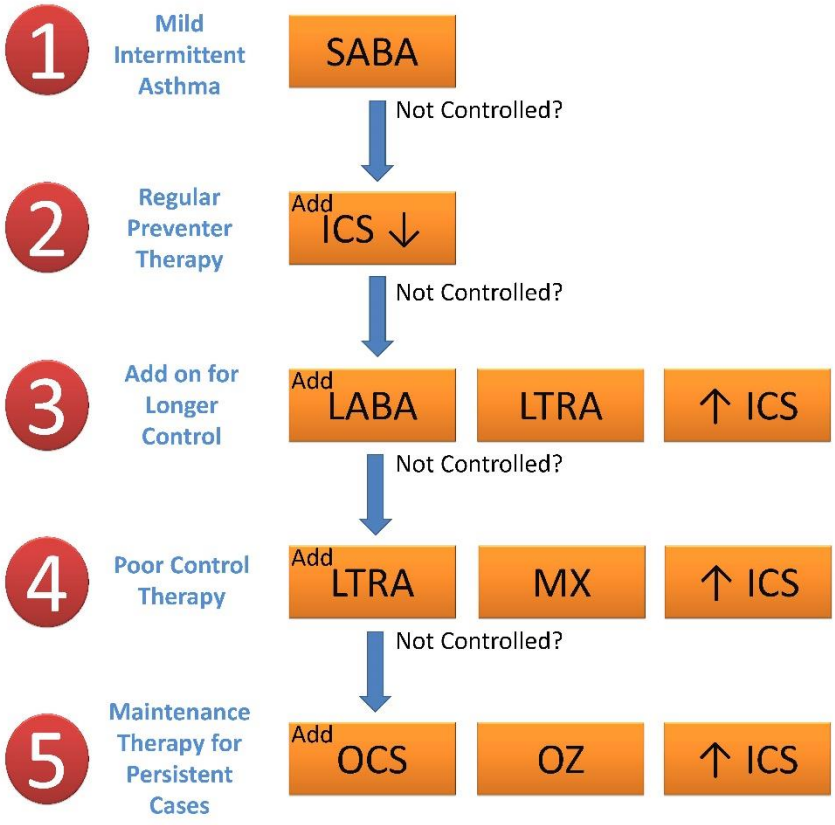
As suggested by the Global Initiative for Asthma (GINA) [139], a five-level step-down approach is widely recognized among the medical practitioners (figure 1.5). The five-level step-down approach of GINA assigns two types of drug classes for managing asthma:

- *Relievers* (bronchodilators): cause immediate dilatation effects on the airway's obstruction, mainly by acting on lungs smooth muscle.
- *Controllers* (preventers): provide long-term control of symptoms, mainly by suppressing the underlying disease process.

β2-agonists and anticholinergics are considered to be bronchodilator relievers. As per the GINA approach (figure 1.5), relievers are first used as monotherapies to control mild-to-moderate asthma. Asthma controllers include corticosteroids, anti-leukotrienes and anti-IgE. Controllers can be used as add-ons for moderate-to-severe uncontrolled asthma (figure 1.5). Theophylline is casually classified as both a bronchodilator and a reliever. The following section of the thesis will briefly discuss each therapeutic class.

Figure 1.5 Global Initiative for Asthma (GINA) recommendations for the stepwise approach in controlling asthma symptoms and minimizing future morbidity.

SABA: short acting B-agonists, ICS: inhaled corticosteroids, LABA: long acting B-agonists, LTRA: leukotriene receptor antagonists, MX: methylxanthines (theophylline), OCS: oral corticosteroids, OZ: Omalizumab. Figure reproduced from Youssef *et. al.* 2016 [15] (open access article under the terms of the Creative Commons Attribution 3.0 License, no permission required).



1.1.6.1 Corticosteroids

Currently, most popular protocols for managing asthma involve the use of corticosteroids and β-agonists [1]. Anti-inflammatory corticosteroids, which are one of most trusted treatments for asthma, were introduced in mid-20th century [130]. The principal mode of action of corticosteroids in asthma is through their direct anti-inflammatory effect in different white blood cells including T cells, mast cells and eosinophils. Among leukocytes, corticosteroids suppress chemotaxis and adhesion, and prevent inflammatory cytokines recruitment [140]. *In vitro*, corticosteroids reduce human ASM proliferation directly [141] by stimulating p21 gene expression [142], an important regulator of cell cycle progression. Moreover, corticosteroids improve the vast majority of vascular remodeling aspects in asthma, reducing angiogenesis, excess blood flow, and vascular leakage [143]. This is mainly mediated by decreasing VEGF activity within the airway wall cells [144].

Various studies describe contradicting effects of corticosteroids on the lung epithelial abnormalities in asthmatics. Dorscheid *et al.* [145] reported that Dexamethasone treatment resulted in increased epithelial apoptosis and shedding. Similar results were obtained when treating guinea pigs with Budesonide, which did not improve the tracheal epithelium [146]. In contrast, some *in vivo* studies showed that inhaled corticosteroids (ICS) treatment resulted in improvement of epithelial damage in severe asthmatics [147,148].

ICS has been used for the past couple of decades. Its idea dates to the nineteenth century when the hand-held glass bulb nebulizer was used, however, pressurized metered-dose inhaler (pMDI) came to the clinic in 1956. After seeing his daughter suffering while using the hand-held nebulizer, George Maison, a medical consultant at 3M Pharmaceuticals, had advocated the use of pMDI. In 1959, George Maison and Irvine Porush were awarded a patent on the first pMDI [149].

1.1.6.2 β-adrenergic agonists (LABAs and SABAs)

Long acting β-agonists (LABAs), such as Formoterol [150] and Salmeterol [151], offer a longer period of bronchodilation compared to the short acting beta agonists (SABAs), *e.g.* Salbutamol [152] and Terbutaline [153]. LABAs persist in the airway tissues for long periods due to their lipophilic nature and they provide a good umbrella of asthma bronchodilation and control, particularly at night [150,151]. However, until recently, the medical literature lacked supporting studies reporting the positive effect of β_2 agonists on the chronic airway remodeling [154]. Addition of a β-agonist to the corticosteroid therapy allows a 'steroid-sparing' effect; i.e. maintains asthma control using lower doses of corticosteroids [155]. LABAs are not used as monotherapies anymore and they must be used in combination with ICS [156], because there have been cases of severe exacerbations and death when LABAs are administrated solely.

1.1.6.3 Antimuscarinic agents (SAMAs and LAMAs)

Inhaled antimuscarinic agents, also known as inhaled anticholinergics, are considered another alternative bronchodilator group to β-agonists. The bronchodilation effect is functionally mediated *via* muscarinic receptors subtypes M1, M2, and M3, although five muscarinic receptors have been revealed in the lungs M1, M2, M3, M4, and M5 [157]. It is widely known that parasympathetic stimulation *via* the Vagus nerve leads to immediate smooth muscle contraction and mucus secretion in the airways [158]. It is also suggested that M receptors interact with β2 adrenergic receptors on the airways smooth muscle, leading to a reduced bronchodilator response of the β-agonists [159]. For years, in both adults and children, short acting antimuscarinic agents (SAMA) use, e.g. Ipratropium [160], has been limited to acute asthma management, in addition to inhaled SABA [161,162]. LAMA (Long acting antimuscarinic agents), e.g. Tiotropium [163], appear to have more benefits in difficult-to-control asthma. Adding Tiotprium to the standard asthma therapy significantly reduces asthma symptoms and highly increases the clinical outcomes [164,165].

1.1.6.4 Targeted therapies

Over the last 40 years, there has been a marked increase in the development of targeted treatments for asthma - anti-leukotrienes, anti-IgE, anti-interleukins, and anti-TNF-α [166]. Obviously, as more of the biological basis of asthma is uncovered, more effective targeted asthma treatments might be developed.

1.1.6.4.1 Anti-leukotrienes

Leukotrienes are lipid eicosanoids with a wide range of biological activities. They are derived from arachidonic acid through the enzymatic action of 5-lipooxygenase, and play a crucial role in asthma inflammatory pathogenesis, and in other allergic diseases such as allergic rhinitis, rhinosinusitis, atopic dermatitis and urticaria [167]. Leukotrienes include three main types: cysteinyl leukotrienes (CysLTs), LTB4, and LTG4. LTG4 is the metabolite of LTE4 in which the cysteinyl moiety has been oxidized to an α-keto-acid [168]. Since, very little is known about the LTG4 putative leukotriene, most clinical research studies focus on CysLTs and LTB4. CysLTs are strong bronchoconstrictors that powerfully affect airway remodeling, whereas, LTB4 is a strong chemo-attractant for most leukocytes subsets [169]. Over the last 20 years, since leukotriene antagonists were introduced to the clinic for asthma management, Montelukast [170,171] and Zafirlukast [172] are the most frequently used drugs in this class.

1.1.6.4.2 Anti-IgE

Now, Omalizumab, which is the only approved targeted monoclonal antibody against IgE, is used to treat allergic asthma in clinical practice. It can significantly decrease serum IgE levels (up to 99%) within two hours following subcutaneous administration, and diminish serum, sputum and tissue eosinophilia [173]. Recently, Omalizumab has also been reported to have steroid-sparing effect, reducing the rate of asthma exacerbations up to 50%, and hence, improving the quality of life [174]. However, nearly 45% of patients treated with Omalizumab had adverse reaction at the

local injection site; which is considered the most commonly observed adverse event for Omalizumab. Some other minor upper respiratory tract infections and sinusitis have also been reported as well. Patients treated with Omalizumab display a very low (0.09%) frequency of anaphylaxis reaction. Importantly, there are no data reporting any correlation between cancer and Omalizumab treatment [175].

1.1.6.4.3 Anti-ILs

Three interleukin pathways are of physiological importance for asthma; IL-5, IL-9, and IL-4/IL-13 pathways. IL-5 is pivotal for both eosinophils differentiation and maturation in the bone marrow. Subsequently, it controls eosinophil mobilization, activation and survival [176]. Hence, antagonizing IL-5 has been proposed to be beneficial asthma therapy, particularly for predominantly eosinophilic asthma. A number of anti-IL-5 monoclonal antibodies are in the process of development for allergic diseases; Reslizumab [177], Mepolizumab [178] and Benralizumab [179]. IL-9 is one of the T helper 2 (Th2) pro-inflammatory cytokines that promote mast cell proliferation and T-cell growth [180]. In mouse models, IL-9 causes several common features of chronic asthma; excessive mucus production, eosinophilic airway inflammation, smooth-muscle cell hyperplasia, and AHR [181]. Activated mast cells, eosinophils, basophils and dendritic cells secrete IL-4 and IL-13. IL-4 and IL-13 both play an important role in asthma mainly by enhancing IgE production. They also control mast cells growth and development, eosinophil recruitment, and AHR [182]. The first trial aimed at antagonizing the IL-4 used a soluble recombinant human IL-4Ra, altrakincept, which blocked the binding of IL-4 to its cellular receptors [183]. Several humanized IL-13-neutralising antibodies have entered asthma phase I/II clinical trials - anrukinzumab [184], QAX576 [185], and CAT354 [186].

1.1.6.4.4 Anti-TNF-α

TNF-α, a cytokine produced by Th1 cells and macrophages, has diverse biological functions. TNFα shows crucial, and previously extensively documented, role in Crohn's disease rheumatoid arthritis, and psoriasis pathogenesis. The association between TNF α increase and these diseases progression had inspired studies aiming to extend anti- TNF-α therapies also for the treatment of severe asthma and COPD [187]. Infliximab and Golimumab, two anti-TNF-α mAbs, and Etanercept, a decoy soluble TNF-α receptor, which are both able to biologically neutralize TNF-α cytokine, and blunt the immune response, and thereby abolishing TNF-α effects in asthma [188].

1.2 Cystic Fibrosis

1.2.1 Gene and protein abnormalities

Cystic fibrosis (CF) is considered the most common genetic disorder in Caucasians. One in every 3000 individuals is affected by CF in western countries [189]. CF disorder was first described in 1936 and 1938 by Fanconi and Andersen respectively [190], however, only in 1989, the underlying defect of the cystic fibrosis transmembrane regulator (*CFTR*) gene, 180 kb pairs on chromosome 7q31.2, was discovered by Francis Collins and Lap-Chee Tsui [191–193]. CFTR protein is involved in chloride secretion and is expressed in most epithelial cells including those of the respiratory and digestive systems. Consequently, the lung, liver, pancreas and intestines of the CF patients are the most affected organs. CF patients' organs are affected based on the degree of the mutation of the *CFTR*, on its protein expression, its importance in the functioning of the organ and whether other types of proteins are present in cells which can compensate for the lack of CFTR. Among all the 1800 CFTR mutations which have been found, only 23 mutations are most commonly detected in affected individuals [194,195]. The most common disease-causing mutation, called F508del, is a deletion of phenylalanine at position 508 (del stands for deletion). According to Cystic Fibrosis

Canada, it is estimated that one in every 3,600 children born in Canada has CF and about 92% them have at least one copy of this mutation.

The various mutations of *CFTR* can be categorized to six classes [196,197]. Table 1.1 summarizes the worldwide frequencies of *CFTR* mutations [198]. A severe presentation of CF disease, with extensive lung and pancreatic disease, is generally caused by Class I, II and III mutations, meanwhile, Class IV, V and VI are associated with milder forms of CF disease.

• Class I mutations: nonsense mutations, frame-shift mutations, and abnormal mRNA splicing resulting in a truncated CFTR protein.

• Class II mutations: folding and maturation defects resulting in premature degradation of CFTR protein (e.g. F508del).

• Class III mutations: defective nucleotide binding, low levels of channel gating activity (e.g. G551D).

• Class IV mutations: defective conductance preventing free flow of ions through CFTR protein.

• Class V mutations: *CFTR* transcripts are normally produced, but translation is defective resulting in a low level of normal protein.

• Class VI mutations: abnormally high turnover of CFTR protein at the membrane.

Table 1.1 The worldwide frequencies of CFTR mutations

Mutation	Frequency Worldwide
ΔF508	66%–70%
G542X	2.4%
G551D	1.6%
N1303K	1.3%
W1282X	1.2%
All others	27.5%

The CFTR protein is a relatively huge protein containing 1480 amino acids with 5 domains: 2 membrane spanning domains which form the channel, 1 regulatory domain and 2 nucleotide binding domains. CFTR protein, being a member of the ATP-binding cassette (ABC) transporter family, is functionally positioned in cellular membranes where it exerts its role. CFTR is mainly known for being a chloride channel, but it is also involved in fluid secretion. It negatively regulates sodium transport by acting on the amiloride-sensitive epithelial Na channel (ENaC) [199,200].

1.2.2 *CFTR* gene deficiencies associated pathologies

The most common signs and symptoms of cystic fibrosis are salty-skin, poor weight gain, poor growth, chronic coughing, accumulation of thick mucus, chronic chest infections and shortness of breath [201].

Several organ pathologies are associated with *CFTR* disease and, notably, the most common manifestations are associated with the digestive and the respiratory systems. Thick mucus

in the pancreatic ducts prevents the release of digestive enzymes into the digestive tract. Most CF patients have pancreatic insufficiency, and functionally, they can be treated with pancreatic enzyme replacement therapy to help with the proper digestion of food [202].

CF patients commonly have malabsorption of fat due to high levels of inflammation [203] and abnormal pH levels in the intestine [204]. Thick mucus chronically present in the intestines of CF patients is considered one of the other causes of malabsorption as well. Undernourished patients, with low body mass index (BMI), have more severe CF lung disease and higher risk of both morbidity and mortality [205].

Additionally, thick mucus impacts the development of the vas deferens in males with CF, which impairs fertility. In countries where there is no screening for CF before or after birth [206], the congenital bilateral absence of the vas deferens in CF males can be firstly diagnosed in infertile men carrying mild *CFTR* mutations who come to be treated for infertility [207].

1.2.3 *CFTR* gene deficiencies mediated lung disease

The lung is in continuous contact with the environment, and thus, must have a barrier system to maintain sterility within the living organism. In effect, the respiratory system of the host has various mechanisms of defense against invading pathogens. The first line of defense is a physiological, and 'physical', barrier in which the airway epithelial cells are lined with a thin layer of fluid called the airway surface liquid (ASL). Cilia are continuously moving hair-like structures located on the surface of the epithelium of the airways. On the apical side of the epithelia lies the periciliary layer (PCL) which enrobes the cilia. Having low viscosity and some fluidity, the PCL can facilitate proper ciliary movement. The PCL also helps with lubricating the cell surface and enabling effective cough clearance. Being in the midst, the PCL acts as a barrier between the mucosal layer and the surface of the epithelium cells, thus it can entrap any foreign particles

coming from the exterior. A layer of mucus formed of glycosylated proteins (MUC5AC and MUC5B) covers the PCL [208,209], additionally, other types of mucins (MUC1 and MUC4) are also expressed on the epithelial cell surface. Over the PCL, all mucins form brush-like structures, thus, these anchored mucins physically act as an extra barrier against pathogens [210]. Underneath the mucus, the cilia move in a coordinated fashion to push the mucus with any trapped foreign bodies resting on top. Mucociliary clearance depends on normal mucus composition and production rates, healthy ciliary beating, together with voluntary coughing made by the host. Altogether, these factors play a large role in maintaining the sterility of the lung. Ineffective mucus clearance allows large amounts of bacteria to replicate within the lung epithelial lining which, in turn, triggers the host's innate immune defense cells; neutrophils and macrophages, to be recruited to the ASL following pathogen detection.

The two main hypotheses explaining CFTR dysfunction and lung infections in CF are; the "high salt hypothesis" and the "low volume hypothesis". The "high salt hypothesis" or "compositional hypothesis" propose that mutated CFTR causes higher salt levels in the ASL, than in normal conditions, which would inactivate antimicrobial factors which are abundant in ASL (e.g. defensins, lactoferrin), enzymes (e.g. lysozyme), proteases (e.g. neutrophil elastase), protease inhibitors (e.g. secretory leukocyte protease inhibitor, α1-antitrypsin) [211]. These antimicrobial factors can interrupt bacterial growth within 24 hours of exposure. In *in vitro* experiments [212], increasing the salt concentration of the ASL from non-CF cells caused inhibition of bacterial killing. The ASL obtained from nasal epithelium of CF patients had higher concentrations of salt compared to those from healthy individuals [212]. Applying fluid with low salt concentrations to the surface of CF cells restored normal bacterial killing.

The second hypothesis; "low volume hypothesis", propose that the over-activation of ENaC in CF disease leads to increased sodium absorption which results in increased chloride

absorption into epithelial cells *via* alternate chloride channels, such as the calcium-activated chloride channel (CaCC). In turn, this increased flux of ions into the cells induces osmosis pressure towards the epithelium, thus reducing the height of the ASL. Dehydration causes the mucosal layer to become more thickened and, hence, the ciliary beat is restricted by the low volume of the PCL and the overall mucociliary clearance is impaired [213]. Matsui *et al.* [213] observed that the salt content of the ASL is not higher in CF, instead its composition is different, and they also showed that the fluid from the ASL was absorbed into CF airway epithelial cells but not in normal cells. Their study also showed that the mucociliary movement is impaired in CF and this decrease in PCL causes the mucosal layer mucins (MUC5AC and MUC5B) to interact with those anchored to the cell surface. The mucins "sticky glue" makes mucus clearance very difficult and this thick layer of mucus provides a perfect environment for pathogen infection [214].

1.2.3.1 Pulmonary bacterial infections

Bacterial lung infections which are innocuous to healthy individuals chronically and persistently affect CF patients. In CF, the chronic lung infections are considered the greatest health care problem in terms of clinic visits and hospitalizations, treatments required, and eventually lead to the morbidities and mortalities related to the respiratory system. *Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacia* are common pathogens infecting the lungs of CF patients, however, *Pseudomonas aeruginosa* is the most prominent respiratory bacterial pathogen in CF, causing the most serious illness in those patients [215]. During younger age, CF patients become infected with *Staphylococcus aureus* and *Haemophilus influenzae*, on the other hand, older patients are colonized by *Pseudomonas aeruginosa*. It has been shown that 59% of Canadian CF adults are colonized with *Pseudomonas aeruginosa* [216]. The reason for that is the thick mucus layer in the lungs which favorize the colonization of *Pseudomonas aeruginosa* in the lungs where it remains away from the host defense mechanisms and any administrated antibiotics. Some bacterial

populations can be eradicated from the lungs while in the stage of initial infections, however, some colonies remain and adapt themselves to the CF lung environment by modifying their gene expression over time to confer antibiotic resistance [217]. Colonies of bacteria in the lung eventually form 'biofilms', or aggregates, by which they switch to starvation mechanisms to survive nutrient depleted environment. Biofilms are highly resistant to antibiotic treatments which results in more difficulty with providing effective therapy to CF patients [218].

1.2.4 Animal models of CF disease

The first generated mouse model KO for *Cftr* was done at University of North Carolina by inserting the neomycin resistance gene into exon 10 of this gene. Although these mice had a clear phenotype of intestinal obstruction and other CF maladies related to the digestive tract, they failed to produce any phenotype related to the respiratory system [219]. Another drawback was the mixed genetic background of these mice, so the researchers started to backcross the mice to C57BL/6J mice and generate *Cftr* KO mice on B/6 background [220]. The *Cftr* KO mice produced by this backcrossing displayed strong lung inflammatory phenotypes even in the absence of infections. Other reports have described thick mucus lining around the airways [221], apparent lung disease along with the destruction of lung structure [221], strong infiltration of immune cells into the airways [222] and over expression of inflammatory genes in the lung specimens [222].

Other *Cftr* KO mouse models were made to express specific *Cftr* mutations such as F508del mutation, $Cftr^{tm2Cam}$ [223], but they have very low survival rate to the age of maturity (less than 5%). Additionally, different F508del mice were generated with better survival rates $Cftr^{tm1Kth}$ (40% survival rate) [224], and $Cftr^{tm1Eur}$ (90% survival rate) [225]. *Cftr* KO mice were also generated to express other mutations for example; G551D mutation ($Cftr^{tm2Hgu}$, 65% survival rate) [226], G480C mutation

(*Cftr*^{tm3Hgu}, survival rate similar to wild type mice) [227], and R117H mutation (*Cftr*^{tm2Uth}, survival rate 95%) [228].

Chronic intestinal obstruction of some of the generated *Cftr* KO mice has resulted in various intestinal phenotypes, hence, these mice needed a liquid Peptamen diet not only to avoid the intestinal blockage, but also to enhance the absorption of nutrients without the necessity to supplement the diet with enzymes. To bypass the problems related to the intestinal phenotypes, and consequently the need for special diets, gut corrected *Cftr*^{tm1unc}-Tg$^{(FABPCFTR)}$ mice, which can locally express CFTR in the gut, were generated [229]. In the intestine, these *Cftr* KO mice express the CFTR protein under the control of human fatty acid binding protein (FABP) promoter. Despite not having severe intestinal pathology, unfortunately, these mice showed no chronic lung phenotype which resembles the disease in CF patients. Based on the current mentioned examples of generating *Cftr* KO mice, it is clearly evident that each generated mouse model demonstrated different phenotypes of CF disease [230], consequently, each research group needs to select the model which best fits into the scope of their research. The differences of the resulting phenotypes of these mouse models mimicking CF disease are important to consider when comparing results from various studies [231–234].

1.2.5 Therapies for CF

There is no cure for CF, however, several treatment approaches are used. Over decades, the management of CF has been improved significantly. In the past, CF infants were unlikely to live beyond their first year, nowadays, CF patients are likely to live well beyond the age of adulthood [235]. The treatment regimens for CF patients involve oral pills, injections and inhaled medications to address thick mucus, acute and chronic infections, malabsorption problems, respiratory and digestive issues. Despite numerous therapies introduced to fight the wide range of

pathologies in CF, patients may still experience respiratory failure, consequently, the only solution remaining will be lung transplantation which has its own complications. Overall, it is strongly believed that the CF therapy is in large need for many pharmaceutical advances.

1.2.5.1 Antibiotics

Antibiotic therapy remains the cornerstone of CF therapy. Numerous CF patients always administer one or more antibiotics, even when healthy, to prophylactically prevent infections. Whenever a CF patient presents clinically with an acute exacerbation of pneumonia, or suspected pneumonia, antibiotics are necessary to treat the morbidity and reduce the mortality [236].

Tobramycin, a broad-spectrum aminoglycoside covering gram positive and negative bacteria, is the recommended anti-pseudomonal antibiotic to treat CF patients in nebulized form [237]. Chronic and frequent aminoglycoside antibiotics usage can cause kidney failure, hearing loss and damage to the balance system in the inner ear [238]. Macrolides, particularly azithromycin, which spectrum covers gram positive and negative bacteria but not *Pseudomonas aeruginosa*, have been used in improving lung function and decreasing pulmonary exacerbation events and have been recommended for CF patients [239].

1.2.5.2 Mucus breakdown therapies

In addition to the thick mucosal layer in CF lungs, high amounts of DNA, resulting mostly from necrotic neutrophils, are also present [240]. Dornase alfa which is inhaled recombinant human deoxyribonuclease (DNase) helps break down the excessive amounts of DNA in mucus and has been proven to benefit CF patients. Dornase alfa reduce the rate of lung function decline compared to non-treated patients, and additionally, the number of pulmonary exacerbations was reduced [240,241].

1.2.5.3 Anti-inflammatory drugs

Many clinical trials demonstrated that using inhaled corticosteroids in CF showed insufficient evidence of benefit to CF patients [242]. Thus, there are no anti-inflammatory drugs routinely prescribed to CF patients, however, they are used to treat pulmonary exacerbations for short periods of time.

Ibuprofen, a non-steroidal anti-inflammatory drug (NSAIDs), lowers the levels of pro-inflammatory prostaglandins derived from the PUFA arachidonic acid (AA) by inhibiting the enzyme cyclooxygenase-2 (COX-2). Ibuprofen use for CF is still debated. Some studies support the use of Ibuprofen in CF as it retards lung function deterioration in patients [243], while others describe no benefit in administrating Ibuprofen [244]. Ibuprofen poses a risk of gastrointestinal bleeding in all patients treated with it including the CF patients [245].

1.2.5.4 Therapies involving correcting protein defects

"Correctors" are molecules used to assist the folding of the mutated CFTR protein. "Potentiators" are used to improve channel gating depending on the type of CF mutation. Ivacaftor is a leading oral medication taken by mouth and is used as a potentiator for the G551D mutation by improving the defect in channel gating. Although Ivacaftor improved, to significant levels, the lung functions in CF, the major drawback of this drug is that it can only help 5% of patients who have this specific *CFTR* mutation.

In July 2015, the FDA approved Ivacaftor/Lumacaftor (brand name: Orkambi) [246] for CF treatment, and in 2018, the combination of Ivacaftor/Tezacaftor (brand name: Symdeko) was approved by FDA with a list price of $292,000 per year [247]. Lumacaftor (VX-809) acts as a chaperone during protein folding thus resulting in elevating the number of CFTR proteins that are trafficked to the surface of the cells [248]. Tezacaftor can help CF patients with F508del mutation by moving the CFTR protein to on the cell surface [249]. Until a comprehensive solution is discovered

for all the disease genotypes, the current therapies routinely used for CF patients focus on controlling lung bacterial infections and improving mucus clearance by reducing mucus viscosity.

1.2.5.5 Therapies involving correcting gene defects

Ultimately for curing CF, scientists can directly correct the mutated *CFTR* by inserting a normal copy of this gene into the lungs [250]. Virtually, gene therapy seems to be an attractive solution because only 5–10% of the normal amount of *CFTR* gene expression is needed [251].

As of right now, gene therapy is still in research stages because the lung has not been proven to be the a good candidate organ for gene delivery due to its wide range of defense mechanisms [252]. Moreover, thick mucosal layer and viscous PCL hinders gene therapy to reach epithelial cells. It has been demonstrated that non-viral delivery systems can overcome the problems of viral vectors which have many health issues in gene therapy [253]. Other research has demonstrated that delivering *CFTR* mRNA is more successful than attempting to insert the large DNA fragment of *CFTR* [254]. Clinically, gene therapy still requires more years of investigation before it can become available to treat CF patients [252,255].

1.3 Fenretinide

1.3.1 Retinoids

Retinoids, among which Fenretinide is a synthetic derivative, are the family of chemical compounds that are vitamers of vitamin A. Retinoids have several forms: retinol, retinal, retinoic acid, retinyl ester and the provitamin carotenoids such as β-carotene [256]. The metabolism of retinoids, which occurs after ingestion and upon their entry into the circulatory system, is a complicated process involving many steps. Two major biochemical reactions are involved in the metabolism of retinoids; esterification and hydrolysis. Once in the blood stream, the retinoids bind to retinol binding protein (RBP) because of being very hydrophobic. For further transport in the

blood, the retinol-RBP complex is bound to transthyretin. It was shown that the binding with transthyretin prevented the loss of the retinol-RBP complex through renal filtration since the size of RBP is small [257].

Once the complex arrives at the cell surface, the retinoid-RBP binds to STRA6 which enables the cellular uptake of the retinoid. Depending on the site of action, the retinoid can be further processed prior to its biological utilization. For example, in the eye retinol is converted to retinal, which is the active retinoid in the eye, and then to retinoic acid for further effects in cells of the eye [258]. Beside their function in maintaining vision, development, and reproduction, retinoids are involved in cell growth, differentiation and apoptosis, hence their therapeutic applications were exploited in the treatment of cancer [259], however, their use in patients was limited due to the risk of toxicity [260].

1.3.2 Fenretinide: A vitamin A synthetic derivative

The use of dietary retinoids has resulted in many limitations; hence, synthetic variants of retinoic acid were synthesized to overcome these limitations. Fenretinide [N-4-hydroxyphenyl retinamide], a vitamin A synthetic derivative, was first synthesized in 1960s by R.W. Johnson Pharmaceuticals (now part of Johnson and Johnson) [261]. Fenretinide has a chemical structure similar to retinoic acid except for containing an amide linked 4 hydroxyphenyl group, which replaces the carboxyl polar end group of retinoic acid. The addition of the bulky, and lipid soluble, 4-hydroxyphenyl group is responsible for the beneficial properties associated with the molecule of Fenretinide, as compared to the other retinoid compounds such as retinoic acid [256,259,261].

Fenretinide was proven to be effective against cancer cells and was found to have limited toxicity [262]. The first report of Fenretinide as an anticancer agent was published in 1979 where it inhibited breast cancer in rats [263]. Subsequently, numerous studies have investigated the use of

Fenretinide in many other clinical settings. Fenretinide showed promising physiologic effects and beneficial pharmacologic actions in acne, psoriasis, rheumatoid arthritis [261], and Stargardt's disease [264]. Furthermore, our laboratory has shown that Fenretinide can correct the inflammatory responses in allergic asthma [265], cystic fibrosis [266,267] and spinal cord injury [268].

1.3.3 Fenretinide formulations

The previously used Fenretinide formulation was large capsules with Fenretinide powder suspended in corn oil which, despite being added to a fat-soluble vehicle, had low absorption. This dosage form was adequate for macular degeneration treatment trials [269], where low doses of the medication were required, but not for other clinical trials investigating Fenretinide in other clinical settings. Also, the big size of the capsule used for this formulation had a bad impact on patient compliance, especially when multiple capsules were needed to achieve a high dose. Several, past and current, studies are focused on reformulating the drug, as micelles [270], liposomes [271], nanoparticles [272], microspheres [273], hydrophilic conjugates [274], organo-gels [275], or oral mucoadhesive patches [276], to increase its bioavailability [262].

Recently, a once-a-day novel oral dry formulation of Fenretinide (LAU-7b), with high bioavailability, was developed by Laurent Pharmaceuticals Inc. and it provides new promise for its potential application in many diseases associated with chronic inflammation. LAU-7b was recently tested in adult cystic fibrosis patients chronically infected with *Pseudomonas aeruginosa* in a phase Ib clinical trial study, showing an excellent safety and promising pharmacokinetic and pharmacodynamic results (NCT02141958). Currently, LAU-7b is being tested in APPLAUD (A double-blind, randomized, Placebo-controlled, Phase 2 study of the efficacy and safety of LAU-7b in the treatment of Cystic Fibrosis in adults) for the treatment of patients suffering from cystic fibrosis (NCT03265288).

The first experimental part (chapter two) of this thesis project involved testing both the treatment efficacy and the medication dose of this novel formulation of Fenretinide (LAU-7b) in a mouse model of allergic asthma. The second and third experimental parts (chapter three and four) examined further the use of LAU-7b formulation in genetically altered mouse models for allergic asthma and Cystic fibrosis, respectively. Studying the novel formulation of Fenretinide (LAU-7b) using different animal models is a substantial step before, and during, the application of this medication clinically in both diseases.

Fenretinide use in the clinical studies has demonstrated an excellent safety profile, unlike naturally derived retinoids that cause liver toxicity in large doses or with prolonged exposure. Natural retinoids are metabolized in the liver to retinyl esters, stored in the liver, and subsequently causes hepatic toxicity. In contrast, it has been shown that Fenretinide and its major metabolites are preferentially stored in fat tissues, such as the mammary glands, in both animal models [263] and human studies [277]. This fact is highly advantageous not only to prevent Fenretinide hepatotoxic accumulation, but also to provide beneficial characteristics for the medication use in prevention and treatment of breast cancer, obesity and type II diabetes [278]. Having limited toxicity and very good patient tolerance, the adverse reactions to the drug appears after four weeks of treatment. The most common side effect experienced by patients is minor visual impairment, diminished dark adaptation, or nyctalopia (night blindness), which is believed to be a consequence of low levels of serum retinol. These adverse reactions were reversed when a three-day drug interruption was added into the treatment schedule (NCT02141958).

1.3.4 Mechanisms of action of Fenretinide

In cancer studies, treatment with Fenretinide caused a reduction in serum retinol and RBP levels [279]. Fenretinide does not inhibit the absorption of retinol [280], yet somehow the levels of

retinol were decreased after treatment with Fenretinide, most likely because of the effect on RBP, which is required for retinol transport [281].

Resembling retinol, Fenretinide binds to RBP because both molecules have high chemical structure similarity. Nevertheless, one major difference is that Fenretinide-RBP complex does not bind to transthyretin because of the modification of the hydroxyl group in Fenretinide [281]. It was suggested that Fenretinide-RBP complex is excreted through the kidneys, leading to lower levels of circulating RBP in serum and interfering with the transport of retinol, thus explaining the decrease in retinol levels with uninterrupted Fenretinide administration [281]. The rapid filtration of Fenretinide-RBP complex also explains why Fenretinide has a lower toxicity than the other retinoids.

Inside the cell, retinol is oxidized to retinoic acid. Retinoic acid binds to nuclear receptors, called retinoic acid receptors (RARs). There are several isoforms of RARs (α, β and γ) which bind both all-trans retinoic acid and 9-cis retinoic acid. The RARs bind to retinoic acid receptor elements (RAREs) on DNA to regulate the transcription of hundreds of genes [282]. Retinoid X receptors (RXR) α, β and γ selectively bind the cis form of retinoic acid with high affinity, in contrast to RAR which binds both cis and trans isoforms [283].

The regulation of gene expression by retinoids is a quite complex process. In the absence of ligands, the RXR heterodimerizes with RAR or PPAR (peroxisome proliferator-activated receptors (PPARs) and all are bound to hormone response elements complexed with corepressor protein. Binding of agonist ligands to RXR (or agonist ligands to RAR) results in dissociation of the corepressor and recruitment of coactivator protein, which, in turn, promotes the transcription of the downstream target genes [284].

Fenretinide was found to be a potent activator ligand for RARγ and a moderate activator for RARβ, in contrast, it acts as a repressor ligand for RARα and RXRα, so its pharmacological

effects are not identical to retinoic acid and the genes expressed can be different even when the same receptor is targeted [285]. Beside RAR, Fenretinide has been shown to act as an agonist for PPARγ receptor; which is a well-known transcription factor [286]. Additionally, not all cells express the same spectrum of receptors, for instance, macrophages express RARα and β, and RXRα and β [287]. Thus, the effect of Fenretinide is suggested to be specific for each cell type.

It was shown that Fenretinide can also act independently of RAR on cancer cells, affecting breast cancer cells which are resistant to retinoic acid treatment [288]. Then, it was observed that the induction of apoptosis in cancer cells by Fenretinide involved in part the increase of ceramide levels [289]. Collectively, the effect of Fenretinide in many cells appears to be independent of RAR activation.

In 2008, our group published for the first-time that CF patients, and a CF mouse model, have defects in ceramide levels [266]. Fenretinide treatment corrected the total ceramide levels in CF mice, and it enabled the CF mice to clear lung infections with *Pseudomonas aeruginosa* similarly to the wild type mice. In 2017, our laboratory published that Fenretinide reverses the imbalance of ceramides, by upregulating the levels of VLCCs (e.g. C24:0) and downregulating the levels of LCCs (e.g. C16:0) in CF mice [290]. This effect is achieved by downregulating Cers5 enzyme which produces LCCs when in homodimer conformation and VLCCs when in heterodimer conformation (with Cers2 enzyme) [290].

The studies presented in this thesis will demonstrate the effects of treatment with the novel formulation of Fenretinide (LAU-7b) in both allergic asthma (Chapter 2 and Chapter 3) and CF (Chapter 4). Here (Chapter 2), for the first time, we demonstrate that very long chain ceramides are diminished in genetically hyperresponsive A/J strain of mice in two different models of allergic asthma and can be corrected by treatment with Fenretinide. We also demonstrated (Chapter 3), for the first time, that Fenretinide significantly lowered the expression of *Ormdl3*, which directly

inhibits the first rate-limiting step of *de novo* ceramide biosynthesis, hence, protected the mice from HDM-induced allergic asthma. It is interesting that Fenretinide can protect the mice against allergic asthma by lowering the expression of *Ormdl3*, which we link by the results of the current study to the elevation of VLCC.

Teichgräber *et al.* [291] showed that accumulation of C16:0 causes death of pulmonary epithelial cells, increases susceptibility of CF KO mice to *Pseudomonas aeruginosa*, and results in overall age-dependent chronic lung inflammation in CF mice, however, in that report, C24:0 was not examined. In chapter 4 of this thesis, we were able to demonstrate that treating CF KO mice with Fenretinide for 21 days, which corrected the levels of both C16:0 and C24:0, protected against age-related deterioration of these mice as demonstrated by our physiology and histology results.

Based on the above, it is obvious that Fenretinide can exert its potential therapeutic effects *via* a wide range of biological mechanisms; the fact which opens many doors for numerous clinical applications. Based on the molecular and physiological nature of the disease, the study design needs to focus on one facet of the multiple mechanisms of Fenretinide. Collectively, dissecting the mechanisms by which Fenretinide can work in more than one chronic inflammatory disease (e.g. lowering the airway hyperresponsiveness and inflammation in asthma and CF) would provide a more comprehensive understanding of the full spectrum of activities of this promising drug.

1.4 Rationale, Hypothesis and Objectives

In 2008, Dr. Radzioch's laboratory discovered that the plasma of CF patients had a much lower concentration of very long chain ceramides (VLCCs: C24:0 and C26:0) [266]. Interestingly, our laboratory found that this imbalance in fatty acids and ceramides can be normalized using low doses of Fenretinide; FEN (N-(4-hydroxy-phenyl) retinamide) [266,290]. The subsequent study by Garic *et al.* [290] revealed that the downregulation of VLCCs in the plasma of CF patients and in CFBE41o- epithelial cells is accompanied by the increase in long chain ceramides (LCCs: C14 and C16:0). Furthermore, FEN treatment decreases the production of the inflammatory cytokines and chemokines. The *Cftr* KO mice treated with FEN following infection with *P. aeruginosa* showed a 10-fold decrease in the bacterial load of infected lungs [266].

FEN is lipid soluble, and insoluble in water, and the drug delivery to the mice had been challenging because of its low bioavailability, even when delivered with lipid-enriched food. The efficacy testing of this drug required developing better drug formulation, which was successfully achieved in 2015 by Laurent Pharmaceuticals Inc., a spin-off company which undertook the development of a new clinical formulation of the drug (Patent 780/16089.3). The development of LAU-7b, a dry formulation of FEN with superior bioavailability (20-fold better than the corn-oil formulation previously developed for cancer studies by Johnson & Johnson which was approved by FDA but rejected by Health Canada), opened a new possibility for basic and clinical research assessing its efficacy. Subsequently, our laboratory investigated both the safety and efficacy of LAU-7b in treating CF disease in mice and in patients (Phase I, Phase II trial APPLAUD in progress in 26 US CF Centers and 6 Canadian CF centers).

Since FEN treatment improves the resolution of lung infection and inflammation in *Cftr* KO mice, **our laboratory hypothesized that LAU-7b might also be very helpful in the control of**

lung inflammation caused by allergic asthma. However, many questions remain to be answered regarding the mechanism of action of this drug in asthma and its posology before LAU-7b can be used to treat patients suffering from allergic asthma.

OBJECTIVES AND HYPOTHESES:

CHAPTER 2: The results illustrated in this Chapter document the establishment of a reliable model of allergic asthma which allows testing the efficacy of the drug in preventing the pathological changes which occur in allergic mice (mice sensitized and challenged with allergen). **The main objective** of the designed studies was to find the optimal dose of the drug, route of administration and to establish the optimal duration of treatment which would allow us to demonstrate significant efficacy of the drug to treat the allergic asthma. **Specifically, we hypothesized that:** 1) The lungs of allergic mice will display regulatory imbalance in fatty acids and ceramides; 2) The treatment with a very low dose of LAU-7b (10 mg/kg), and for a relatively short time (9 days), will protect against allergic asthma induced by various allergens in atopic A/J mice which are genetically predisposed to airway hyperresponsiveness (AHR); 3) The optimized protocol of treatment with LAU-7b will normalize the distribution of ceramides composition in the lungs of atopic, and genetically prone to AHR, A/J mice and improve their inflammatory markers. The generated results addressing the above hypotheses are documented in **Chapter 2.**

CHAPTER 3: The main objective of the studies described in this chapter was to understand the mechanism of action of this drug. Given that one of the candidate regions identified in GWAS studies contained both *ZPBP2* gene and *ORMDL3* gene (involved in modulating ceramide biosynthesis), we focused our studies on the importance of the modulation of fatty acid and ceramide metabolism in allergic asthma. **We hypothesised that:** 1) Deletion of *Zpbp2* gene on A/J atopic genetic background will be able to validate the protective effect of the ablation of

Zpbp2 gene and the decrease in the expression of *Ormdl3* gene; 2) The normalizing effect of LAU-7b on the imbalance of the LCCs and the VLCCs is associated with its ability to inhibit expression of the *Ormdl3* gene. The experimental data addressing these two hypotheses are illustrated in **Chapter 3.**

CHAPTER 4: Lung physiology deteriorates with age, even in healthy people. The lung physiology parameters are always expressed as a score which is age-corrected (FEV1). Since we have seen significant improvement in lung physiology parameters in both allergic asthma and *Cftr* KO mouse models following treatment with LAU-7b, we wondered if the age-dependent deterioration in lung physiology could be prevented if the mice are treated with LAU-7b. Therefore, the main **objective** of the studies designed in this chapter was to assess if the age-dependent deterioration in lung physiology parameters is reversible in CF mice. **We hypothesized that:** 1) *Cftr* KO mice, compared to their littermate controls, will show significantly different lung physiology parameters due to chronic inflammation-induced changes in the lungs; 2) The *Cftr* gene deletion-associated difference in lung physiology will be more pronounced in older mice than younger mice; 3) The protective effect of LAU-7b treatment will prevent age-dependent deterioration of lung physiology parameters in *Cftr* KO mice. The results of the studies addressing these hypotheses are illustrated in **Chapter 4.**

Preface to Chapter 2

Fenretinide has been shown to correct the inflammatory responses in CF and allergic asthma. A novel oral dry formulation of Fenretinide with high bioavailability (LAU-7b) was developed by Laurent Pharmaceutical Inc. Our laboratory reported aberrant levels of the long chain ceramides (LCCs) and the very long chain ceramides (VLCCs) in both *Cftr* KO mice and CF patients carrying mutations in *Cftr* gene. We also investigated the safety and therapeutic potential of Fenretinide in treating CF disease and we discovered that the imbalance in levels of VLCCs and LCCs can be normalized following the treatment with LAU-7b.

Currently, LAU-7b is being tested in a phase II clinical trial in CF patients (NCT03265288), however, prior to the study described in this Chapter, LAU-7b has not been tested in any animal model of allergic asthma or asthmatic patients. The preclinical efficacy study results are needed to apply for the approval of a solid clinical protocol to treat patients who have allergic asthma, especially severe asthma, which is not adequately controlled by the currently available medications. Ceramide metabolism and the effect of LAU-7b treatment on the metabolism of ceramides has not been previously studied in the context of allergic asthma.

The hypotheses tested in the experiments illustrated in Chapter 2 are: 1) Treatment with a 10mg/kg/day dose of LAU-7b will be able to improve lung physiology in allergic mice; 2) Mice suffering from allergic asthma will display the imbalance between VLCCs and LCCs; and, 3) Treatment with a 10 mg/kg/day dose of LAU-7b will be able to correct the aberrant VLCCs/LCCs ratio in allergic mice.

The experimental work of Chapter 2 allowed the establishment of the protocol of treatment with LAU-7b in two allergic asthma mouse models (OVA and HDM). We also investigated the effect of Fenretinide on the levels of the LCCs and VLCCs in allergic asthma.

Chapter 2. Efficacy of optimized treatment protocol using LAU-7b formulation against ovalbumin (OVA) and house dust mite (HDM) -induced allergic asthma in atopic hypersensitive A/J mice

Manuscript submitted to Pharmaceutical Research:

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2.1 Abstract

Purpose: To assess the efficacy of the novel clinical formulation of fenretinide (LAU-7b) for the treatment of allergic asthma. To study the association between LAU-7b treatment in allergic asthma and the modulation of very long chain ceramides (VLCC).

Methods: We used two allergens (OVA and HDM) to induce asthma in mouse models and we established a treatment protocol with LAU-7b. The severity of allergic asthma reaction was quantified by measuring the airway resistance, quantifying lung inflammatory cell infiltration (Haematoxylin and eosin stain) and mucus production (Periodic acid Schiff satin). IgE levels were measured by ELISA. Immunophenotyping of T cells was done using Fluorescence-activated cell sorting (FACS) analysis. The analysis of the specific species of lipids and markers of oxidation was performed using mass spectrometry.

Results: Our data demonstrate that 10 mg/kg of LAU-7b was able to protect OVA- and HDM-challenged mice against increase in airway hyperresponsiveness, influx of inflammatory cells into the airways, and mucus production without affecting IgE levels. Treatment with LAU-7b significantly increased percentage of regulatory T cells and $CD4^+$ IL-10-producing T cells and significantly decreased percentage of $CD4^+$ IL-4-producing T cells. Our data also demonstrate a strong association between the improvement in the lung physiology and histology parameters and the drug-induced normalization of the aberrant distribution of ceramides in allergic mice.

Conclusion: 9 days of 10 mg/kg of LAU-7b daily treatment protects the mice against allergen-induced asthma and restores VLCC levels in the lungs and plasma.

Keywords: fenretinide, LAU-7b, allergic asthma, very long chain ceramides

2.2 Abbreviations

Table 2.1 List of abbreviations used in the manuscript (optimized treatment protocol using LAU-7b formulation against ovalbumin (OVA) and house dust mite (HDM) -induced allergic asthma in atopic hypersensitive A/J mice)

AA	Arachidonic acid
AHR	Airway hyperresponsiveness
DHA	Docosahexaenoic acid
ELISA	Enzyme-linked immunosorbent assay
FEN	Fenretinide
H&E	Haematoxylin and eosin
HDM	House dust mite
i.n.	Intranasal
i.p.	Intraperitoneal
MCh	Methacholine
MDA	Malondialdehyde
NT	Nitrotyrosine
OVA	Ovalbumin
p.o.	*per os*
PAS	Periodic acid Schiff
VLCC	Very long chain ceramides

2.3 *Introduction*

Allergic asthma is a complex heterogeneous disease affecting the airways of millions of people worldwide and this number is estimated to increase to 400 million by 2025 (1). The main features of allergic asthma are structural thickening of the alveolar epithelial and sub-epithelial layers, excessive mucus production, recruitment of inflammatory cells to the airways, airway hyperresponsiveness (AHR) and elevated IgE levels (2).

There is currently no cure for asthma. Nonetheless, the symptoms of asthma are treatable, allowing patients to achieve an acceptable quality of life. Current treatment protocols for managing asthma involve the use of inhaled and oral corticosteroids, selective β-2 agonists and leukotriene receptor antagonists (3). Unfortunately, in some patients, the treatments are not fully effective, and resistance to steroids develops over time. According to the Global Initiative for Asthma (GINA) 2019 statistics, 17% of asthmatics have difficult-to-treat asthma and 3.7% of asthmatics have severe asthma, consequently, identifying newer drugs and a better understanding of asthma molecular pathophysiology are required (1,4). Therefore, novel approaches and medications are needed to target the etiological basis of this debilitating disease, and to provide more effective control over life-threatening symptoms of severe and chronic allergic asthma.

Fenretinide (FEN), [N-(4-hydroxyphenyl)retinamide, 4-HPR], a vitamin A semi-synthetic derivative, has been shown by our laboratory to correct the inflammatory responses in cystic fibrosis (5,6), allergic asthma (7) and spinal cord injury (8).

Recently, a once-a-day novel oral dry formulation of fenretinide (LAU-7b), with high bioavailability, was developed by Laurent Pharmaceutical Inc. and it provides new promise for its potential application in many diseases associated with chronic inflammation. LAU-7b was recently tested in adult cystic fibrosis patients chronically infected with *Pseudomonas aeruginosa* in a

phase Ib clinical trial study, showing an excellent safety and promising pharmacokinetic and pharmacodynamic results (NCT02141958). Currently, LAU-7b is being tested in APPLAUD (A double-blind, randomized, Placebo-controlled, Phase 2 study of the efficacy and safety of LAU-7b in the treatment of Cystic Fibrosis in adults) for the treatment of patients suffering from cystic fibrosis (NCT03265288). However, the novel clinical formulation of fenretinide (LAU-7b) has not been yet tested in patients who have allergic asthma, or any other murine model of allergic asthma. Moreover, the preclinical efficacy study results are needed to apply for the approval of Clinical Protocol to treat patients who have allergic asthma, especially those with severe allergic asthma which is not well controlled with steroids.

Mouse models of allergic asthma reproducing the symptoms of the disease allow studying the etiology of asthma and testing efficacy and safety of novel therapies. Ovalbumin (OVA), house dust mite (HDM), fungi and ragweed are among the common allergens that have been used to induce allergic asthma in murine models. Using the OVA-induced model of allergic asthma, our laboratory demonstrated that treatment of mice with oral fenretinide (60 mg/kg body weight for 30 days) was able to correct lung physiology and various pro-inflammatory events including lung infiltration with inflammatory cells despite that no effect on IgE level was observed (7). Testing the efficacy of novel therapies using at least two different models of allergic asthma aids in preparing sufficient documentation acceptable for final approval by the regulatory agencies for testing the candidate drug in patients with asthma. Therefore, the preclinical anti-allergic asthma treatment protocol which we have initially developed using the OVA-model of allergic asthma (7) has been validated in the current study with a second model of allergic asthma using a natural human allergen. Since HDM represents the most common allergen causing allergic asthma in the Western world (9), we have chosen HDM-induced allergic asthma model for the validation studies.

We hypothesized that utilizing either fenretinide or its novel oral formulation (LAU-7b), using a low dose of 5 and 10 mg/kg, delivered intraperitoneal (i.p.) or by *per os* gavage (p.o.), will be sufficiently effective to protect against allergic asthma symptoms in atopic and airway hyperresponsive mice of the A/J strain. Overall, our study has evaluated the efficacy of LAU-7b for 9 days and it describes the optimized dosage of the drug and demonstrates excellent efficacy of 9-day long treatment protocol which has not been tried before.

2.4 Methods

2.4.1 Animal Models of OVA- and HDM-induced Allergic Asthma

Eight-week-old (20–25 g) A/J mice of both sexes were purchased from Jackson laboratories, Maine, USA. Mice were maintained on twelve hours light and twelve hours dark cycle. All experimental procedures were approved by the Animal Care Committee of McGill University Health Centre, Montreal, QC, Canada. At the age of eight weeks, mice were sensitized by i.p. injection of either OVA or HDM allergen every week for three weeks (figure 1).

For the OVA model; 100 µg of OVA (Albumin from chicken egg white, Cat: A5503, Sigma Aldrich, Saint Louis, MO, USA) adsorbed to 1.5 mg aluminum hydroxide adjuvant (Imject Alum, Cat: 77161, Thermo Scientific, Pierce, Rockford, IL, USA), in a total volume of 200 µL sterile PBS was used for sensitization. One week following the third systemic sensitization with OVA, daily challenges for three consecutive days were achieved by aerosol exposure to 1% OVA solution for 30 minutes.

For the HDM model; 5µg of HDM protein extract (*Dermatophagoides Pteronyssinus*, Cat: XPB82D3A2.5, Greer lab, Lenoir, NC, USA), adsorbed to 1.5 mg of aluminium hydroxide adjuvant (Imject Alum, Pierce, Rockford, IL, USA), in a total volume of 200 µL sterile PBS was

used for sensitization. One week following the third systemic sensitization with HDM, daily challenges for three consecutive days were administered by intranasal (i.n.) exposures to 1mg/ml of HDM using 15µL PBS per nostril.

To compare i.n and i.p. routes of allergen sensitization, mice were sensitized by 25µg HDM (in 15µL PBS) intranasally every week for three weeks. One week following the third local sensitization with HDM, consecutive three daily challenges were achieved i.n. and i.p by exposures to 1mg/ml of HDM using 15µL PBS per nostril. These experiments were performed to compare the effect of two routes (i.n. and i.p) of HDM sensitization on lung infiltration and lipid profiles (supplementary data).

2.4.2 Fenretinide Formulation

To test the effectiveness of fenretinide i.p. doses, we used fenretinide powder (CAS: 65646-68-6, Cedarburg Pharmaceuticals Inc., Grafton, WI, USA) dissolved in 95% ethanol (Cat: P016EA95, Greenfield Specialty Alcohols Inc., Brampton, ON, CA). Fenretinide stock solution aliquots of concentration 20 mg/mL were prepared in 95% alcohol and kept at -20^{o}C, until preparing the working solution of 1 mg/mL on the day of each i.p. injection. Any unused portion of the drug was discarded. Depending on the experimental protocol executed, each mouse was i.p treated with fenretinide either with 5 or 10 mg/kg body weight.

To test the effectiveness of LAU-7b *per os* (p.o.) doses, mice were gavaged with the contents of LAU-7b capsules reconstituted in methylcellulose. The LAU-7b capsules containing 100 mg of the active pharmaceutical ingredient (API) of N-(4-hydroxyphenyl)retinamide were obtained from Laurent Pharmaceuticals Inc. Methyl cellulose (Cat: M7027, Sigma Aldrich, Saint Louis, MO, USA) at a concentration of 0.5% in milliQ purified water was used as a drug vehicle to disperse the content of LAU-7b capsule (API and the necessary excipients). The hard-shell

gelatin capsules were opened, and the contents of each capsule were dispersed in 10 mL of 0.5% methylcellulose solution (vehicle) to prepare fenretinide stock suspension aliquots of concentration 10 mg/mL. Stock aliquots were kept at -20^{o}C until further dilution using the same vehicle to achieve the final solution of the drug at the concentration of 1 mg/mL. The final solution was kept for a maximum of 72 hours at 4^{o}C and any unused portion of the drug prepared for any given day was discarded. Feneretinide was administered by gavage at 10mg/kg.

2.4.3 Mouse Groups and Batches

As summarized in table 2.2, mice were sensitized with either OVA or HDM. Following sensitization, mice were divided into the following groups: non-treated and PBS-challenged (or negative controls; PBS), non-treated and allergen-challenged (or positive controls; OVA or HDM), treated and allergen-challenged (or treated mice with fenretinide or LAU-7b). OVA challenged mice were treated with either fenretinide i.p. 5 or 10 mg/kg or LAU-7b 10 mg/kg p.o. HDM challenged mice were only treated with LAU-7b 10 mg/kg p.o. For simplification; the mouse groups are presented as “PBS”, and “OVA” or “HDM”, and “FEN” or “LAU-7b”.

Another group of mice was neither allergen-sensitized, nor challenged but the mice in this group were treated with fenretinide i.p. 10 mg/kg or LAU-7b 10 mg/kg p.o. The data collected from these mice were only used as controls for lipid levels evaluation and are presented as “FEN-CTR” or “LAU-7b-CTR”.

Additionally, a group of mice was neither sensitized to an allergen, nor challenged or drug treated. The plasma collected from this group “IgE-CTR” was used as naïve controls to establish the baseline level of IgE and it was only used as a control group for IgE levels evaluation. Each figure legend summarizes the number of mice in each experimental group. The results collected from all experiments were pooled together to make final graphs.

Table 2.2 Mouse groups and batches used in the manuscript (optimized treatment protocol using LAU-7b formulation against ovalbumin (OVA) and house dust mite (HDM) -induced allergic asthma in atopic hypersensitive A/J mice)

Study groups and their abbreviations used in this study. Mice were sensitized with either ovalbumin (OVA) or house dust mite (HDM) and treated with either Fenretinide 5 or 10 mg/kg or the novel formulation of Fenretinide (LAU-7b) 10 mg/kg. Oral treatments are abbreviated p.o. and intraperitoneal injections are abbreviated i.p.

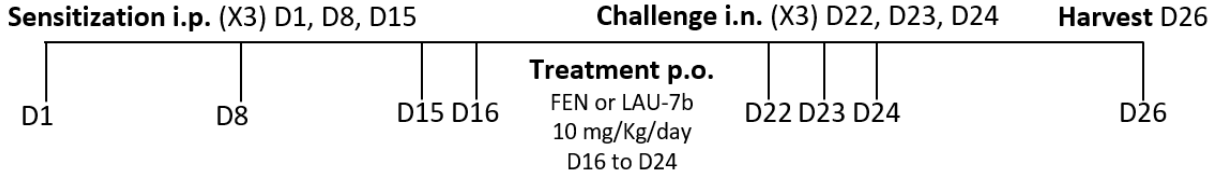
Group Name	**Sensitization**	**Challenge**	**Treatment**	**Group Description**
PBS	OVA or HDM	PBS	Vehicle	Negative controls for OVA or HDM model
OVA	OVA	OVA	Vehicle	Positive controls for OVA model
HDM	HDM	HDM	Vehicle	Positive controls for HDM model
FEN 5 or 10	OVA	OVA	Fenretinide 5 or 10 mg/kg	Fenretinide i.p. 5 or 10 mg/kg treatment for OVA model
LAU-7b / OVA	OVA	OVA	LAU-7b 10 mg/kg	LAU-7b p.o. 10 mg/kg treatment for OVA model
LAU-7b / HDM	HDM	HDM	LAU-7b 10 mg/kg	LAU-7b p.o. 10 mg/kg treatment for HDM model
FEN-CTR or LAU-7b-CTR	N/A	N/A	Fenretinide 10 mg/kg or LAU-7b 10 mg/kg	Controls for lipid levels evaluation
IgE-CTR	N/A	N/A	N/A	Controls for IgE baselines

2.4.4 Fenretinide Treatment Protocols

Two doses of fenretinide (5 or 10mg/kg) i.p. were tested in OVA-sensitized and challenged mice. LAU-7b was tested at a dose of 10mg/kg p.o. using both allergic asthma models (OVA and HDM- sensitized and challenged mice). We tested a "sequential" protocol (figure 1) where the allergen-sensitized mice were treated with either fenretinide i.p. or LAU-7b p.o. for 9 days starting from the day after the third sensitization until the day of the last challenge. Harvesting of animals for both models was done at 48 hours following the third allergen challenge.

Figure 2.1 Illustration of i.p Fenretinide treatment protocol and p.o LAU-7b treatment protocol used to treat mice with allergic asthma.

5 or 10 mg/kg of intraperitoneal Fenretinide or 10 mg/kg oral LAU-7b was used to treat the mice daily starting from day 16 to 24. Harvesting the mice was done at 48 hours with respect to the third challenge. i.p., i.n., p.o. are intraperitoneal, intranasal, and oral routes of allergen administration, respectively.

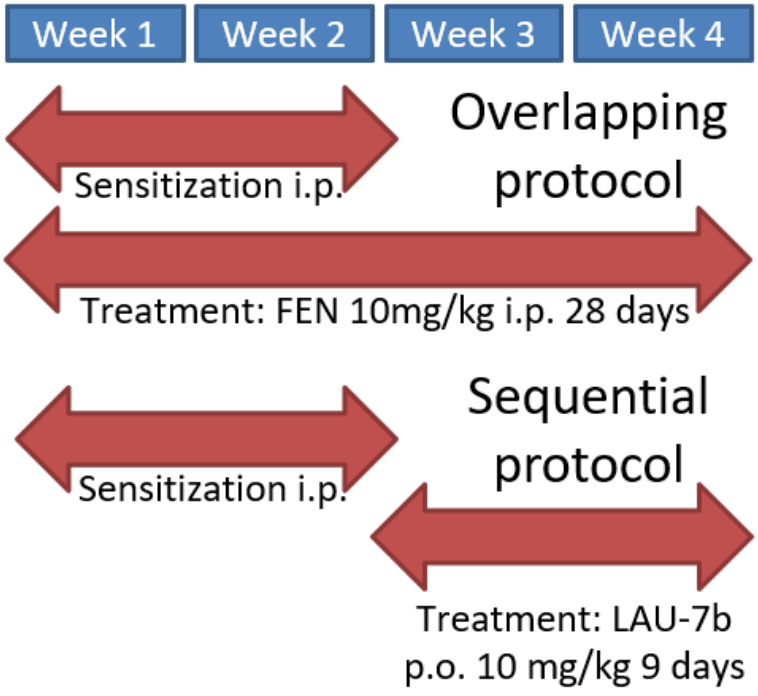


We also used another "overlapping" protocol to investigate whether the IgE levels would get inhibited if fenretinide treatment would be administrated at the same time as the first sensitization (figure 2). We injected the mice i.p. for 28 consecutive days with fenretinide starting from the first sensitization day. Like the sequential protocol, mice undergoing the overlapping

protocol were exposed to 3 sensitizations (one every week) and 3 consecutive daily challenges on the fourth week. Harvest was done at 48 hours with respect to the third challenge. The overlapping protocol of treating the mice with fenretinide for 28 days was only used to pool results for IgE levels in plasma.

Figure 2.2 Illustration of the models used in 28-day p.o. treatment protocol and in 9-day p.o LAU-7b treatment protocol.

Both protocols consisted of 3 weekly i.p. sensitizations of the corresponding allergen. Treatment starts on the day of the first sensitization in the 28-day protocol and therefore the period of sensitization overlaps with the treatment protocol, whereas in the 9-day treatment protocol using LAU-7b the treatment starts the day after the third sensitization. The 28-day protocol of Fenretinide treatment was only used to establish if the IgE levels could be modulated if the treatment started at the time of sensitization. It is not clinically relevant treatment protocol.



2.4.5 Lung Resistance Measurements

Airway resistance was measured using a Buxco plethysmograph system (Buxco Research System, Wilmington, NC, USA), ventilators, and nebulizers (Harvard Apparatus, Holliston, MA, USA). Standard invasive lung resistance measurement was done at the day of harvest. Mice were anesthetized using a cocktail of ketamine (100 mg/kg, DIN: 02374994), xylazine (10 mg/kg, DIN: 02169592) and acepromazine (3 mg/kg, Cat: A7111, Sigma Aldrich, Saint Louis, MO, USA), and then the trachea was opened, and connected to the ventilator. A nebulizer was used to administer ascending doses of methacholine MCh (6.25, 12.5, 25, 50 mg/mL) (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.

2.4.6 Lung Histology Analysis

The left lung from each mouse was inflated with 10% buffered formalin (Cat: SF100-4, Fisher Scientific, Nepean, ON, CA) and then kept in formalin for fixation for 48 hours. Fixed lungs were subsequently rinsed in PBS, placed in the cassettes and embedded in paraffin (Formula R, Cat: 3801450, Leica Biosystems, Concord, ON, CA). Paraffin blocks were then sliced into 4 μm thick sections using Leica microtome. Lung sections were deparaffinized, hydrated, and stained with two histology stains to examine the effect of fenretinide on the airway tissues. Haematoxylin and eosin (H&E) staining was used to assess lung tissue inflammation and recruitment of different inflammatory cells to the airways. Periodic acid Schiff (PAS) staining was used to assess airway mucin and other poly saccharides production by the goblet cells. Quantification of drug treatment effect was done by counting the number of infiltrated inflammatory cells/or goblet cells hyperplasia in at least 4 airways/mice. The total numbers were averaged and normalized using the perimeter of the airway basement membrane.

2.4.7 Lipids and Markers of Oxidation Analysis

Lipid analysis was done in blood plasma and lung tissues collected following the final allergen challenge. 50 µL plasma preserved in 500 µL of 1 mM butylated hydroxyanisole (BHA) in chloroform:methanol solution (2:1 vol/vol) was used for the analysis of lipids in plasma. 25 mg mashed lung tissue preserved in 1 mL of the mentioned BHA solution was used for the analysis of lipids in the lungs. All BHA preserved samples were kept at -80^{o}C until analysis. Classical isolation of lipids was done as previously described by Folch (10), then the levels of different lipid species were measured using high-performance liquid chromatography - tandem mass spectrometry as we have previously described in detail (5).

2.4.8 IgE levels in plasma Analysis

On the day of the harvest, the blood was collected into tubes containing 10 µL EDTA (Invitrogen, Cat: 15575-038, Grand Island, NY, USA) using intra-cardiac puncture. Plasma was collected after spinning of the blood at 3000 rpm for 10 minutes at 4^{o}C. 25 µL of plasma was used for IgE measurements/quantification by ELISA following the manufacturer's instructions (BD OptEIA kit, Cat: 555248, BD Biosciences, San Diego, CA, USA).

2.4.9 Immunophenotyping of lung T cells

Lungs were injected with 1 mL of digestion buffer, 3 U/mL collagenase D, and 10 mg/mL DNase I through the trachea. The lungs were digested for 30 min at 37 °C and the resulting cell suspension were filtered as previously described in our report (11). T-cells were stained with the following specific antibodies purchased from BioLegend (CD45: PerCP, CD4: FITC, CD25: PE, IFNy: Pacific Blue, IL4: PE-Cy7, IL-10: APC) and fixable viability dye (FVD: eFluor780) and FOXP3: APC were purchased from eBioScience. After incubation in fixation/permeabilization buffer for 30 min at RT, lung cells were stained with FoxP3-APC (17577382, eBioScience) in

Perm/Wash buffer for 1 h at RT, washed in Perm/Wash buffer, then resuspended in FACS buffer as previously described (11). Samples were analyzed using a Fluorescence-activated cell sorting (FACS) Canto II on the same day. The flow cytometry data were analyzed using FlowJo version 10.1 software.

2.4.10 Statistical Analysis

The number of mice of each group is indicated in the legend of each figure. Data were pooled out from at all independent experiments for each analysis (at least 2 for each experiment). Data were analyzed using GraphPad Prism 6 (version 6.01; GraphPad Software Inc., San Diego, CA, USA).

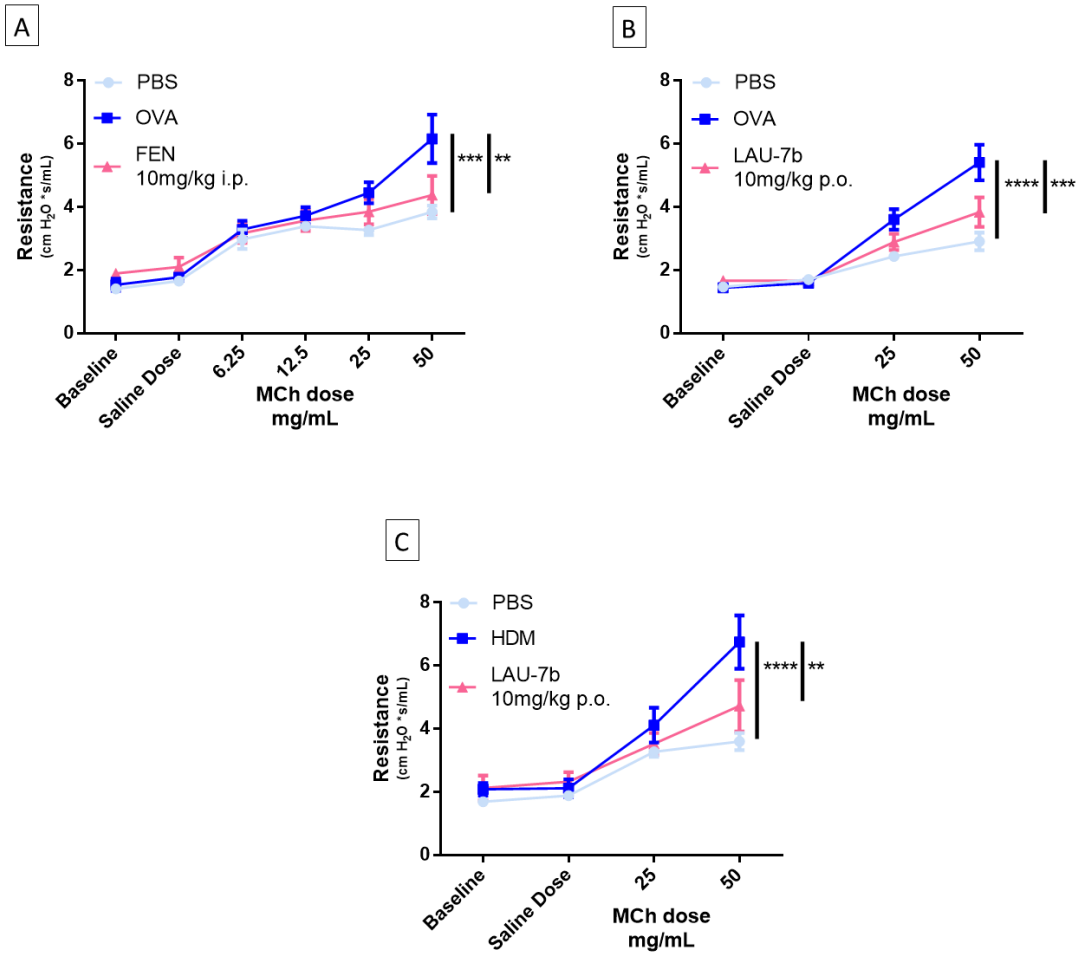
2.5 Results

2.5.1 Fenretinide protects the allergen-challenged mice from methacholine-induced airway hyperresponsiveness

The overall protective effects of the dose of 10 mg/kg of either fenretinide i.p. or LAU-7b p.o. on allergen-induced airway resistance was assessed using the invasive standard method (figure 3). Assessment of airway responsiveness demonstrated a significant increase in lung resistance in OVA-challenged mice compared to PBS challenged mice at escalating doses of MCh up to 50 mg/mL. Lung physiology of mice sensitized and challenged with HDM was assessed at doses of 25 and 50 mg/mL of MCh. Mice injected intraperitoneally with fenretinide 10mg/kg or treated *per os* with LAU-7b 10 mg/kg for 9 days and challenged with OVA (figure 3A and 3B) or HDM (figure 3C) displayed significantly lower airway hyperresponsiveness than allergen challenged non-treated mice.

Figure 2.3 Fenretinide (FEN) and the novel formulation of FEN (LAU-7b) at concentration of 10mg/kg prevent airway hyperresponsiveness (AHR) in Ovalbumin (OVA) and house dust mite (HDM) mouse models, respectively

(A) Metacholine (MCh) challenge causes a dose–response increase (6.25, 12.5, 25, 50 mg/mL) of AHR in OVA mice compared with PBS mice. Treatment with FEN 10 mg/kg i.p. before OVA challenge prevented AHR against MCh in allergic mice. (B) Treatment with LAU-7b 10 mg/kg p.o. before OVA challenge prevented AHR against MCh (25, 50 mg/mL) in allergic mice. (C) Treatment with LAU-7b 10 mg/kg p.o. before HDM challenge prevented AHR against MCh (25, 50 mg/mL) in allergic mice. Statistical significance was calculated by Two-way ANOVA (Tukey's multiple comparisons test), $n \geq 5$ mice in each group, data are presented as mean ± SEM, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

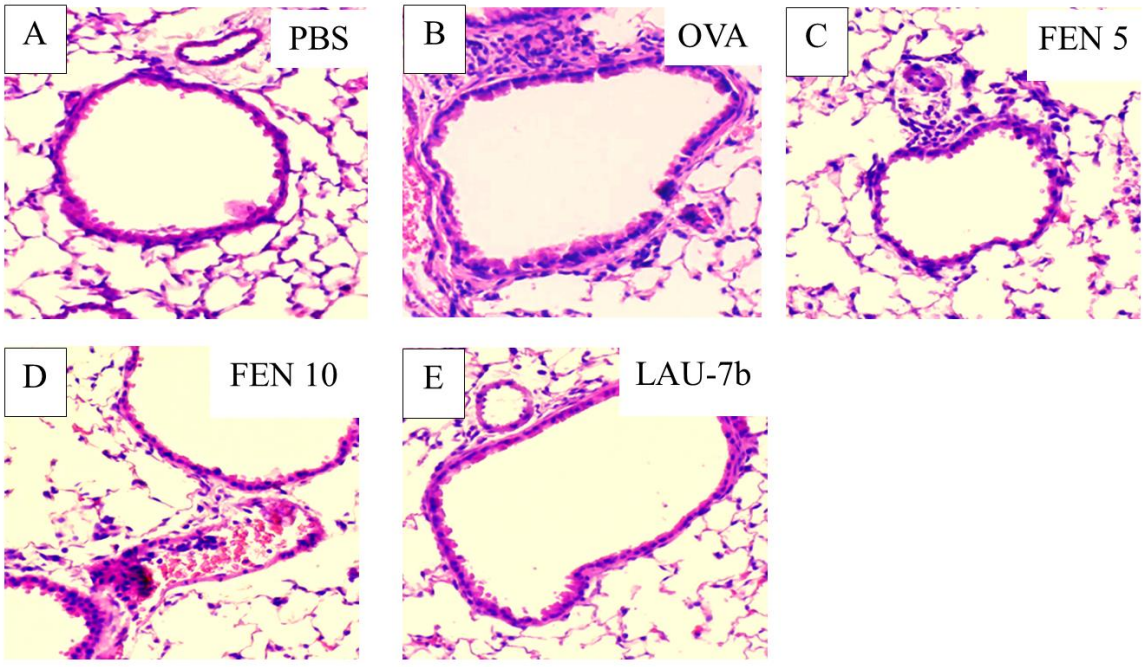

A
PBS
OVA
FEN
10mg/kg i.p.
Resistance
(cm H2O *s/mL)
***
**
Baseline
Saline Dose
6.25
12.5
25
50
MCh dose
mg/mL
B
PBS
OVA
LAU-7b
10mg/kg p.o.
Resistance
(cm H2O *s/mL)
****
***
Baseline
Saline Dose
25
50
MCh dose
mg/mL
C
PBS
HDM
LAU-7b
10mg/kg p.o.
Resistance
(cm H2O *s/mL)
****
**
Baseline
Saline Dose
25
50
MCh dose
mg/mL

2.5.2 Fenretinide prevented the influx of inflammatory cells to the airways of allergen-challenged mice

As shown by H&E staining, both OVA- and HDM-challenged and vehicle treated mice displayed a marked influx of inflammatory cells around the airways, compared to PBS- challenged and vehicle treated mice (figure 4 and 5). The oral dry formulation of fenretinide LAU-7b was able to significantly decrease the recruitment of inflammatory cells into the airways in both allergen models. Quantification of the inflammatory cells coming from the blood vessels to the lung airways showed a significant reduction in the number of infiltrating cells in fenretinide treated mice compared to untreated mice (figure 4 and 5). Treatment with 5 and 10 mg/kg fenretinide i.p. for 9 days was able to protect OVA-challenged mice against the influx of inflammatory cells (figure 4F). Similarly, treatment with 10 mg/kg LAU-7b p.o. for 9 days was able to protect OVA- (figure 4F) and HDM- (figure 5D) challenged mice against the influx of inflammatory cells.

Figure 2.4 Fenretinide (FEN) and LAU-7b prevent recruitment of inflammatory cells to the airways of ovalbumin (OVA) mouse model

Lung sections from (A) PBS–treated animals and challenged with PBS, (B) PBS–treated animals and challenged with OVA, (C) 5 mg/kg, (D) 10 mg/kg i.p. FEN–treated animals and challenged with OVA, and (E) 10 mg/kg p.o. LAU-7b–treated animals and challenged with OVA. Quantification of inflammatory cells around the airways for (F) OVA-FEN 5, 10 mg/kg i.p. and OVA-LAU-7b 10 mg/kg p.o. Inflammation surrounding at least four airways for each mouse was quantified by counting the number of inflammatory cells and dividing by the square of the perimeter of the basement membrane (Pbm^2). Statistical significance was calculated by One-way ANOVA (Tukey's multiple comparisons test). For PBS n = 9, OVA n = 9, FEN 5 mg/kg i.p. n = 8, FEN 10 mg/kg i.p. n= 9, LAU-7b 10 mg/kg p.o. n = 8. **** $p<0.0001$


A
PBS
B
OVA
C
FEN 5
D
FEN 10
E
LAU-7b

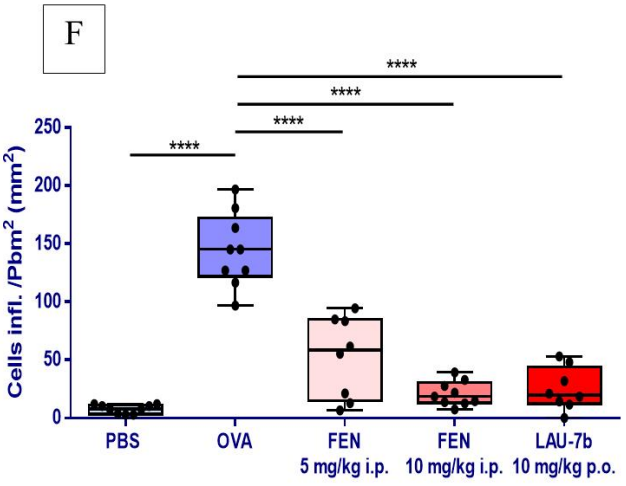
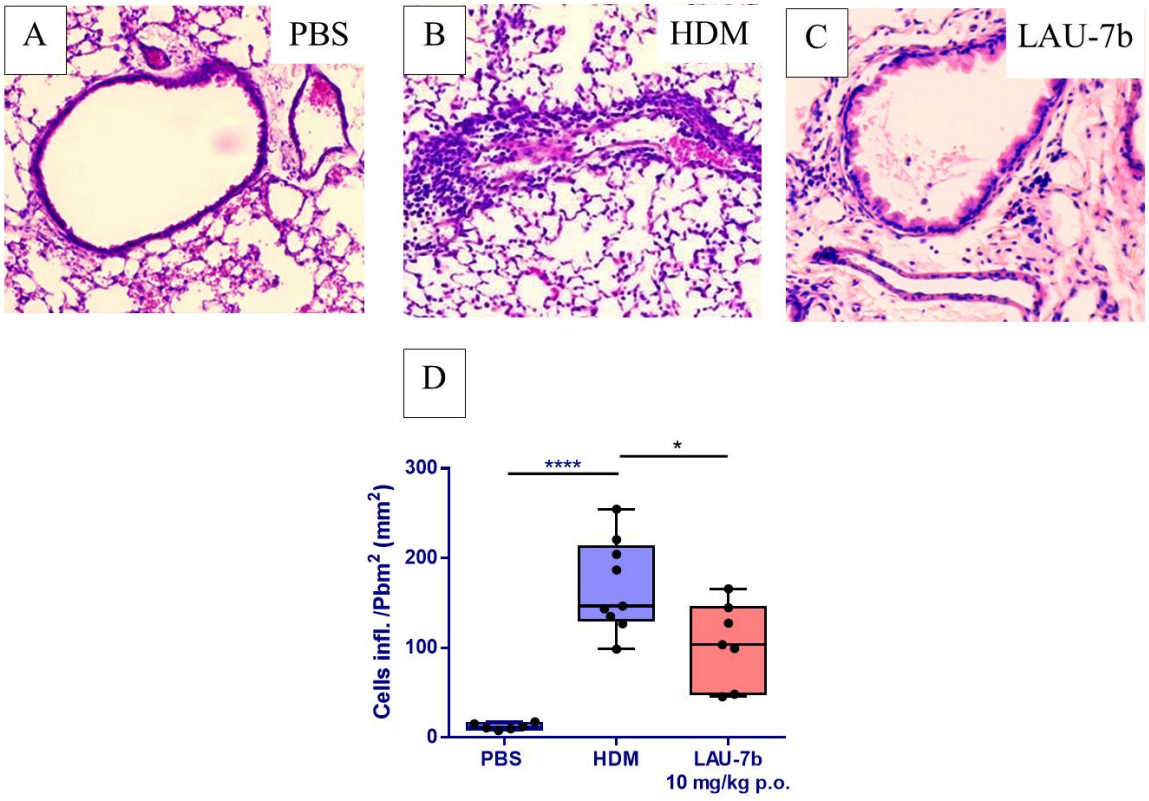

F
****
****
****
****
250
200
150
100
50
0
Cells infl. /Pbm2 (mm2)
PBS
OVA
FEN
5 mg/kg i.p.
FEN
10 mg/kg i.p.
LAU-7b
10 mg/kg p.o.

Figure 2.5 LAU-7b prevents recruitment of inflammatory cells to the airways of house dust mite (HDM) exposed mice

Lung sections from (A) PBS–treated animals and challenged with PBS, (B) PBS–treated animals and challenged with HDM, and (C) 10 mg/kg LAU-7b–treated animals and challenged with HDM. (D) Quantification of inflammatory cells around the airways for HDM- LAU-7b 10 mg/kg p.o. Inflammation surrounding at least four airways for each mouse was quantified by counting the number of inflammatory cells and dividing by the square of the perimeter of the basement membrane (Pbm^2). Statistical significance was calculated by One-way ANOVA (Tukey's multiple comparisons test). For HDM- LAU-7b 10 mg/kg p.o. PBS n = 6, HDM n = 9, LAU-7b 10 mg/kg p.o. n= 7. * $p<0.05$, **** $p<0.0001$

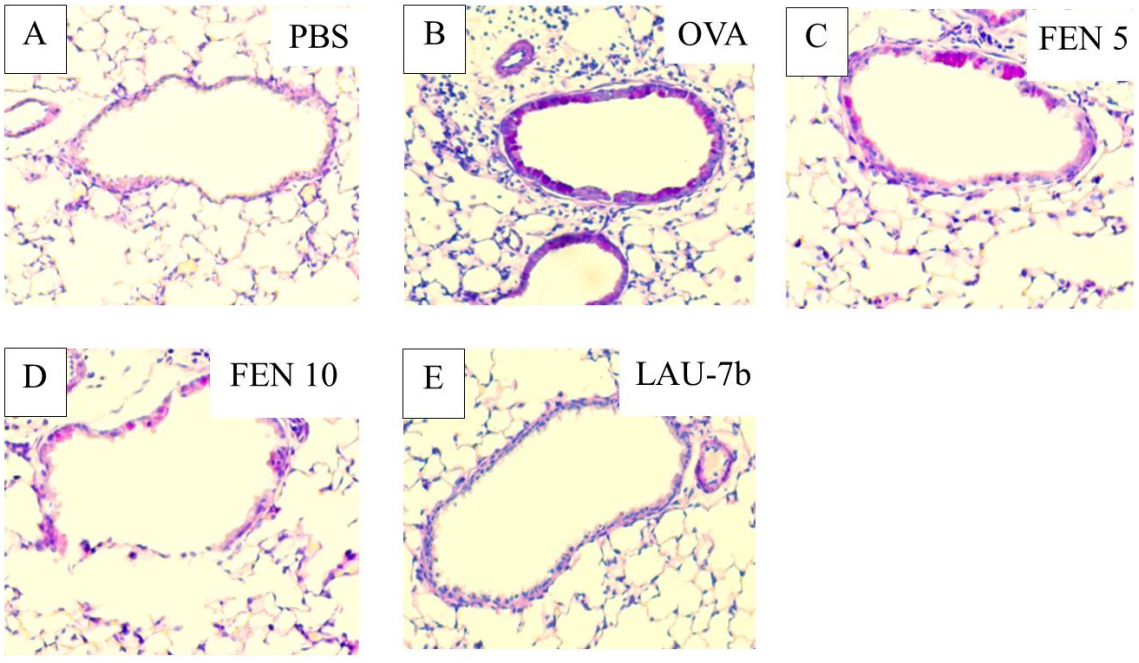

A
PBS
B
HDM
C
LAU-7b
D
****
*
300
200
100
0
Cells infl. /Pbm2 (mm2)
PBS
HDM
LAU-7b
10 mg/kg p.o.

2.5.3 Fenretinide decreased goblet cell hyperplasia in the airways of allergen-challenged mice

PAS staining assesses goblet cells hyperplasia and mucin production after allergen challenge. OVA- and HDM- challenged and untreated mice clearly displayed PAS-positive cells around the airways, compared to PBS- challenged and untreated mice (figure 6 and 7). Fenretinide 10 mg/kg i.p. or LAU-7b 10 mg/kg p.o. for 9 days was able to dramatically prevent goblet cells hyperplasia and mucin production around the airways in both allergen models. Again, quantification of PAS-positive cells around the airways showed a significant reduction in treated and challenged mice compared to untreated and challenged mice (figure 6 and 7). Treatment with 5 and 10 mg/kg fenretinide i.p. for 9 days was able to protect OVA-challenged mice against mucus production and Goblet cells hyperplasia (figure 6F). Likewise, mice treated for 9 days orally with 10 mg/kg LAU-7b formulation showed a very significant decrease in goblet cells hyperplasia as compared to OVA- (figure 6F) and HDM- (figure 7D) challenged and untreated mice.

Figure 2.6 Fenretinide (FEN) and LAU-7b the novel formulation of FEN prevent goblet cell hyperplasia of ovalbumin (OVA) mouse model

Lung sections from (A) PBS–treated animals and challenged with PBS, (B) PBS–treated animals and challenged with OVA, (C) 5 mg/kg, (D) 10 mg/kg FEN–treated animals and challenged with OVA, and (E) 10 mg/kg LAU-7b–treated animals and challenged with OVA. Quantification of periodic acid Schiff–positive cells around the airways for (F) OVA-FEN 5, 10 mg/kg i.p. and OVA- LAU-7b 10 mg/kg p.o. The number of periodic acid Schiff–positive cells in at least four airways from each animal in each group was determined and normalized by the perimeter of the airway basement membrane (Pbm). Statistical significance was calculated by One-way ANOVA (Tukey's multiple comparisons test). For PBS n = 9, OVA n = 9, FEN 5 mg/kg i.p. n = 8, FEN 10 mg/kg i.p. n= 9, LAU-7b 10 mg/kg p.o. n = 8. **** $p<0.0001$


A
PBS
B
OVA
C
FEN 5
D
FEN 10
E
LAU-7b

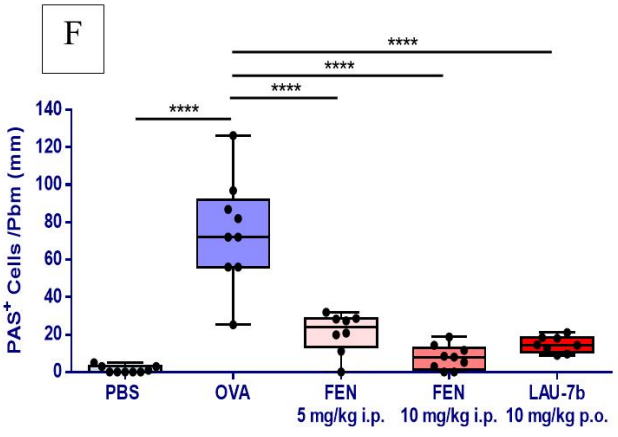
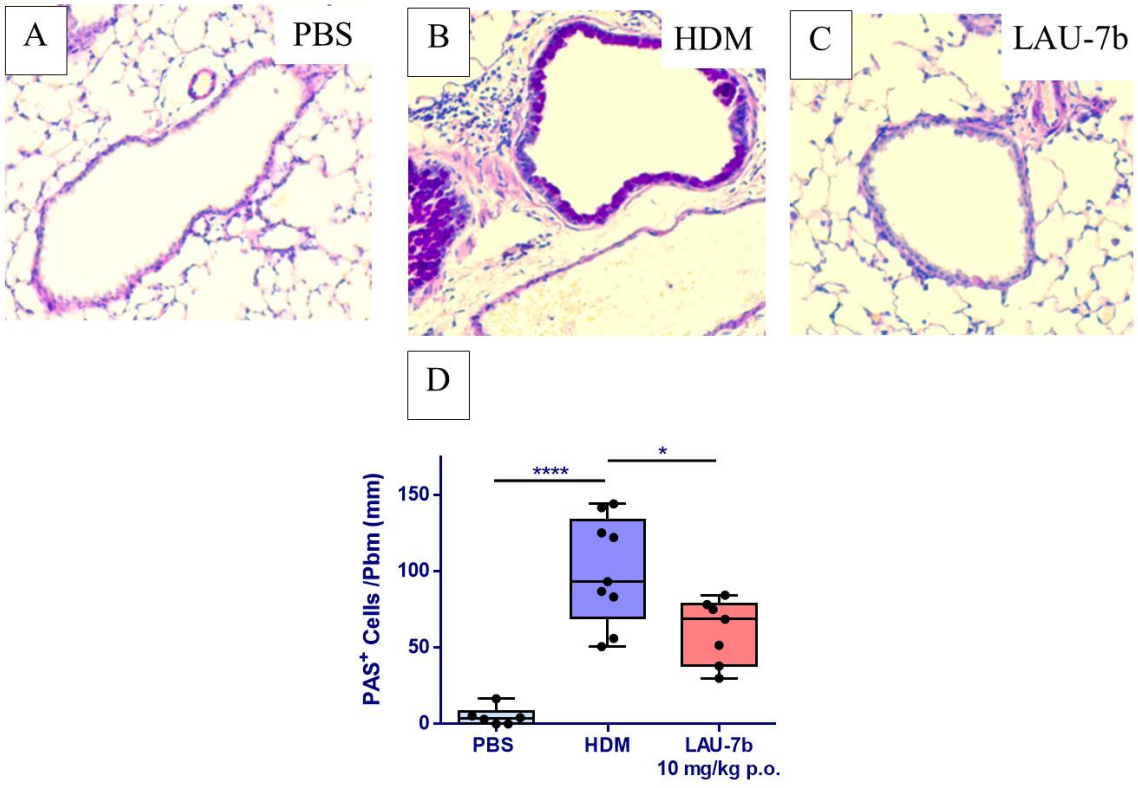

F
****
****
****
****
140
120
100
80
60
40
20
0
PAS+ Cells /Pbm (mm)
PBS
OVA
FEN
5 mg/kg i.p.
FEN
10 mg/kg i.p.
LAU-7b
10 mg/kg p.o.

Figure 2.7 LAU-7b the novel formulation of Fenretinide (FEN) prevents goblet cell hyperplasia of house dust mite (HDM) mouse model

Lung sections from (A) PBS–treated animals and challenged with PBS, (B) PBS–treated animals and challenged with HDM, and (C) 10 mg/kg LAU-7b–treated animals and challenged with HDM. (D) Quantification of periodic acid Schiff–positive cells around the airways for HDM- LAU-7b 10 mg/kg p.o. The number of periodic acid Schiff–positive cells in at least four airways from each animal in each group was determined and normalized by the perimeter of the airway basement membrane (Pbm). Statistical significance was calculated by One-way ANOVA. For HDM- LAU-7b 10 mg/kg p.o. PBS n = 6, HDM n = 9, LAU-7b 10 mg/kg p.o. n= 7. * $p<0.05$, **** $p<0.0001$


A
PBS
B
HDM
C
LAU-7b
D
****
*
PAS+ Cells /Pbm (mm)
150
100
50
0
PBS
HDM
LAU-7b
10 mg/kg p.o.

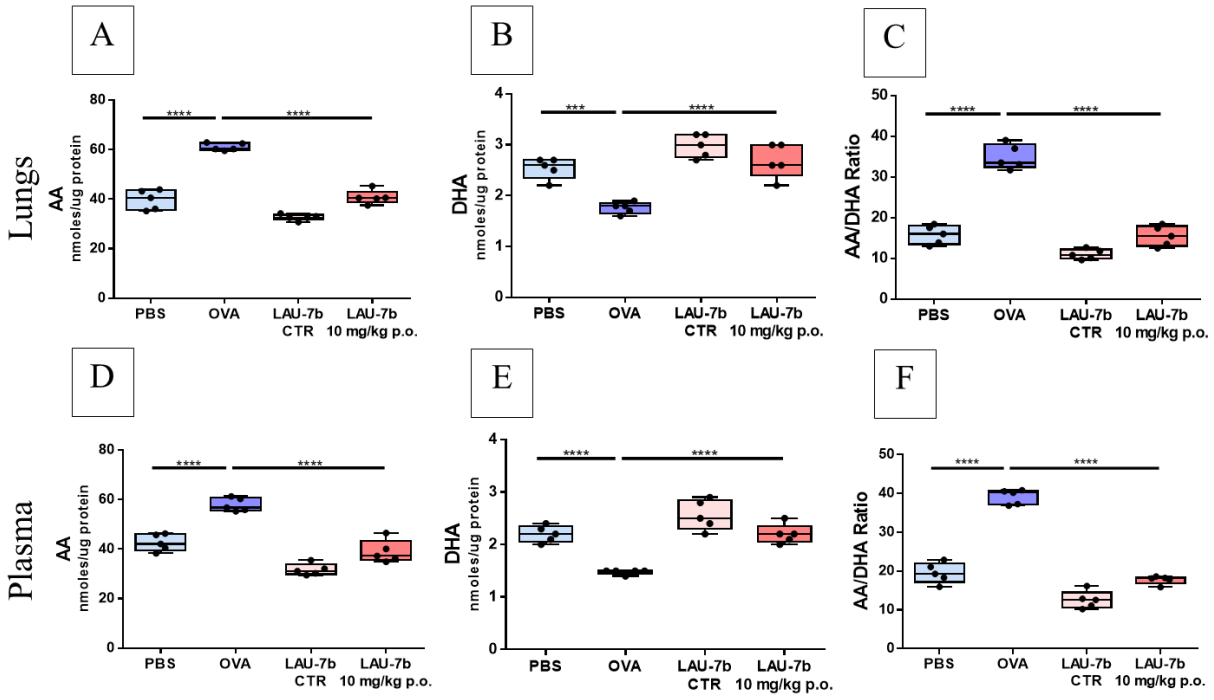
2.5.4 Lipid profile changes, after allergen challenges, were corrected by fenretinide treatment

Lipid profile analysis revealed changes in levels of arachidonic acid (AA), docosahexaenoic acid (DHA), and the ratio AA over DHA. After OVA sensitization, AA levels were significantly elevated and DHA levels were significantly decreased, and consequently, the overall ratio AA/DHA was increased after sensitization (figure 8). Treatment with 10 mg/kg of LAU-7b was able to dramatically decrease AA levels and increase DHA levels to the levels observed in non-allergic animals. The overall ratio of AA/DHA has been significantly improved in LAU-7b treated mice compared to untreated mice and was comparable to the AA/DHA ratio in healthy control mice.

We also investigated the effects of LAU-7b treatment (FEN-LAU-7b), but without OVA sensitization or challenge. Our results revealed that treatment with LAU-7b in naïve mice was even able to increase DHA and decrease AA and AA/DHA as compared to the sensitized and unchallenged mice (PBS) (figure 8).

Figure 2.8 LAU-7b prevents changes in the ratio of arachidonic acid (AA) to docosahexaenoic acid (DHA) in lungs and plasma of ovalbumin-challenged (OVA) mice

Top panel (A-C) for lipid analysis in lungs and bottom panel (D-F) for lipid analysis in plasma. After OVA challenge, mice have elevated levels of AA and reduced levels of DHA both in lungs (A and B) and plasma (D and E). The overall ratio of AA/DHA (C and F) were increased after OVA challenge. LAU-7b 10 mg/kg p.o. treatment corrected the AA, DHA, and AA/DHA imbalances. The baseline of lipid levels in lungs and plasma after LAU-7b 10 mg/kg p.o. treatment (without allergen sensitization or challenge) is demonstrated by the LAU-7b-CTR group of mice. Statistical significance was calculated by One-way ANOVA. Selected n = 5 in all groups, all points are shown. *** $p<0.001$, **** $p<0.0001$


A
B
C
D
E
F
Lungs
Plasma
AA
nmoles/ug protein
DHA
nmoles/ug protein
AA/DHA Ratio
PBS
OVA
LAU-7b CTR
LAU-7b 10 mg/kg p.o.
****
***

Next, we tested the levels of ceramides most abundant in the lungs and plasma. Our results demonstrated that C24:0 and C26:0 ceramides were significantly lower, compared to controls, in the lungs and plasma of mice sensitized and challenged with OVA (figure 9) or HDM (figure 10). The levels of long-chain ceramides C14:0 and C16:0 were significantly elevated in allergic mice compared to control mice (figure 9 and 10). Treatment with 10 mg/kg/day with LAU-7b p.o. for 9 days successfully normalized the imbalance in ceramides species by increasing the diminished levels of very long chain (C24:0 and C26:0) ceramides and decreasing levels of long-chain (C16:0 and C14:0) ceramides.

Figure 2.9 Changes in the levels of different ceramide species in lungs of mice challenged with OVA and treated with FEN 10 mg/kg i.p.

(A-B) Levels of long chain ceramides C14 and C16, (C) level of Ceramide-1-phosphate species (C1P), (D-E) levels of very long chain ceramides C24 and C26, and (F) level of Sphingosine-1-phosphate species (S1P). After OVA challenge, the long chain ceramides and C1P were significantly increased, and the very long chain ceramides and the S1P were significantly decreased. FEN 10 mg/kg i.p. treatment corrected the ceramides imbalances. The baseline of ceramide levels in lungs after FEN 10 mg/kg i.p. treatment (without allergen sensitization or challenge) is demonstrated by the FEN-CTR group of mice. Statistical significance was calculated by One-way ANOVA. $n \geq 5$ in all groups, all points are shown. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$

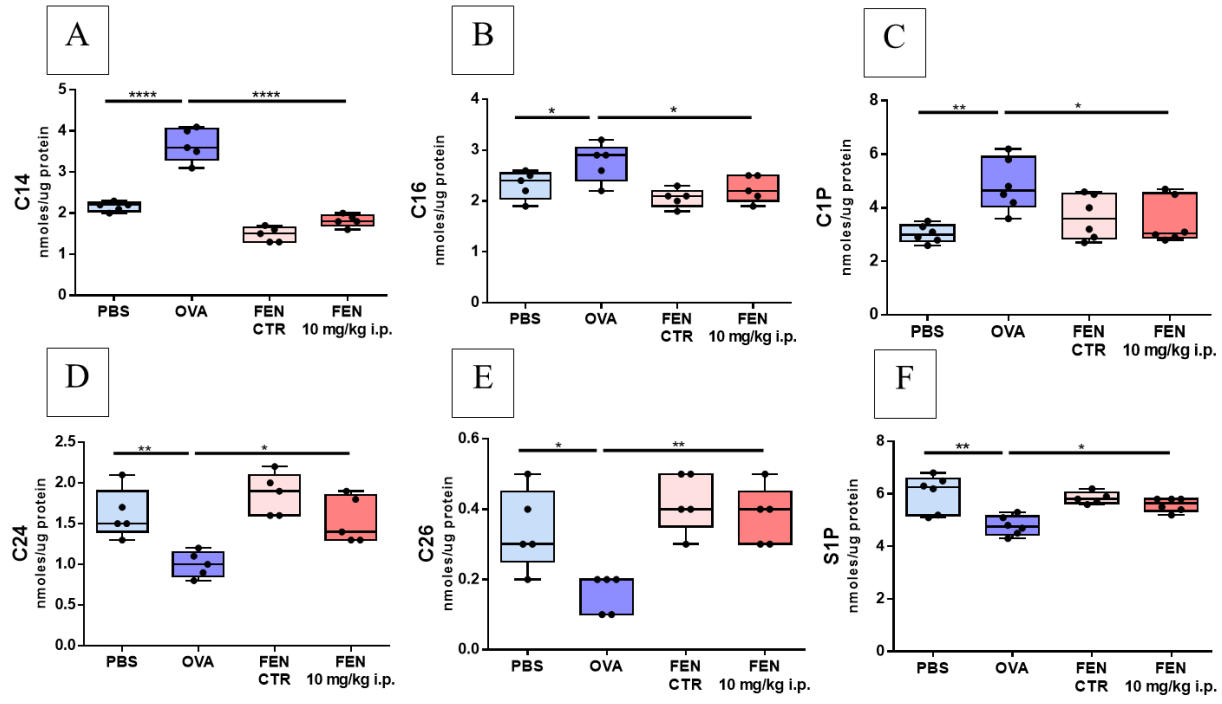
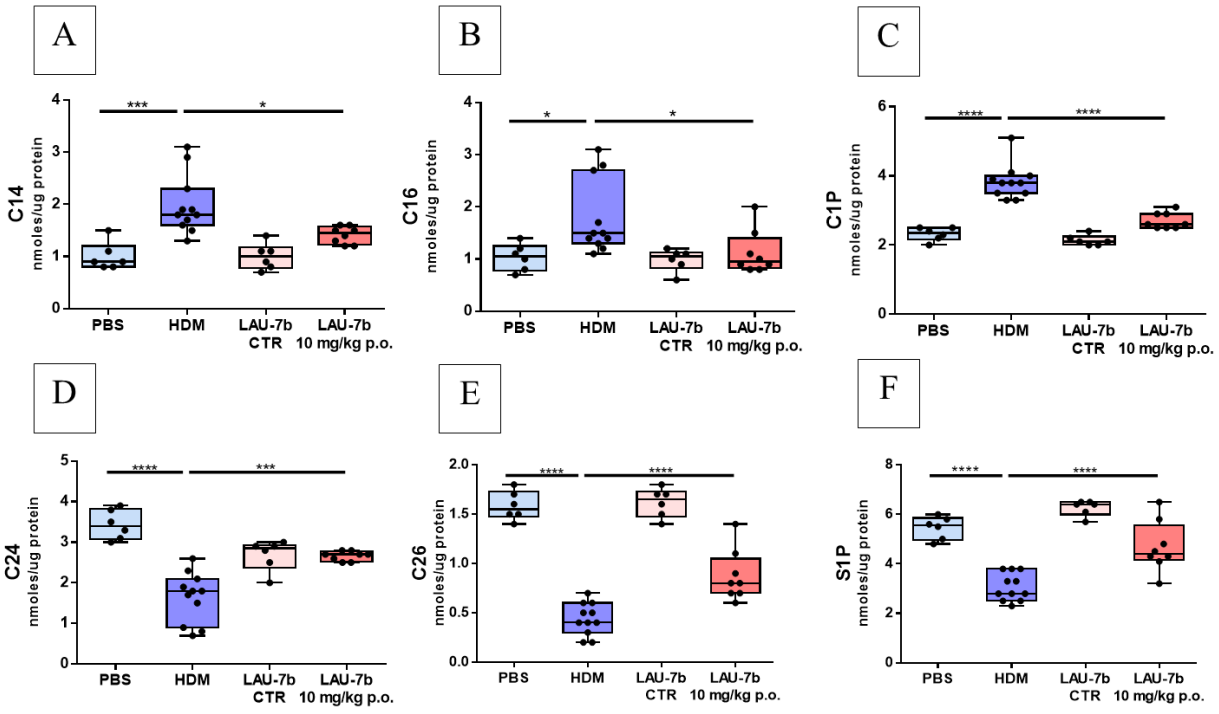

A
C14
nmoles/ug protein
****
****
PBS
OVA
FEN CTR
FEN 10 mg/kg i.p.
B
C16
nmoles/ug protein
*
*
C
C1P
nmoles/ug protein
**
*
D
C24
nmoles/ug protein
**
*
E
C26
nmoles/ug protein
*
**
F
S1P
nmoles/ug protein
**
*

Figure 2.10 Changes in the levels of different ceramide species in lungs of mice challenged with HDM and treated with LAU-7b 10 mg/kg p.o.

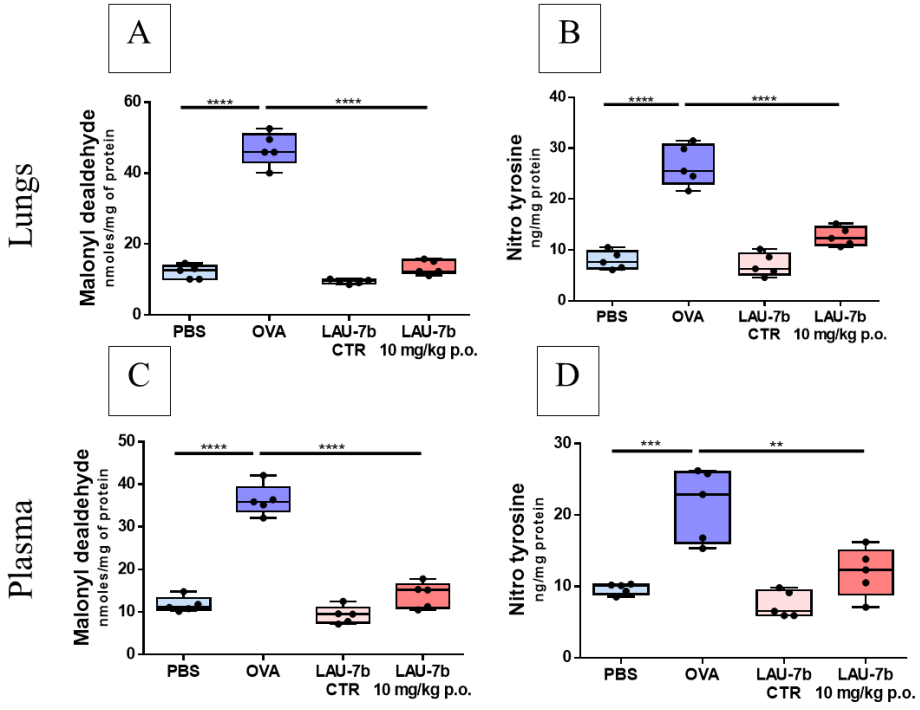
(A-B) Levels of long chain ceramides C14 and C16, (C) level of Ceramide-1-phosphate species (C1P), (D-E) levels of very long chain ceramides C24 and C26, and (F) level of Sphingosine-1-phosphate species (S1P). After HDM challenge, the long chain ceramides and C1P were significantly increased, and the very long chain ceramides and the S1P were significantly decreased. LAU-7b 10 mg/kg p.o. treatment corrected the ceramides imbalances. The baseline of ceramide levels in lungs after LAU-7b 10 mg/kg p.o. treatment (without allergen sensitization or challenge) is demonstrated by the FEN-CTR group of mice. Statistical significance was calculated by One-way ANOVA. $n \geq 6$ in all groups, all points are shown. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$


A
B
C
D
E
F
C14
C16
C1P
C24
C26
S1P
nmoles/ug protein
PBS
HDM
LAU-7b CTR
LAU-7b 10 mg/kg p.o.

Malondialdehyde (MDA), a marker of lipid oxidation, and nitrotyrosine (NT), a marker of protein oxidation, were both significantly elevated in the allergic animals (sensitized and challenged) both in the lung (figure 11A and 11B) and plasma (figure 11C and 11D) samples. Daily treatment with 10mg/kg of LAU-7b was able to completely normalize MDA and NT levels both in mice lungs and plasma.

Figure 2.11 LAU-7b treatment prevents the elevation of malonyldialdehyde (MDA) and nitrotyrosine (NT) in lungs and plasma of ovalbumin-challenged (OVA) mice

Top panel (A-B) for analysis of lung samples and bottom panel (C-D) for analysis of plasma samples. After OVA challenge, mice have elevated levels of MDA and NT both in lungs (A and B) and plasma (C and D). 9 days of LAU-7b 10 mg/kg p.o. treatment corrected the MDA and NT imbalances. The baseline of MDA and NT levels in lungs and plasma after LAU-7b treatment (without allergen sensitization or challenge) is demonstrated by the LAU-7b-CTR group of mice. Statistical significance was calculated by One-way ANOVA. Selected n = 5 in all groups, all points are shown. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$


A
B
C
D
Lungs
Plasma
Malonyl dealdehyde
nmoles/mg of protein
Nitro tyrosine
ng/mg protein
PBS
OVA
LAU-7b CTR
LAU-7b 10 mg/kg p.o.
****
***
**

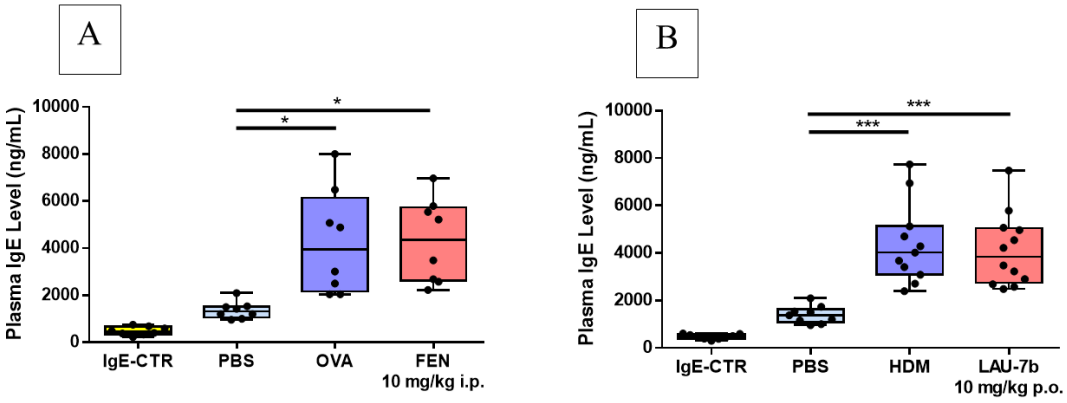
2.5.5 IgE levels were not affected by treatment with fenretinide or LAU-7b.

Naïve mice (IgE-CTR) have lower IgE levels than sensitized and unchallenged mice, which in turn were 2- to 8- fold lower than allergen sensitized and challenged mice. Treatment with LAU-7b for 9 days did not affect significantly the elevated IgE levels in LAU-7b treated allergic mice before and during allergen challenge compared to the untreated allergic mice (figure 12A).

To further confirm that the treatment with this drug does not modulate IgE levels even if treatment was administered at the time of mice sensitization, we have treated a subset of mice with the drug starting from the first day of sensitization. Our results also showed that starting drug treatment for 28 days at the beginning of sensitization process (i.e. drug treatment was overlapping with the period of allergen sensitization) still did not affect the levels of IgE in the plasma (figure 2 and 12B).

Figure 2.12 Elevated IgE levels, after challenge with OVA (overlapping protocol) or HDM (sequential protocol), were not affected when mice were treated with FEN 10 mg/kg i.p. or LAU-7b 10 mg/kg p.o., respectively

(A) Overlapping protocol: IgE levels before and after OVA challenge and FEN 10 mg/kg i.p. treatment for 30 days starting from the first sensitization day. (B) Sequential protocol: IgE levels before and after HDM challenge and LAU-7b 10 mg/kg p.o. treatment for 9 days after the third sensitization. The baseline of IgE levels in lungs without allergen sensitization or challenge is demonstrated by the IgE-CTR group of mice. After allergen (OVA or HDM) challenge, IgE levels are significantly elevated, compared to the PBS challenged mice. IgE levels are unaffected after treatment with FEN 10 mg/kg i.p. or LAU-7b 10 mg/kg p.o. Also, IgE levels are still unaffected whether the drug was administrated with the first allergen sensitization or after the third allergen sensitization. Statistical significance was calculated by One-way ANOVA. $n \geq 8$ in all groups, all points are shown. * $p<0.05$, *** $p<0.001$

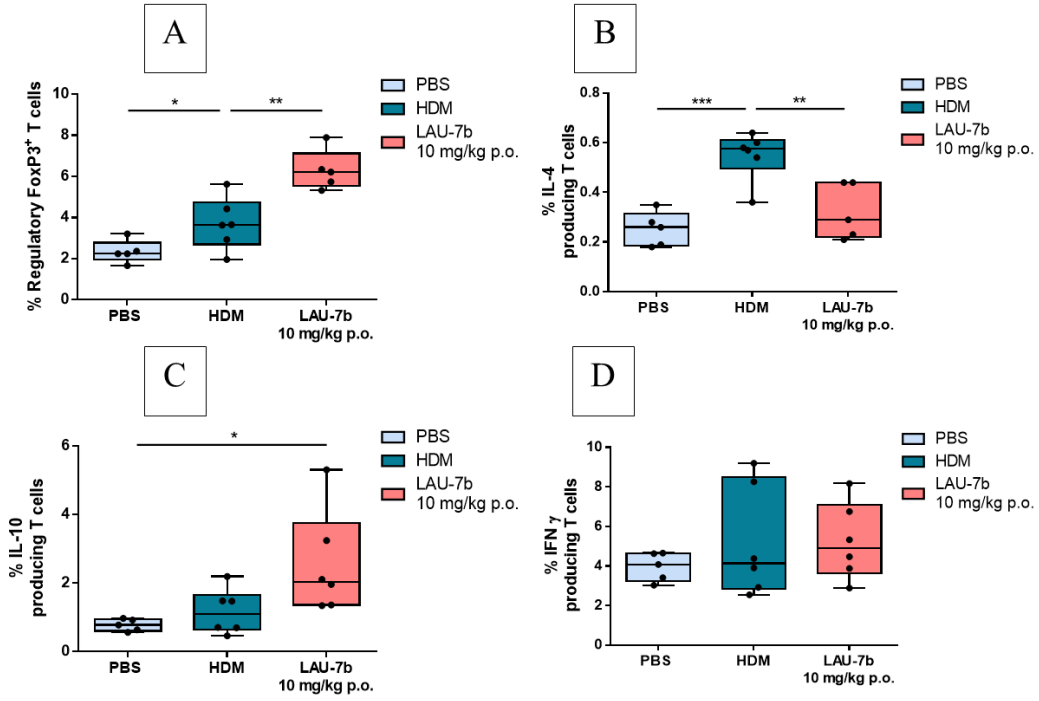

A
Plasma IgE Level (ng/mL)
10000
8000
6000
4000
2000
0
*
*
IgE-CTR
PBS
OVA
FEN
10 mg/kg i.p.
B
Plasma IgE Level (ng/mL)
10000
8000
6000
4000
2000
0
***
***
IgE-CTR
PBS
HDM
LAU-7b
10 mg/kg p.o.

2.5.6 HDM-challenged mice treated with LAU-7b exhibit increased percentage of regulatory T cells ($CD45^+CD4^+CD25^+Foxp3^+$) and decreased percentage of IL4-producing T cells ($CD45^+CD4^+IL4^+$)

The immunoregulatory effect of Fenretinide on HDM-challenged mice was assessed by using flow cytometry on samples collected from the lungs (figure 13). Immunophenotyping of the mice post sacrifice demonstrated that LAU-7b treatment significantly increased the percentage of regulatory T cells ($FoxP3^+$ T cells) and IL10-producing T cells and decreased IL4-producing T cells compared to non-treated mice in the lungs. However, IFNγ-producing T cells were not affected by treatment with LAU-7b.

Figure 2.13 Elevated regulatory T cells and decreased IL4-producing T cells in LAU-7b treated mice.

After HDM challenge, the percentages of regulatory T cells and IL4-producing T cells are increased. LAU-7b treatment significantly increased the percentage of regulatory T cells and IL10-producing T cells and decreased IL4-producing T cells compared to non-treated mice. IFNγ-producing T cells were not affected by treatment with LAU-7b. Statistical significance was calculated by One-way ANOVA. $n \geq 5$ in all groups, all points are shown. * $p<0.05$, ** $p<0.01$, *** $p<0.001$


A
% Regulatory FoxP3+ T cells
PBS
HDM
LAU-7b
10 mg/kg p.o.
B
% IL-4
producing T cells
C
% IL-10
producing T cells
D
% IFN γ
producing T cells

2.6 Discussion

Up to 10% of the population in developed countries are affected by allergic asthma and this high percentage is associated with a large socioeconomic burden (12). Animal models play a substantial role in helping us to understand the pathogenesis of allergic asthma and, consequently, discovering new and effective therapies. Different allergens have been used in allergic asthma studies e.g. OVA, HDM *Dermatophagoides pteronyssinus* (Der p, or European dust mite) or *Dermatophagoides farinae* (Der f, or American dust mite), *Aspergillus fumigatus*, *Alternaria alternata*, cockroach extracts, ragweed and other pollen grains, and latex (13).

OVA-sensitized and challenged mice represent a very well characterized model of allergic asthma. OVA- sensitized and challenged mice are classically used for allergic asthma studies since many years and it is a useful allergic asthma model allowing to study airway remodeling, lung inflammation and an affordable model frequently used in pharmacological evaluation of efficiency of potential new drugs (9). According to the Asthma and Allergy Foundation of America (aafa.org), egg allergy is considered the second most common ingested food allergen in young children after cow's milk allergen. Although OVA can induce airway inflammation and AHR in mice, it does not induce airway inflammation nor AHR in humans. Depending on the route of exposure, OVA can induce food, but not airway, allergies in humans. For this reason, it has been questioned whether this model can successfully mimic the real human inhaled allergens (9).

On the other hand, HDM is the most common inhaled human allergen worldwide as it is estimated that 50–85% of all asthmatics carry antibodies against HDM allergens (14). Additionally, as compared to OVA which contains one main protein allergen (> 98% albumin), 23 allergens (Der p1 - Der p23) have been characterized from the extract of HDM. The most critical component of HDM extract is Der p1, cysteine protease, which is responsible for the disruption of

the airway's tight junctions, cytokine, chemokine and growth factor production, eosinophil and mast cell degranulation, fibroblast maturation and collagen production (allergen.org). Johnson *et al.* (15) showed that mice continuously exposed to HDM, but not OVA, had demonstrated persistent eosinophilic airway inflammation which suggests that repeated exposure to OVA could have led to allergic tolerance.

Our laboratory has previously demonstrated that oral treatment of OVA-sensitized and challenged mice with fenretinide corrected several pro-inflammatory phenotypes (7). However, using HDM, the current study has focused on the optimization of allergic asthma treatment protocol (figure 1) using the clinical formulation of fenretinide (LAU-7b) which is currently being used for CF patients. The LAU-7b formulation has never been used before to test its efficacy against allergic asthma neither in mice nor in humans.

Despite excellent anticancer activities of fenretinide *in vitro*, the results were less impressive in many cancer clinical trials (16-18) most likely due to the low bioavailability of the administrated fenretinide oral formulations. The most promising results of this drug were reported in pediatric retinoblastoma and hematological malignancies, and these results prompted many attempts to improve the formulation of this drug, so the sufficient levels of the drug in the blood could be achieved (19-22). These formulations were approved by FDA for phase I and II clinical trials in cancer, however, those oral formulations were never approved by the Health Canada regulatory agency.

Because of very low aqueous solubility, poor pharmacokinetic properties of previously existing fenretinide formulations lead to poor bioavailability and limit further development of the drug in clinical trials. According to Lipinski's Rule of 5, RO5, the logP of a compound intended for oral administration should be lower than 5. If administrated orally, a more lipophilic compound,

with LogP > 5, will have a very low aqueous solubility which heavily compromises its bioavailability and the overall drug action. Fenretinide has a LogP equal to 6.15 (https://chemaxon.com); this fact resulted in a couple of challenges regarding its solubility.

We previously showed that oral treatment with 60mg/kg of fenretinide was successfully able to prevent diverse inflammatory phenotypes in a mouse model of allergic asthma (7). Given that fenretinide has low bioavailability when administered orally, we reasoned that a lower dosage could be used when administered i.p. Accordingly, we performed a series of pilot experiments testing lower doses (5 to 40mg/kg) of fenretinide administered i.p. (data not shown). Our results here demonstrate that a daily dose of fenretinide as low as 5 mg/kg by i.p. route is effective against allergic asthma in mice (figure 4 and figure 6).

To administer fenretinide p.o., we used the oral dry formulation (LAU-7b, developed by Laurent Pharmaceutical Inc. Mice were treated with LAU-7b at 10mg/kg daily. We wanted to test both the treatment efficacy and the medication dose of this novel formulation in a mouse model of allergic asthma. As demonstrated by our histology results, treatment with LAU-7b at 10 mg/kg protected the mice against the infiltration of immune cells (figure 4 and figure 5) and prevented mucus production (figure 6 and figure 7) after allergen challenge to the comparable extent as fenretinide administration i.p. at the same daily dose and duration of treatment. These results demonstrate comparable efficacy of fenretinide treatment administered p.o. or administered i.p. and that the LAU-7b formulation represents a very good oral dosage form to be proposed for the clinical trial in asthmatic patients. LAU-7b bioavailability was already assessed in CF patients (NCT02141958) and in a dosage of 200 mg per day per person in humans (approx. 3-4 mg/kg) results in the plasma 1-2 μM of Fenretinide at the peak of its activity (data not shown).

Medication compliance is one of the critical challenges in asthma management (23), and proper adherence to prescribed frequency and dosage is associated with a lower risk of severe disease exacerbations. Since allergic asthma is a chronic disease, if it is possible to achieve the treatment targets within the shorter period, the overall patient compliance is much better. The results of the current study demonstrated that oral treatment with LAU-7b for 9 days effectively protects sensitized subjects against allergic asthma symptoms when challenged repeatedly to the allergen. Our studies demonstrated that the treatment for 9 days was as effective as the treatment which was administered for 28 days and the oral LAU-7B formulation is suitable to prevent and resolve asthma symptoms.

Ceramides can act as both building blocks in the cell membranes and as signaling molecules (24). Ceramide imbalance is implicated in many lung diseases including asthma (25,26) and cystic fibrosis (27,28). Depending on the length of the acyl side chain, the most abundant species of ceramides are long-chain (C14:0-C20:0) or very long chain (C22:0-C26:0) ceramides (29). The studies performed by our laboratory demonstrated that very long chain ceramides are diminished in cystic fibrosis (30) and asthma (31). Here for the first time we prove that very long chain ceramides are diminished in genetically hyperresponsive A/J strain of mice in two different models of allergic asthma and can be corrected by fenretinide treatment. Our data demonstrated that very long chain (C24:0 and C26:0) ceramides levels were diminished during the response to an allergen in sensitized animals and can be successfully restored by treatment of animals for 9 days with 10mg/kg LAU-7b formulation (figure 10, $p<0.0001$ and $p<0.001$).

Our group (30) has shown that fenretinide increases the levels of VLCC and decrease the levels of LCC by the transcriptional downregulation of ceramide synthase 5 (Cers5) enzyme. Ceramide synthase 2 (Cers2) forms heterodimers with Cers5 producing VLCCs, whereas

Cer5:Cers5 homodimers produce LCCs and Cers2:Cers2 homodimers display very low or no catalytic activity (32). Very few studies described the interaction between ceramides and their target proteins (24,27). LCC indirectly activate protein phosphatase 2A (PP2A) by binding to its inhibitor I2PP2A (Inhibitor-2 of PP2A), thereby stopping its inhibitory effects on PP2A. PP2A are a family of enzymes composed of 3 subunits, having different isoforms, which can assemble in different combinatorial associations to control different cell functions (24). Additionally, LCC form large channels in the outer mitochondrial membrane which lead to permeabilization, release of cytochrome c and initiation of apoptosis (33). VLCC counteract the formation of these pores, made by LCC, thus inhibiting apoptosis (34). As of 2019, the direct intracellular targets of VLCC remain unknown.

Currently, the inhaled corticosteroids (ICS) are considered the cornerstone and the preferred choices for the maintenance of allergic asthma. ICS should be used "regularly" at the lowest effective dose to maintain a good control of allergic asthma, rather than "as needed, prn, *pro re nata*" mode of administration, which is common for beta2-adrenergic agonists (e.g. salbutamol, the blue or reliver puffer). ICS have very low systemic activity than do oral corticosteroids, rather they act mostly topically (35), hence, ICS are preferred more than oral corticosteroids (36,37).

Inhaled fluticasone (the orange or controller puffer), mometasone, beclomethasone and budesonide are safe and effective steroidal drugs that treat the inflammatory component of allergic asthma. Administration of steroids controls the inflammation by directly regulating the transcription of up to 100 genes, nonetheless, many other genes are indirectly regulated by several interactions with other transcription factors (38). Glucocorticoids increase the transcription of annexin-1 (phospholipase A2 inhibitor) and Ikβ-α (inhibitor of NF-kβ). Steroids decrease the

transcription of several inflammatory cytokines (IL-1, 2, 3, 4, 5, 6, 9, 11, 12, 13, 16, 17, 18, TNFα, GM-CSF) and chemokines (IL-8, RANTES, MIP-1, MCP-1, MCP-3, MCP-4, eotaxin) (39).

The main clinical context of using systemic corticosteroids is in treating acute exacerbations of allergic asthma (37). The Canadian guidelines suggest 25–50 mg of prednisone daily for 7–14 days in case of an acute exacerbation of allergic asthma (36). Less commonly, the systemic corticosteroids are used as long-term maintenance therapy because of the significant side effects. Over the short term, systemic corticosteroids administration cause fluid retention, glucose intolerance, increased blood pressure, increased appetite, mood alterations, and weight gain. Over long terms, administration of systemic corticosteroids results in adrenal axis suppression, necrosis of the hip, cataracts, dermal thinning, diabetes, glaucoma, hypertension, myopathy and osteoporosis (40). Even more importantly, in some patients, long term administration of steroids leads to resistance and they can no longer be efficiently controlled with steroids therapy.

Our laboratory (30) and several other research groups, have previously published that Fenretinide administration is considered to be clinically very safe over long-term periods, as equally being therapeutically effective. A clinical trial (41) recruited 2,867 women over a 5-years period to assess the safety and efficacy of Fenretinide in preventing secondary breast malignancies. In terms of tolerability, out of 2,667 women treated daily only 63 patients (4.4%) discontinued Fenretinide because of the possible adverse events. In terms of efficacy, the trial showed that Fenretinide is potentially effective in preventing future malignancies in premenopausal women. Another phase 2 trial (42) assessed Fenretinide for its safety and efficacy in slowing age-related macular degeneration (AMD) over a period of 2 years. Besides being therapeutically effective in retarding age-related AMD progression, Fenretinide established high safety profile in chronic

dosing regimens. Using the novel formulation of Fenretinide, LAU-7b, in clinical trial phase Ib (NCT02141958) for CF patients, only one candidate withdrew in the 3rd (last) cycle due to elective surgery to clean up the infected sinuses. The withdrawal did not occur due to any side effects related to the administration of LAU-7b. Based on the number of patients involved in all these studies and the prolonged period of drug administration, Fenretinide tolerability can be considered sufficiently high to justify testing its long-term administration in asthmatic or CF patients. Collectively, because of its high tolerability and wide safety margins, Fenretinide would be a suitable drug choice for chronic long-term administration.

Our data (figure 8) demonstrate very strong potentials of Fenretinide to act as an anti-inflammatory drug. Arachidonic acid (AA) and docosahexaenoic acid (DHA) play important roles in allergic asthma, where AA act as a proinflammatory fatty acid and DHA as an anti-inflammatory fatty acid. We observed in our models of allergic asthma that the AA/DHA ratio is significantly skewed in the proinflammatory direction. Treatment with Fenretinide, or LAU-7, have corrected effectively the imbalances of AA and DHA which happened due to allergen sensitization and challenge in our model. Similar results were reported by Kanagaratham *et al.*(7) in allergic asthma and Guilbault *et al.* in CF (6)

AA promotes the production of the inflammatory cytokine IL-8 through COX-2 and NF-kB pathways (43). *In vitro* studies have shown that Fenretinide prevented the production of IL-8 from LPS-stimulated human lung epithelial cells (44). Moreover, the results reported by Kanagaratham *et al.* (7) showed that pre-treatment of cells with Fenretinide before LPS stimulation dampened the transcription of several cytokines and chemokines (*Ccl2 (MCP-1), Ccl5 (RANTES), Ccl7 (MCP-3), Ccl11 (eotaxin-1), Cxcl1 (KC), Cxcl2 (MIP2-α), Cxcl9 (MIG), Cxcl10 (IP-10), Il-6, iNOS, Pdgf,* and *Tnf-α*), hence, it can inhibit the inflammation in cellular levels.

During our experiments, all the mice were challenged with HDM via the i.n. route of administration. We sensitized the mice with HDM via the i.p. route of administration; on the other hand, it has been described in the literature that sensitizing the mice by inhalation (i.n.) better mimics the way in which humans are sensitized to allergens. To address this point, we compared both routes of allergen administration (i.n. and i.p.) during the sensitization timepoints. We observed that sensitization of mice by i.n. route did not differ from i.p. as demonstrated by our histology and lipid results (supp. figure 1 and figure 2). Moreover, sensitization given i.p. showed much lower variability and higher significance (supp. figure 2). Our analysis demonstrated higher significance when assessing ceramides species profiles between mice sensitized i.p. and challenged i.n. with HDM ($p<0.0001$ {C24:0} and $p<0.001$ {C14:0, C16:0, C26:0}) compared to the mice which were sensitized i.n. and challenged i.n. ($p<0.0001$ {C24:0}, $p<0.01$ {C26:0}, $p<0.05$ {C14:0}, ns {C16:0}) with HDM. The difference in C16:0 ceramide level between i.n. sensitized and i.n. challenged mice with HDM show the trend toward similar difference as observed in i.p. sensitized mice but was not statistically significant (supp. figure 2) most likely due to higher variability from mouse to mouse when sensitizing i.n., so higher n per group or increase number of i.n. challenges would be recommended when using this route of sensitization. Based on those results, and to reduce the amount of HDM allergen administrated (5µg i.p. compared to 25 µg i.n.), we performed all our sensitizations for the HDM experiments using the i.p. route of administration.

Immunoglobulin E (IgE) is one of the mediators of the immediate hypersensitivity reaction that underlie several atopic diseases including allergic asthma (45). However, the complete understanding of the IgE levels regulation and the exact contribution of increased production of IgE to overall allergic asthma severity requires further investigation. Neutralization of the

circulating serum IgE, by humanized anti-IgE antibodies, is a new approach for the treatment of atopic diseases. However, due to lack of long-term safety and efficacy, treating patients with anti-IgE antibodies is usually considered a last resort according to the current asthma therapeutic guidelines which principally rely on medications not correcting the levels of IgE (3).

We had previously observed that allergic asthma could be successfully treated with 60mg/kg of fenretinide without affecting IgE levels (7). Therefore, we were wondering whether it was not because the IgE was already elevated prior to the initiation of the treatment (during sensitization with allergen). We have tested an overlapping protocol, within which drug treatment starts from the first allergen sensitization, and for 28 days until harvest (figure 12). Our results demonstrate that starting fenretinide treatment since the beginning of the allergic model did not modulate the increase in plasma IgE associated with sensitization. Our current results are consistent with our previously analyzed effects of treatment with fenretinide using OVA allergic asthma model (7). Specific IgE levels against either OVA or HDM were not assessed, so we are unable to conclude if specific IgE levels are affected. Interestingly, symptoms of allergic asthma can be treated even if the IgE levels remain high. Our data demonstrate that allergic asthma symptoms can be treated successfully without neutralization or decrease of IgE.

In addition to restoring ceramide balance in the lungs of treated mice, we demonstrated that LAU-7b promotes regulatory T cell responses and downregulates inflammatory IL4-producing T cells and does not affect IFNγ-producing T cells (figure 13). The interplay between T cell phenotype and ceramides is presently unclear. Whether Fenretinide is acting directly or indirectly on T cells to promote a more regulatory phenotype remains unclear and is a subject for further study.

2.7 Conclusion

The novel formulation of fenretinide, LAU-7b, has significant protective effects against both OVA- and HDM-induced allergic asthma. Our protocol using LAU-7b, which consists of only 9 days of treatments at 10mg/kg, was able to restore to normal levels the aberrant ratio of omega-3 to omega-6 fatty acids, inhibited inflammatory cells migration to the lungs, diminished production of mucins, and significantly improved lung physiology parameters without affecting IgE levels. Treatment with LAU-7b also restored the diminished very long ceramides species and diminished the levels of long-chain ceramides in sensitized, challenged and LAU-7b treated mice both when the allergen used was OVA or HDM. All these findings demonstrate the outstanding efficacy of the LAU-7b therapy and provide robust preclinical data justifying testing the efficacy of this drug in a clinical trial. The clinical trial would validate its efficacy in patients suffering from allergic asthma, especially in those patients with severe asthma symptoms who are not responding anymore to current therapies.

2.8 Supplementary Data

Figure 2.14 (Supplementary figure) Intraperitoneal (i.p.) and intranasal (i.n.) routes of house dust mite (HDM) sensitization and challenge and the effect of LAU-7b the novel formulation of FEN

Lung sections from (A) i.p. and (B) i.n. sensitized animals with HDM and challenged with PBS, (C) i.p. and (D) i.n. sensitized animals with HDM, challenged with HDM, and untreated with LAU-7b, (E) i.p. and (F) i.n. sensitized animals with HDM, challenged with HDM, and treated with LAU-7b. Both routes of allergen administration, i.p. (5µg HDM) or i.n. (25µg HDM), were capable of producing similar lung infiltration which was successfully corrected by treatment with LAU-7b. $n \geq 4$ for the PBS groups and $n \geq 6$ for the HDM and LAU-7b groups.

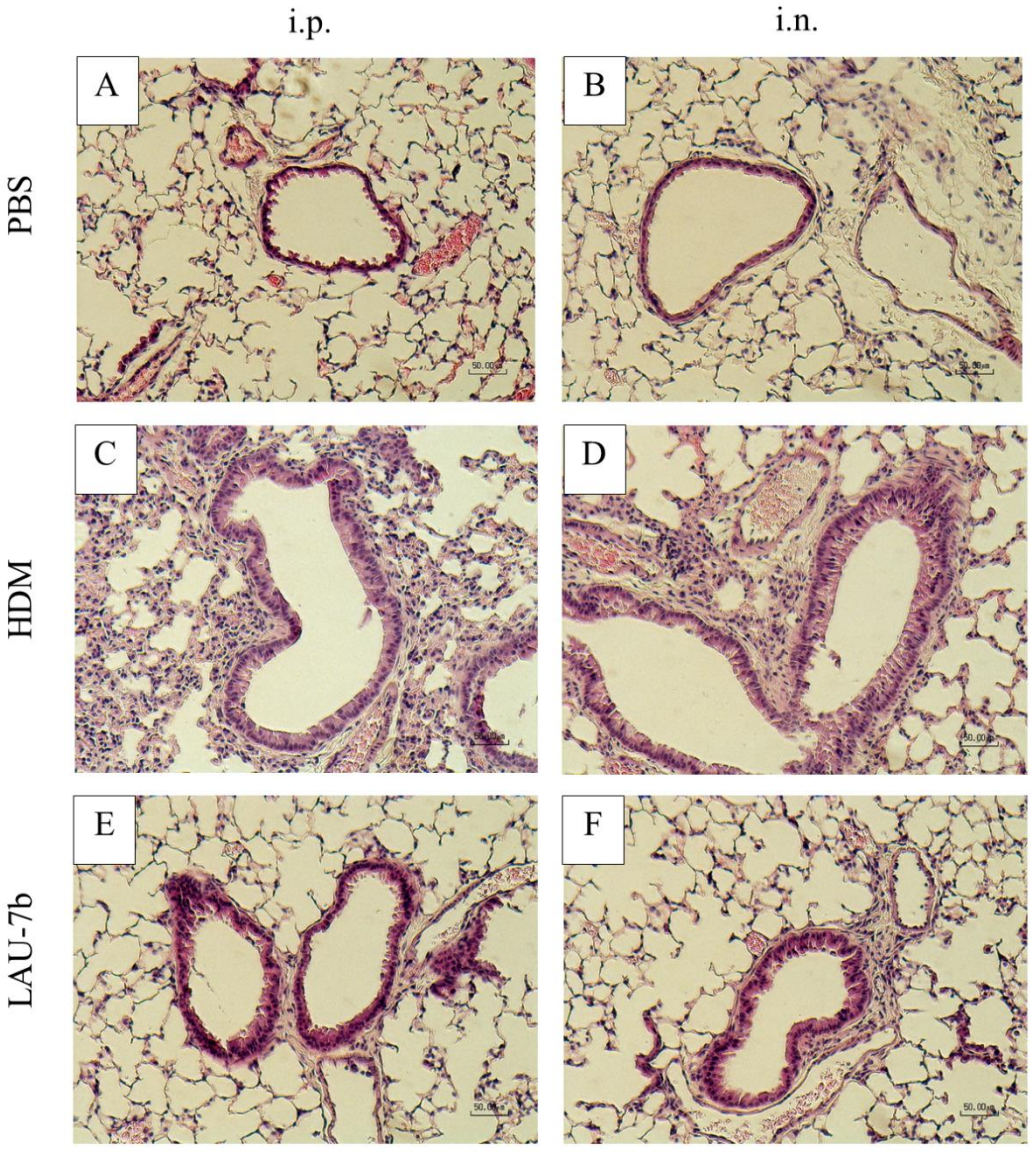
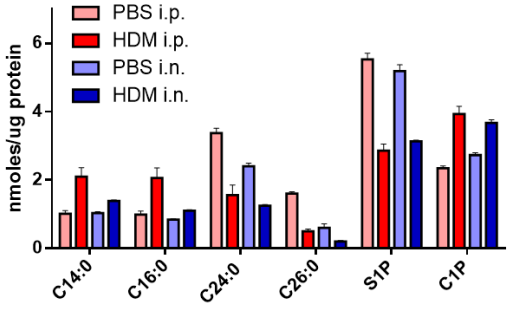

i.p.
i.n.
PBS
HDM
LAU-7b
A
B
C
D
E
F

Figure 2.15 (Supplementary figure) Analysis of different lipids species in lungs of mice challenged with house dust mite (HDM) and sensitized with HDM intraperitoneally (i.p.) and intranasally (i.n.)

(A) Lipid analysis in lungs and (B) results of statistical analysis of each route of HDM allergen administration. After HDM challenge, mice have elevated levels of long chain ceramides species C14:0, C:16:0 and C1P, and diminished levels of very long ceramide species C24:0, C26:0 and S1P. Both i.p. and i.n. routes of HDM administration, during sensitization, resulted in similar changes in lipid profiles in lungs and plasma. sensitizations given i.p. showed lower variability and higher significance than sensitizations given i.n. for all species of lipids measured. n ≥ 7 in all groups.

A



B

PBS i.p. vs HDM i.p.	*p* value	Sig.	PBS i.n. vs HDM i.n.	*p* value	Sig.
C14:0	0.0006	***	C14:0	0.0136	*
C16:0	0.0007	***	C16:0	0.1443	ns
C24:0	< 0.0001	****	C24:0	< 0.0001	****
C26:0	0.0005	***	C26:0	0.0042	**
S1P	< 0.0001	****	S1P	< 0.0001	****
C1P	< 0.0001	****	C1P	< 0.0001	****

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Preface to Chapter 3

Chapter 3 contains the results of the experiments designed to validate the effect of ORMDL sphingolipid biosynthesis regulator 3 (*Ormdl3*) gene on allergic asthma susceptibility. *Ormdl3* is involved in modulating ceramide biosynthesis by inhibiting the serine palmitoyl transferase (SPT) enzyme which is the rate limiting step in the *de novo* ceramide biosynthesis. It was shown that the deletion of *Zona pellucida* binding protein 2 (*Zpbp2*) *gene* in C57BL/6 mice led to a decrease in the expression of *Ormdl3* gene, which is mapped downstream of *Zpbp2*. Therefore, to assess the effect of the identified locus, we used *Zpbp2* knockout (KO) mice on an A/J genetic background, an atopic strain of mice that is genetically predisposed to allergic asthma due to inherited atopy and airway hyperresponsiveness (AHR).

Chapter 3 contains experimental data that provide a logical extension of Chapter 2. In Chapter 2, we have optimized the treatment protocol with LAU-7b and we have shown that LAU-7b was able to correct the imbalance of the long chain ceramides (LCCs) and the very long chain ceramides (VLCCs) in allergic asthma. In chapter 3, we hypothesized that the impact of *Zpbp2* and *Ormdl3* asthma susceptibility genes is associated with the imbalance of ceramides and can be validated using A/J mice (atopic and displaying AHR). The results illustrated in Chapter 3 were generated to specifically address the following hypotheses:

1) A/J KO mice with *Zpbp2* gene deletion would have lower AHR measured following exposure to MCh, and LAU-7b treatment can be effective in lowering AHR in *Zpbp2* KO A/J mice.

2) The normalizing effect of the treatment with LAU-7b on the imbalance of the LCCs and VLCCs is associated with the modulation of *Ormdl3* gene expression in WT mice.

Chapter 3. Treatment of allergic asthma with Fenretinide downregulates *Ormdl3* expression and normalizes ceramides imbalance

Manuscript to be submitted to the American Journal of Respiratory Cell and Molecular Biology

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3.1 Abstract

Background: Zona pellucida binding protein 2 (*Zpbp2*) and ORMDL sphingolipid biosynthesis regulator 3 (*Ormdl3*), mapped downstream of *Zpbp2,* were identified as two genes associated with airway hyperresponsiveness (AHR). *Ormdl3* gene product has been shown to regulate the biosynthesis of ceramides. Allergic asthma was shown to be associated with an imbalance between very long chain ceramides (VLCCs) and long chain ceramides (LCCs). We hypothesized that Fenretinide (FEN) can prevent the allergic asthma-induced augmentation of *Ormdl3* gene expression, normalize aberrant levels of VLCCs and LCCs, and treat allergic asthma symptoms.

Methods: To accomplish this, we induced allergic asthma, by house dust mite (HDM), in A/J WT mice and *Zpbp2* KO mice expressing lower levels of *Ormdl3* mRNA than WT. We investigated the effect of a novel formulation of FEN, LAU-7b, on the AHR, inflammatory cell infiltration, mucus production, IgE levels, and ceramides levels.

Results: Although lower *Ormdl3* expression, observed in *Zpbp2* KO mice, was associated with lower lung resistance, allergic *Zpbp2* KO mice were not protected from inflammatory cell infiltration, mucus accumulation or aberrant levels of VLCCs, LCCs and fatty acids induced by HDM. LAU-7b treatment protects both the *Zpbp2* KO and WT mice. The treatment significantly lowers the gene expression of *Ormdl3,* normalizes the aberrant ratio between VLCCs and LCCs and corrects all the other phenotypes associated with allergic asthma following HDM challenge, except the elevated levels of IgE.

Conclusion: Overall, our results demonstrate that LAU-7b treatment prevents the augmentation of *Ormdl3* expression and ceramides imbalance induced by HDM challenge and protects both WT and *Zpbp2* KO mice against allergic asthma symptoms.

Keywords: LAU-7b, Fenretinide, Asthma, *Zpbp2*, *Ormdl3*, Sphingolipids, very long chain ceramides; VLCCs, and long chain ceramides; LCCs.

3.2 Introduction

Allergic asthma causes global health burdens as it affects millions of people worldwide. Being a complex disease, in addition to environmental exposures, several genetically controlled factors greatly influence the predisposition and severity of allergic asthma (1). Genome wide association studies (GWAS) have highlighted the 17q21 locus, which contains several susceptibility genes including *Zona pellucida* binding protein 2 (*ZPBP2*) and ORMDL sphingolipid biosynthesis regulator 3 (*ORMDL3*), on human chromosome 17 and their orthologs *Zpbp2* and *Ormdl3* on mouse chromosome 11, as a well-established susceptible locus for airway hyper responsiveness (2).

ZPBP2 localizes to the sperm acrosome, facilitating the binding of the spermatozoa to the oocyte's *zona pellucida*, and so, it is highly expressed in testes in both mice and humans (3). ZPBP2 expression has also been detected, in lower levels, in human somatic airway epithelial cells (4). Recently, our group (5), and Miller *et al.* (6), have published that *Zpbp2* gene deletion attenuated AHR in C57BL/6 mice, nevertheless, the functional impact of *Zpbp2* on allergic asthma remains undiscovered. *Ormdl3,* which is involved in modulating the biosynthesis of ceramides, has been mapped downstream of *Zpbp2* and it was recently shown that the deletion of *Zpbp2* in C57BL/6 mice led to a decrease in the levels of *Ormdl3* expression (6). Compared to ZPBP2, more studies have been published pertinent to ORMDL3 and allergic asthma. It has been shown that increased expression of *Ormdl3* positively correlates with airway hyper-reactivity (7). Moreover, elevated levels of ORMDL3 inhibit sphingolipid biosynthesis and result in inhibition of long chain

ceramide (LCC; C16:0) and four species of very long chain ceramides (VLCCs; C22:0, C24:0, C24:1 and C24:2) although the exact molecular steps are still under investigation (8).

Fenretinide (FEN), a vitamin A derivative, corrects the aberrant inflammatory responses and improves lung functions in both cystic fibrosis (CF) (9-12) and allergic asthma (13). Recently, a novel clinical oral formulation of Fenretinide (LAU-7b), with excellent bioavailability, was developed by Laurent Pharmaceutical Inc. and was tested using daily single oral capsules (100 mg, 200 mg and 300 mg) in CF patients in a Phase Ib clinical trial study (NCT02141958). The results of the Phase I clinical trial demonstrated very promising pharmacokinetics and pharmacodynamics of LAU-7B. Currently, LAU-7b is being tested in a Phase II clinical trial in CF patients (NCT03265288). However, LAU-7b capsules have not yet been tested in patients suffering from allergic asthma.

In our study, we developed KO mice for *Zpbp2* gene which carry a deletion of exons 1-3 on the A/J genetic background. Unlike C57BL/6, the A/J strain of mice is not only atopic but also expresses genes regulating AHR. Allergic asthma developed in this strain of mice is characterized by a very strong inflammatory response, a dramatic increase in airway hyperplasia, and a very strong increase in airway resistance which was measured following exposure to methacholine (MCh). Therefore, we have used this strain of mice, which represents a particularly good model, to investigate the protective effects of LAU-7b treatment against HDM-induced asthma. We aimed to validate the importance of the *Zpbp2* gene in allergic asthma, so we hypothesized that A/J KO mice with the *Zpbp2* gene deletion would be associated with lower airway resistance, measured following exposure to MCh. Our group has shown that Fenretinide improves lung function in CF by elevating the levels of VLCCs (9). Because the *Ormdl3* gene product regulates the biosynthesis of ceramides, we expected that changes in *Ormdl3* gene expression will correlate with changes in the distribution of specific species of ceramides. We hypothesized that the allergic asthma induced

augmentation of *Ormdl3* can be prevented by treatment with LAU-7b in the A/J hyperresponsive strain of mice. Furthermore, we hypothesized that there is an association between the LAU-7b - induced improvement in lung physiology and the normalization of relative abundance of VLCCs and LCCs in the lungs.

3.3 *Materials and Methods*

3.3.1 Animal Model

Heterozygous B6.127S7-*Zpbp2*^{tm1Zuk}/J mice (14) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and backcrossed to A/J inbred mice purchased from Jackson Laboratory (Bar Harbor, Maine, USA) for more than ten generations. N10 *Zpbp2* KO mice were intercrossed to generate homozygous KO A.127S7-*Zpbp2*^{tm1Zuk} mice, referred to from this point on as KO.

Genotyping was done was done using the CFX384 Touch Real-Time PCR Detection System, SsoFast EvaGreen Supermix (BioRad), the *Zpbp2* forward primer 5'-GGT TTG CTT CGC TCT GTA-3', and the *Zpbp2* reverse primer 5'-CAA GCA TCA CGG CTC AG-3'. Mice were maintained on 12 hours light and 12 hours dark cycles. All experimental procedures were approved by the Animal Care Committee of McGill University Health Center, Montreal, QC, Canada.

3.3.2 Sensitization, Challenge, and Harvest Protocol

At the age of eight weeks, male and female KO and WT mice were sensitized by intraperitoneal (i.p.) injection of HDM allergen on a weekly basis for three weeks. 5µg of HDM protein extract (*Dermatophagoides Pteronyssinus*, Cat: XPB82D3A2.5, Greer lab, Lenoir, NC, USA) adsorbed to 1.5 mg of aluminum hydroxide adjuvant (Imject Alum, Pierce, Rockford, IL, USA) in a total volume of 200 µL sterile PBS was used for sensitization. One week following the

third systemic sensitization with HDM, three daily successive challenges were achieved by intranasal (i.n.) exposures to 15 µL of 1 µg/µL HDM protein extract prepared in PBS. The animals were harvested 48 hours after the third challenge.

3.3.3 Mouse Groups and Batches

Following sensitization, the mice were split into the following groups: allergic-PBS/treated-unchallenged mice (HDM-PBS-PBS), allergic-PBS/treated-challenged mice (HDM-PBS-HDM), and allergic-LAU-7b/treated-challenged mice (HDM-LAU-7b-HDM), for simplification; the three groups are presented as "PBS", "HDM", and "LAU-7b", respectively. Eight different experiments batches using *Zpbp2* WT and KO mice on the A/J background (n = 217) were tested. The results collected from all 8 experiments were pooled together to make the final graphs.

3.3.4 LAU-7b Treatment Protocol

An oral dose of 10 mg/kg LAU-7b was tested in HDM sensitized and challenged mice. Allergen sensitized mice were treated with LAU-7b orally for 9 days by daily gavage starting from the day of the third sensitization until the day prior to harvest. 0.5% methyl cellulose in milliQ purified water was used as a drug vehicle to re-disperse the active ingredient (FEN) in the LAU-7b capsule plus the necessary excipients.

3.3.5 Lung Resistance Measurements

Airway resistance was measured using a Buxco plethysmograph system, ventilators, and nebulizers (Harvard Apparatus, Holliston, MA, USA) as previously described (5,13). For the non-invasive whole-body plethysmograph, WBP; the baseline lung resistance was measured without anesthesia at the age of eight weeks before the first sensitization. A nebulizer was used in both non-invasive and invasive lung experiments to administer ascending doses of MCh (Acetyl β-

methyl choline, Cat: A2251, Sigma Aldrich, Saint Louis, MO, USA). Doses of 25 and 50 mg/mL MCh were used for non-invasive WBP lung measurements and doses of 25, 50, and 100 mg/mL MCh were used for invasive lung measurements. The maximum resistance for each mouse at each dose of MCh was determined.

3.3.6 Lung Histology Analysis

During harvest, the left lung from each harvested mouse was inflated with 10% buffered formalin (Fisher Scientific, Nepean, ON) and then kept in formalin for fixation for 48 hours. Paraffin blocks and slide sectioning were done as previously described (13). Haematoxylin and eosin (H&E) staining was used to assess lung tissue recruitment of different inflammatory cells around airways. Quantifying LAU-7b effects was done by counting the number of infiltrated inflammatory cells among at least 4 airways/mice, averaged, and normalized versus the perimeter of the airway basement membrane. The Periodic acid Schiff (PAS) stain was used to visualize goblet cell hyperplasia in the lungs. PAS positive cells in the airways were counted and normalized by dividing the counts by the perimeter of the basement membrane (Pbm). Per mouse, at least 4 airways were counted as previously described (13).

3.3.7 Lipids and Markers of Oxidation Analysis

Lipid analysis was done using 25 mg of macerated lung tissue from each mouse. On the day of the harvest, lungs were preserved in butylated hydroxy anisole (BHA) solution until analysis. Classical isolation of lipids was done as previously described by Folch (15), and the levels of different lipid species were measured using high-performance liquid chromatography (HPLC) tandem mass spectrometry (MS) as previously described (10).

3.3.8 Gene Expression Measurements

RNA was extracted from snap frozen lungs using the RNeasy Mini kit (Qiagen) following the manufacturer's instructions. The concentration of the extracted RNA was measured using the Nanodrop 2000 device. Five hundred ng of RNA was reverse transcribed into cDNA for each analyzed sample using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's instructions. Levels of expression of *Ormdl3* mRNA were measured by using the CFX384 Touch Real-Time PCR Detection System and SsoFast EvaGreen Supermix (BioRad). Primers were designed using the NCBI primer BLAST online tool. *Ormdl3* forward primer 5'-AGG AAG TTC TTA ACC ATC AC-3' and *Ormdl3* reverse primer 5'-AAG GAC ACA GTG TTG AGT AT-3'. *Il-4* forward primer 5'-CGA GCT CAC TCT CTG TGG TG-3' and *Il-4* reverse primer 5'-TGA ACG AGG TCA CAG GAG AA-3'. *Il-5* forward primer 5'-CTC TGT TGA CAA GCA ATG AGA CG-3' and *Il-5* reverse primer 5'-TCT TCA GTA TGT CTA GCC CCT G-3'. *Il-13* forward primer 5'-CCT GGC TCT TGC TTG CCT T-3' and *Il-13* reverse primer 5'-GGT CTT GTG TGA TGT TGC TCA-3'. *Ccl11* forward primer 5'-CCA GAC ATT CGG CGG TTG-3' and *Ccl11* reverse primer 5'-CAG CAG CAG GCA CAT CAG-3'

3.3.9 IgE Measurements in Serum

After blood was collected into tubes containing EDTA, samples were spun at 3000 rpm for 10 min. The obtained plasma was stored at -80^{o}C until the day of analysis. Serum IgE levels were measured using an enzyme-linked immunosorbent assay (ELISA) IgE mouse kit (BD OptEIA Biosciences) following the manufacturer's instructions.

3.3.10 Statistical Analysis

Data were pulled out from at least 3 independent experiments for each analysis. Data were analyzed using GraphPad Prism 6 (version 6.01; GraphPad Software Inc., San Diego, CA, USA).

An ANOVA test was used for analyzing the results of more than two groups and a *t* test was used for analyzing the results of two groups. *p* values of less than 0.05 were considered statistically significant. The number of mice (n) used for each analysis is written in each figure caption.

3.4 Results

3.4.1 Gene Expression and Basal Lung Function Analysis

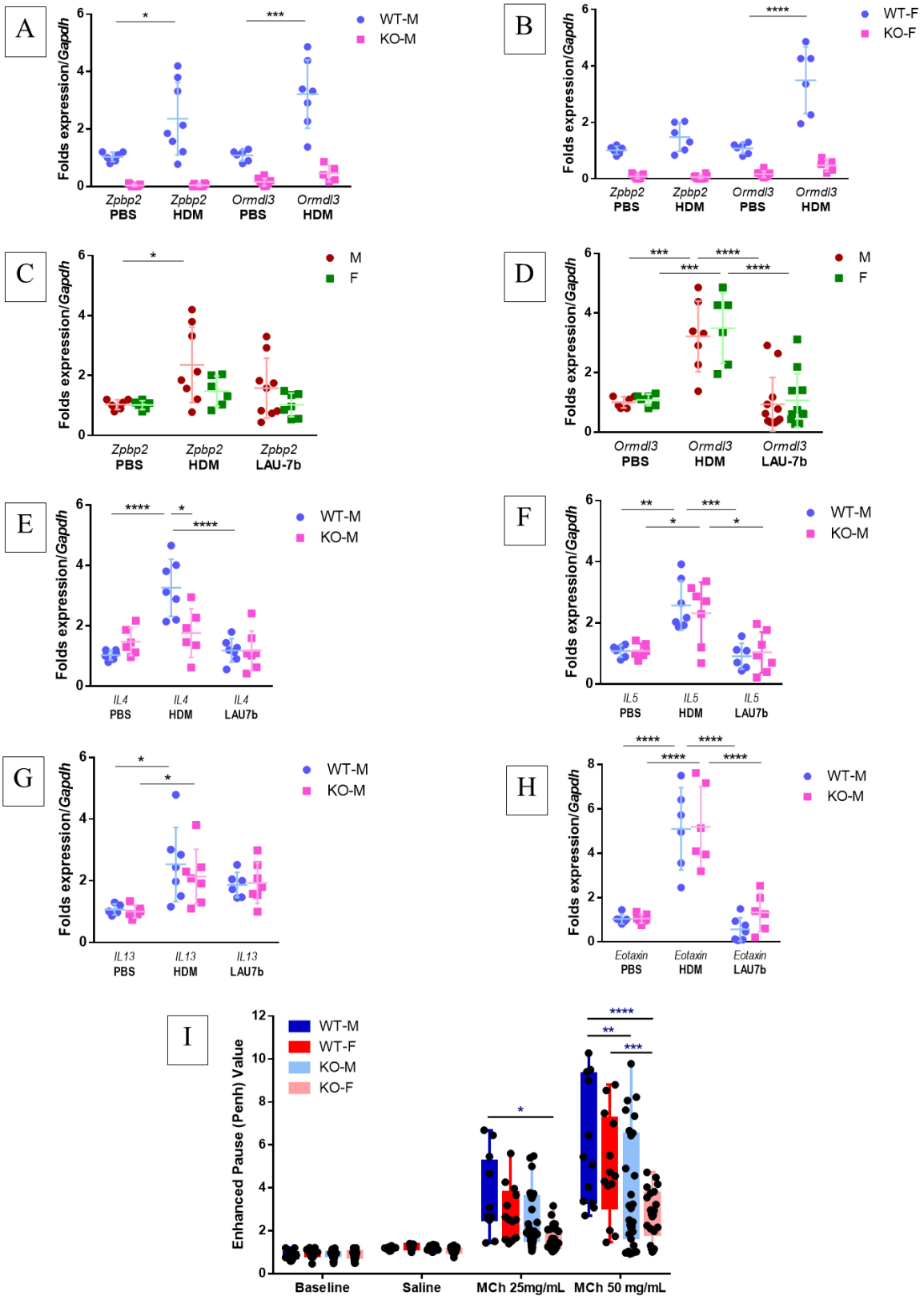
We confirmed that, by removing exons 1-3 of the 5' region of *Zpbp2*, the mRNA gene expression was abolished (figure 1A and 1B) using lung tissue collected from the A/J *Zpbp2* KO mice we developed. It has been previously reported (6) that deletion of *Zpbp2* has downregulated *Ormdl3* expression in lung epithelial cell lines. Therefore, we wanted to evaluate if *Ormdl3* gene expression might be decreased in the lungs of *Zpbp2* KO A/J mice. In WT control mice, PBS-challenged mice demonstrated very low gene expression of *Ormdl3* compared to the gene expression of HDM-challenged mice (figure 1A and 1B). Furthermore, in *Zpbp2* KO mice, *Ormdl3* gene expression was also abolished (half-fold expression in *Zpbp2* KO mice compared to 2-4-folds expression in WT mice) even after HDM challenges (figure 1A and 1B). Then, we treated the WT mice with LAU-7b, and we measured *Zpbp2* and *Ormdl3* gene expressions. Our data show that LAU-7b treatment significantly decreased the expression of *Ormdl3* in both WT males and females, compared to HDM-challenged and PBS-treated mice (figure 1D).

We also tested the gene expression of four of the TH-2 immune pathway associated genes (figure 1E-1H); *Il-4* (differentiate naive Th0 into Th2), *Il-5* (activate eosinophils), *Il-13* (regulate the production of IgE) and *Ccl11 (Eotaxin-1*; enhances the infiltration of eosinophils into the airways). Our results show that after HDM sensitization and challenge, the expression of *Il-5, Il-13 and Eotaxin-1* was significantly elevated in WT and *Zpbp2* KO mice, meanwhile, the expression of *Il-4* was significantly elevated in WT, but not *Zpbp2* KO mice. LAU-7b treated WT

mice have significantly lower expression of *Il-4*, *Il-5*, and *eotaxin-1* genes, but not *Il-13*, compared to littermate WT controls. Non-invasive whole-body plethysmography (WBP) was used to measure the baseline respiratory functions before starting the HDM sensitization and without sacrificing the mice (figure 1I). At MCh dose of 25 mg/mL, both WT males and females have significantly higher lung enhanced pause values (Penh) than KO males and females, respectively.

Figure 3.1 *Zpbp2* and *Ormdl3* gene expression and basal lung function in WT versus *Zpbp2* KO mice

Fold-change expression of *Zpbp2* and *Ormdl3* genes quantitated using qPCR for WT and *Zpbp2* KO A) male mice and B) female mice. Fold-change expression of C) *Zpbp2* and D) *Ormdl3* genes quantitated using qPCR for LAU-7b-treated WT male mice and female mice. LAU-7b treatment and fold-change expression of E) *Il-4*, F) *Il-5*, G) *Il-13* and H) *Eotaxin-1* genes in *Zpbp2* KO and WT mice. All mice were sensitized with house dust mite (HDM) and challenged with either PBS or HDM. Treatment group is marked as (LAU-7b). I) Non-invasive whole-body plethysmography (WBP) for WT and Zpbp2 KO mice. Folds change in lung resistance in response to increasing doses of inhaled MCh in naïve WT and KO males and females. The doses of MCh used were 25 mg/mL and 50 mg/mL. Unpaired *t* test was used for analyzing 2 mouse groups and a two-way ANOVA test was used for analyzing more than two mouse groups, n equal at least 5 mice in each group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$


A
Folds expression/Gapdh
WT-M
KO-M
Zpbp2 PBS
Zpbp2 HDM
Ormdl3 PBS
Ormdl3 HDM
B
WT-F
KO-F
C
M
F
Zpbp2 LAU-7b
D
Ormdl3 LAU-7b
E
IL4 PBS
IL4 HDM
IL4 LAU7b
F
IL5 PBS
IL5 HDM
IL5 LAU7b
G
IL13 PBS
IL13 HDM
IL13 LAU7b
H
Eotaxin PBS
Eotaxin HDM
Eotaxin LAU7b
I
Enhanced Pause (Penh) Value
Baseline
Saline
MCh 25mg/mL
MCh 50 mg/mL

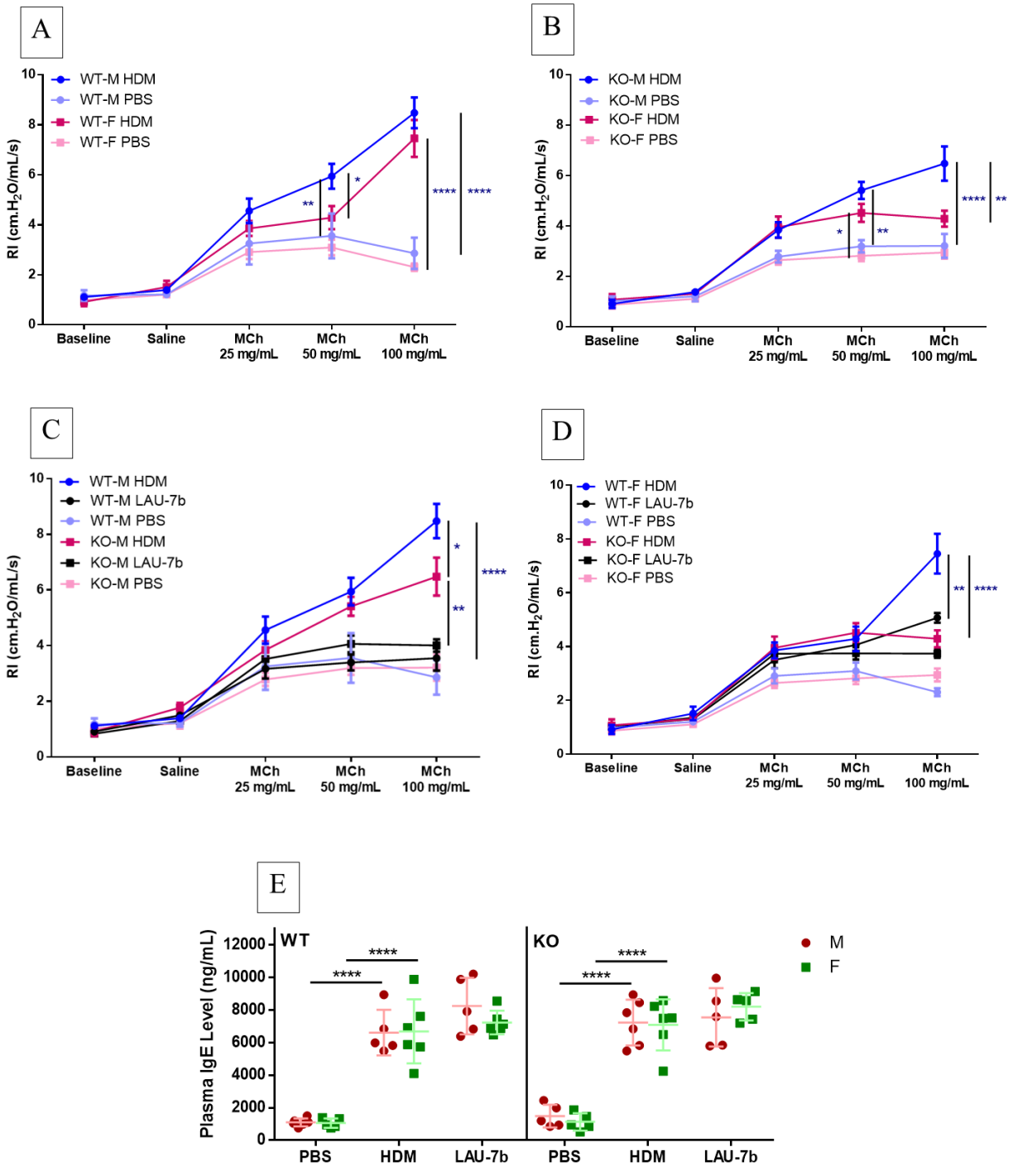
3.4.2 Assessment of Airway Hyperresponsiveness and IgE Levels

The results of WBP were confirmed by classical invasive measurement of lung resistance on the day of harvest (figure 2A-2D). HDM challenge significantly increased the lung resistance values of all mouse groups; males, females, WTs and KOs, at MCh dose of 50 mg/mL and 100 mg/mL, compared to PBS-challenged mice. Our data demonstrate that WT males have higher lung resistance than WT females; similarly, KO males have higher lung resistance than KO females (figure 2A and 2B). Lung responsiveness to nebulized MCh revealed that WT (M and F) and KO (M) mice gavaged with 10 mg/kg LAU-7b for 9 days had significantly lower lung resistance than PBS-treated mice (figure 2C and 2D). Allergic KO females display very low increase in lung resistance following HDM challenge (non-significant from PBS), and the treatment does not improve the lung physiology any further, which under these circumstances is not surprising in this group of mice.

The protective effects of LAU-7b against increased airway hyperresponsiveness after allergen challenge prompted us to evaluate its potential against IgE production which is also associated with allergic asthma. After HDM challenge, the titer of IgE was increased by 4- to 10-folds in WT and KO mice (figure 2E). IgE measurements show that WT and KO, male and female groups, were not statistically significant one from another. Nonetheless, LAU-7b treatment did not lower the levels of IgE, caused by HDM challenge in WT and KO, male and female mice, compared to PBS-treated and HDM challenged mice.

Figure 3.2 Classical invasive measurements of lung resistance and measurements of IgE levels of *Zpbp2* KO and WT mice

A) Classical invasive measurements of lung resistance in A) WT and B) *Zpbp2* KO mice. All mice were sensitized with house dust mite (HDM) and challenged with either PBS or HDM. Treatment group is marked as (LAU-7b). The doses of MCh used were 25 mg/mL, 50 mg/mL and 100 mg/mL. LAU-7b treatment and airway hyper-responsiveness in MCh nebulized male C) and female D) mice. Two-way ANOVA, n equal at least 11 mice in each group. E) Treatment with LAU-7b did not affect the levels of IgE in *Zpbp2* KO and WT mice. Two-way ANOVA, n equal at least 5 mice for each group. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$


A
WT-M HDM
WT-M PBS
WT-F HDM
WT-F PBS
RI (cm.H2O/mL/s)
Baseline
Saline
MCh 25 mg/mL
MCh 50 mg/mL
MCh 100 mg/mL
B
KO-M HDM
KO-M PBS
KO-F HDM
KO-F PBS
C
WT-M HDM
WT-M LAU-7b
WT-M PBS
KO-M HDM
KO-M LAU-7b
KO-M PBS
D
WT-F HDM
WT-F LAU-7b
WT-F PBS
KO-F HDM
KO-F LAU-7b
KO-F PBS
E
Plasma IgE Level (ng/mL)
WT
KO
PBS
HDM
LAU-7b
M
F

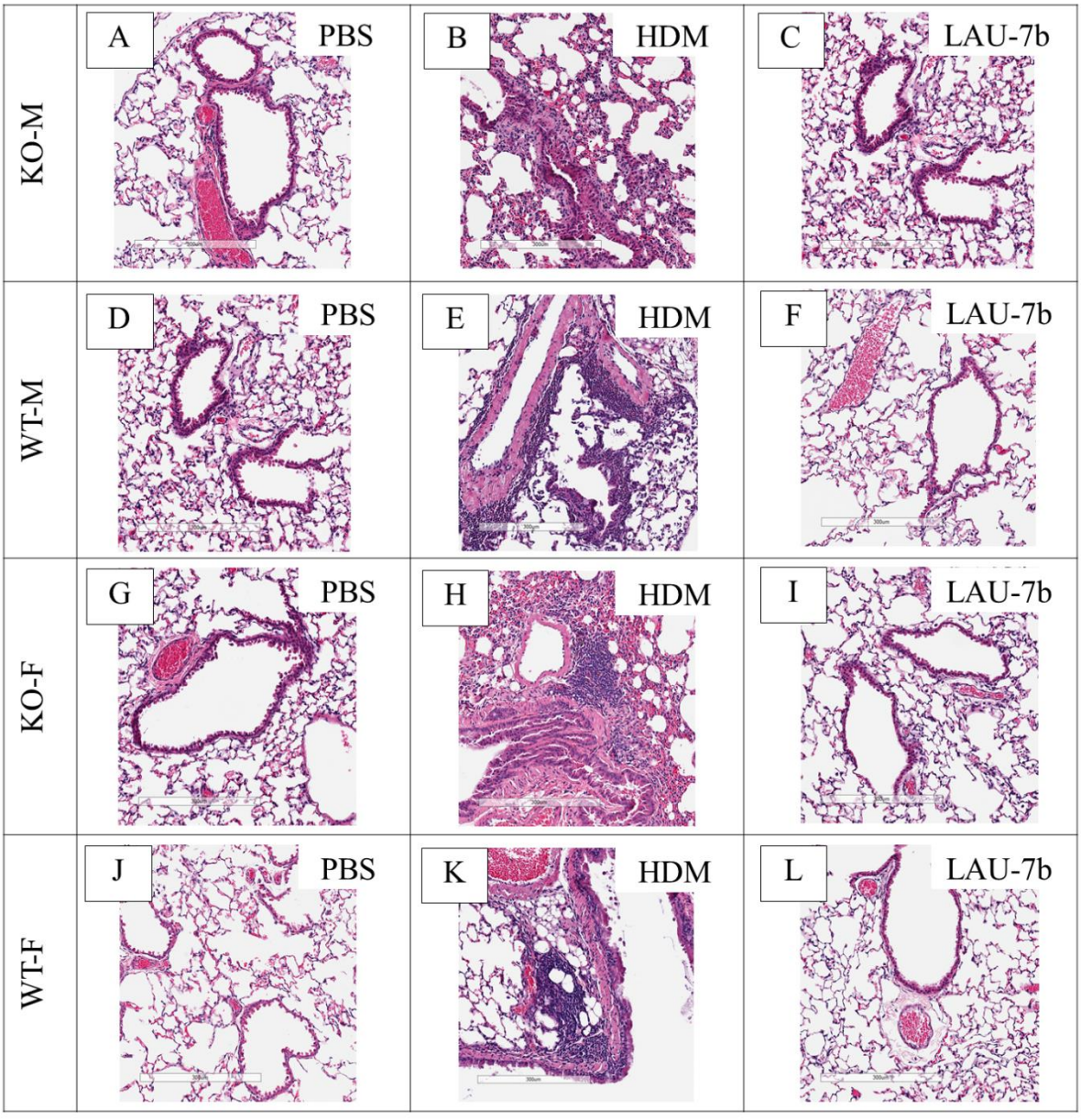
3.4.3 Evaluation of Inflammatory Cells Infiltration and Mucus Production in the Airways

To visualize and quantify the inflammatory cells infiltration into the airways after HDM challenge, we used H&E staining (figure 3). The lung sections of both WT and KO mice showed significant incoming inflammatory cells after HDM challenge compared to PBS challenged mice (figure 3A-3L). We also quantified our H&E staining results by counting and normalizing the recruited cells around the lung airways (figure 3M). Our data demonstrated that there was equally strong inflammatory cells infiltration into the airways of both WT and KO, male and female, mice following HDM allergen challenge, despite that KO mice had displayed much lesser lung resistance than WT mice. As shown in the representative pictures from each mouse group, after LAU-7b treatment significantly lowered the recruitment of inflammatory cells in both WT and KO, male and female, mice.

Mucus hypersecretion, and the subsequent plugging of the airways, has long been recognized as a common phenotype of allergic asthma. The mucus production was not investigated before in the *Zpbp2* KO mouse model, so in our study we wanted to examine if the deletion of this gene would result in any effects that could be visualized by the PAS stain (figure 4) and corrected with LAU-7b treatment. Our lung sections of both WT and KO mice markedly show mucus production by goblet cells after HDM challenge compared to PBS challenged mice (figure 4A-4L). No significant differences between WT and KO, male or female mice were observed. LAU-7b treatment significantly decreased the production of mucus by goblet cells in both WT and KO, male and female, mice (figure 4M).

Figure 3.3 Infiltration of airways by inflammatory cells in untreated and LAU-7b treated mice; Haematoxylin and eosin (H&E) staining

All mice were sensitized with house dust mite (HDM) and challenged with either PBS or HDM. Treatment group is marked as (LAU-7b). Panel A-C) *Zpbp2* KO males, D-F) WT males, G-I) *Zpbp2* KO females, and J-L) WT females. M) LAU-7b treated mice have significantly lower lung cell infiltration compared to the lungs of untreated mice. Quantification was done by counting the number of inflammatory cells around each airway and normalizing it by division over the square of the perimeter "in millimeter" of the airway basement membrane. Measurements were done using at least 4 different airways for each mouse from each group. n equal at least 7 mice for each group, Two-way ANOVA. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$


KO-M
WT-M
KO-F
WT-F
A
PBS
B
HDM
C
LAU-7b
D
PBS
E
HDM
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G
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L
LAU-7b

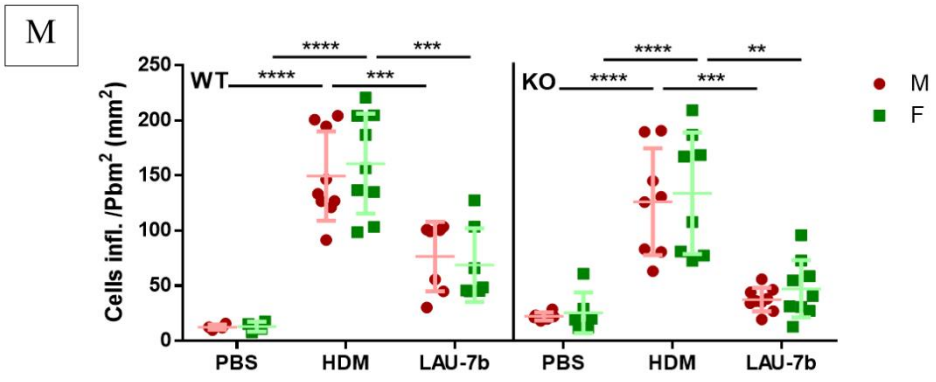
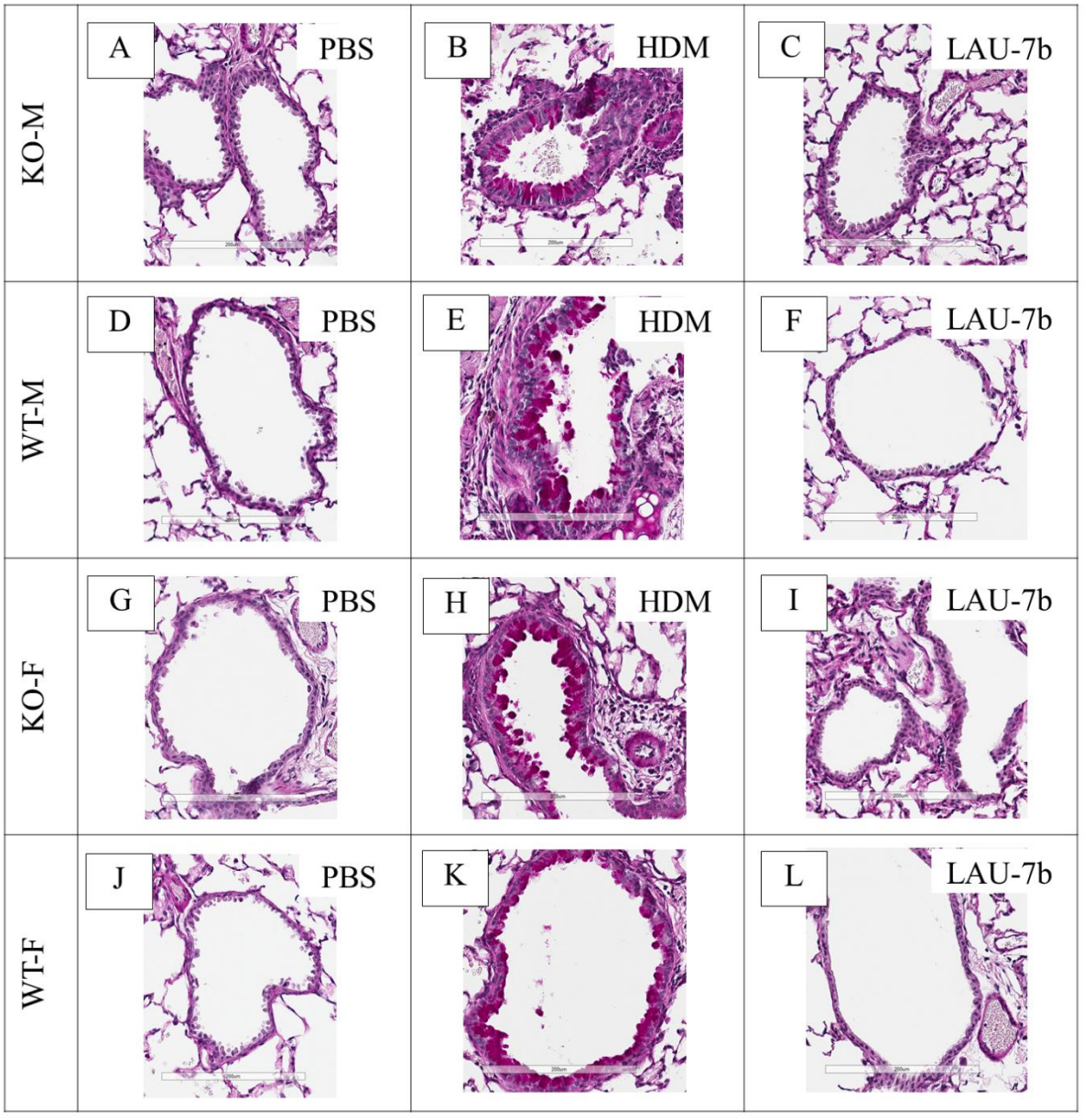
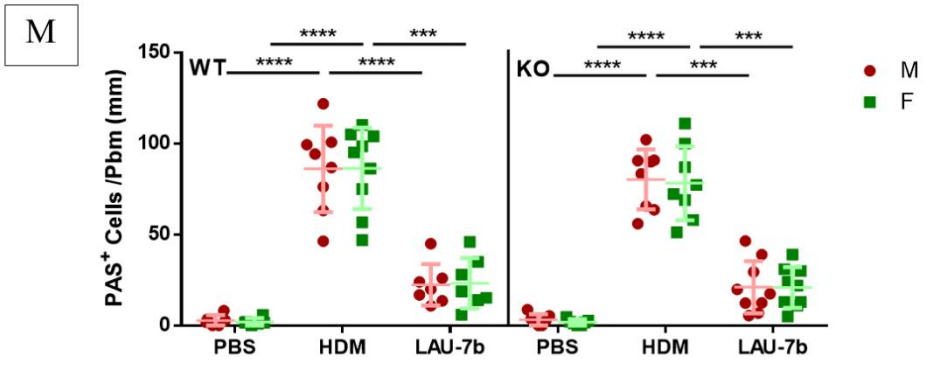

M
Cells infl. /Pbm2 (mm2)
250
200
150
100
50
0
WT
KO
****
***
**
PBS
HDM
LAU-7b
M
F

Figure 3.4 Mucus production by Goblet cells in untreated and LAU-7b treated mice. Periodic acid Schiff (PAS) staining

All mice were sensitized with house dust mite (HDM) and challenged with either PBS or HDM. Treatment group is marked as (LAU-7b). Panel A-C) *Zpbp2* KO males, D-F) WT males, G-I) *Zpbp2* KO females, and J-L) WT females. M) LAU-7b treated mice have significantly lower production of mucus by Goblet cells compared to untreated mice. Quantification was done by counting the number of PAS positive cells around each airway and normalizing it by division over the perimeter "in millimeter" of the airway basement membrane. Measurements were done using at least 4 different airways for each mouse from each group. n equal at least 7 mice for each group, Two-way ANOVA. *** $p<0.001$, **** $p<0.0001$


KO-M
WT-M
KO-F
WT-F
A
PBS
B
HDM
C
LAU-7b
D
PBS
E
HDM
F
LAU-7b
G
PBS
H
HDM
I
LAU-7b
J
PBS
K
L
LAU-7b


M
WT
KO
****
***
PAS+ Cells /Pbm (mm)
150
100
50
0
PBS
HDM
LAU-7b
M
F

3.4.4 Analysis of Ceramides, Fatty Acids, and Markers of Oxidation

We evaluated the levels of malondialdehyde (MD), a marker of lipid oxidation, and nitro tyrosine (NT), a marker of protein oxidation, in our mice because they are markers of cellular stress and damage which happen after allergen challenge. After HDM challenge, both MD and NT significantly increased in male and female KO and WT mice (figure 5A and 5B). No significant differences were observed between males and females, or KO and WT mice. LAU-7b significantly normalized the levels of MD and NT in the HDM-challenged and treated mice compared to HDM-challenged and untreated mice.

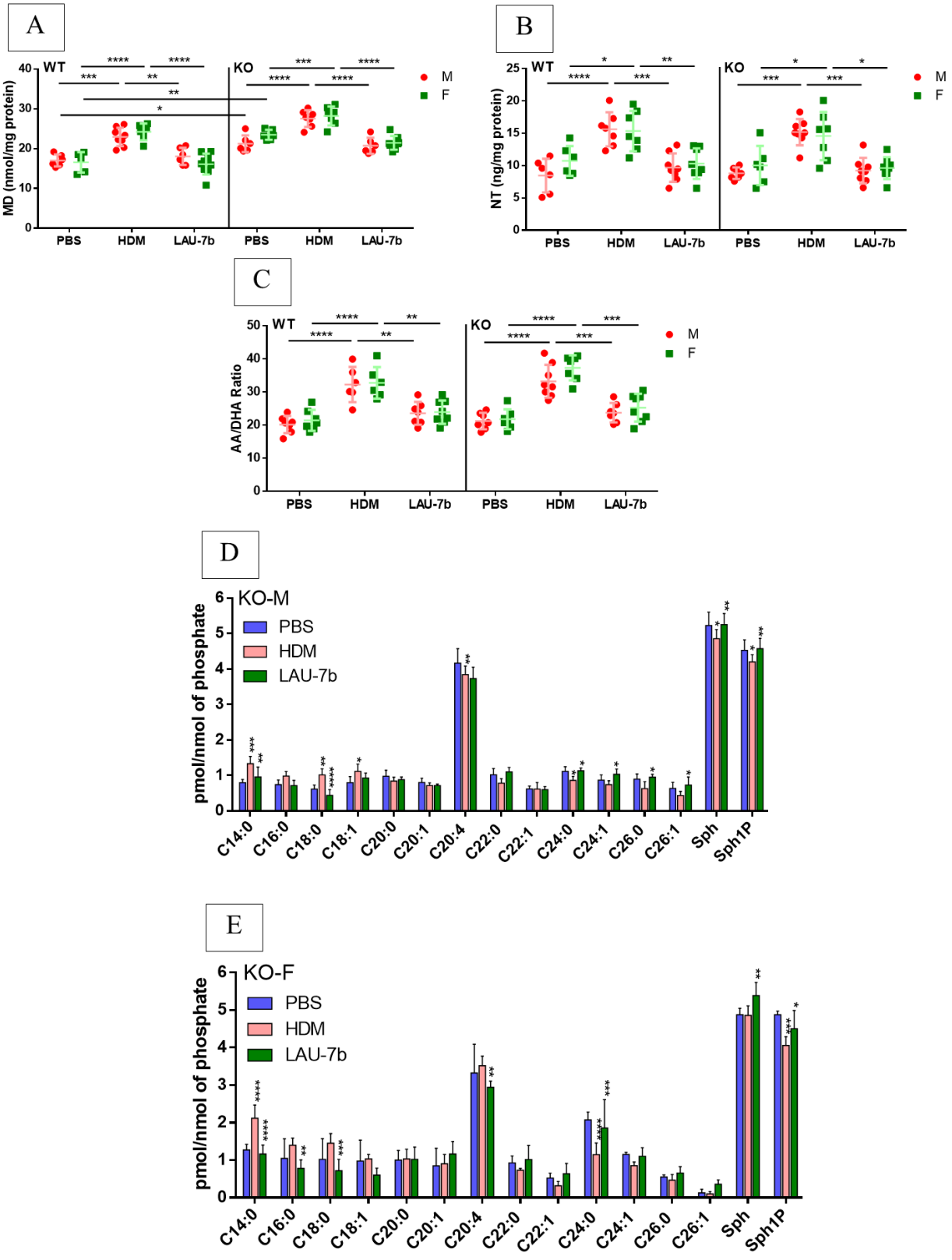
Furthermore, fatty acid analysis (figure 5C) reveals significant elevation in arachidonic acid/ docosahexaenoic acid ratio (AA/DHA ratio) following HDM challenge which is typically associated with inflammation. Nevertheless, this ratio is significantly lowered after treatment with LAU-7b in HDM sensitized and challenged mice compared to the untreated HDM sensitized and challenged mice. Likewise, no significant differences in AA/DHA ratio were observed between males and females, or KO and WT mice.

The lipidomic analysis of the lungs (figure 5D and 5E and figure SUPP.) revealed that the relative levels of VLCCs (C22:0, C22:1, C24:0, C24:1, C26:0, C26:1) were diminished in HDM-challenged KO mice (females = 24.07%, lower than males = 34.45%) compared to PBS-challenged KO mice (females = 34.44%, lower than males = 37.93%). LAU-7b treated KO mice displayed higher levels of VLCCs compared to untreated KO mice in both males and females; 40.70% and 40.24%, respectively. In KO mice, treatment with LAU-7b resulted in significantly elevated levels of C24:0, C24:1, C26:0 and C26:1 in males, and C24:0 in females. Similarly, the treatment with LAU-7b resulted in significantly reduced levels of LCCs C14:0 and C18:0 in KO males, and C14:0, C16:0 and C18:0 in KO females.

Moreover, compared to KO mice, the assessment of VLCCs demonstrated that WT male and female mice have higher levels of VLCCs (figure SUPP.). Total VLCCs levels in PBS-challenged WT mice demonstrated a percentage of 41.85% in males and 43.07% in females. After challenge with HDM, the total VLCCs levels were reduced to 39.92% in males and to 31.06% in females. As shown here, the VLCCs values obtained from male and female KO mice were lower than the values obtained for male and female WT mice for both PBS- and HDM-challenged groups. However, as observed in KO mice, LAU-7b treatment restored the levels of VLCCs to those typically observed in WT male and female mice, up to 42.43% and 47.89%, respectively, which is even higher than the levels of VLCCs detected before HDM-challenge (in PBS mouse groups).

Figure 3.5 LAU-7b treatment corrected the aberrant levels of markers of oxidation, fatty acids and sphingolipids in *Zpbp2* KO and WT mice

Analysis of A) malondialdehyde (MD), marker of lipid oxidation, B) nitro tyrosine (NT), marker of protein oxidation, and C) arachidonic acid/ docosahexaenoic acid ratio (AA/DHA ratio). Analysis of sphingolipid species in D) *Zpbp2* KO male and E) *Zpbp2* KO female mice. All mice were sensitized with house dust mite (HDM) and challenged with either PBS or HDM. Treatment group is marked as (LAU-7b). Two-way ANOVA, n equal at least 6 mice for each group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$


A
WT
KO
MD (nmol/mg protein)
PBS
HDM
LAU-7b
M
F
B
NT (ng/mg protein)
C
AA/DHA Ratio
D
KO-M
pmol/nmol of phosphate
C14:0
C16:0
C18:0
C18:1
C20:0
C20:1
C20:4
C22:0
C22:1
C24:0
C24:1
C26:0
C26:1
Sph
Sph1P
E
KO-F

3.5 Discussion

Several genome-wide association studies (GWAS) had investigated the genetic bases of allergic asthma extensively to explain why some people are susceptible to asthma while other people are not. In the first reported GWAS study for asthma susceptibility, Moffatt *et al.* (2) identified the 17q21 locus, containing several genes such as *ORMDL3* and *ZPBP2,* as being associated with asthma. The importance of this region was subsequently replicated in several subsequent studies (16-18). In this study, we developed a mouse model with a deletion of the *Zpbp2* gene on an atopic A/J background which is genetically susceptible to the development of allergic asthma and displays airway AHR even at the basal level (without any allergen exposure).

Our main objective was threefold. First, we aimed to evaluate the effect of the *Zpbp2* gene deletion on lung physiology and inflammatory cells infiltration into the lungs, following HDM challenge and sensitization, in *Zpbp2* KO and littermate WT control A/J mice. Secondly, we aimed to investigate the effects of LAU-7b treatment against HDM-induced allergic asthma in *Zpbp2* KO and WT littermate A/J mice. Our third objective was to test the expression of *Zpbp2* and *Ormdl3* genes following LAU-7b treatment to evaluate if there might be an association between the modulation of the expression of these genes and the changes in the regulation of relative ratios of VLCCs and LCCs as observed in allergic asthma. It has been described that Fenretinide elevated the levels of VLCCs and reduced the levels of LCCs (9) in CF KO mice with chronic lung inflammation. Therefore, we assessed all ceramide levels in this allergic asthma mouse model to establish if the normalization of VLCC and LCC levels in allergic asthma might occur following treatment with Fenretinide. We also postulated that the modulation of ceramides synthesis, associated with Fenretinide treatment, is mediated by the regulation of *Ormdl3* gene expression.

We have previously published (5) that the deletion of the *Zpbp2* gene in C57BL/6 mice resulted in a reduction of AHR in females, but not males, on baseline levels. However, the deletion of the *Zpbp2* gene in our previous study (5) did not significantly affect the AHR of male or female mice after OVA sensitization and challenge, compared to WT littermate controls, perhaps because the C57BL/6 strain of mice is genetically resistant to developing allergic asthma. Miller *et al.* (6) have similarly reported that *Zpbp2* KO C57BL/6 male and female mice challenged with HDM had significantly reduced AHR compared to WT controls. Our results here demonstrated that deletion of the *Zpbp2* gene on the A/J background has resulted in a significant reduction of baseline Penh values in male and female mice (figure 1I), compared to WT controls. Similarly, we observed a significant reduction in AHR (shown by the lung resistance values, figure 2A and 2B) of *Zpbp2* KO male and female mice compared to WT mice. By using an A/J hyperresponsive strain of mice, and inducing allergic asthma by HDM, we have obtained a good segregation of different mouse groups (KO and WT, males and females) in terms of AHR. Our data demonstrates for the first time that in the genetically very susceptible and atopic mice this gene plays important role in the control of lung physiology in allergic asthma. These data are consistent with our previous findings (5) and the report by Miller *et al.* (6) on the impact of *Zpbp2* gene deletion and AHR done using the C57BL/6 non-atopic hyporesponsive strain of mice.

Sex discordance is a well-established factor taken into consideration when observing discrepancies in airway hyperresponsiveness between allergic males and females. Additionally, this disparity appears to be affected by the age of the asthmatic individual (19). Changes in asthma prevalence, based on sex, coincide with alterations of sex hormones throughout life (in the case of males and females) and menstruation status (in case of females), consequently, in allergic asthma, studying mice of different sexes draws a comprehensive picture of the disease (20). At a younger age, males have an increased prevalence of asthma compared to females (19). Conversely, by

adulthood, evidence suggests that allergic asthma has a higher incidence in females (21), however, because females experience continuous alterations in the levels of sex hormones throughout life, this conclusion can not be inclusive. In our current study, we have assessed the deletion of the *Zpbp2* gene in both male and female mice, compared to WT littermate control mice of the same sex and age. Consistent with the previously published data (22,23), our data show that WT males have higher Penh values and lung resistance than WT females; similarly, KO males have higher Penh values and lung resistance than KO females (figure 1I and figure 2A and 2B). Nonetheless, no differences were observed on the level of cell infiltration to the lungs (figure 3), mucus production (figure 4), lipid levels (figure 5) or gene expression (figure 1) in the lungs of mice of both sexes except for *Zpbp2* gene expression (figure 1C) which has shown to be non-significantly lower in female WT mice when compared to male WT mice. Regardless of the sex of the studied mouse group, LAU-7b treatment significantly decreased lung resistance measurements, recruitment of inflammatory cells, mucus production and normalized altered distribution of ceramides and fatty acids in the airways of mice.

HDM sensitization and challenge significantly enhanced the recruitment of inflammatory cells into the lungs (figure 3), caused hyperplasia of the lining of the airways (figure 3), and increased the production of mucus (figure 4) in *Zpbp2* KO and WT mice. Interestingly, the ablation of the *Zpbp2* gene, although it affected the lung resistance, did not result in the modulation of inflammatory response following allergen challenge. Although *Zpbp2* KO mice have reduced AHR after HDM challenge, the inflammatory response demonstrated by the recruitment of cells into the airways and excessive mucus production was still induced. Altogether, in WT and *Zpbp2* KO mice, males and females, LAU-7b treatment had resulted in significantly lower cell infiltration, no hyperplasia and inhibited mucus production compared to PBS-treated mice of the same genotype and sex.

To determine whether *Zpbp2* deficiency influenced the levels of IgE, a common marker of allergy, we quantified IgE in plasma which was statistically elevated after HDM challenge in KO males and females to similar levels present in WT mice (figure 2E). Although LAU-7b treatment did not correct elevated plasma IgE levels either in KO males or in females (findings were similar to our previously published data (5,13)), it was still able to control the inflammatory response to the allergen in the lungs and normalize lung physiology of the allergen-sensitized and challenged animals. These findings suggest that even in the most severely affected patients who are not responding to either steroids or to anti-IgE therapy anymore, this type of treatment might still be effective.

Several studies have investigated how *Ormdl3* plays a significant role in allergic asthma (24). In yeast, ORM proteins negatively regulate sphingolipid synthesis by forming a conserved complex with the serine palmitoyl transferase (SPT) enzyme, thus, inhibiting the first rate-limiting step of *de novo* ceramide, and all other sphingolipids, biosynthesis (25). Furthermore, in a mouse study, myriocin, an inhibitor of the SPT enzyme, reduced the *de novo* sphingolipid synthesis and increased bronchial reactivity (26). Likewise, increased airway responsiveness and airway remodeling have been reported in C57BL/6 transgenic mice overexpressing *Ormdl3* after OVA sensitization and challenge (27). Similarly, C57BL/6 mice lacking *Ormdl3* were protected from developing AHR and airway eosinophilia induced by *Alternaria alternate* (28). Several studies have been published in regard to *Ormdl3* and allergic asthma, nevertheless, the full molecular picture has not been elucidated yet.

Further, the expression of *ORMDL3* is not independent of the expression of other genes present in the 17q21 chromosomal locus. For example, it has been reported that *ZPBP2 and ORMDL3 genes* are co-regulated together as closely associated cis-haplotype elements (29). In our study, we report that there is significantly lower *Ormdl3* gene expression in A/J *Zpbp2* KO mice

than in the littermate WT controls (figure 1D). The finding that knocking out of the Z*pbp2* gene in A/J mice has markedly reduced AHR can be, at least partially, attributed to the downregulation of two hyperresponsiveness susceptibility genes (*Ormdl3* and *Zpbp2*), since this strain of mice expresses genes which make them both atopic (loci on chromosome 4) and increase AHR (loci on chromosome 12). We have previously reported (5) that ablation of *Zpbp2* in C57BL/6 mice did not significantly change the levels of *Ormdl3* gene expression. This apparent difference in the impact of *Zpbp2* ablation on *Ormdl3* gene expression between the two strains of mice might be explained by the differences between the C57BL/6J and A/J genetic backgrounds. Moreover, modeling allergic asthma was not similar in the two studies which makes the two studies difficult to compare.

The correlation between the gene expression of *Ormdl3* and the levels of ceramide species was recently investigated in-depth in several studies. It has been reported that elevated levels of ORMDL3 inhibit sphingolipid biosynthesis resulting in inhibition of both long chain ceramide (LCC; C16:0) and four species of very long chain ceramides (VLCC; C22:0, C24:0, C24:1 and C24:2) (8). By contrast, Zhang *et al.* (30) had reported that *ORMDL3* gene silencing in A549 and NHBE cell lines resulted in a marked increase in the levels of C24:0, C24:1, C26:1 and sphingosine-1-phosphate species. Recently, Debeuf *et al.* (31) reported that the transgenic mice overexpressing *Ormdl3* had significantly reduced levels of C24:0 and C24:1, by contrast, loss of *Ormdl3* in mice resulted in elevated levels of C24:0 and C24:1. Interestingly, our results demonstrated that LAU-7b treatment had lowered the expression of *Ormdl3* (figure 1D) and, overall, had protected the sensitized and challenged WT mice against allergic asthma. It is well-established that *Ormdl3* overexpression inhibits *de novo* sphingolipids biosynthesis, therefore, the LAU-7b induced downregulation of *Ormdl3* gene expression in WT mice may explain, at least in part, the reason why we observed the increase in the levels of VLCCs in LAU-7b treated mice

(figure 5). Although *Zpbp2* KO mice have lower AHR after HDM sensitization and challenges, the inflammatory reaction after allergen exposure still occurred to its full capacity. It is intriguing that LAU-7b treatment was able to protect the *Zpbp2* KO mice against the inflammatory reactions after HDM sensitization and challenges. This effect can not be explained by the ability of LAU-7b to modulate *Ormdl3* expression since our *Zpbp2* KO mouse model has ablated expression of *Zpbp2* and reduced expression of *Ormdl3*. These findings demonstrate that LAU-7b acts in *Zpbp2* KO mice *via* an alternative pathway, for example by its ability to block ERK1/2 protein phosphorylation which we have previously reported to be modulated by Fenretinide treatment in the context of macrophage response to endotoxins (32).

Our data show that after HDM challenge, *Zpbp2* (in males) and *Ormdl3* (in males and females) gene expression were significantly elevated (figure 1A and 1B). Beside *Zpbp2* and *Ormdl3*, we also tested four important genes (*Il-4, Il-5, Il-13 and Eotaxin-1*) that are overexpressed by T helper type 2 (Th2) cells in allergic asthma (figure 1E-1H). IL-4 is the central cytokine in allergic asthma because it promotes the differentiation of naive Th0 lymphocytes into Th2 lymphocytes in a positive feedback loop (33). IL-5 is the main cytokine involved in activating eosinophils (34), on the other hand, eotaxin-1 is a chemokine which further helps the eosinophils migrate into the airways after allergen challenge in asthma (35). IL-13 is a key regulatory cytokine in the production of IgE and has similar biological effects to IL-4 (36).

After challenge with HDM, both *Zpbp2* KO and WT mice had significantly increased expression of *Il-5, Il-13* and *Eotaxin-1* genes compared to PBS-challenged control mice (figure 1E-1H). However, for these 3 genes, no significant differences were observed between *Zpbp2* KO and WT mice. These findings coincide with non-significant differences between the two mouse strains in terms of elevated IgE levels (figure 2E) and an increased number of inflammatory cells recruited into the airways (figure 3) after allergen challenge. In our *Zpbp2* KO mouse model, the

expression of *Il-4* was not elevated significantly following HDM challenge compared to PBS KO-controls, unlike HDM challenged WT mice, which show significant elevation of *Il-4* levels. Similarly, Miller *et al.* (6) reported a lack of significant increase in IL-13 levels in *Zpbp2* KO mice after HDM challenge compared to *Zpbp2* KO controls. The results of the studies reported by Debeuf *et al.* (31) also demonstrated the lack of a significant increase in the levels of IL-13 or IL-5 after HDM challenge when the mice were either overexpressing, or had displayed the loss of, *Ormdl3*. Levels of IL-4 were not reported in these two cited above studies. Taken together, there is no evidence linking the alteration of *Zpbp2* or *Ormdl3* genes with modulation of *Il-4, Il-5, Il-13 or Eotaxin-1* expression.

Interestingly, our studies clearly demonstrate that treatment of allergen-sensitized and allergen-challenged mice with LAU-7b had lower expression of *Il-4* (WT mice), *Il-5* and *Eotaxin-1* (WT and KO mice) compared to PBS-treated mice. By contrast, both *Zpbp2* KO and WT mice treated with LAU-7b did not show significant reduction of *Il-13* expression. Maintenance of high *Il-13* expression levels seen after LAU-7b treatment shown in our study may explain why the IgE levels remained elevated in both *Zpbp2* KO and WT mouse groups, and also document that even without normalization of IL-13 levels and IgE levels it was possible to significantly diminish the lung inflammation and correct lung physiology when the allergic subjects were treated with LAU-7b.

Clinically, quick-relief medicines, e.g. short-acting inhaled beta2-agonists, can stop asthma symptoms in minutes *via* dilating the airways, but they do not control the airway inflammation. The long-term control medicines currently recommended for allergic asthma are inhaled corticosteroids and antileukotrienes. However, prolonged treatment with corticosteroids eventually results in the development of resistance to corticosteroids. Based on the posology used in our study, a similar treatment protocol using LAU-7b capsules should be likewise effective in

treating allergic asthma-triggered inflammation of the airways, with greater patient compliance at once-a-day dosing. Interestingly, the allergic asthma symptoms can be perfectly controlled even if the IgE levels are not modulated by drug treatment. Overall, the preclinical data presented here for Fenretinide, the active pharmaceutical ingredient of LAU-7b capsules, provide robust grounds that justify testing the efficacy of this dosage form in a clinical trial, especially among asthmatics whose severe asthma is no longer treatable using the currently available therapies.

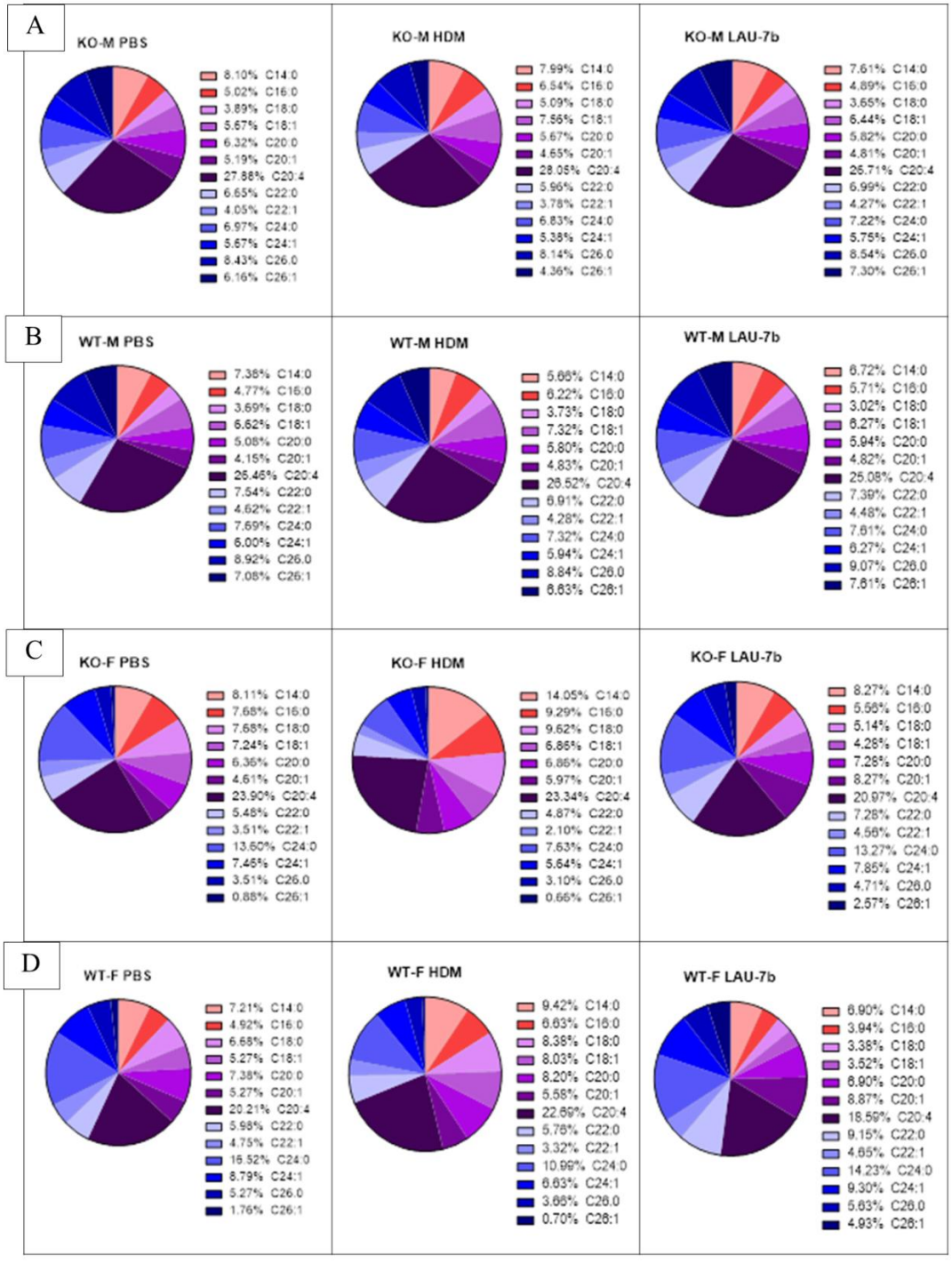
3.6 Conclusion

LAU-7b treatment, in a dose of 10 mg/kg/day for 9 days, protects both *Zpbp2* KO mice, which have significantly reduced *Ormdl3* expression, and their WT littermate controls from HDM-induced allergic asthma. Interestingly, in WT mice, LAU-7b had significantly lowered the expression of *Ormdl3,* and hence, it can be effective in allergic asthma treatment by elevating the levels of VLCCs and decreasing the levels of LCCs.

3.7 Supplementary Data

Figure 3.6 Pie charts of ceramides levels in *Zpbp2* KO and WT, male and female, mice

Analysis of (long chain ceramides) LCCs and (very long chain ceramides) VLCCs species in A) *Zpbp2* KO male, B) WT male, C) *Zpbp2* KO female, and D) WT female mice. All mice were sensitized with house dust mite (HDM) and challenged with either (PBS) or (HDM). Treatment group is marked as (LAU-7b). Relative levels of VLCCs were diminished in HDM-challenged KO male and female mice compared to PBS-challenged KO mice. LAU-7b treated KO mice displayed higher levels of VLCCs and reduced levels of LCCs compared to untreated KO mice in both males and females. Compared to KO mice, the assessment of VLCCs demonstrated that WT male and female mice have higher levels of VLCCs for both PBS- and HDM-challenged groups. As observed in KO mice, LAU-7b treatment restored the levels of VLCCs to those typically observed in WT male and female mice after HDM challenge.


A
KO-M PBS
8.10% C14:0
5.02% C16:0
3.89% C18:0
5.67% C18:1
6.32% C20:0
5.19% C20:1
27.88% C20:4
6.65% C22:0
4.05% C22:1
6.97% C24:0
5.67% C24:1
8.43% C26:0
6.16% C26:1
KO-M HDM
7.99% C14:0
6.54% C16:0
5.09% C18:0
7.56% C18:1
5.67% C20:0
4.65% C20:1
28.05% C20:4
5.96% C22:0
3.78% C22:1
6.83% C24:0
5.38% C24:1
8.14% C26:0
4.36% C26:1
KO-M LAU-7b
7.61% C14:0
4.89% C16:0
3.65% C18:0
6.44% C18:1
5.82% C20:0
4.81% C20:1
26.71% C20:4
6.99% C22:0
4.27% C22:1
7.22% C24:0
5.75% C24:1
8.54% C26:0
7.30% C26:1
B
WT-M PBS
7.36% C14:0
4.77% C16:0
3.69% C18:0
6.62% C18:1
5.08% C20:0
4.15% C20:1
25.46% C20:4
7.54% C22:0
4.62% C22:1
7.69% C24:0
6.00% C24:1
8.92% C26:0
7.08% C26:1
WT-M HDM
5.66% C14:0
6.22% C16:0
3.73% C18:0
7.32% C18:1
5.80% C20:0
4.83% C20:1
26.52% C20:4
6.91% C22:0
4.28% C22:1
7.32% C24:0
5.94% C24:1
8.84% C26:0
6.63% C26:1
WT-M LAU-7b
6.72% C14:0
5.71% C16:0
3.02% C18:0
6.27% C18:1
5.94% C20:0
4.82% C20:1
25.08% C20:4
7.39% C22:0
4.48% C22:1
7.61% C24:0
6.27% C24:1
9.07% C26:0
7.81% C26:1
C
KO-F PBS
8.11% C14:0
7.68% C16:0
7.68% C18:0
7.24% C18:1
6.36% C20:0
4.61% C20:1
23.90% C20:4
5.48% C22:0
3.51% C22:1
13.60% C24:0
7.46% C24:1
3.51% C26:0
0.88% C26:1
KO-F HDM
14.05% C14:0
9.29% C16:0
9.62% C18:0
6.86% C18:1
6.86% C20:0
5.97% C20:1
23.34% C20:4
4.87% C22:0
2.10% C22:1
7.63% C24:0
5.64% C24:1
3.10% C26:0
0.66% C26:1
KO-F LAU-7b
8.27% C14:0
5.56% C16:0
5.14% C18:0
4.26% C18:1
7.26% C20:0
8.27% C20:1
20.97% C20:4
7.26% C22:0
4.56% C22:1
13.27% C24:0
7.85% C24:1
4.71% C26:0
2.57% C26:1
D
WT-F PBS
7.21% C14:0
4.92% C16:0
6.68% C18:0
5.27% C18:1
7.38% C20:0
5.27% C20:1
20.21% C20:4
5.98% C22:0
4.75% C22:1
16.52% C24:0
8.79% C24:1
5.27% C26:0
1.76% C26:1
WT-F HDM
9.42% C14:0
6.63% C16:0
8.38% C18:0
8.03% C18:1
8.20% C20:0
5.58% C20:1
22.69% C20:4
5.76% C22:0
3.32% C22:1
10.99% C24:0
6.63% C24:1
3.66% C26:0
0.70% C26:1
WT-F LAU-7b
6.90% C14:0
3.94% C16:0
3.38% C18:0
3.52% C18:1
6.90% C20:0
8.87% C20:1
18.59% C20:4
9.15% C22:0
4.65% C22:1
14.23% C24:0
9.30% C24:1
5.63% C26:0
4.93% C26:1

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Preface to Chapter 4

In Chapter 2 and Chapter 3 of this thesis, we have tested a novel clinical formulation of Fenretinide (LAU-7b) to assess its efficacy against allergic asthma induced in A/J mice. The treatment with LAU-7b dramatically improved lung physiology parameters in A/J mice suffering from allergic asthma. Although we have previously shown that LAU-7b treatment improves the ability of *Cftr* KO mice to fight against *Pseudomonas* lung infection and decreases chronic inflammation, we have not previously tested lung physiology parameters in *Cftr* KO mice compared to their littermate controls. Our carefully selected *Cftr* KO mouse model (not expressing any alternative chloride channels) provides a unique experimental model allowing us to test for a direct link between *Cftr* gene ablation, ageing, and diminished lung physiology parameters. It is not possible to test this hypothesis in CF patients because their lung physiology starts deteriorating when they become young adults and at that point most of them are chronically infected with various pathogens.

In Chapter 4, we hypothesized that the *Cftr* gene deletion is associated with progressive age-dependent deterioration in the lungs. The results illustrated in Chapter 4 were generated to specifically address the following hypotheses:

1) *Cftr* KO mice, compared to their littermate controls, will show significantly different lung physiology parameters due to chronic inflammation-induced changes in the lungs of *Cftr* KO mice. Hence, the *Cftr* gene deletion-associated difference in lung physiology will be more pronounced in older mice than younger mice.

2) The protective effect of LAU-7b treatment might prevent chronic inflammation-associated deterioration of lung physiology parameters in *Cftr* KO mice.

Chapter 4. Protective effect of treatment with LAU-7b against age-related deterioration in *Cftr* knock-out mice

Manuscript to be submitted to the Journal of Cellular and Molecular Life Sciences

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4.1 Abstract

Background: Cystic fibrosis (CF) is a progressive disease that causes continuous decline in lung capacity with age. Fenretinide has been shown to correct the inflammatory responses in several diseases including CF. A novel clinical formulation of Fenretinide (LAU-7b), with excellent bioavailability, was recently developed by Laurent Pharmaceutical Inc. Our study aimed to investigate the age-dependent deterioration in lung function in CF and the effects of treatment with LAU-7b.

Methods: Non-invasive whole-body plethysmography (WBP) was done to measure the baseline lung functions of the *Cftr* knockout (KO) and wild type (WT) mice at the age of 2 and 4 months. Mice were then treated for 21 days with PBS or LAU-7b at a dose of 10mg/kg/day, initiated at 4 and 7 months. At the end of treatment, we performed standard airway resistance measurements. Haematoxylin and eosin staining visualized inflammatory cell infiltration and airway hyperplasia at the ages of 2, 4 and 7 months. The analysis of lipids and markers of oxidation in plasma and lungs was performed using mass spectrometry.

Results: The 4- and 7-month-old KO mice had significantly higher lung enhanced pause (Penh) and resistance values than age-matched WT mice and 2-month-old KO mice. Likewise, analysis of ceramides showed that PBS-treated mice had higher levels of long chain ceramides (LCCs; C14-C18) and lower levels of very long chain ceramides (VLCCs; C24-C26) compared to LAU-7b treated mice. *Cftr* KO mice displayed markedly greater inflammatory cell infiltration and epithelial hyperplasia at the ages of 2, 4 and 7 months compared to WT. LAU-7b treatment significantly diminished this cellular infiltration and epithelial hyperplasia compared to PBS-treated mice.

Conclusion: Our results demonstrate a progressive age dependent decline in lung function in *Cftr* KO mice. Treatment with LAU-7b corrects the lipid imbalance observed in the aging KO and WT mice and, more importantly, inhibits the age-dependent deterioration in lung physiology and histopathology.

Keywords: Cystic fibrosis, *Cftr* knockout mice, LAU-7b (Fenretinide), airways inflammation, ceramides imbalance.

4.2 Introduction

Cystic fibrosis (CF) is caused by a defect in the CF transmembrane conductance regulator (CFTR) protein, which functions as a chloride channel regulator. Dysfunction of the CFTR protein results in pancreatic insufficiency, intestinal obstruction, male infertility, and severe pulmonary complications which are the main cause of morbidity and mortality in CF patients (1). CF-related symptoms worsen with age, and patients usually experience a progressive decline in lung function with every additional year of living. This increases the risk of complications related to CF lung disease (2).

Some CF patients remain relatively healthy during their childhood (3) and may remain undiagnosed until disease symptoms appear, or they are investigated for apparent infertility. While each person's experience with CF is different, based on the disease severity and the patient's lifestyle, it is noteworthy that CF patients can remain relatively healthy if they are adequately managed by medications (4). Several treatments have been developed to help manage the disease pathology and the resultant symptoms. Collectively, these therapeutic approaches for CF frequently allow the patients to achieve an acceptable quality of life and longer survival rates (5). However, there is still no cure for CF.

Since the discovery of the *Cftr* gene (1), a number of *Cftr* knock out (KO) murine models have been developed. However, only a few of these models display the CF lung phenotype (10-12). The original *Cftr*^{m1UNC} KO mice on the mixed 129/J C57BL/6 genetic background failed to develop lung disease because of the compensatory activity of alternate non-CFTR chloride channels in the airway epithelium (19). In contrast, C57BL/6J *Cftr*^{m1UNC}/*Cftr*^{m1UNC} KO mice, described by Kent *et al.* (15), provide a reproducible model of lung disease which mimics many characteristics of human CF lung disease. Our laboratory participated in developing and characterizing various backcrosses of *Cftr* KO mice (15-18), including the C57BL/6J *Cftr*^{m1UNC}/*Cftr*^{m1UNC} KO mice (for simplification, this strain of mice is abbreviated here as *Cftr* KO). This strain was confirmed to not express the alternate non-CFTR chloride channels in the airway epithelium. Hence, these congenic *Cftr* KO mice consistently develop spontaneous and progressive lung disease which can be observed as early as 3 weeks of age.

Fenretinide (FEN), a vitamin A derivative, has been shown by our laboratory to mitigate inflammatory responses in CF (6-8). A novel once-a-day oral formulation of FEN, with excellent bioavailability, was recently developed. This formulation (called LAU-7b) has potential applications not only in CF disease but also in other inflammatory diseases including allergic asthma (9), which can also be found in CF patients. LAU-7b was tested in a phase Ib clinical trial study (NCT02141958) in adult CF patients who were chronically infected with *Pseudomonas aeruginosa*. In this study, LAU-7b showed excellent safety with promising pharmacokinetic and pharmacodynamic features. LAU-7b is currently being further evaluated in APPLAUD (A double-blind, randomized, Placebo-controlled, Phase 2 study of the efficacy and safety of LAU-7b in the treatment of CF in adults, NCT03265288) for the treatment of patients suffering from CF. In addition, the different potential mechanisms of action of FEN benefits in CF lung disease, are still under investigation.

Ceramides are structural components of the cellular membrane, which help maintain membrane integrity, and also act as bioactive signaling molecules produced upon various stimuli such as inflammation (20). An imbalance of ceramide levels (21,22) has been associated with several lung diseases including CF (7,8) and recently, allergic asthma (23-25). Our laboratory has previously reported that *Cftr* KO mice, compared to WT controls, have upregulated levels of long chain ceramide species (LCCs: C14:0, C16:0, C18:0). While on the other hand, very long chain ceramides, VLCCs: C22:0, C24:0, C26:0, are significantly downregulated (8). Treatment with FEN successfully corrected the imbalance of LCCs and VLCCs in *Cftr* KO mice (8). However, the protective effects of FEN, or its new formulation (LAU-7b), on lung physiology and histopathology had not been investigated.

In this study, we explored the lipidomic signature of aging lungs by measuring the sphingolipids, fatty acids, and oxidation markers in CF mice. In addition, we hypothesized that LAU-7b treatment can inhibit the age-dependent deterioration in lung physiology and histopathology observed in aging *Cftr* KO mice. We thus monitored the lung physiology and histopathology of 2, 4- and 7-month-old mice and assessed the effectiveness of daily oral LAU-7b treatment in correcting the lung disease observed in naïve uninfected *Cftr* KO mice and WT littermate controls.

4.3 Materials and Methods

4.3.1 Animal model

Cftr KO and WT sex-matched mice at the age of 2 months (n for KO and WT = 14 and 16, respectively), 4 months (n for KO and WT = 15 and 19, respectively) and 7 months (n for KO and WT = 17 and 20, respectively) were kept at 1-4 mice/cage in 12-hour light/dark cycles. All pups were genotyped at 2 weeks of age, as previously described (6). The animals were kept in cages

under pathogen-free conditions and fed with irradiated diet after weaning at 4 weeks of age. All experimental procedures with the mice were conducted in accordance and approval of the Animal Care Committee of the McGill University Health Center, Montreal, Quebec, Canada.

4.3.2 Experimental groups and treatment with LAU-7b

Mice in the different age groups were split into the following groups: PBS treated KO, LAU-7b treated KO, PBS treated WT, and LAU-7b treated WT. Oral LAU-7b (10 mg/kg) was tested in naïve, uninfected, and Methacholine (MCh)-challenged mice. The mice received LAU-7b once daily for 21 days by oral gavage starting at the age of 4 months and 7 months until the day before endpoint. Clinical grade LAU-7b capsules were generously provided by Laurent Pharmaceutical Inc. and 0.5% methylcellulose was used as the drug vehicle. The resulting stock suspension aliquots of FEN at a concentration of 10 mg/mL were kept at -20^{o}C. Further dilution using 0.5% methylcellulose was done to achieve a final concentration of the drug at 1 mg/mL which was used to gavage the mice. Mice were gavaged based on their body weight, and each mouse received a dose of FEN corresponding to 10 mg/kg/day.

4.3.3 Lipids and markers of oxidation analysis

Analysis of lipids was done using approximately 50 µL of plasma or 25 mg of macerated lung tissue preserved in 500 µL of 1 mM butylated hydroxyanisole (BHA) in chloroform:methanol solution (2:1 vol/vol). All BHA preserved samples were kept at -80^{O}C until analysis. Classical isolation of lipids was done as previously described by Folch (26), and the levels of different lipid species were measured using high-performance liquid chromatography (HPLC) tandem mass spectrometry (MS), as previously described (6).

4.3.4 Lung resistance measurements

Airway resistance was measured using a Buxco plethysmograph system (Buxco Research System, Wilmington, NC, USA), ventilators, and nebulizers (Harvard Apparatus, Holliston, MA, USA). For the non-invasive whole-body plethysmograph (WBP) the baseline lung resistance was measured at the age of 2 months without anesthesia and without sacrificing the mice.

The standard invasive lung resistance measurement was done on the day of harvest at the age of 4 and 7 months. On the day of the harvest, mice were anesthetized using a cocktail of ketamine (100 mg/kg, DIN: 02374994), acepromazine (3 mg/kg, Cat: A7111, Sigma Aldrich, Saint Louis, MO, USA) and xylazine (10 mg/kg, DIN: 02169592) and then the mice were connected to the ventilator through a tracheotomy. A nebulizer was used to administer ascending doses (25 mg/mL to 200 mg/mL) of methacholine (MCh, Acetyl β-methyl choline, Cat: A2251, Sigma Aldrich, Saint Louis, MO, USA) and the lung resistance was measured. The results presented here are collected from all the experiments and have been pooled together to generate the final data.

4.3.5 Lung histology analysis

During harvest, the left lung from each mouse was inflated with buffered formalin 10% (Fisher Scientific, Nepean, ON, Canada) and then kept in formalin for a 48-hour fixation. Fixed formalin lungs were embedded in paraffin blocks (Leica Biosystems, Concord, ON, Canada), and were then sectioned into 4 μm thick section slides. Lung sections were deparaffinized, hydrated, and stained with haematoxylin and eosin (H&E) staining to assess lung tissue inflammation and recruitment of different inflammatory cells to the airways.

4.4 Results

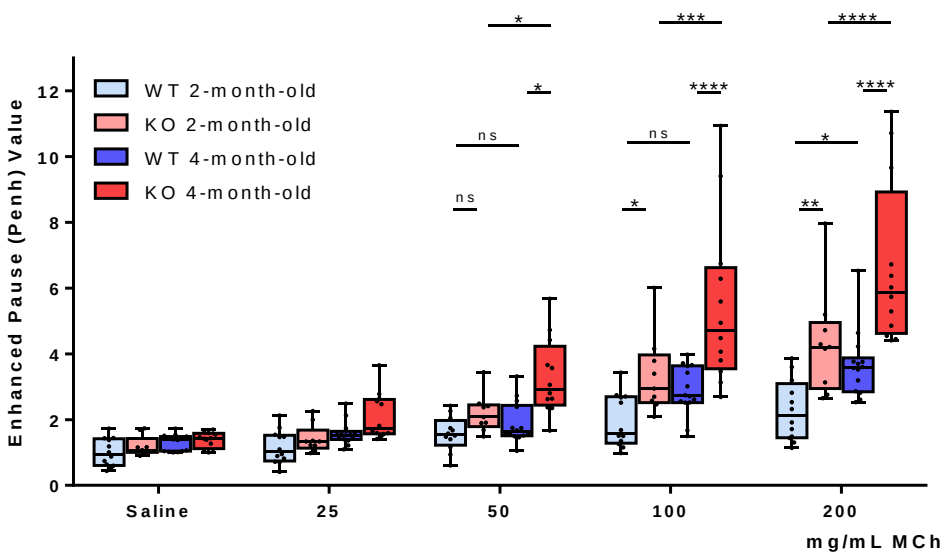
4.4.1 Two- and four-month-old *Cftr* KO mice have higher enhanced pause (Penh) values compared to their littermate controls

At the age of 2 and 4 months, a non-invasive WBP was used to measure the MCh-induced changes in airway function without sacrificing the mice (Figure 1). Our data shows that the *Cftr* KO mice have significantly higher values of Penh, an index of bronchoconstriction, than WT mice at the age of 2 (at doses of 100 and 200 mg/mL MCh) and 4 months (at doses of 50, 100 and 200 mg/mL MCh). While at the age of 4 months, *Cftr* KO and WT mice demonstrated a significant difference in baseline lung respiratory function at 50 mg/mL MCh, higher MCh concentrations (100 and 200 mg/mL) were needed to demonstrate the impact of *Cftr* gene ablation in younger mice (2 months).

Furthermore, 4-month-old *Cftr* KO mice have significantly higher Penh values than 2-month-old *Cftr* KO mice (50, 100 and 200 mg/mL MCh). Additionally, WT mice at the age of 4 months show significantly higher Penh values (200 mg/mL MCh) than WT mice at the age of 2 months.

Figure 4.1 Non-invasive Whole-Body Plethysmography (WBP) measurements in *Cftr* KO and WT mice

Penh values, as measured by WBP, in response to increasing doses of inhaled MCh in naïve WT and *Cftr* KO mice. WT mice have significantly lower lung resistance than *Cftr* KO mice at the age of 2 and 4 months. Used MCh doses = 25-200 mg/mL (all points shown, n equal at least 9 mice in each group, Two-way ANOVA, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).


WT 2-month-old
KO 2-month-old
WT 4-month-old
KO 4-month-old
Enhanced Pause (Penh) Value
0
2
4
6
8
10
12
Saline
25
50
100
200
mg/mL MCh
ns
*
*
ns
*
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ns
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4.4.2 Markers of oxidation, fatty acids and ceramides levels in *Cftr* KO mice were corrected by LAU-7b treatment

Given that our laboratory has previously reported imbalances in the relative levels of ceramides in 2-month-old *Cftr* KO mice, compared to WT mice (6,20), here we examined a panel of lipids and oxidation markers in the *Cftr* KO and WT mice to investigate the imbalances which occur in older mice at the age of 7 months. Both malondialdehyde (MD), a conventional marker of lipid oxidation, and nitrotyrosine (NT), a common marker of protein oxidation, were significantly increased in *Cftr* KO mice compared to WT mice in the plasma (figure 2A) and lungs (figure 2D). LAU-7b treatment was able to significantly correct the levels of MD and NT in *Cftr* KO mice.

Lipid profile analysis revealed that the levels of Arachidonic acid (AA), an inflammatory omega-6 fatty acid, were significantly higher in *Cftr* KO mice compared to WT mice; by contrast, the levels of docosahexaenoic acid (DHA), an anti-inflammatory omega-3 fatty acid, were significantly lower in *Cftr* KO mice compared to their littermate WT controls. After LAU-7b treatment, AA levels were significantly lowered and DHA levels were significantly elevated in the plasma (figure 2B and 2C) and the lungs (figure 2E and 2F).

We also measured the ceramides and sphingosine species which are particularly abundant in the plasma (figure 3A) and lungs (figure 3B) of the *Cftr* KO and WT mice. Our results demonstrated that the levels of LCCs (C14:0, C16:0, C18:0, C18:1, C20:1 and C20:4) were significantly elevated in the *Cftr* KO mice; on the other hand, the levels of VLCCs (C24:0, C24:1 and C26:0), sphingosine and spingosine1P species were significantly decreased in the *Cftr* KO mice compared to the WT controls. Treatment with 10 mg/kg/day LAU-7b for 21 days successfully corrected the imbalances of ceramides in the plasma and lungs of the *Cftr* KO mice by significantly

lowering the levels of LCCs and significantly elevating the levels of VLCCs, sphingosine and spingosine1P species.

Moreover, in 7-month-old WT mice, LAU-7b treatment was able to significantly decrease the elevated levels of C14:0, C16:0 and C18:1 ceramide species. In addition, LAU-7b treatment significantly elevated the levels of C24:0, C24:1, C26:0, sphingosine and spingosine1P species, which were decreased in the plasma and lungs of this group of mice.

It has been encouraging that even the mice with already fully developed lung disease and severe impairment in the composition of important lipids and inflammatory mediators and regulators can still be successfully treated with LAU-7b reversing these changes. Therefore, it was logical to assess next the impact of the treatment with LAU-7b on the lung physiology and histology.

Figure 4.2 LAU-7b treatment corrects the imbalances of markers of oxidation and fatty acids in the plasma and the lungs of *Cftr* KO mice

Analysis in plasma (A-C) and lungs (D-F). Both Nitrotyrosine NT and Malondialdehyde (MD) are significantly elevated in PBS treated *Cftr* KO mice compared to PBS treated WT mice. Meanwhile, after LAU-7b treatment, the levels of both MD and NT are decreased significantly in the plasma (A) and lungs (D) of the *Cftr* KO mice compared to the PBS treated *Cftr* KO group. (B and E) Arachidonic acid (AA) is significantly higher in PBS treated *Cftr* KO mice than PBS treated WT mice. Simultaneously, AA is higher in PBS treated *Cftr* KO mice compared to LAU-7b treated *Cftr* KO mice. (C and F) Docosahexaenoic (DHA) is significantly lower in PBS treated *Cftr* KO than PBS treated WT. After treatment, the level of DHA is significantly increased in LAU-7b treated *Cftr* KO mice compared to the PBS treated mice (n equal at least 6 mice in each group, Two-way ANOVA, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).

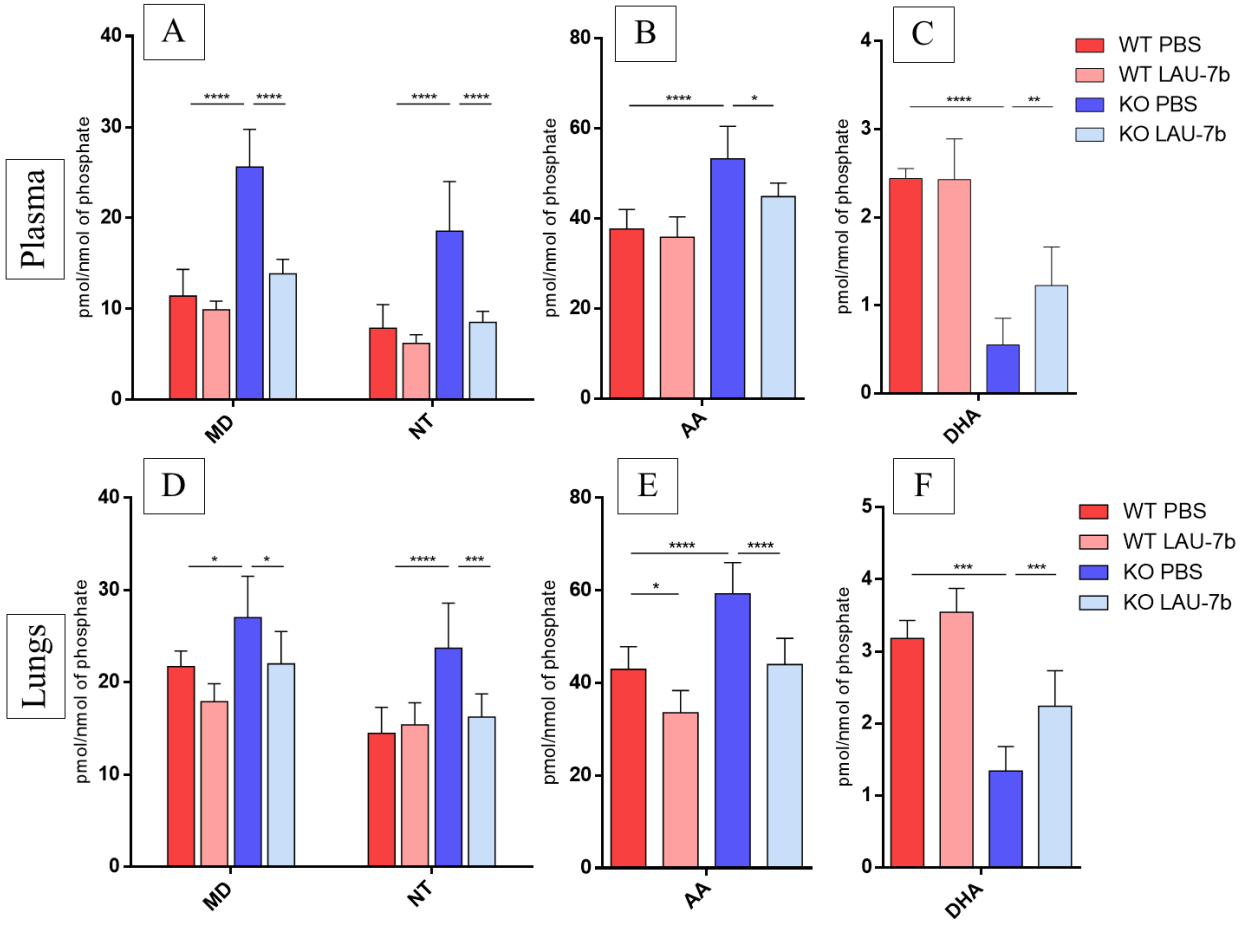
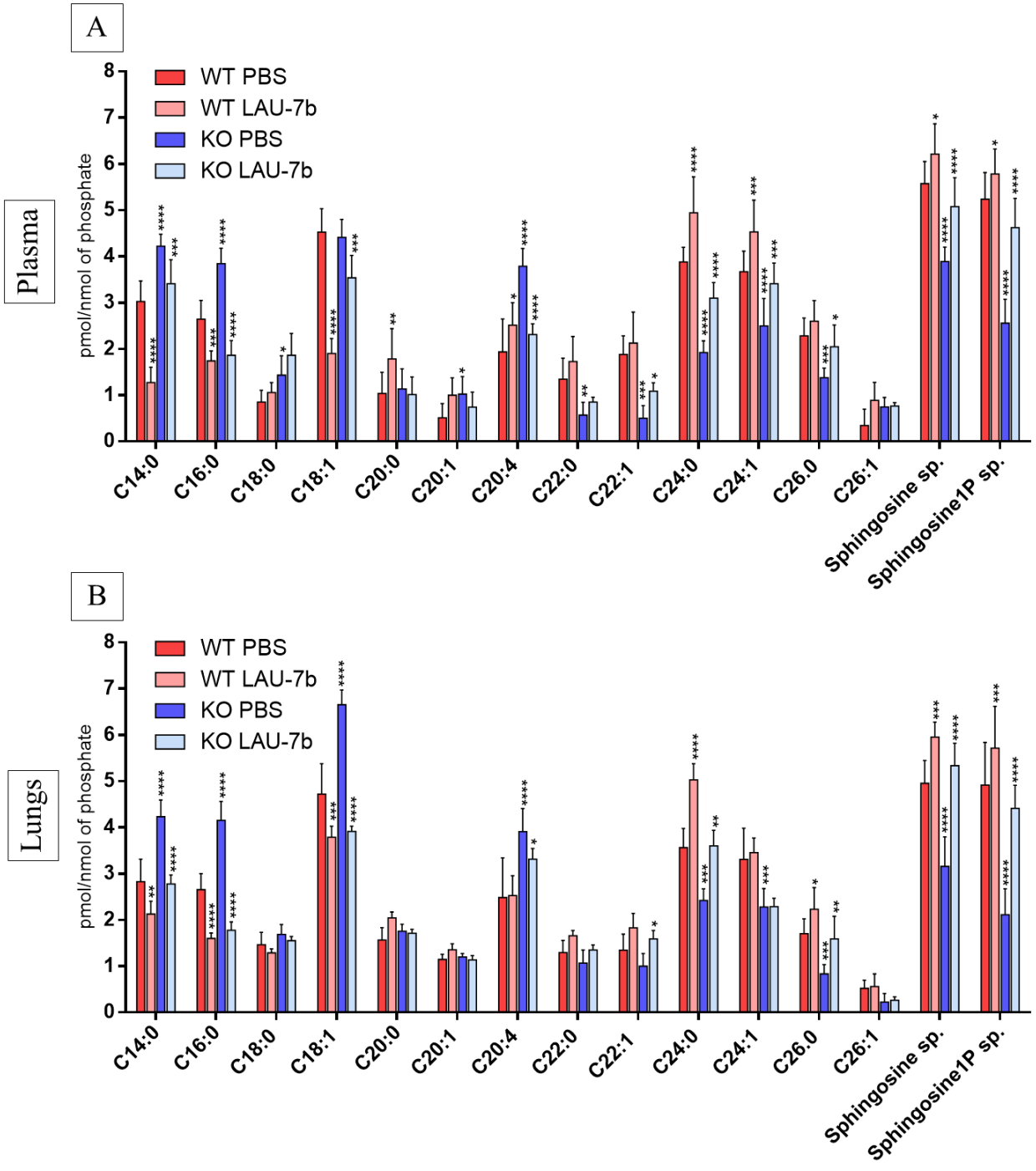

Plasma
Lungs
A
B
C
D
E
F
pmol/nmol of phosphate
MD
NT
AA
DHA
WT PBS
WT LAU-7b
KO PBS
KO LAU-7b

Figure 4.3 LAU-7b treatment corrects the imbalances of sphingolipids in the plasma and the lungs of *Cftr* KO and WT mice

Full panel analysis of ceramides and sphingosine species in plasma (A) and lungs (B). Long chain ceramide species (LCCs); C14:0, C16:0, C18:0, C18:1, C20:1 and C20:4 were significantly increased in the *Cftr* KO mice compared to the WT controls. By contrast, the levels of very long chain ceramides (VLCCs); C24:0, C24:1, C26:0, and sphingosine and spingosine1P species were significantly decreased in the *Cftr* KO mice compared to the WT controls. Treatment with LAU-7b successfully corrected the imbalances of ceramide and sphingosine species levels in *Cftr* KO and WT mice by significantly lowering the levels of LCCs and significantly elevating the levels of VLCCs, sphingosine and spingosine1P species (n equal at least 7 mice in each group, Two-way ANOVA, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).


A
Plasma
WT PBS
WT LAU-7b
KO PBS
KO LAU-7b
pmol/nmol of phosphate
C14:0
C16:0
C18:0
C18:1
C20:0
C20:1
C20:4
C22:0
C22:1
C24:0
C24:1
C26:0
C26:1
Sphingosine sp.
Sphingosine1P sp.
B
Lungs
WT PBS
WT LAU-7b
KO PBS
KO LAU-7b
pmol/nmol of phosphate
C14:0
C16:0
C18:0
C18:1
C20:0
C20:1
C20:4
C22:0
C22:1
C24:0
C24:1
C26:0
C26:1
Sphingosine sp.
Sphingosine1P sp.

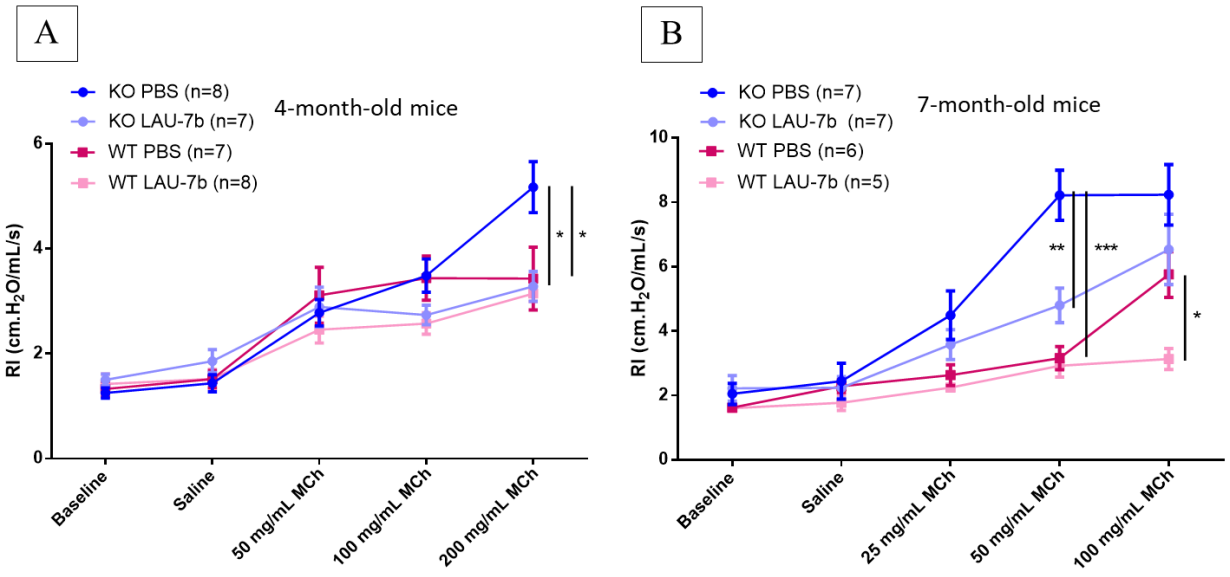
4.4.3 Four- and seven-month-old *Cftr* KO mice have higher airway resistance that can be corrected by LAU-7b treatment

Age-related deterioration of lung physiology demonstrated by WBP was confirmed by the classical invasive measurement of lung resistance on the day of harvest for both 4- and 7-month-old mice (figure 4). At the age of 4 months, KO mice have significantly higher lung resistance than WT mice when the lungs are challenged with 200 mg/mL MCh. At the age of 7 months, the KO mice (at lower doses of MCh; 50 mg/mL) had significantly higher lung resistance when compared to WT mice.

The protective effects of 10 mg/kg LAU-7b for 21 days on lung resistance was assessed at the age of 4 and 7 months. The LAU-7b treated *Cftr* KO had significantly lower lung resistance than PBS-treated *Cftr* KO mice at both 4 and 7 months of age. Interestingly, LAU-7b treated WT 7-month-old mice had significantly lower lung resistance than PBS-treated WT mice (at 100 mg/mL MCh).

Figure 4.4 Classical invasive measurements of lung resistance in WT and *Cftr* KO mice and the protective effects of LAU-7b treatment

LAU-7b treatment was able to significantly lower the lung resistance for both (A) 4-month-old and (B) 7-month-old *Cftr* KO mice Used MCh doses = 25-100 mg/mL for 7-month-old mice, and 50-200 mg/mL for 4-month-old mice (n equal to at least 5 mice in each group, Two-way ANOVA, * $p<0.05$, ** $p<0.01$, *** $p<0.001$).


A
KO PBS (n=8)
KO LAU-7b (n=7)
WT PBS (n=7)
WT LAU-7b (n=8)
4-month-old mice
RI (cm.H2O/mL/s)
Baseline
Saline
50 mg/mL MCh
100 mg/mL MCh
200 mg/mL MCh
B
KO PBS (n=7)
KO LAU-7b (n=7)
WT PBS (n=6)
WT LAU-7b (n=5)
7-month-old mice
RI (cm.H2O/mL/s)
Baseline
Saline
25 mg/mL MCh
50 mg/mL MCh
100 mg/mL MCh

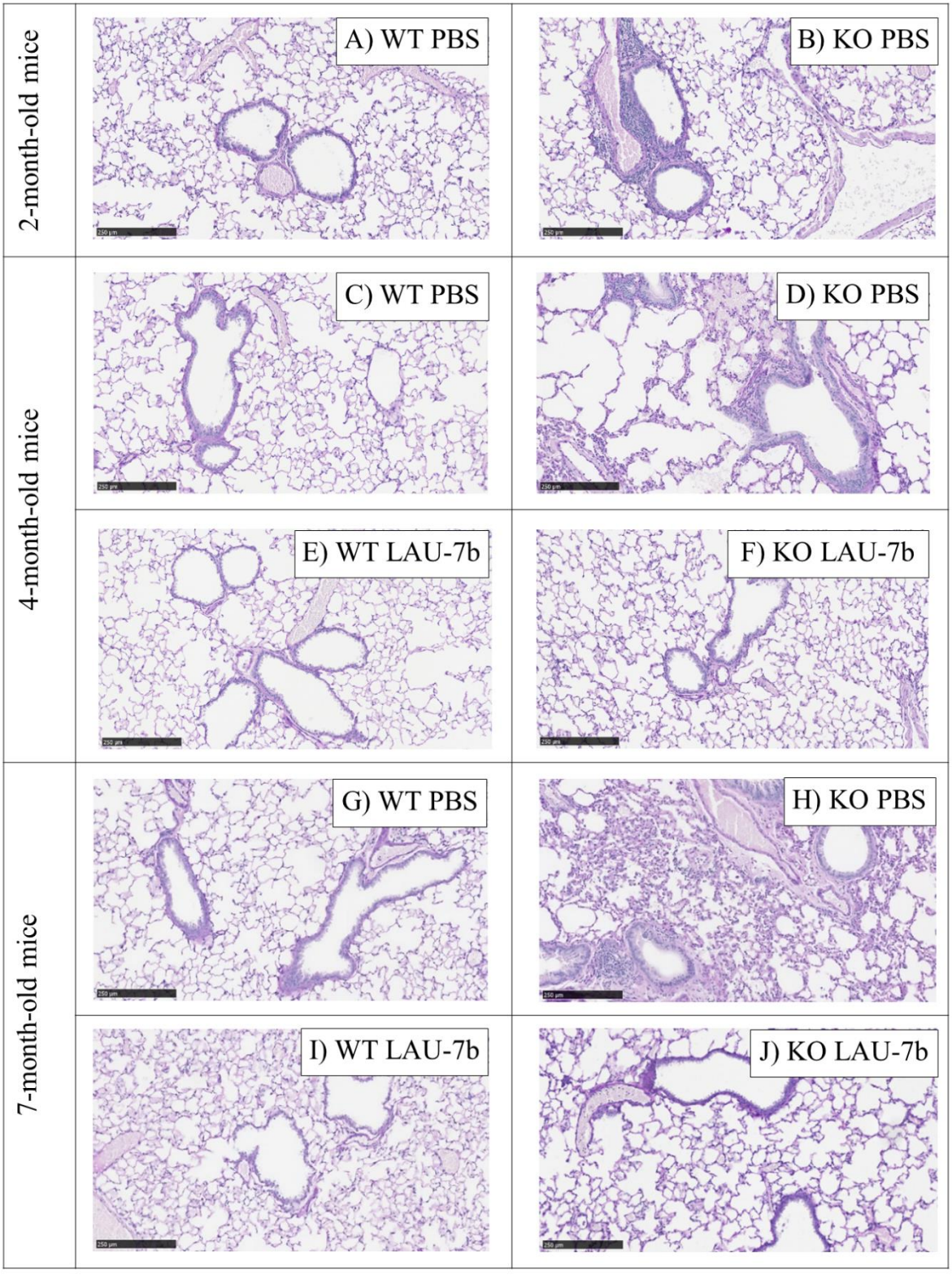
4.4.4 LAU-7b treatment decreased the influx of inflammatory cells and the epithelial cells hyperplasia of young and old *Cftr* KO mice

As shown by H&E staining, our *Cftr* KO mouse model displays a marked increase of inflammatory cell infiltration and notable hyperplasia of the lung airway epithelium at the ages of 2, 4 and 7 months. The 7-month-old mice lung sections demonstrated the most prominent age-dependent deterioration as compared to 4- and 2-month-old lung groups. LAU-7b treatment significantly lowered cellular infiltration to the lungs and minimized the airway epithelium hyperplasia in the *Cftr* KO mice, compared to the PBS-treated *Cftr* KO mice, at both 4 and 7months of age (figure 5).

Additionally, the lung sections of 7-month-old WT mice showed notable epithelial hyperplasia of the lung airways and loose background matrix of the alveoli surrounding those airways. LAU-7b treatment of 7-month-old WT mice was able to remarkably lower the epithelial cell hyperplasia and restore the lung tissue matrix to the levels observed in 2-month-old mice.

Figure 4.5 LAU-7b treatment prevents the age-dependent influx of inflammatory cells and lung hyperplasia

Representative pictures of H&E staining of mouse lung tissues collected from 2-, 4, and 7-month-old WT and *Cftr* KO mice. Left panel for WT mice and right panel for *Cftr* KO mice. A-B) 2-month-old mice, C-F) 4-month-old mice, and G-J) 7-month-old mice. 4- and 7-month-old mice were treated for 3 weeks with 10mg/kg LAU-7b prior to harvest. n equal at least 6 in each group, numbers of fields examined per mouse ≥ 5 fields.


2-month-old mice
A) WT PBS
B) KO PBS
4-month-old mice
C) WT PBS
D) KO PBS
E) WT LAU-7b
F) KO LAU-7b
7-month-old mice
G) WT PBS
H) KO PBS
I) WT LAU-7b
J) KO LAU-7b

4.5 Discussion

Among Caucasians, CF is the leading life-limiting genetic disease (4). Although no definitive cure for CF has been discovered up to this moment, several medications such as mucolytics, bronchodilators, steroids, and antibiotics, can be used to control the disease symptoms, acute exacerbations and/or to treat the related complications (31). Although steroids are able to lower the inflammation observed in CF, long-term administration of oral (32) or inhaled (33) steroids has been associated with many adverse effects. Therefore, testing other medications is required for controlling CF in the future. CF mouse models provide relevant and convenient venues to study the disease pathology and test possible treatments for it. Very few mouse studies have investigated the CF disease pathology as a function of age (29, 30), possibly because not many mice who develop the CF phenotype can survive to an older age. In our study, we used a C57BL/6J *Cftr*^{m1UNC}/*Cftr*^{m1UNC} KO (*Cftr* KO) mouse model (17) which provides a relevant picture of the lung disease occurring clinically in CF patients.

Fenretinide, [FEN, N-4-hydroxyphenyl retinamide], has shown promising physiologic effects and beneficial anti-inflammatory actions in CF (6-8) and allergic asthma (9). In CF, FEN was shown to correct the imbalance in ceramides by elevating the levels of VLCCs and decreasing the levels of LCCs (8). FEN also corrected the imbalances of AA (pro-inflammatory fatty acid) and DHA (anti-inflammatory fatty acid) observed in CF by increasing the levels of DHA and decreasing the levels of AA (7). In allergic asthma, FEN has corrected the AA/DHA ratio, downregulated the levels of MD and NT, inhibited the production of mucus, and diminished the recruitment of inflammatory cells into the airways after allergen challenge (9). Our current study aimed to test the treatment with LAU-7b, a novel oral formulation of FEN, in *Cftr* KO mouse groups of 2 different ages: 4 and 7 months old. To achieve this, we examined lung physiology and

histopathology, and measured oxidation markers (MD and NT), fatty acids (AA and DHA) and ceramides of *Cftr* KO mice compared to WT mice.

Teichgraber *et al.* (30) previously reported that 32-week-old *Cftr* KO mice have more than a 3-fold increase of lung ceramide levels (specifically C16:0 species) compared to 8-week-old KO mice. However, in the latter study, no results were reported for other LCCs or VLCCs. Our laboratory has previously reported (8) that 2-month-old *Cftr* KO mice, compared to WT littermates, have elevated LCCs and diminished VLCCs in the lungs and the plasma. Nevertheless, the ceramide levels of older mice were not examined in that study. When comparing the ceramide levels analyzed by Garic *et al.* (8) (2 months) and the results of our current study, we find that the 7-month-old *Cftr* KO mice have markedly higher levels of LCCs than the younger *Cftr* KO mice, whereas, the levels of VLCCs appear to be unremarkably diminished with age. The full functional picture which explains how the balance between LCCs and VLCCs affects lung inflammation is still unclear. Nonetheless, higher LCCs levels and lower VLCCs levels have been associated with lung inflammation in both mice and humans (20). In our study, treatment with LAU-7b corrected the imbalance of ceramide species which were observed in both *Cftr* KO and WT mice as a function of age.

The overall preclinical therapeutic outcomes obtained by the current study are threefold. First, administrating 10 mg/kg/day of LAU-7b for 3 weeks to the *Cftr* KO mice at the age of 4 months prevented the development of CF manifestations as demonstrated by the lung physiology (figure 4) and histopathology (figure 5). Treating *Cftr* KO mice with LAU-7b lowered their lung resistance values to levels similar to those of WT mice. Furthermore, LAU-7b treatment was able to dramatically diminish the recruitment of inflammatory cells into the airways and it also lowered the hyperplasia of the airway epithelium. Obviously, controlling the early development of CF pathologies at a younger age would allow better long-term control of the disease (34).

Second, when the same treatment period and dose of LAU-7b was administrated at the age of 7 months, it was also able to reverse CF pathologies, though not equally to what occurs if the drug is administrated at the age of 4 months. The Penh values of the *Cftr* KO mice at age of 2 and 4 months (figure 1), and the lung resistance measurements at the age of 4 and 7 months (figure 4) showed substantial loss of lung capacity as a function of age. Treatment with LAU-7b successfully cleared the lungs of the incoming inflammatory cells (figure 5). Additionally, LAU-7b significantly reduced the lung resistance of the *Cftr* KO treated mice as compared to the non-treated mice (figure 4). However, because of the cumulative physiological deterioration of the lungs when the mice survived to an older age (7 months), the lung resistance values remained higher than the range of resistance values of younger WT mice or younger treated *Cftr* KO mice (figure 4).

Third, to our surprise, LAU-7b treatment also lowered the lung resistance in WT mice, which normally increases with aging (figure 4B). The changes at the level of lung resistance measurements were consistent with the effect of the treatment on the histopathology (figure 5G and 5I). The lungs of LAU-7b treated 7-month-old WT mice resembled the lung histology of 2-month-old WT mice and did not display abnormal airway hyperplasia, which is typically observed with the older WT mice. This can also be explained by the modulation of mucin production by LAU-7b. It is important to note that mucus, in CF patients, consists mainly of DNA from dead neutrophils as well as mucins produced by goblet cells. Mucus accumulation not only creates a favorable environment for the proliferation of pathogens, but the MUC5AC mucin is also responsible for the pathological plugging of airways in CF patients. Indeed, as recently shown by our laboratory, FEN treatment prevented the accumulation of MUC5AC mucin in *P. aeruginosa* infected WT and CFTR KO mice (38). Overall, clearance of the MUC5AC mucin in the lungs of

CF mice by FEN might contribute to less airway epithelium hyperplasia and less severe impairment of lung function.

Taken as a whole, the results of this study show for the first time that treatment with LAU-7b can inhibit age-dependent deterioration in lung physiology, histopathology, ceramides and fatty acids imbalances observed in the aging *Cftr* KO mice. For more than 20 years, FEN has been widely investigated in the clinic and it has shown very good safety profiles (35) for long administration periods (36) when it was administered daily for 5 years at a dose of 200 mg among a large breast cancer cohort. In addition, FEN toxicity studies in children reported that FEN has manageable toxicity and minimal side effects (37). Based on the reported safety profile and the tolerable side effects of FEN, our preclinical efficacy data provides a strong rationale for testing long-term administration of this medication at regular cycles with breaks as in the current clinical trial (NCT02141958), in young and old CF patients. Because CF is a life-long disease, the once-per-day pharmaceutical formulation of FEN, LAU-7b, represents a convenient candidate medication which can clinically meet the needs of CF patients. The data available from our current report warrant further investigation of the LAU-7b formulation clinically, over long-term periods, for an optimum control of age-dependent CF morbidities.

4.6 Highlights

- *Cftr* KO mouse model display clear age-dependent progressive deterioration demonstrated by the increase in lung resistance, cell infiltration and epithelium hyperplasia of the lung airways, and the imbalance of lipids in both lungs and plasma.
- Treatment of *Cftr* KO mice for 21 days with 10 mg/kg LAU-7b improved dramatically the lung physiology and histopathology and corrected the imbalances of ceramides when the treatment is administrated at the age of 4 and 7 months.

- To our surprise, the treatment also reversed the progressive age-related changes in the lung of 7-month-old WT mice which normally occur with age.

4.7 Funding

This research has been supported by Cystic Fibrosis Canada Grant #494470 to BP, DR; ENOCH Molecular, cellular and clinical approach to healthy ageing grant ENOCH; Registration number: CZ.02.1.01/0.0/0.0/16_019/0000868 MH, DR, JdS. CIHR grant FRN115117 #2433990; RI-MUHC Account 4925 to DR.

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Chapter 5. General Discussion

The projects presented in Chapter 2, Chapter 3 and Chapter 4 of this thesis were conducted as separate and consecutive projects on allergic asthma and cystic fibrosis (CF). These three projects are linked and build upon one another. The results presented in Chapter 2 illustrate the optimization of a treatment protocol using Fenretinide and its novel formulation (LAU-7b) against ovalbumin (OVA) and house dust mite (HDM) -induced allergic asthma. Chapter 3 illustrates results generated to test the efficacy of LAU-7b treatment against allergic asthma in *Zpbp2* knockout A/J mice along with studying the effects of our medication on the expression of *Ormdl3* gene. Chapter 4 describes the results generated an investigation of the effects of LAU-7b treatment on the age-dependent deterioration in lung functions in CF.

The general discussion presented here in Chapter 5 reports the current clinical guidelines for the management of allergic asthma and CF. Many of the drugs listed here in this chapter can be classified into more than one functional category. It is important to note that the clinical introduction of any new medication into the pharmaceutical market would question its superiority over the other medications currently present in clinic. Accordingly, based on our findings in the previous chapters of this thesis, here we have highlighted the promising effects of Fenretinide, and LAU-7b, as compared to the other medications currently recommended in allergic asthma (Table 5.1) and CF (Table 5.2).

5.1 Allergic Asthma

Allergic asthma is a heterogeneous respiratory disorder characterized by reversible airflow limitation, airway inflammation and chronic airway hyperresponsiveness (AHR). The goal of therapy is to maintain asthma control and prevent exacerbations. The current Canadian guidelines, published in 2019, follow the 'Global Initiative for Asthma (GINA)' Strategies that were updated in 2017 (www.ginasthma.org). The cornerstone of asthma management is inhaled therapy that

maximizes the delivery of drugs to the respiratory tract and minimizes systemic side effects. Inhaled medications include bronchodilators and anti-inflammatory agents. Other medications are available by the oral route, including anti-inflammatories, or by injection, such as biologic therapies. In allergic asthma, treatment is reviewed every 3–6 months and if control is achieved, a stepwise reduction in treatment is done by 25-50% over 3 months [292].

5.1.1 Allergic Asthma Therapies: Does Fenretinide have enough competitive edges?

5.1.2 Short-Acting and Long-Acting Inhaled Beta2-agonists

Beta2-adrenergic agonists work on the sympathetic system beta2-adrenergic receptors in the lungs smooth muscles and dilates the airways, hence, facilitate the airflow. Beta2-adrenergic agonists include salbutamol (the blue or reliefer puffer), which is a short-acting beta2-adrenergic agonist (SABA), and salmeterol and formoterol, which are long-acting beta2-agonists (LABAs).

Unlike SABAs or LABA's, which work as agonists on only single type of sympathetic receptors; beta2-adrenergic receptors, Fenretinide has been shown to have pleiotropic effects. Although it was not shown that Fenretinide act as a direct bronchodilator, Fenretinide can significantly lower the overall lung resistance measurements and AHR in mice. Airway hyperresponsiveness is defined as increased sensitivity of the airways to an inhaled constrictor. Certain inhaled stimuli, such as allergens, result in significantly increased AHR and elevated airway resistance [293].

Our results illustrated in Chapter 2 and Chapter 3 demonstrated that the mice which administrated Fenretinide, or its formulation LAU-7b, following allergen sensitization and challenge, had lower AHR and reduced lung resistance and enhanced pause values compared to PBS-treated mice. Similar results were obtained in Chapter 4 of this thesis in context of CF.

Likewise, our results were homogenous with the previously published reports of Kanagaratham *et al.* [265]. Based on the complexity and heterogenicity of the genetic nature of allergic asthma, we do not believe that mutation in a single genetic locus exclusively account for the AHR phenotype, nonetheless, Fenretinide demonstrated outstanding efficacy in reducing AHR and lowering the lung resistance measurements.

We have done a pilot experiment to investigate the effects of Fenretinide in allergic asthma when its administration was started on the day of the challenge, not before. Our histology results (Youssef *et al.*, unpublished data) showed that Fenretinide was able to reduce the time needed to clear the airways, from the incoming inflammatory cells, from 14 days (PBS-treated) to 4 days (LAU-7b-treated) after the allergen challenge. However, no experiments for AHR or lung resistance measurements were done in this pilot experiment. Thereupon, while Fenretinide can not be recommended as an immediate rescue medication after allergen challenge (like SABAs), we have preliminary data suggesting that Fenretinide can accelerate the resolution of what has happened in the lungs much faster than in the case of not administrating it.

According to the GINA guidelines, salbutamol is the agent of first choice for the treatment of acute allergic asthma exacerbations. It can also be used for the prevention of exercise-induced asthma. However, in both clinical contexts, SABAs are to be used *pro re nata,* prn, or "as needed" rather than on a fixed schedule. Although SABAs are potent bronchodilators, they no effect on the late (inflammatory) phase of the disease exacerbation [294]. The current guidelines recommend that if an asthmatic patient uses the reliefer puffer two or more times per week (including any situations used to prevent or treat exercise-induced symptoms), s/he needs to initiate therapy with a controller (anti-inflammatory agent).

Salmeterol and formoterol are long-acting beta2-agonists (LABAs) used for regular treatment of allergic asthma and they need to be administrated on a twice-daily basis. These drugs

are prescribed only in patients who already take inhaled corticosteroids (ICS). Adding LABAs to the ICS allows to lower the dose of the ICS, additionally, it reduces the incidence of asthma exacerbations in comparison with an increased dose of ICS without a LABA [295]. According to the current guidelines of GINA, physicians should only consider adding a LABA when low-dose ICS fail to provide adequate control of asthma. In this way, most of the commercial devices available for LABA medications combine LABA and ICS products in one device to prevent the use of LABAs as monotherapy. The regular use of LABA as a monotherapy is not recommended as it has been associated in asthmatics with an increased risk of death [156].

5.1.3 Inhaled Corticosteroids

Currently, inhaled corticosteroids (ICS) are considered the most commonly used anti-inflammatories, the cornerstone of allergic asthma therapy, and the preferred choices for the maintenance of allergic asthma.

Our data presented in Chapter 2 and Chapter 3 demonstrate very strong potentials of Fenretinide to act as an anti-inflammatory drug. Arachidonic acid (AA) and docosahexaenoic acid (DHA) play important roles in allergic asthma, where AA act as a proinflammatory fatty acid and DHA as an anti-inflammatory fatty acid. We observed in our models of allergic asthma that the AA/DHA ratio is significantly skewed in the proinflammatory direction. Treatment with Fenretinide, or LAU-7, have corrected effectively the imbalances of AA and DHA which happened due to allergen sensitization and challenge in our model. Similar results were reported by Kanagaratham *et al.*[265] in allergic asthma and Guilbault *et al.* in CF [267]

AA promotes the production of the inflammatory cytokine IL-8 through COX-2 and NF-kB pathways [296]. However, *in vitro* studies have shown that Fenretinide prevented the production of IL-8 from LPS-stimulated human lung epithelial cells [297]. Moreover, the results reported by

Kanagaratham *et al.* [265] showed that pre-treatment of cells with Fenretinide before LPS stimulation dampened the transcription of several cytokines and chemokines (*Ccl2 (MCP-1), Ccl5 (RANTES), Ccl7 (MCP-3), Ccl11 (eotaxin-1), Cxcl1 (KC), Cxcl2 (MIP2-α), Cxcl9 (MIG), Cxcl10 (IP-10), Il-6, iNOS, Pdgf,* and *Tnf-α*), hence, it can inhibit the inflammation in cellular levels.

In Chapter 3, we have shown that after HDM challenge, both *Zpbp2* KO and WT mice had significantly increased expression of *Il-5, Il-13* and *Eotaxin-1* genes compared to PBS-challenged control mice. Meanwhile, Fenretinide treated mice had lower expression of *Il-4* (WTs), *Il-5* and *Eotaxin-1* (WTs and KOs) compared to PBS-treated mice. IL-4 is the central cytokine in allergic asthma because it promotes the differentiation of Th2 lymphocytes [298]. IL-5 is the main cytokine involved in activating eosinophils [299], on the other hand, eotaxin-1 is a chemokine which further helps the eosinophils to migrate into the airways after allergen challenge in asthma [300]. By downregulating the gene expression of *Il-4, Il-13* and *Eotaxin-1,* Fenretinide significantly lowered the inflammatory events in allergic asthma, notably the number of inflammatory cells recruited into the airways.

Recently, it has been shown that in allergic asthma overexpression of *ORMDL3*, or treatments with the SPT inhibitor myriocin, induces inflammation by promoting IL-6 and IL-8 production in airway epithelial cells [301]. Silencing of *ORMDL3* led to steroid-independent inhibition of IL-6 and IL-8 release and reduced endoplasmic reticulum stress after IL-1B stimulation [301]. Our results presented in Chapter 3 showed that Fenretinide treatment downregulated the expression of *Ormdl3* in WT mice compared to PBS-treated mice after allergen challenge. Thus, by downregulating *Ormdl3* expression, we believe that Fenretinide could exert, at least in part, some of its anti-inflammatory effects in allergic asthma.

By contrast, another recent study, by Kiefer *et al.* [302], reported that the induction of TNFα and iNOS, and the expression of proinflammatory cytokines IL-1β and IL-6 were not different

between the *Ormdl3*-overexpressing mouse model and WT controls used in their study. However, the latter study has reported that *Ormdl3*-overexpressing transgenic mice show significant reduction in total ceramide levels and significant reduction of specific ceramide species C22:0, C24:0, C24:1 and C24:2, compared to WT mice [302]. The results of our studies presented in Chapter 2 and Chapter 3 demonstrate that Fenretinide treatment in allergic asthma lowers the production of long chain ceramide species, LCCs, C16:0 and C18:0, and normalizes the levels of very long chain ceramide species, VLCCs, C24:0 and C26:0, by elevating them, thus it can counteract the imbalances of ceramides caused by *Ormdl3* overexpression. Ceramides have been known as signaling molecules produced mostly upon various exogenous stimuli e.g. inflammation [88], so by correcting the imbalances of ceramides, we propose that Fenretinide could accomplish its anti-inflammatory effects in both allergic asthma and CF.

On the other hand, the examples of the ICS include: inhaled fluticasone (the orange or controller puffer), mometasone, beclomethasone and budesonide, which are all safe and effective steroidal drugs that treat the inflammatory component of allergic asthma. Pharmaceutically, ICS have very low systemic activity compared to oral corticosteroids, rather they act mostly topically [303]; hence, ICS are preferred more than oral corticosteroids [292,304]. ICS should be used regularly at the lowest effective dose rather than "as needed" to maintain a good control of allergic asthma. Administration of steroids controls the inflammation by directly regulating the transcription of up to 100 genes, nonetheless, many other genes are indirectly regulated by several interactions with other transcription factors [305]. Glucocorticoids increase the transcription of annexin-1 (phospholipase A2 inhibitor) and Ikβ-α (inhibitor of NF-kβ). Steroids decrease the transcription of several inflammatory cytokines (IL-1, 2, 3, 4, 5, 6, 9, 11, 12, 13, 16, 17, 18, TNFα, GM-CSF) and chemokines (IL-8, RANTES, MIP-1, MCP-1, MCP-3, MCP-4, eotaxin) [306].

5.1.4 Leukotriene Receptor Antagonists

Montelukast is a leukotriene receptor antagonist (LTRAs). Leukotrienes are eicosanoids derived from AA through the enzymatic action of 5-lipooxygenase. They play a crucial role in asthma inflammatory pathogenesis because they are strong bronchoconstrictors and powerfully result in the remodeling of the airways over the long run [169].

Although montelukast has some anti-inflammatory properties, evidences suggest that it is not as effective as low-dose ICS for improving symptoms or preventing exacerbations [307]. Consequently, montelukast is a second-line monotherapy (or as a combination therapy) after ICS for asthma control [294,307]. The monograph of Singulair™ (montelukast) states: *"Patients should be advised to continue taking Singulair while their asthma is controlled, as well as during periods of worsening asthma"*. Medication compliance is one of the critical challenges in the management of chronic diseases, and proper adherence to prescribed frequency and dosage is associated with a lower risk of disease exacerbations. Since allergic asthma, and CF, are chronic diseases, if it is possible to achieve the treatment targets within shorter periods, the overall patient compliance is much better. By contrast to the necessity of taking montelukast regularly, we have used Fenretinide for only 9 days in allergic asthma (Chapter 2 and Chapter 3) and 21 days in CF (Chapter 4) and our results showed that Fenretinide can provide optimal control of inflammatory symptoms in few days (or weeks) in both diseases.

5.1.5 Systemic Corticosteroids

The main clinical context of using systemic corticosteroids is in treating acute exacerbations of allergic asthma [304]. The Canadian guidelines suggest 25–50 mg of prednisone daily for 7–14 days in case of an acute exacerbation of allergic asthma [292]. Less commonly, the systemic corticosteroids are used as long-term maintenance therapy because of the

significant side effects. Over the short term, systemic corticosteroids administration cause fluid retention, glucose intolerance, increased blood pressure, increased appetite, mood alterations, and weight gain. Over long terms, administration of systemic corticosteroids results in adrenal axis suppression, necrosis of the hip, cataracts, dermal thinning, diabetes, glaucoma, hypertension, myopathy and osteoporosis [308].

By contrast to the well-established evidences in regard to the significant side effects of long term corticosteroid administration, our laboratory [290] and several other research groups, have previously published that Fenretinide administration is considered to be clinically very safe over long-term periods, as equally being therapeutically effective. A clinical trial [309] recruited 2,867 women over a 5-years period to assess the safety and efficacy of Fenretinide in preventing secondary breast malignancies. In terms of tolerability, out of 2,667 women treated daily only 63 patients (4.4%) discontinued Fenretinide because of the possible adverse events. In terms of efficacy, the trial showed that Fenretinide is potentially effective in preventing future malignancies in premenopausal women. Another phase 2 trial [269] assessed Fenretinide for its safety and efficacy in slowing age-related macular degeneration (AMD) over a period of 2 years. Besides being therapeutically effective in retarding age-related AMD progression, Fenretinide established high safety profile in chronic dosing regimens. Using the novel formulation of Fenretinide, LAU-7b, in clinical trial phase Ib (NCT02141958) for CF patients, only one candidate withdrew in the 3rd (last) cycle due to elective surgery to clean up the infected sinuses. The withdrawal did not occur due to any side effects related to the administration of LAU-7b. Based on the number of patients involved in all these studies and the prolonged period of drug administration, Fenretinide tolerability can be considered sufficiently high to justify testing its long-term administration in asthmatic or CF patients. Collectively, in terms of chronic long-term administration, Fenretinide would be a

superior choice, as compared to systemic corticosteroids, because of its high tolerability and wide safety margins.

5.1.6 Anticholinergic Agents (SAMA and LAMA)

SAMAs, short-acting muscarinic antagonists, are used to lower the mucus production in the airways of asthmatic patients, additionally, they are considered another alternative bronchodilator group to β-agonists [157].

Fenretinide has shown to diminish the mucus production in both asthma and CF. We have demonstrated in Chapter 2 that challenging the mice with OVA or HDM caused a visible increase in PAS positive cells. In Chapter 3, we have shown that Fenretinide was able to lower the mucus production in both *Zpbp2* KO and WT mice after HDM challenge, compared to control mice. Our conclusions, in both chapters, drawn from the visual inspection of the airways were validated by quantifying goblet cell hyperplasia. Our data provide an evidence that treating the mice with Fenretinide efficiently prevented the accumulation of mucus in both OVA and HDM sensitized and challenged mouse models.

In Chapter 4 of this thesis, our chronic model of *Cftr* KO mice did not display significant production of mucus when the lung sections were stained by PAS, probably because these mice were not infected by *P. aeruginosa*. In this study, we have explored the progressive deterioration of lung capacity, as a function of age, in the *Cftr* KO mice, however, the acute mucus production phenotype was not apparent in our uninfected *Cftr* KO mouse model.

Similar results were obtained by Garic *et al.* in context of CF and acute *P. aeruginosa* infection (Garic *et al.*, manuscript accepted for publication). Garic *et al.* have shown that Fenretinide prevented the lipopolysaccharide-induced increase of MUC5AC gene expression

(responsible for the pathological plugging of the airways), without affecting the level of MUC5B gene (responsible for a protective role against bacterial infection) in the lung goblet cell line.

Ipratropium is a SAMA that is used as an add-on therapy to beta2-agonists for management of acute asthma.While SAMAs are used in acute allergic asthma management, LAMA (long acting antimuscarinic agents), e.g. Tiotropium [163] are used only as long term controllers. Anticholinergic agents represent an alternative for patients who experience tremor or tachycardia from using beta2-agonists. Although the onset of action is delayed compared to beta2-agonists, the bronchodilator and mucus inhibitory effect lasts longer.

5.1.7 IgE-Neutralizing Antibody

Omalizumab, a monoclonal antibody against IgE, is considered for patients with moderate to severe persistent asthma who have had a positive skin test. Omalizumab is prescribed as an add-on therapy only for asthmatics whose symptoms are inadequately controlled with high-dose ICS[310]. By contrast to Omalizumab, Fenretinide does not protect against allergic asthma by lowering the levels of IgE.

During our experiments, we wanted to explore if treatment with Fenretinide would lower the IgE levels as seen in its other protective effects against allergic asthma (decreasing AHR, lowering mucus production and diminishing the recruitment of inflammatory cells into the airways). Our data illustrated in Chapter 2 and Chapter 3 show that Fenretinide does not affect the elevated levels of IgE in response to allergen challenge, unlike the other phenotypes which Fenretinide was able to have a significant impact on.

We were wondering whether it was not because the IgE was already elevated prior to the initiation of the treatment (during sensitization with allergen). So, in Chapter 1, we have tested an overlapping protocol, within which drug treatment starts from the first allergen sensitization, and

for 28 days until harvest. Our results demonstrated that starting Fenretinide treatment since the beginning of the allergic model did not modulate the increase in plasma IgE associated with sensitization. Our results presented here in this thesis were consistent with our laboratory previous report of Fenretinide treatment using OVA allergic asthma model [265]. Interestingly, even in the presence of high levels of IgE, the influx of inflammatory cells, the hyperplasia of the airways, and the mucus production associated with allergen exposure was prevented.

5.1.8 Immunotherapy

Immunotherapy are either subcutaneous immunotherapy (SCIT) or sublingual immunotherapy (SLIT). Compared to immunotherapy, therapy with Fenretinide has provided a strong evidence of clear efficacy and long-term safety (previously discussed hereabove).

A Cochrane review [311] included 88 trials for SCIT. SCIT was recommended for asthmatics with identified allergies to reduces both asthma symptoms and the use of asthma medications and improves bronchial hyperreactivity. One trial included in this Cochrane review found the benefit of SCIT is possibly comparable to ICS. The possibility of local or systemic adverse effects of SCIT (such as anaphylaxis) must be considered in deciding the benefit of therapy using SCIT [311].

The role of sublingual immunotherapy (SLIT) in the treatment of asthma is less clear [312], with most available evidence being in mild asthmatics. People receiving SLIT were less likely to experience serious unwanted side effects as with SCIT [312]. Currently in Canada, SLIT therapy is available for ragweed and tree allergies. SLIT for house dust mite as a perennial allergen may be beneficial for asthma symptoms, but further studies are necessary.

Both the SCIT and the SLIT lack long-term efficacy and safety studies, consequently, they find their places last in the cadre of the GINA recommendations in managing allergic asthma. Moreover, each immunotherapy is effective only against the allergen it was prepared from (mono-

valent). Since many asthmatics are sensitized against several allergens in real life [313], the mono-valent immunotherapy would provide very little benefits for them. Until future studies can provide strong grounds for their clinical use, we do not believe that any international guidelines would recommend them routinely in asthmatics [314].

5.2 Cystic Fibrosis

CF is the most leading life-limiting genetic disease among Caucasians. According to Cystic Fibrosis Canada (cysticfibrosis.ca), about 4,000 people have CF in Canada. CF mostly affects the lungs, pancreas, and intestine of people who carry CF mutation. In accordance with the causing mutation, patients have different degrees of symptoms [315].

5.2.1 Fenretinide Use in Cystic Fibrosis

In Chapter 4, we tested the lung physiology and histopathology of 2-, 4- and 7-months old mice and we have demonstrated that the Cftr KO mice demonstrated progressive decline in lung functions with age. We applied oral daily LAU-7b treatment, for a period of 21 days (10 mg/kg body weight), to the mice at age of 4- and 7-months and we demonstrated that treatment with LAU-7b can inhibit the age-dependant deterioration in lung physiology, histopathology and correct the lipid imbalances observed in the aging KO and WT mice.

In CF, the threshold of forty years of age has been employed by some studies to describe the ‘‘long-term survivors’’ or ‘‘older’’ patients [316]. Currently, the emerging older CF population presents an opportunity to evaluate the challenges which are related to long-term survivors. When the CF patient becomes old, new challenges are introduced to his life (non-CFTR-related), over the burdens of the CF disease itself (CFTR-related) [317]. Examples of the (non-CFTR-related) burdens which have been introduced to the CF elderly are depression and anxiety that often increase in adulthood presumably due to increasing co-morbidities and personal responsibilities

[318]. Longevity exposes the patient organs for a longer duration to the abnormal CFTR function. Many susceptible tissues will sooner or later be diseased and manifest new (CFTR-related) symptoms. Examples includes pancreatic dysfunction which results in diabetes in 50% of the patients [319] and CF bone disease which manifest later on as osteopenia and osteoporosis [320]. The majority of CF mortalities are due to lung diseases. Lung function tests worsens each year and the patients' sputum microbiology is also variable over the life-time of patients. For example, the prevalence of chronic P. aeruginosa is approximately 30% by age of 15 years, however, by age of 30 years it doubles [317].

We have shown in Chapter 4 that 7-month-old Cftr KO mice have markedly higher levels of LCC, whereas, the levels of VLCC appears to significantly lower than WT mice. LAU-7b treatment successfully corrected the imbalances of ceramides species which were observed in both Cftr KO and WT mice as a function of age.

Contradicting findings have been published about ceramide levels in CF. In contrast to our finding, the study by Gulbins group reported an accumulation of ceramide in CF; however only C16:0 species were measured in this study and no results were reported for other LCC or VLCC [291]. Other UK investigators assessed the ceramide levels in lungs by immunochemistry with two antibodies and HPLC/MS and they found increase in the levels of ceramides [321]. In this study, the immunochemistry results indicated that CF lung tissues had higher levels of staining compared to controls; nevertheless, the authors reported variability in staining between the two antibodies. Also, the results of HPLC/MS of this study demonstrated changes in only four species of ceramides (elevated C16:0, C18;0, C20:0 and unchanged C22:0) in the CF lungs. Another study group [322] analyzed the ceramide species, using MS, in two 16HBE14o(-) cell populations; either expressing CFTR or expressing the antisense control gene construct. Four ceramide species were found to be

elevated (DHC16:0, C22:0, C24:0, C26:0) when no CFTR was expressed while 2 species were reduced (C18:0 and C18:1) compared to cells expressing CFTR.

There may be more than one answer to the question why several studies report different results regarding the ceramide levels in CF. The first suggestion is because of the use of different models; either murine or cellular models presenting the CF disease. Different cell types or sources of cells (patient samples vs. immortalized cell lines) may respond differently to stress and ceramide production may be different within each cell type. As for animal models, *Cftr* KO mice have different phenotypes which may lead to differences in the ceramides composition. Secondly, different groups use different methods of ceramide analysis. Using antibodies for the detection of ceramides can be misleading as the anti-ceramides antibodies bind to many species of ceramides and lipids in general, so the pool of ceramides needs to be purified from other lipids first to generate meaningful results. Using antibodies for detecting ceramides can reduce the costs for the quantification of total amounts of ceramides, nonetheless, it cannot provide individual results for each ceramide species nor the concentration of each species present in the sample.

MS offers precise identification of each ceramide species; therefore, it represents the best method for accurate quantification of ceramides. In our studies presented in Chapter 4, we have analyzed all the species of ceramides by MS, hence the results demonstrated here would give a comprehensive picture of the alteration in sphingolipids composition in CF. Furthermore, in our study, we used C57BL/6$^{H/M}$-Cftr KO mouse model [222] which provides an outstanding picture of the lung disease occurring clinically in CF patients. Although very few studies investigated the disease pathology in old CF mice [291,323], possibly because not many mice can survive to older age, we were able to breed and maintain a colony of the Cftr KO mice sufficient to perform our experiments at 2-, 4-, and 7-month timepoints.

5.2.2 Therapies and Treatment Options Currently Used in Cystic Fibrosis

Since the early discovery of CF in the 1930s, the clinical management of CF has focused on treating symptoms and/or delaying end-organ effects. Cystic fibrosis transmembrane conductance regulator (CFTR) modulators are a promising new class of medications which help restore the function of the mutated CFTR protein [324]. Since its approval by the FDA in 2012, it has been proven that Ivacaftor substantially improve the symptoms of CF patients with the G551D mutation by potentiating the ion current through the chloride channel [325]. It was also found to improve insulin secretion in CF patients [326]. The effects of this drug on mucus, inflammation and oxidation are yet to be fully described. There is no doubts that CF patients with G551D mutation in the *Cftr* gene can benefit from this treatment, whereas for other mutation the treatments even with combination of potentiators and correctors do not correct lung disease sufficiently despite very high cost of the medications [325].

F508del results in folding defects leading to decreased CFTR at the cell surface, as well as gating defects causing decreased conductance [327]. Lumacaftor can partially correct the folding defect in F508del-CFTR, resulting in slightly increased surface protein, but Ivacaftor is also needed to improve conductance [327,328]. In 2015, the FDA approved lumacaftor/ivacaftor (Orkambi®) for patients with CF of age 12 years or older and homozygous for F508del. The efficacy of the treatment with Orkambi turned out to be disappointing since a significant number of CF patients did not respond well to it. In 2018, the Canadian Agency for Drugs and Technologies in Health (CADTH)-Canadian Drug Expert Committee recommended that lumacaftor/ivacaftor not be reimbursed for the treatment of CF patients because the magnitude of improvement was of uncertain clinical significance based on a systematic review of RCTs and pivotal studies. Currently, triple combination of correctors and potentiators is being tested

(NCT03525444 and NCT03525548) and the future will show if the clear improvement in lung disease and chronic inflammation which CF patients suffer from can be significantly diminished. While steroids can be prescribed for CF patients to decrease the lung inflammation, long-term administration of steroids in CF has been associated with many adverse effects. A Cochrane systematic review article [329] summarized three CF clinical trials; one trial for three months and two trials over the period of 4 years. Although the oral steroid (in low doses equivalent to prednisolone 1-2 mg/kg) given every other day lowered the lungs inflammation, there were serious adverse effects such as cataracts and growth retardation. These adverse effects led to one trial termination earlier than what have been planned to [329]. Another systematic review [242] gathered clinical evidences from 13 trials which assessed the effectiveness of regularly administrating inhaled corticosteroids in CF children and adults. Evidences from these clinical trials failed to establish whether long-term inhaled corticosteroids are beneficial in CF, however, there is some evidence that they may cause harm [242]. Therefore, the development and testing of other anti-inflammatory medications is needed to improve this chronic and a life-lasting disease.

Ibuprofen is a widely used over-the-counter anti-inflammatory, analgesic and antipyretic drug. It was found that Ibuprofen reduced the levels of NF-κB activation in CF epithelial cells [330], however, the production of IL-8 was not affected. Further, Ibuprofen shows important effects in young CF patients by preventing the decline of lung function [331]. However, since CF is a chronic and life-long disease, the risk of gastrointestinal bleeding [332] and acute renal failure [333] related to the continuous intake of non-steroidal anti-inflammatory drugs remains an important factor to consider.

Mucolytics and bronchodilators are used to loosen the thick mucus and expand airways in CF patients, however, these two classes of medications do not treat the disease inflammation [334]. Antibiotics are used to fight lung infections in case of acute exacerbation episodes, meanwhile,

some CF patients need to administer the antibiotics on regular basis to prevent any possible future infections [335]. Long-term, or recurrent, administration of antibiotics is not advised clinically as it usually results in bacterial resistance and overall treatment failure.

Recombinant human DNase (rhDNase) is used to breakdown the thick mucus which is frequently present in the airways of CF patients [336]. Accordingly, the environment for pathogens is interrupted and the patients have fewer bacterial populations after rhDNase treatment without the need of antibiotics. Moreover, rhDNase was shown to have some anti-inflammatory effects by preventing the increase of IL-8 and neutrophil elastase in young uninfected CF patients [337]. Hypersensitivity against rhDNase is considered the most known limitation against its utilization in CF patients.

Compared to the previously mentioned medications currently recommended for CF patients, we believe that Fenretinide is a good candidate for managing CF disease. Chapter 4 concluded that LAU-7b can address many significant issues in aged Cftr KO mice as a model for CF disease. Treatment with the formulation of Fenretinide, LAU-7b, corrected the altered composition of ceramide species, diminished AA, MD and NT, elevated DHA, lowered lung resistance, decreased cell infiltration and epithelial hyperplasia. The evidence presented in this thesis demonstrates the potential therapeutic benefits of LAU-7b, the oral formulation of Fenretinide, even in the older CF subjects (as illustrated using aged *Cftr* KO mice). These preclinical data require further clinical trials to fully assess the efficacy of LAU-7b in elderly patients who have CF.

Table 5.1 Summary of the biological effects of Fenretinide in comparison to the current routine treatments for asthmatic patients

	Fenretinide	B2 agonists SABA/LABA	Steroids (local/systemic)	Leukotriene antagonists LTRA	Anticholinergics (SAMA/LAMA)	IgE Therapy	Mucolytics	Immunotherapy
Anti-inflammatory	*		*	*				*
Broncho-dilatation		*			*			
Airway hyper-responsiveness	*		*	*				
Correction of ceramide levels	*							
Anti-oxidant	*							
Reduction of Mucus	*				*		*	
Reduction of IgE levels						*		

Table 5.2 Summary of the biological effects of Fenretinide in comparison to the current routine treatments for CF patients

	Fenretinide	CFTR protein correctors	Antibiotics	Steroids	Non-steroidal anti-inflammatory drugs	rhDNase	Mucolytics	Bronchodilators
Anti-inflammatory	*			*	*	*		
Anti-infective	*		*			*		
Anti-oxidant	*							
Correction of ceramide levels	*							
Correction of CFTR protein								
Reduction of Mucus	*					*	*	
Broncho-dilatation								*

Chapter 6. Conclusion and Future Directions

6.1 Conclusion

Chapter 1 of this thesis has demonstrated that 10 mg/kg of p.o. LAU-7b or 5 or 10 mg/kg of i.p. FEN for 9 days is able to correct diverse pro-inflammatory events, improve lung physiology and histology phenotypes, and restore the levels of VLCCs in the lungs and plasma, in both OVA- and HDM-sensitized asthmatic A/J mice.

In Chapter 2, we demonstrated that the loss of *Zpbp2* gene renders the A/J mice less sensitive to airway hyper-responsiveness as measured by MCh. Additionally, LAU-7b oral treatment in dose of 10 mg/kg for 9 days corrected diverse pro-inflammatory events and improved lung physiology in HDM-sensitized *Zpbp2* A/J mice. Our findings show that LAU-7b lowers the expression of *Ormdl3*, which may explain in part the elevation of VLCCs, and protects the WT mice against allergic asthma symptoms.

The experimental work of Chapter 4 has shown that *Cftr* KO B6 mouse model displays clear age-dependent progressive deterioration demonstrated by the increase in lung resistance, number of cell infiltration and epithelium hyperplasia of the lung airways at age of 2, 4 and 7 months. We demonstrated that the treatment of *Cftr* KO B6 mice for 21 days with LAU-7b at dose of 10 mg/kg improves dramatically lung physiology and histopathology when the treatment is administrated at the age of 4 and 7 months. LAU-7b treatment also reverses the progressive age-related changes in the lung which normally occur with age in 4- and 7-month-old WT B6 mice. The changes at the level of histopathology are consistent with the effect of the treatment on the lung physiology.

6.2 Future Directions

The work presented in this thesis was done as consecutive projects on allergic asthma and CF. The results presented in Chapter 2 illustrate the optimization of a treatment protocol using LAU-7b against allergic asthma. Chapter 3 illustrates the efficacy of LAU-7b treatment against allergic asthma in *Zpbp2* knockout A/J mice. In Chapter 4, we tested the efficacy of LAU-7b treatment against age-dependent deterioration in lung functions in CF. Since the thesis was mainly built up on three divisions, the experimental work of this thesis can be exploited to other research projects in three directions.

First, assessment of LAU-7b efficacy in a chronic mouse model of allergic asthma can be done using longer protocols of successive allergen exposures (> 2 months). Chronic mouse models better mimic allergic asthma in humans, because people are exposed to allergens over a long period of time. Considering that the asthmatics are sometimes sensitized to more than one allergen, we suggest testing LAU-7b in mouse models with different allergens. Different protocols of LAU-7b administration can also be tried in allergic asthma mouse models, e.g., administration of LAU-7b could be tested with or after the timepoint of allergen challenge. Nebulized/inhaler dosage form of Fenretinide can be developed to facilitate the medication delivery locally into the lungs. Topical dosage form of Fenretinide (as cream or ointment) can be evaluated as an anti-inflammatory medication against atopic skin conditions (e.g., atopic and contact dermatitis). LAU-7b efficacy in other chronic respiratory diseases can be investigated (e.g., chronic obstructive lung disease, COPD, and allergic rhinitis).

Second, the evaluation of LAU-7b treatment in transgenic mouse models overexpressing *Ormdl3* and knockout for *Ormdl3* would give a more precise picture of the molecular mechanisms of action of LAU-7b in relation to the ceramide imbalances in these mice. Beside *Zpbp2* and

Ormdl3, the assessment of the possible effects of LAU-7b on the expression of other genes present in the 17q21 chromosomal locus can be done (e.g., Gasdermin B (*Gsdmb*) and IKAROS family zinc finger 3 (*Ikzf3*)).

Third, different protocols of LAU-7b administration can be tried in the *Cftr* KO mouse models. For example; the administration of LAU-7b continuously, with periods of rest, throughout the life of the mouse, can be tested. This protocol will allow a better investigation of the long-term protection of LAU-7b in CF disease. Additionally, rather than knocking down the whole gene, mouse models which carry *Cftr* mutations, e.g., d508 *Cftr* mutation, can be assessed a long with LAU-7b treatment in both young and old mice.

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Appendix

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Animal Protocols

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December 4, 2014

Animal Certificate

This is to certify that Dr. Danuta Radzioch, Department of Medicine, Montreal General Hospital (RI-MUHC), currently holds an approved Animal Use Protocol # 2012-7191 with McGill University and its Affiliated Hospitals' Research Institutes for the following project:

Animal Use Protocol Title:
Genetic and epigenetic control of the ZPBP2/ORMDL3/GSDMB domain and susceptibility to asthma

Start date: January 1, 2015

Expiration date: January 1, 2016

McGill University and Affiliated Hospitals Research Institutes recognize the importance of animal research in our efforts to further our knowledge of natural processes, diseases and conservation. Research, educational and testing projects are conducted with full commitment to the wellbeing of the animal subjects. In order to limit animal use to meritorious research or educational projects, the institution relies on stringent peer review processes, along with assessment of ethical issues by the Animal Care Committee. McGill University recognizes that the use of animals in research, teaching and testing carries significant responsibilities. The institution will continue to develop and maintain guidelines and regulations, following the high standards established by the Canadian Council on Animal Care. It is committed to conducting the highest-quality research and to providing animals with the best care.

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McGill

April 15, 2016

Animal Certificate

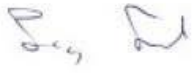
This is to certify that **Dr. Danuta Radzioch**, MUHC / Glen site, currently holds an approved **Animal Use Protocol # 2015-7636** with McGill University and its Affiliated Hospital's Research Institutes for the following project:

Animal Use Protocol Title: Genetic and epigenetic control of the ZPBP2/ ORMDL3/ GSDMB domain and susceptibility to asthma.

Start date: January 1, 2016

Expiration date: December 31, 2016

McGill University and Affiliated Hospitals Research Institutes recognize the importance of animal research in our efforts to further our knowledge of natural processes, diseases and conservation. Research, educational and testing projects are conducted with full commitment to the wellbeing of the animal subjects. In order to limit animal use to meritorious research or educational projects, the institution relies on stringent peer review processes, along with assessment of ethical issues by the Animal Care Committee. McGill University recognizes that the use of animals in research, teaching and testing carries significant responsibilities. The institution will continue to develop and maintain guidelines and regulations, following the high standards established by the Canadian Council on Animal Care. It is committed to conducting the highest-quality research and to providing animals with the best care.



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February 24, 2017

Animal Certificate

This is to certify that **Dr. Danuta Radzioch**, MUHC RI/ Glen site, currently holds an approved **Animal Use Protocol #2015-7636** with McGill University and its Affiliated Hospital's Research Institutes for the following project:

Animal Use Protocol Title: Genetic and epigenetic control of the ZPBP2/ ORMDL3/ GSDMB domain and susceptibility to asthma.

Start date: January 1, 2017

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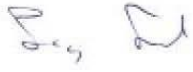

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February 24, 2017

Animal Certificate

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Animal Use Protocol Title: Genetic and epigenetic control of the ZPBP2/ ORMDL3/ GSDMB domain and susceptibility to asthma.

Start date: January 1, 2017

Expiration date: December 31, 2017

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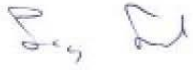

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January 10, 2018

Animal Certificate

This is to certify that **Dr. Danuta Radzioch, Department of Medicine, RI-MUHC (Glen Site),** currently holds an approved Animal Use Protocol # **2015-7636** with McGill University and its Affiliated Hospitals' Research Institutes for the following project:

Animal Use Protocol Title: Genetic and epigenetic control of the ZPBP2/ORMDL3/ GSDMB domain and susceptibility to asthma - (was MGH AUP #7191)

Start date: January 1, 2018

Expiration date: January 1, 2019

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May 7, 2019

Animal Certificate

This is to certify that **Dr. Danuta Radzioch, Department of Medicine, (RI MUHC) Glen site,** currently holds an approved Animal Use Protocol # **2015-7636** with McGill University and its Affiliated Hospitals' Research Institutes for the following project:

Animal Use Protocol Title: Genetic and epigenetic control of the ZPBP2/ORMDL3/GSDMB domain and susceptibility to asthma

Start date: January 1, 2019

Expiration date: January 1, 2020

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July 31, 2014

Animal Certificate

This is to certify that Dr. Danuta Radzioch, Department of Medicine, Montreal General Hospital (RI-MUHC), currently holds an approved Animal Use Protocol # 2001-3874 with McGill University and its Affiliated Hospitals' Research Institutes for the following project:

Animal Use Protocol Title: Étude Clinique du Fenretinide Pour la Fibrose Kystique (Clinical Study of fenretinide for cystic fibrosis) / Regulation of the lung inflammation in Cftr-knockout mice

Start date: June 1, 2014

Expiration date: June 1, 2015

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July 17, 2018

Animal Certificate

This is to certify that **Dr. Danuta Radzioch, Department of Medicine, RI-MUHC (Glen Site),** currently holds an approved Animal Use Protocol # **2015-7740** with McGill University and its Affiliated Hospitals' Research Institutes for the following project:

Animal Use Protocol Title: Role of B-cell activating factor (BAFF) in the regulation of the lung inflammation in Cftr-knockout mice and their ability to combat chronic infections with Pseudomonas.

Start date: April 1, 2018

Expiration date: April 1, 2019

McGill University and Affiliated Hospitals Research Institutes recognize the importance of animal research in our efforts to further our knowledge of natural processes, diseases and conservation. Research, educational and testing projects are conducted with full commitment to the wellbeing of the animal subjects. In order to limit animal use to meritorious research or educational projects, the institution relies on stringent peer review processes, along with assessment of ethical issues by the Animal Care Committee. McGill University recognizes that the use of animals in research, teaching and testing carries significant responsibilities. The institution will continue to develop and maintain guidelines and regulations, following the high standards established by the Canadian Council on Animal Care. It is committed to conducting the highest-quality research and to providing animals with the best care.

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Contributions by the PhD candidate to other publications

- Manuscript accepted for publication: *BBA - Molecular and Cell Biology of Lipids*. Dušan Garić, Juan B. De Sanctis, Juhi Shah, Daciana Catalina Dumut, **Mina Youssef** and Danuta Radzioch. "Fenretinide mimics CFTR-induced correction of DHA/AA imbalance and blocks LPS-induced MUC5AC overexpression without affecting MUC5B." (2019).

* Contribution: Preparation of LAU-7b doses and executed the mice experiments.

- Dušan Garić, Shao Tao, Eisha Ahmed, **Mina Youssef**, Cynthia Kanagaratham, Juhi Shah, Bruce Mazer, and Danuta Radzioch. "Depletion of BAFF cytokine exacerbates infection in *Pseudomonas aeruginosa* infected mice." *Journal of Cystic Fibrosis* 18, no. 3 (2019): 349-356.

* Contribution: Executed the mice experiments.

- Cynthia Kanagaratham, Victoria Chiwara, Bianca Ho, Sanny Moussette, **Mina Youssef**, David Venuto, Lucie Jeannotte *et al.* "Loss of the zona pellucida-binding protein 2 (Zpbp2) gene in mice impacts airway hypersensitivity and lung lipid metabolism in a sex-dependent fashion." *Mammalian genome*29, no. 3-4 (2018): 281-298.

* Contribution: Executed the mice experiments, participated in data collection and analysis.

- Xu Yong Zhong, Cynthia Kanagaratham, **Mina Youssef**, and Danuta Radzioch. "New frontiers in cancer chemotherapy—targeting cell death pathways." *Cell biology—new insights. Rijeka: InTech* (2016): 93-140.

* Contribution: Participated in editing the article.

- Mohamed I. Saad, Taha M. Abdelkhalek, Moustafa M. Saleh, Maher A. Kamel, **Mina Youssef**, Shady H. Tawfik, and Helena Dominguez. "Insights into the molecular mechanisms of diabetes-induced endothelial dysfunction: focus on oxidative stress and endothelial progenitor cells." *Endocrine* 50, no. 3 (2015): 537-567.

* Contribution: Participated in writing and editing the article.

- Nadia A Abd El Moneim, Hisham El Masry, **Mina Mamdouh Sorial (Mina Youssef)**, Taha I. Hewala, Amira Embaby, and Salah Sheweita. "A molecular case-control study on the association of melatonin hormone and rs# 10830963 single nucleotide polymorphism in its receptor MTNR1B gene with breast cancer." *Middle East Journal of Cancer* 6, no. 1 (2015): 11-20.

* Contribution: Designed the study, executed all the experimental work, collected the data, performed the analysis and wrote the manuscript (M.Sc. thesis publication).