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1.1 Title Page

Treatment of allergic asthma with Fenretinide formulation (LAU-7b) downregulates *Ormdl3* expression and normalizes ceramides imbalance

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1.2 Running Title

LAU-7b efficacy in asthmatic *Zpbp2* KO and WT A/J mice

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List of Abbreviations:

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
KO	Knockout
LCCs	Long chain ceramides
MCh	Methacholine
MDA	Malondialdehyde
NT	Nitrotyrosine
Ormdl3	ORMDL sphingolipid biosynthesis regulator 3
OVA	Ovalbumin
PAS	Periodic acid Schiff
VLCCs	Very long chain ceramides
WT	Wildtype
Zpbp2	*Zona pellucida* binding protein 2

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1.3 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:** 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 AHR, allergic *Zpbp2* KO mice were not protected from inflammatory cell infiltration, mucus accumulation or aberrant levels of VLCCs and LCCs 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 VLCCs and LCCs and corrects all the other phenotypes associated with allergic asthma following HDM challenge, except the elevated levels of IgE. **Conclusion:** 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.

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1.4 Significance Statement

Number of words in the Significance Statement: 79 (80 max.)

- Compared to A/J WT mice, KO mice with *Zpbp2* gene deletion have lower AHR and lower levels of *Ormdl3* expression.

- The novel oral clinical formulation of Fenretinide (LAU-7b) effectively lowers the AHR, protects against inflammatory cell infiltration and mucus accumulation induced by HDM in both *Zpbp2* KO and WT A/J mice.

- LAU-7b prevents *Ormdl3* overexpression in WT allergic mice and corrects the aberrant levels of VLCCs and LCCs in both WT and *Zpbp2* KO allergic mice.

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1.5 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 (Burbank et al. 2017). 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 hyperresponsiveness (AHR) (Moffatt et al. 2007). The 17q21 locus importance was evaluated in an ethnically diverse population but ethnic-based differences were not found (Kothari et al. 2018).

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 (Torabi et al. 2017). ZPBP2 expression has also been detected, in lower levels, in human somatic airway epithelial cells (Moussette et al. 2017). Recently, our group (Kanagaratham et al. 2018), and Miller *et al.* (Miller et al. 2018), 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 (Miller 2018). 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 (Davis et

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al. 2018). Transcriptomic and metabolomic analyses have linked the FEV1/FVC ratio with ORMDL3 and dysregulated lipid metabolism in a study of asthmatic children.

Moreover, elevated levels of ORMDL3 inhibit sphingolipid biosynthesis and result in inhibition of long chain ceramide (LCCs; C16:0) and four species of very long chain ceramides (VLCCs; C22:0, C24:0, C24:1 and C24:2) (Kiefer et al. 2019). Debeuf *et al.* (Debeuf et al. 2019) reported that transgenic mice overexpressing *Ormdl3* had significantly lower levels of VLCCs (C24:0 and C24:1), by contrast, loss of *Ormdl3* in KO mice resulted in elevated levels of C24:0 and C24:1. Beside allergic asthma, several studies showed that the *ORMDL3* locus is also associated with other numerous pathologies e.g. allergic rhinitis, type 1 diabetes, primary biliary cirrhosis, rheumatoid arthritis, ulcerative colitis, Crohn's disease, and ankylosing spondylitis.

Fenretinide (FEN), a vitamin A derivative, corrects the aberrant inflammatory responses and improves lung functions in both cystic fibrosis (CF) (Garić et al. 2017; Guilbault et al. 2008, 2009; Youssef et al. 2016) and allergic asthma (Kanagaratham et al. 2014). In CF, our lab has shown that Fenretinide improves the lung function by elevating the levels of VLCCs and lowering the levels of LCCs (Garić 2017). Recently, a novel clinical oral formulation of Fenretinide (LAU-7b), with excellent bioavailability, 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 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.

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In our study, we developed knockout (KO) mice for *Zpbp2* gene 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 significant increase in airway hyperplasia, and an intense AHR which can be measured following exposure to methacholine (MCh). Therefore, we have used this strain of mice to investigate the protective effects of LAU-7b treatment (10 mg/kg/day for 9 days) 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 decreased AHR measured following exposure to MCh. 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.

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1.6 Materials and Methods

1.6.1 Animal Model

Heterozygous B6.127S7-*Zpbp2*^{tm1Zuk}/J mice (Lin et al. 2007), which carry a deletion of *Zpbp2* exons 1-3, were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and backcrossed for more than ten generations to A/J inbred mice purchased from Jackson Laboratory (Bar Harbor, Maine, USA). N10 *Zpbp2* KO mice were intercrossed to generate homozygous KO A.127S7-*Zpbp2*^{tm1Zuk} mice, referred to from this point on as KO. All experimental procedures were approved by the Animal Care Committee of McGill University Health Center, Montreal, QC, Canada. More information is provided in the online supplement.

1.6.2 Mouse Groups and Batches

Following sensitization (more information is provided in the online supplement), 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.

1.6.3 AHR Measurements

AHR was measured using a Buxco plethysmograph system, ventilators, and nebulizers (Harvard Apparatus, Holliston, MA, USA) as previously described (Kanagaratham 2014, 2018). For the non-invasive whole-body plethysmography (WBP) the baseline enhanced pause (Penh) values were measured without anesthesia at the age of eight weeks before the first sensitization. The invasive AHR measurements were done when sacrificing the mice on the day of the harvest.

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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).

1.6.4 Lung Histology Analysis

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 as previously described (Kanagaratham 2014). 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 (Kanagaratham 2014). Airway smooth muscle (ASM) mass was stained using specific α -smooth muscle actin (α-SMA) antibody as previously described by our laboratory. Masson trichrome stain was used to assess collagen production as previously described.

1.6.5 Lipids and Markers of Oxidation Analysis

Lipid analysis was done using 25 mg of macerated lung tissue from each mouse. Classical isolation of lipids was done as previously described by Folch (Folch et al. 1957), and the levels of different lipid species were measured using high-performance liquid chromatography (HPLC) tandem mass spectrometry (MS) as previously described (Guilbault 2008).

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1.6.6 Gene Expression Measurements

RNA was extracted from snap frozen lungs using the RNeasy Mini kit (Qiagen). Five hundred ng of RNA was reverse transcribed into cDNA for each analyzed sample using the QuantiTect Reverse Transcription kit (Qiagen). 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. More information is provided in the online supplement.

1.6.7 IgE Measurements in Serum

Plasma IgE levels were measured using an enzyme-linked immunosorbent assay (ELISA) IgE mouse kit (BD OptEIA Biosciences) following the manufacturer's instructions.

1.6.8 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.

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1.7 Results

1.7.1 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 (Miller 2018) 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

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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.

1.7.2 Assessment of AHR 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.

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1.7.3 Evaluation of Inflammatory Cells Infiltration, Mucus Production, Smooth Muscle Mass and Collagen 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).

Because remodelling of the airways is an important aspect in the context of human asthma, we wanted to assess the smooth muscle mass and collagen production in our mouse

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model and to evaluate the effect of treatment with LAU-7b. Our data (SUPP. E1) shows that after HDM challenge, there is a slight increase in the mass of the smooth muscles of the mice airways. Similarly, challenge with HDM results in the production of collagen (SUPP. E2), which was evident in the airways. LAU-7b treatment lowered both the mass of the smooth muscles and the collagen production in our KO and WT A/J mice.

1.7.4 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. E3) 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. E3). 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).

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1.8 Discussion

The importance of the 17q21 locus containing genes such as *ORMDL3* and *ZPBP2* was replicated in several studies (Moffatt et al. 2007) (Karunas et al. 2011; Torgerson et al. 2011; Wan et al. 2012). Our main objective was threefold. First, we aimed to evaluate the effect of the *Zpbp2* gene deletion on lung physiology. Secondly, we aimed to investigate the effects of LAU-7b treatment against HDM-induced allergic asthma in *Zpbp2* KO and WT 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.

We have previously published (Kanagaratham et al. 2018) 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 (Kanagaratham et al. 2018) did not significantly affect the AHR of male or female mice after OVA sensitization and challenge perhaps because the C57BL/6 strain of mice is genetically resistant to developing allergic asthma. Miller *et al.* (Miller et al. 2018) 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

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susceptible (high AHR) and atopic mice this gene plays an important role in the control of lung physiology in allergic asthma.

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 (Kanagaratham 2014, 2018)), 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.

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-

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limiting step of *de novo* ceramide, and all other sphingolipids, biosynthesis (Breslow et al. 2010). In a mouse study, myriocin, an inhibitor of the SPT enzyme, reduced the *de novo* sphingolipid synthesis and increased bronchial reactivity (Worgall et al. 2013). Likewise, increased airway responsiveness and airway remodeling has been reported in C57BL/6 transgenic mice overexpressing *Ormdl3* after OVA sensitization and challenge (Miller et al. 2014). Similarly, C57BL/6 mice lacking *Ormdl3* were protected from developing AHR and airway eosinophilia induced by *Alternaria alternate* (Löser et al. 2017).

Further, the expression of *ORMDL3* is not independent of the expression of other genes present in the 17q21 chromosomal locus. It has been reported that *ZPBP2 and ORMDL3 genes* are co-regulated together as closely associated cis-haplotype elements (Verlaan et al. 2009). 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 displaying increased AHR (loci on chromosome 12).

It has been reported that elevated levels of ORMDL3 inhibit sphingolipid biosynthesis resulting in inhibition of both long chain ceramide (LCCs; C16:0) and four species of very long chain ceramides (VLCCs; C22:0, C24:0, C24:1 and C24:2) (Kiefer 2019). By contrast, Zhang *et al.* (Zhang et al. 2018) 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.* (Debeuf 2019) reported that transgenic mice overexpressing *Ormdl3* had significantly reduced levels of C24:0 and C24:1. By contrast, loss of *Ormdl3* in

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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 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 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*.

We tested four 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). 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. Miller *et al.* (Miller 2018) reported a lack of significant increase in IL-13 levels in *Zpbp2* KO mice after HDM challenge compared to *Zpbp2*

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KO controls. The results of the studies reported by Debeuf *et al.* (Debeuf 2019) 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.

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 which is consistent with the previously reported effect on ERK1/2 phosphorylation (Lachance et al. 2013). IL-13 is a key regulatory cytokine in the production of IgE (Rael et al. 2011). Interestingly, both *Zpbp2* KO and WT mice treated with LAU-7b did not show significant reduction of *Il-13* expression which may explain why IgE levels remained elevated in both groups. Interestingly, inhibition of IgE levels was not essential for improving lung physiology in treated animals.

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.

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1.9 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.

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1.11 Authorship Contributions

Designed all the experiments: Youssef, Naumova and Radzioch

Supervised the study: Radzioch

Participated in all the experiments and wrote the manuscript: Youssef

Performed the animal experiments, performed and analyzed the whole-body plethysmography, the airway resistance measurements, the histology work and the IgE measurements: Youssef

Performed the RNA expression analysis: Youssef, Shah and Dumut

Measurements of lipids, ceramides and markers of oxidative stress: De Sanctis and Hajduch

Performed data analyses: Youssef

Participated in the editing of the manuscript: All the authors

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1.13 Figure Legends

Figure 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. Enhanced pause (Penh) values were measured 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. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Figure 2 Classical invasive measurements of lung resistance and measurements of IgE levels of *Zpbp2* KO and WT mice

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

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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. * $p<0.05$, ** $p<0.01$, **** $p<0.0001$

Figure 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. ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

Figure 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. *** $p<0.001$, **** $p<0.0001$

Figure 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. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

1.14 Figures

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Figure 1

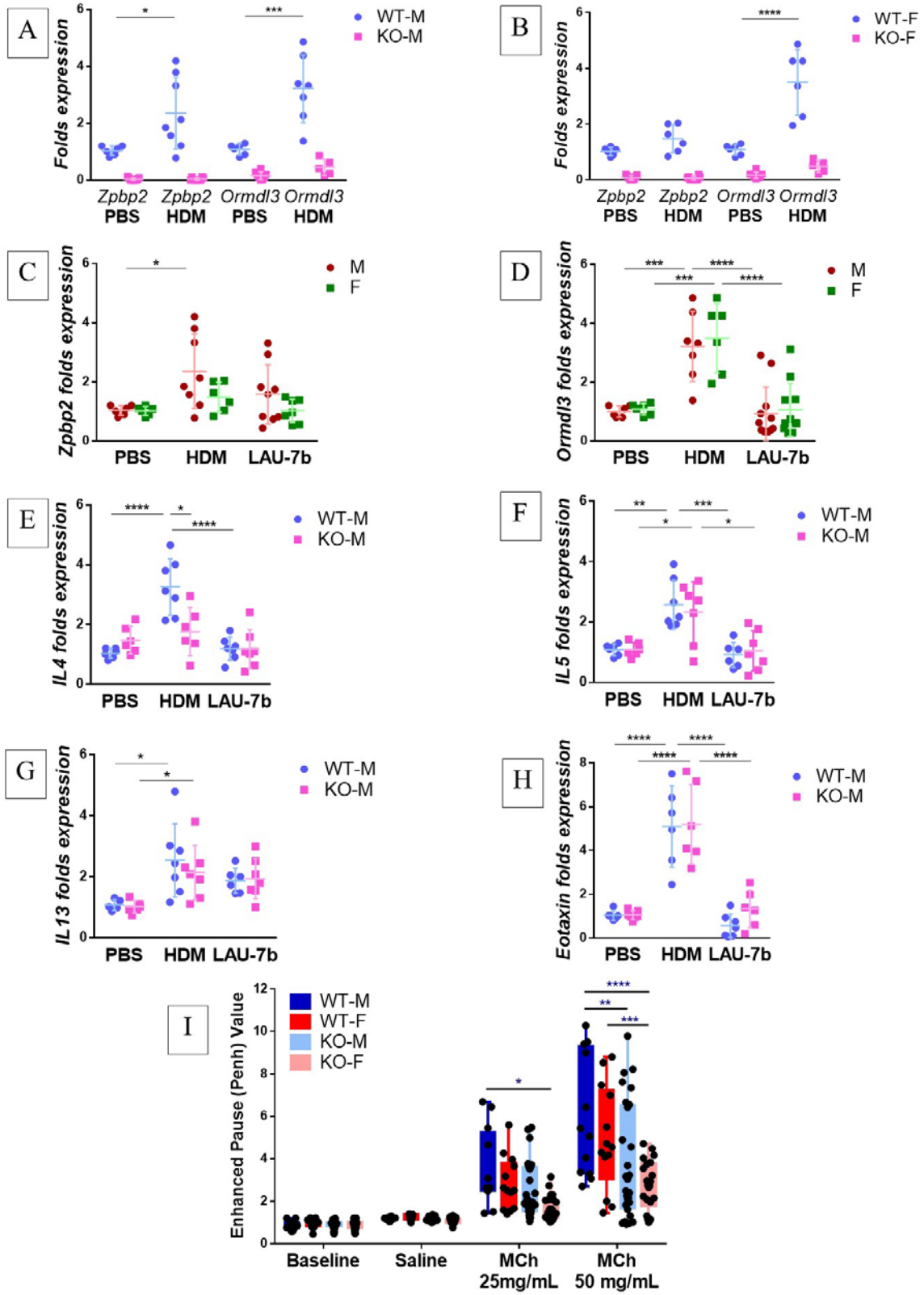

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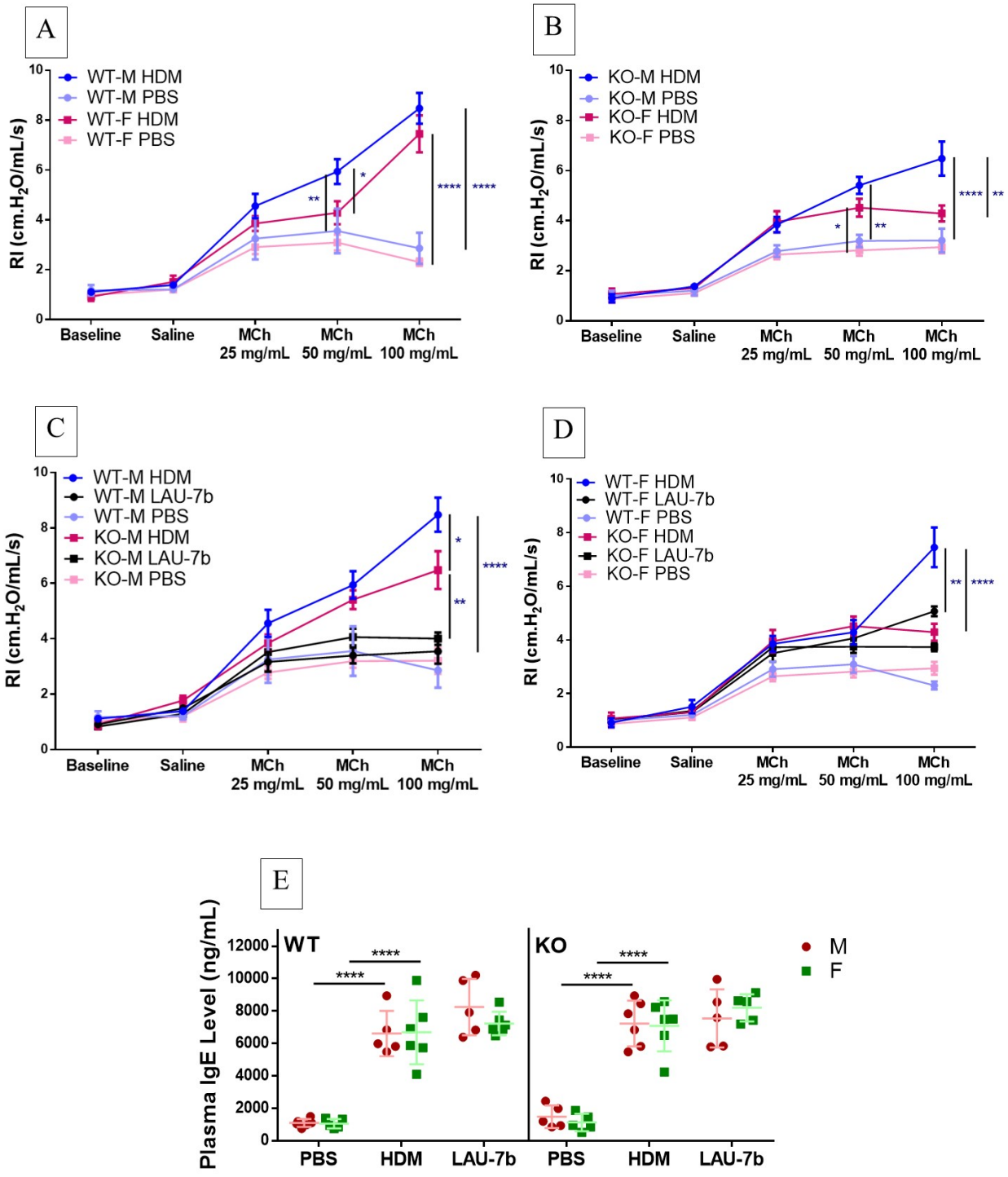
Figure 2



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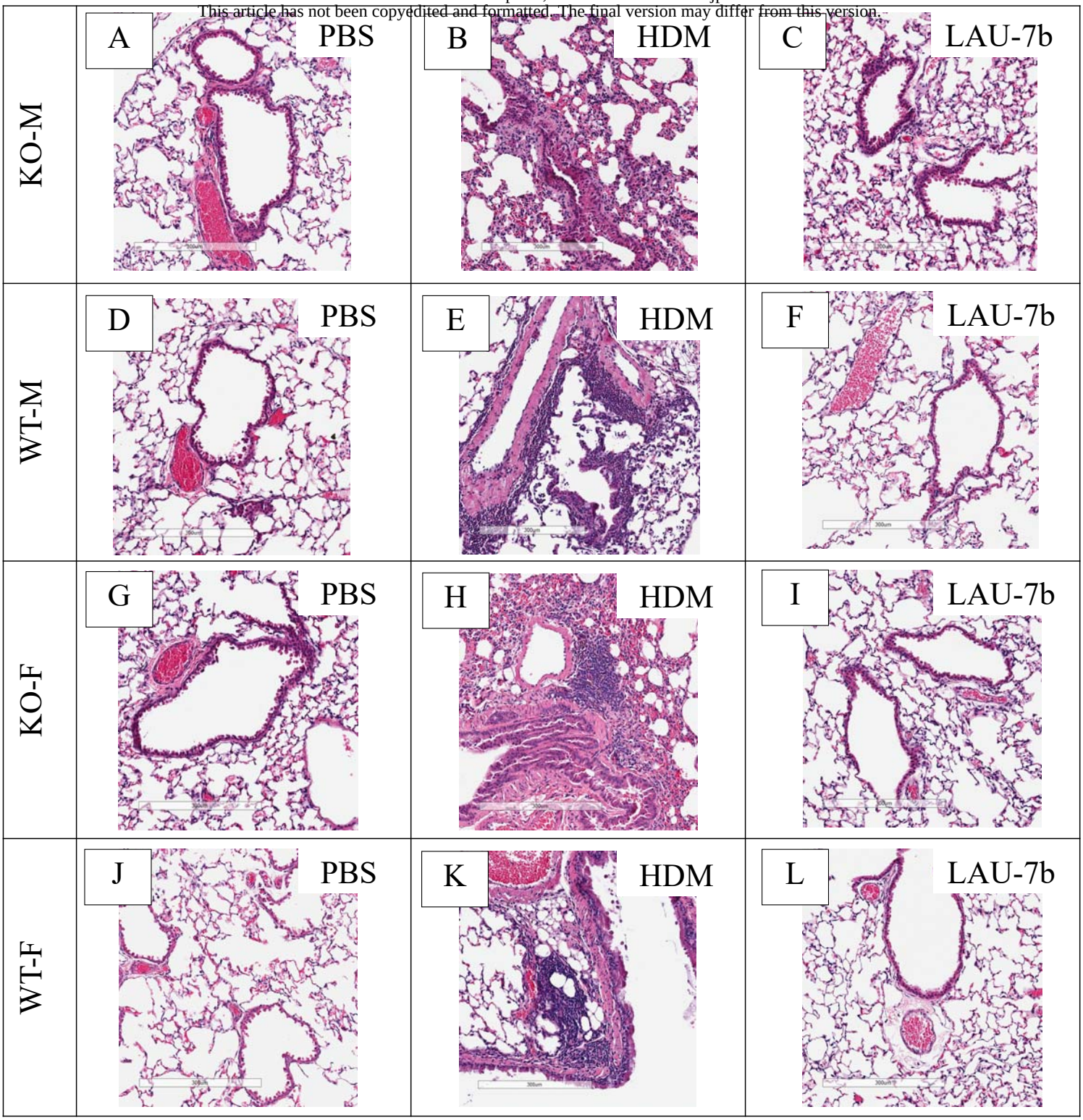

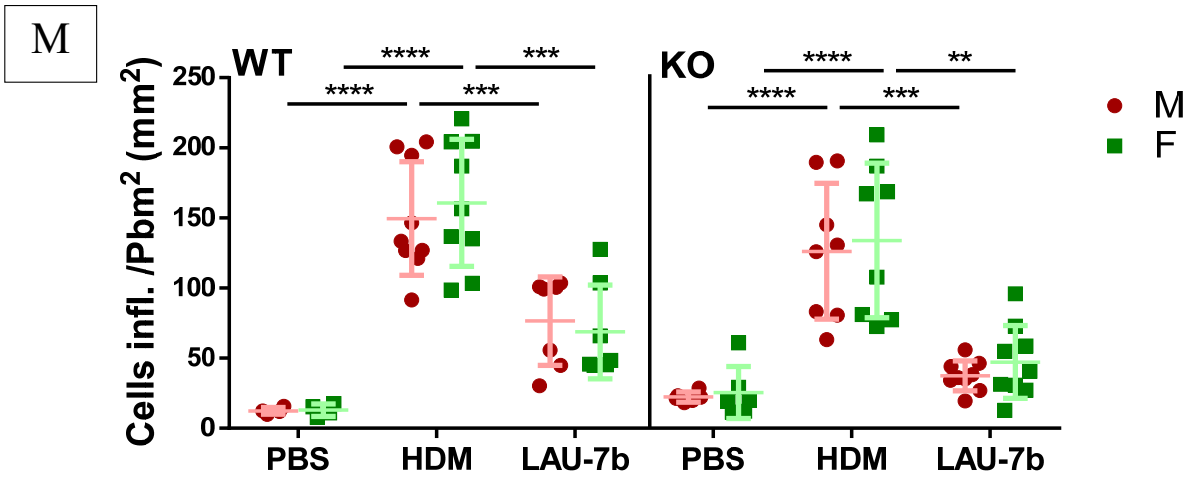

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Figure 4

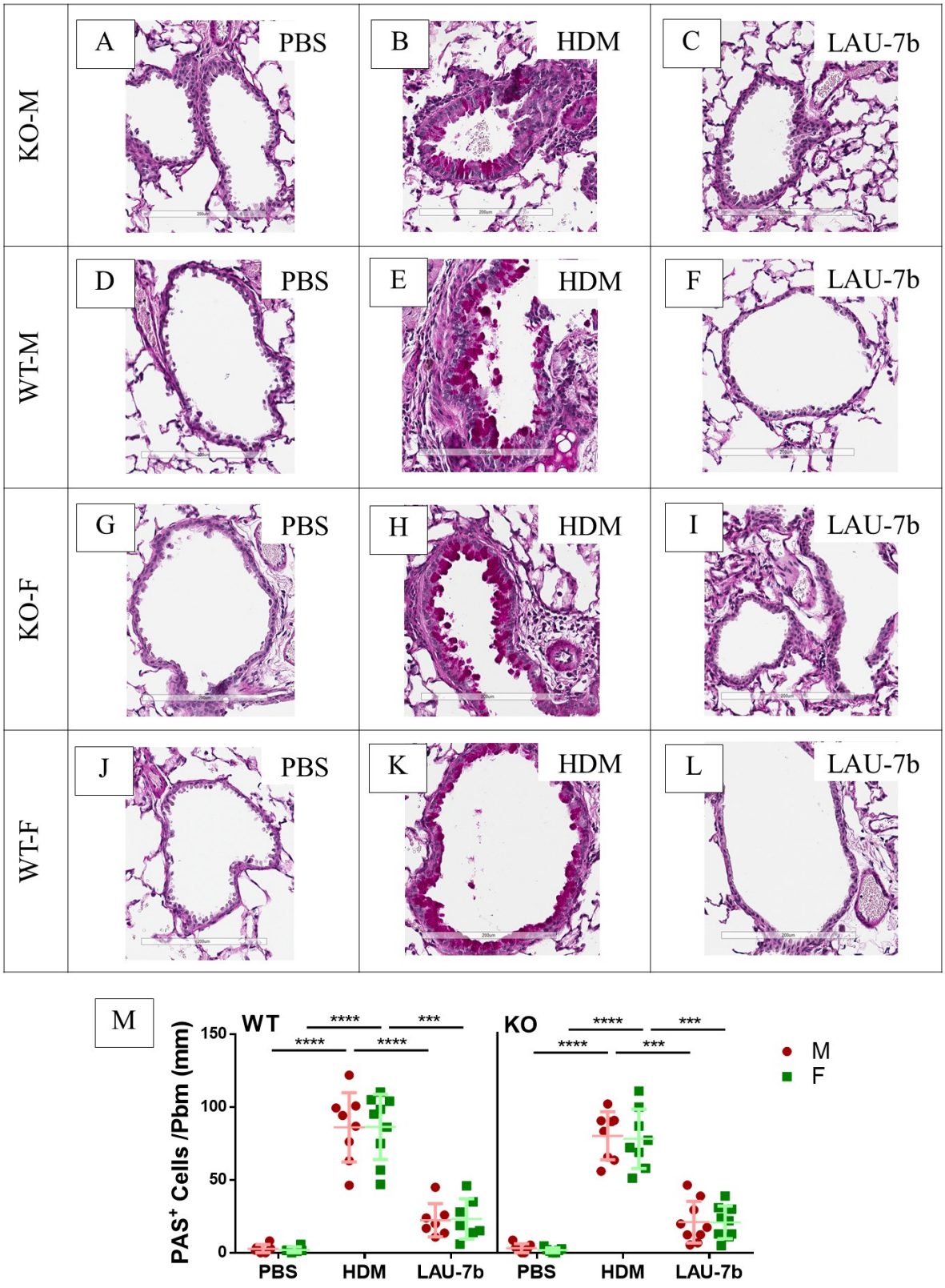

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Figure 5