VIRUSES AND POLLUTANTS

Pathways to ferroptosis in the airway epithelium

Ana Laura Manzano Covarrubias



PROPOSITIONS

Accompanying the PhD dissertation

Viruses and pollutants: Pathways to ferroptosis in the airway epithelium

by

Ana Laura Manzano Covarrubias

1. The detrimental effects of air pollution and respiratory viruses are amplified by shared mechanisms, including oxidative stress, barrier disruption and disturbed cell death pathways. *(This thesis)*

2. Ferroptosis represents a relevant mechanism of epithelial damage during viral respiratory infections. *(This thesis)*

3. Influenza A virus and human adenovirus type C5 induce ferroptosis-related features in lung epithelial cells in a virus- and cell type-specific manner. *(This thesis)*

4. Ferroptosis may contribute to chronic disease progression more broadly than currently appreciated. *(This thesis)*

5. What we breathe over time determines how we breathe. *(This thesis)*

6. "You have brains in your head. You have feet in your shoes. You can steer yourself any direction you choose." – *Dr. Seuss*

7. "There will come soft rains." – *Sara Teasdale*

Viruses and pollutants: Pathways to ferroptosis in the airway epithelium

Ana Laura Manzano Covarrubias

The research presented in this thesis was conducted at the Department of Molecular Pharmacology, Faculty of Science and Engineering, University of Groningen, The Netherlands, and within the framework of the Groningen Research Institute of Pharmacy (GRIP) and the Groningen Research Institute for Asthma and COPD (GRIAC).

This research was financially supported by the Secretaría de Ciencia, Humanidades, Tecnología e Innovación (SECIHTI, formerly CONACYT) and the University of Groningen.

Printing of this thesis was financially supported by the Graduate School of Science and Engineering, Faculty of Science and Engineering, University of Groningen, The Netherlands.







Cover design and chapter cover illustrations: Arturo Hernández
Layout by Ana Laura Manzano Covarrubias
Printed by ProefschriftMaken

Copyright ©2026, Ana Laura Manzano Covarrubias

All rights reserved. No part of this publication may be reproduced, distributed, stored in a retrieval system or transmitted in any form or by any means, whether electronic, mechanical, photocopying, recording or otherwise, without the prior written permission of the author.

university of groningen

Viruses and pollutants: Pathways to ferroptosis in the airway epithelium

PhD thesis

to obtain the degree of PhD of the
University of Groningen
on the authority of the
Rector Magnificus Prof. J.M.A. Scherpen
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Tuesday 15 December 2026 at 16:15 hours

by

Ana Laura Manzano Covarrubias

born on 26 August 1992
Puebla, México

Supervisor(s)
Prof. M. Schmidt
Prof. A.M. Dolga

Co-supervisor(s)
Dr K. Rafie

Assessment Committee
Prof. J.M. van Dijl
Prof. K. Haslinger
Prof. A. Ehrhardt

TABLE OF CONTENTS

Chapter 1	General introduction	**7**
Chapter 2	Unravelling the signaling power of pollutants	**23**
Chapter 3	Therapeutic potential of targeting novel signaling pathways in regulating chronic inflammation in obstructive lung disorders	**61**
Chapter 4	Respiratory viruses induce ferroptosis-like features in lung epithelial cells	**109**
Chapter 5	General discussion	**153**
Appendices		
	Summary	**166**
	Nederlandse samenvatting	**169**
	Resumen en español	**172**
	Acknowledgements/Agradecimientos	**175**
	Curriculum vitae	**182**
	List of publications	**183**



Chapter 1

General introduction

CHRONIC RESPIRATORY DISEASES

Chronic respiratory diseases (CRDs), a group of diseases that affect the airways and lungs, are the sixth leading cause of death in the European Union. Chronic obstructive pulmonary disease (COPD) and asthma are the two most prevalent CRDs globally, with 80% of all CRD deaths caused by COPD [1]. Studies estimate that COPD cases will increase by more than 20% worldwide in the next 25 years [2]. Similarly, asthma cases are expected to rise over the next 25 years, greatly impacting the quality of life and causing debilitating conditions from early childhood [1, 3].

COPD is characterized by progressive and irreversible loss of alveolar structures due to emphysema, mucus hypersecretion, persistent bronchitis, and a predominantly neutrophilic inflammation profile [4]. Asthma is characterized by reversible airway constriction and hyperresponsiveness with a predominant type 2 helper T-cell inflammation profile [4]. Although COPD and asthma differ in their underlying molecular mechanisms, they often overlap and share common triggers and causes of acute exacerbations, which are defined as periods of worsening symptoms that require modifications to a patient's usual medication.

Two of the most common causes of exacerbation are air pollutants and respiratory infections [5]. Air pollution, a complex mixture of particulate matter (PM), gases, and organic compounds, is recognized as an important risk factor for COPD and asthma [6]. PM, the most abundant component of air pollution, negatively affects the epithelial barrier integrity, causes inflammation, increases oxidative stress [7, 8], and makes the respiratory tract more susceptible to other trigger, such as viruses and bacteria [9]. Exacerbations due to respiratory infections are most common in patients in more advanced stages of disease. Common viruses, including influenza, rhinoviruses, respiratory syncytial virus (RSV), and adenoviruses, as well as emerging viruses accelerate lung function decline and increase disease progression in both COPD and asthma [10-13].

Viral infection of the respiratory tract initiates recognition of pathogen-associated molecular patterns (PAMPs) in infected epithelial cells. This activates pattern recognition receptors (PRRs) and downstream pathways such as Toll-like receptors

(TLRs), cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) and retinoic acid-inducible gene I (RIG-I)/mitochondrial antiviral-signaling protein (MAVS). Consequently, infected cells undergo apoptosis and necroptosis to limit viral spread and release pro-inflammatory cytokines. In healthy lungs, these initiate the recruitment of immune cells to aid in the response and resolution of the infection and the activation of airway epithelium progenitor cells involved in tissue repair [14, 15]. In COPD and asthma patients, where there is already an underlying chronic inflammatory profile, the viral-induced cytokine release causes an uncontrolled hyperinflammatory state and unresolved tissue damage [16]. Combined with the cytopathic effect caused by the virus, this further compromises epithelial barrier integrity and facilitates viral spread to the lower respiratory tract and alveolar structures [17]. Consequently, this causes further release of cytokines, interferons (IFNs) and chemoattractants during the exacerbation that often leads to incomplete recovery and accelerate lung function decline (**Figure 1A**) [12, 18].

The respiratory epithelium is therefore an important first line of defense against pathogens and environmental pollutants. In addition to its function as a physical barrier limiting viral and foreign particle entrance to submucosal tissue, the airway epithelium regulates immune responses and participates in tissue repair following injury [19]. Understanding how respiratory viruses and environmental pollutants alter epithelial cell function and induce cell death pathways is therefore important for understanding the detrimental effects of viral- and pollutant-induced exacerbations [12].

REGULATED CELL DEATH IN VIRAL INFECTIONS

Regulated cell death (RCD) is a tightly controlled process essential for maintaining homeostasis and immune regulation. In viral infections, RCD is a mechanism used as an immune response to eliminate potential hubs of viral replication and limit viral progeny spread. Various types of RCD are well known to be triggered during infection, including apoptosis, necroptosis and pyroptosis [20], but less-known RCD types, including ferroptosis, have also been reported as an active RCD mechanism elicited during viral infections (**Figure 1B**) [21].

Apoptosis is the most studied type of RCD. Morphologically it is characterized by chromatin condensation, DNA fragmentation, cell shrinkage, and the formation of apoptotic bodies. It can be initiated by intrinsic and extrinsic pathways, which converge in the permeabilization of the mitochondrial outer membrane (MOMP). This causes the release of cytochrome c and downstream activation of caspases 3, 7, 8 and 9 resulting in a non-inflammatory RCD [21]. Necroptosis and pyroptosis are both lytic types of RCD. Necroptosis is characterized by organelle swelling and increased cell volume that results in loss of membrane integrity and release of cellular contents. It is initiated by various mediators, including TLRs, IFNs, tumor necrosis factor receptor-1 (TNFR1) and intracellular RNA and DNA sensors, which activate the receptor-interacting protein kinase-3 (RIPK3) and mixed lineage kinase domain-like (MLKL) [20, 21]. Activated MLKL forms necroptotic pores that culminate in an inflammatory, lytic type of RCD. Pyroptosis similarly causes cell swelling and membrane rupture, but with characteristic participation of inflammasomes that activate caspase 1. In its active form, caspase 1 cleaves gasdermin D to form pores in the cellular membrane causing a highly inflammatory RCD [20, 21].

Ferroptosis is a type of RCD and far less studied in the context of viral infections. Morphologically, ferroptosis is characterized by organelle and cytoplasmic swelling, condensed mitochondria with little or no cristae and increased membrane density, and moderate chromatin condensation [22-24]. Ferroptosis can be initiated in two ways: first, by the increase of intracellular iron in its ferrous form (Fe^{2+}) and second, by the inhibition of the cysteine/glutamate antiporter system (Xc^-) and glutathione (GSH)/ glutathione peroxidase 4 (GPX4) systems [22]. Iron is transported by transferrin (Tf), which binds to transferrin receptor 1 (TfR1) to mediate its internalization. In the cytoplasm, Fe^{2+} is stored as ferric iron (Fe^{3+}) in the iron storage protein, ferritin, or exported back to the extracellular space by ferroportin (Fpn) [25]. Under ferroptotic conditions, increased iron uptake by TfR1, inhibition of export by Fpn, or increased breakdown of ferritin, known as ferritinophagy, favors the accumulation of free iron in the cytoplasm [26, 27]. Failure to maintain iron homeostasis leads to excessive Fe^{2+}, which participates in the Fenton reaction, resulting in the production of reactive oxygen species (ROS)

that react with polyunsaturated fatty acids (PUFAs) to generate lipid peroxide radicals [26, 27]. Simultaneously, the System Xc- controls the export of glutamate and the import of cysteine, which is essential for the production of GSH, the main antioxidant of the cell. GSH is then needed by GPX4 to reduce lipid peroxide radicals and maintain lipotoxicity under control [25]. Incorporation of oxidized PUFAs into phospholipids in cellular membranes results in RCD caused by defective lipid peroxide repair [28]. Therefore, inhibition of System Xc- or GPX4 contributes to cell death by ferroptosis [29] (**Figure 1C**).

Clearly, not all types of RCD are the same. In the context of viral infections, investigating the different virus-induced cell death pathways may help to further understand underlying mechanisms leading to loss of epithelial barrier integrity and may reveal how they contribute to disease pathogenesis. In the next sections, I further review what is known about ferroptosis and its regulation in the context of virus infections.

FERROPTOSIS IN RESPIRATORY DISEASE

Ferroptosis is closely intertwined with inflammatory pathways and disease development, including neurodegenerative, cardiovascular, and immune diseases [30]. The oxidative stress caused by iron-dependent ROS, lipid peroxides and disruption of the redox homeostasis contribute to chronic inflammation and tissue damage. Growing research suggests that it may play an important role in the pathophysiology of COPD and asthma [31]. Exposure of human bronchial epithelial cells (HBECs) and bronchial epithelial cell line (BEAS-2B) to cigarette smoke (CS) led to iron increase via ferritinophagy. These findings were confirmed in mice exposed to CS. GPX4-deficient mice had higher levels of lipid peroxidation and proinflammatory cytokine release than wild-type mice, and were completely attenuated in GPX4 transgenic mice [32]. Similarly, RSV increases the expression of 12/15 lipoxygenases (LOX) which cause an increase in lipid peroxidation and drives the release of pro-inflammatory CCL3 and CCL5 chemokines for the recruitment of monocytes and neutrophils [33].

Viruses are known to manipulate iron homeostasis during infection [34]. The US2 protein of human cytomegalovirus and the Nef protein of human immunodeficiency virus-1 (HIV-1) reduce TfR1 expression and increased ferritin expression [35, 36]. Similarly, influenza virus disrupts the iron metabolism by altering the expression of Tf and TfR1 and uses its hemagglutinin protein to facilitate the formation of ferritin and nuclear receptor coactivator 4 (NCOA4) condensates for its subsequent degradation by ferritinophagy to benefit viral replication [37].

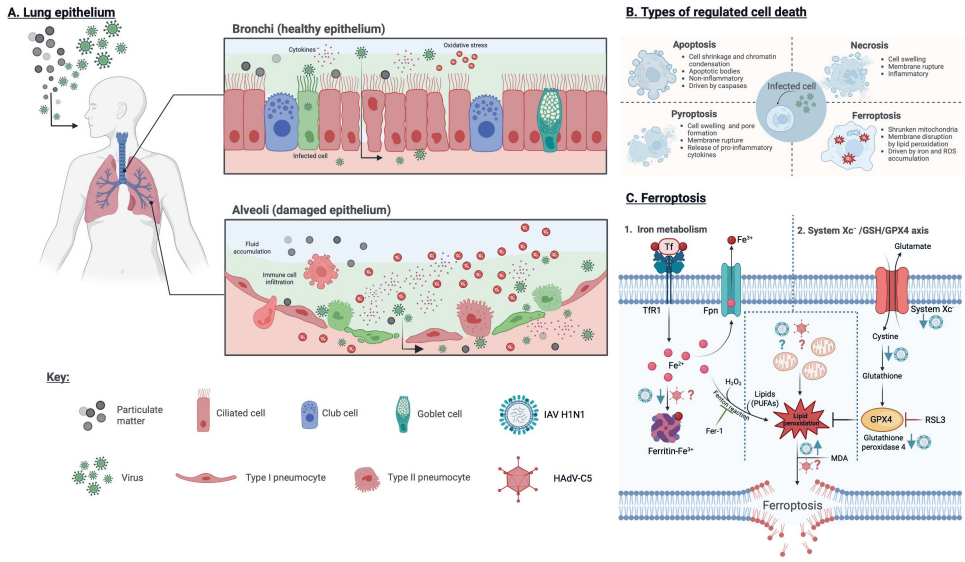


Figure 1. Schematic overview of particulate matter (PM) and viruses as stressors and inducers of cell damage. (A) PM and viruses enter the body via inhalation and reach the upper respiratory tract, where they induce epithelial damage. In healthy lungs, the epithelium barrier limits viral spread and facilitates PM clearance. Cytokine production and oxidative stress are tightly regulated and resolved following infection, thereby minimizing epithelial damage. In contrast, in lungs with underlying chronic disease, the compromised epithelium is more susceptible to additional stressors. PM can easily translocate to into the bloodstream, viral spread is amplified, and excessive cytokine production and oxidative stress further impair barrier integrity. **(B)** Types and main characteristics of regulated cell death induced by viruses. **(C)** Molecular mechanisms of ferroptosis. This type of cell death can be triggered by disruption in (1) iron homeostasis, resulting in increased intracellular iron (Fe^{2+}), or (2) in the System Xc-/glutathione (GSH)/glutathione peroxidase 4 (GPX4) axis, leading to GSH depletion. These events promote the accumulation of reactive oxygen species and lipid peroxidation, ultimately resulting in membrane damage. Influenza A virus (IAV) H1N1 induces ferroptosis by downregulating ferritin, System Xc^-, GSH, GPX4 and increasing lipid peroxidation (blue arrows). Human adenovirus type C5 (HAdV-C5) alterations to ferroptosis pathways remain unknown (red questions marks). Abbreviations: Fer-1, ferrostatin-1; Fpn, ferroportin; MDA, malondialdehyde; RSL3, RAS-selective lethal 3; Tf, transferrin; TfR1, transferrin receptor 1. Figure 1 was created using BioRender (www.biorender.com).

Beyond disruption of iron homeostasis, viruses induce ferroptosis via other pathways [38-40]. Severe acute respiratory syndrome coronavirus (SARS-CoV-2) and HIV-1 downregulate GPX4 expression to enhance cell damage [41-43]. Influenza virus downregulates the expression of the two System Xc- subunits SLC3A2 and SLC7A11, and GPX4, depletes GSH and increases malondialdehyde (MDA), a coproduct of lipid peroxidation [44] (**Figure 1C**). Similarly, monkeypox virus and herpes simplex virus 1 (HSV-1) increase MDA, deplete GSH and cause mitochondria morphological changes consistent with those observed in ferroptotic conditions [45, 46].

Similar findings have been reported in particulate matter (PM)-induced COPD models [47] and in house dust mite (HDM)- and ovalbumin-induced asthma models [48-50]. As the lung epithelium is particularly exposed to viruses, CS, HDM and PM [51], ferroptosis induced by these factors contribute to ROS production and chronic inflammation, which consequently, might lead to airway remodeling, mucus hypersecretion, and destruction of the epithelial barrier [31, 52].

INFLUENZA A VIRUS AND HUMAN ADENOVIRUSES TYPE C

Influenza is a common, contagious, acute viral respiratory infection caused by influenza viruses. There are four types (A-D), of which Type A viruses are the most clinically relevant [53]. In addition to causing four major pandemics, influenza A virus (IAV) is the cause of seasonal influenza, often leading to annual epidemics that represent a significant burden to global healthcare [54]. Typical symptoms include fever, chills, headache, muscle and joint pain, dry cough, sore throat and generalized weakness. In children, additional symptoms such as nausea, vomiting and stomach pain are also common. Usually, symptoms resolve on their own after three to eight days but in some cases, they can persist for a longer period of time. Older adults, as well as patients with chronic diseases, are at increased risk of complications such as bronchiolitis, pneumonia and acute respiratory distress syndrome [55-57]. In COPD and asthma, IAV infection is a common cause of exacerbation often leading to hospitalization and increased risk of secondary infections [16, 53, 58].

IAVs are enveloped viruses that contain eight negative-sense, single-stranded RNA segments that are each attached to nucleoproteins (NP) for their stabilization [59]. In the envelope, hemagglutinin (HA), the most abundant viral protein, binds to sialic acid residues linked to glycoproteins present on the surface of upper and lower airway epithelial cells and alveolar type II cells [53, 60]. After binding, the virus enters the cells by endocytosis and inside the endosome, HA enables the release of the viral genome into the cytoplasm and its subsequent transport into the nucleus for replication [61].

Human adenoviruses (HAdVs) are a large family of more than 110 types of non-enveloped, double-stranded DNA viruses classified into seven different species (A-G) [62]. Due to their large diversity, HAdVs cause a wide range of diseases, including respiratory illness, conjunctivitis, enteritis and urinary tract infection. Since their first identification in 1955 during an outbreak of acute respiratory distress in a military training facility in the Netherlands, HAdVs have been the cause of outbreaks in closed populations, such as college dormitories, hospitals, childcare facilities, assisted-living facilities and military camps [63, 64]. In addition to outbreaks, it is estimated that up to 10% of pediatric respiratory infections and up to 4% of adult respiratory infections are caused by HAdVs [63], but this might be an underestimation due to poor surveillance [65, 66]. HAdV respiratory tract infections are usually mild with symptoms that include fever, sore throat, swollen tonsils and cough that usually resolve on their own within 14 days [67]. In children and immunocompromised patients, severe complications such as gastroenteritis, diarrhea, febrile seizures, encephalitis, encephalomyelitis, aseptic meningitis and pneumonia can also occur [67, 68]. Of special interest are species C HAdVs (HAdV-C5), which have been shown to cause persistent infections that have been associated with the development and/or exacerbation of COPD [69, 70].

The capsid of HAdVs is formed by three major structural proteins (hexon, penton and fiber) and four minor structural proteins (IIa, VI, VIII and IX) [64, 71]. HAdV-C5 uses its fiber protein to attach to the coxsackievirus and adenovirus receptor (CAR), a transmembrane protein of tight junctions located on the basolateral side of epithelial cells [72]. CAR is the primary receptor for HAdV-C5, but CAR-independent entry pathways have been identified including hexon binding to

lactoferrin [73, 74]. Following attachment, the penton protein binds integrins, which act as a co-receptor to trigger viral entry [75]. HAdV-C5 is subsequently internalized by endocytosis, and its genome is translocated into the nucleus. The early region 1A (E1A) gene is the first to be expressed and allows the virus to regulate the cell cycle and induce transcription for its benefit. Interestingly, E1A expression has been detected in airway and alveolar epithelial cells after clinical manifestations of infection have resolved [76]. Persistent expression of E1A causes chronic lung inflammation and has been associated to contribute to the development of COPD and to worsening exacerbations [76-78].

Although ferroptosis is a relatively new field of research in infectious diseases, IAV is among one of the most studied viruses in this context. As outlined above, growing evidence indicates that IAV alters various ferroptosis related proteins including Tf, TfR1, GPX4, NCOA4, ferritin, SLC3A2 and SLC7A11 and increases lipid peroxidation to benefit its replication [37, 44]. However, little is known regarding the underlying mechanisms of HAdV infection and disease in the context of ferroptosis (**Figure 1C**).

SCOPE OF THIS THESIS

The work in this thesis aims to explore mechanisms of pollutant and viral-induced epithelial damage and their contribution to chronic inflammation and tissue damage. In **Chapter 1**, main aspects of the current thesis were introduced. I reviewed IAV H1N1, HAdV-C5, and pollutants as three common causes of exacerbation of COPD and asthma and laid the foundations for the research presented in this thesis.

In **Chapter 2**, I provide an overview of environmental pollutants as a cause of cellular damage. I explore how pollutants, specifically particulate matter, cause oxidative stress and mitochondrial dysfunction that lead to persistent inflammation and protein instability. Importantly, I focus on how these processes disrupt the respiratory epithelium and the crosstalk between pollutant sensing and chronic respiratory diseases.

In **Chapter 3**, novel signaling pathways of chronic inflammation in obstructive lung disorders are reviewed. I highlight the importance of infections as causes of exacerbation and how they contribute to inflammatory responses and accelerate lung damage. Lastly, I evaluate current therapeutics and discuss potential new therapeutic targets for better management of chronic inflammation in obstructive lung disorders.

In **Chapter 4**, I focus on two common respiratory viruses, influenza A virus (IAV), human adenovirus type C5 (HAdV-C5) and ferroptosis as a cell death mechanism during infection. I perform a comparative study investigating ferroptosis features of these two viruses, in bronchial and alveolar epithelial cells. Using IAV as a reference virus, I find that both viruses differentially induce ferroptosis features in a cell-type specific manner. I report alterations in ferritin, increased lipid peroxidation, and marked damage to the mitochondrial network.

Finally, in **Chapter 5,** I summarize the main findings presented in this thesis and discuss implications of my research. The work in this thesis lays the foundation for advancing the understanding of ferroptosis as a novel mechanism involved in viral and pollutant-induced cell damage. Lastly, I conclude with future perspectives and research directions.

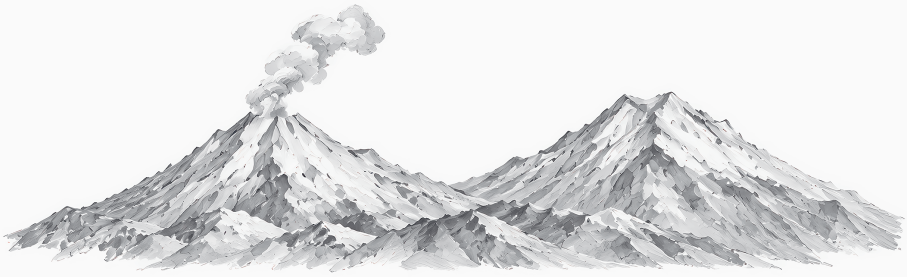
REFERENCES

1. Organization, W.H. (2025) Chronic respiratory diseases in the WHO European Region. https://www.who.int/europe/publications/i/item/WHO-EURO-2025-12340-52112-79990
2. Boers, E., *et al.* (2023) Global Burden of Chronic Obstructive Pulmonary Disease Through 2050. *JAMA Netw Open* 6, e2346598
3. Yuan, L., *et al.* (2025) Global, regional, national burden of asthma from 1990 to 2021, with projections of incidence to 2050: a systematic analysis of the global burden of disease study 2021. *EClinicalMedicine* 80, 103051
4. Postma, D.S. and Rabe, K.F. (2015) The Asthma-COPD Overlap Syndrome. *N Engl J Med* 373, 1241-1249
5. Awad, M.T. and Sankari, A. (2026) Asthma and COPD Overlap. In *StatPearls*
6. Lim, S.S., *et al.* (2012) A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 380, 2224-2260
7. Kayalar, O., *et al.* (2024) Impact of particulate air pollution on airway injury and epithelial plasticity; underlying mechanisms. *Front Immunol* 15, 1324552
8. Lee, Y.G., *et al.* (2021) Effects of Air Pollutants on Airway Diseases. *Int J Environ Res Public Health* 18
9. Arias-Perez, R.D., *et al.* (2020) Inflammatory effects of particulate matter air pollution. *Environ Sci Pollut Res Int* 27, 42390-42404
10. Dransfield, M.T., *et al.* (2017) Acute Exacerbations and Lung Function Loss in Smokers with and without Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* 195, 324-330
11. Soremekun, S., *et al.* (2023) Asthma exacerbations are associated with a decline in lung function: a longitudinal population-based study. *Thorax* 78, 643-652
12. van der Koog, L., *et al.* (2026) Regenerative therapeutics for chronic obstructive pulmonary disease. *Pharmacol Rev* 78, 100124
13. Mohan, A., *et al.* (2010) Prevalence of viral infection detected by PCR and RT-PCR in patients with acute exacerbation of COPD: a systematic review. *Respirology* 15, 536-542
14. Kumar, P.A., *et al.* (2011) Distal airway stem cells yield alveoli in vitro and during lung regeneration following H1N1 influenza infection. *Cell* 147, 525-538
15. Ray, S., *et al.* (2016) Rare SOX2(+) Airway Progenitor Cells Generate KRT5(+) Cells that Repopulate Damaged Alveolar Parenchyma following Influenza Virus Infection. *Stem Cell Reports* 7, 817-825
16. Seemungal, T., *et al.* (2001) Respiratory viruses, symptoms, and inflammatory markers in acute exacerbations and stable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 164, 1618-1623
17. Nie, J., *et al.* (2025) Deep insight into cytokine storm: from pathogenesis to treatment. *Signal Transduct Target Ther* 10, 112
18. Linden, D., *et al.* (2019) Respiratory viral infection: a potential "missing link" in the pathogenesis of COPD. *Eur Respir Rev* 28
19. Raby, K.L., *et al.* (2023) Mechanisms of airway epithelial injury and abnormal repair in asthma and COPD. *Front Immunol* 14, 1201658

20. Jorgensen, I., *et al.* (2017) Programmed cell death as a defence against infection. *Nat Rev Immunol* 17, 151-164
21. Rex, D.A.B., *et al.* (2022) Revisiting Regulated Cell Death Responses in Viral Infections. *Int J Mol Sci* 23
22. Dixon, S.J., *et al.* (2012) Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* 149, 1060-1072
23. Tang, D., *et al.* (2021) Ferroptosis: molecular mechanisms and health implications. *Cell Res* 31, 107-125
24. Yagoda, N., *et al.* (2007) RAS-RAF-MEK-dependent oxidative cell death involving voltage-dependent anion channels. *Nature* 447, 864-868
25. Gao, M., *et al.* (2015) Glutaminolysis and Transferrin Regulate Ferroptosis. *Mol Cell* 59, 298-308
26. Gao, M., *et al.* (2016) Ferroptosis is an autophagic cell death process. *Cell Res* 26, 1021-1032
27. Hou, W., *et al.* (2016) Autophagy promotes ferroptosis by degradation of ferritin. *Autophagy* 12, 1425-1428
28. Dixon, S.J. and Stockwell, B.R. (2019) The Hallmarks of Ferroptosis. *Annu Rev Cancer Biol* 3, 35-54
29. Yang, W.S., *et al.* (2014) Regulation of ferroptotic cancer cell death by GPX4. *Cell* 156, 317-331
30. Chen, X., *et al.* (2021) Ferroptosis in infection, inflammation, and immunity. *J Exp Med* 218
31. Khawas, S. and Sharma, N. (2025) Cell death crosstalk in respiratory diseases: unveiling the relationship between pyroptosis and ferroptosis in asthma and COPD. *Mol Cell Biochem* 480, 1305-1326
32. Yoshida, M., *et al.* (2019) Involvement of cigarette smoke-induced epithelial cell ferroptosis in COPD pathogenesis. *Nat Commun* 10, 3145
33. Salimi, V., *et al.* (2017) Evaluation of the expression level of 12/15 lipoxygenase and the related inflammatory factors (CCL5, CCL3) in respiratory syncytial virus infection in mice model. *Microb Pathog* 109, 209-213
34. Drakesmith, H. and Prentice, A. (2008) Viral infection and iron metabolism. *Nat Rev Microbiol* 6, 541-552
35. Drakesmith, H., *et al.* (2005) HIV-1 Nef down-regulates the hemochromatosis protein HFE, manipulating cellular iron homeostasis. *Proc Natl Acad Sci U S A* 102, 11017-11022
36. Vahdati-Ben Arieh, S., *et al.* (2003) A single viral protein HCMV US2 affects antigen presentation and intracellular iron homeostasis by degradation of classical HLA class I and HFE molecules. *Blood* 101, 2858-2864
37. Ouyang, A., *et al.* (2024) The Hemagglutinin of Influenza A Virus Induces Ferroptosis to Facilitate Viral Replication. *Adv Sci (Weinh)* 11, e2404365
38. Gao, J., *et al.* (2023) When ferroptosis meets pathogenic infections. *Trends Microbiol* 31, 468-479
39. Hong, L., *et al.* (2025) The relationship between ferroptosis and respiratory infectious diseases: a novel landscape for therapeutic approach. *Front Immunol* 16, 1550968

40. Wang, M.P., *et al.* (2022) Ferroptosis in viral infection: the unexplored possibility. *Acta Pharmacol Sin* 43, 1905-1915
41. Cao, D., *et al.* (2020) A Matter of Life or Death: Productively Infected and Bystander CD4 T Cells in Early HIV Infection. *Front Immunol* 11, 626431
42. Han, Y., *et al.* (2022) SARS-CoV-2 Infection Induces Ferroptosis of Sinoatrial Node Pacemaker Cells. *Circ Res* 130, 963-977
43. Wang, Y., *et al.* (2021) SARS-CoV-2 suppresses mRNA expression of selenoproteins associated with ferroptosis, endoplasmic reticulum stress and DNA synthesis. *Food Chem Toxicol* 153, 112286
44. Cheng, J., *et al.* (2022) Swine influenza virus triggers ferroptosis in A549 cells to enhance virus replication. *Virol J* 19, 104
45. Chuai, X., *et al.* (2025) Monkeypox virus induces ferroptosis to facilitate viral replication and promotes inflammatory responses. *Emerg Microbes Infect* 14, 2522877
46. Xu, X.Q., *et al.* (2023) Herpes Simplex Virus 1-Induced Ferroptosis Contributes to Viral Encephalitis. *mBio* 14, e0237022
47. Wang, Y., *et al.* (2022) Hydrogen sulfide alleviates particulate matter-induced emphysema and airway inflammation by suppressing ferroptosis. *Free Radic Biol Med* 186, 1-16
48. Tang, W., *et al.* (2021) Environmental allergens house dust mite-induced asthma is associated with ferroptosis in the lungs. *Exp Ther Med* 22, 1483
49. Yang, N. and Shang, Y. (2022) Ferrostatin-1 and 3-Methyladenine Ameliorate Ferroptosis in OVA-Induced Asthma Model and in IL-13-Challenged BEAS-2B Cells. *Oxid Med Cell Longev* 2022, 9657933
50. Zeng, Z., *et al.* (2022) HDM induce airway epithelial cell ferroptosis and promote inflammation by activating ferritinophagy in asthma. *FASEB J* 36, e22359
51. Wang, Y. and Tang, M. (2019) PM2.5 induces ferroptosis in human endothelial cells through iron overload and redox imbalance. *Environ Pollut* 254, 112937
52. Li, M., *et al.* (2023) Ferroptosis triggers airway inflammation in asthma. *Ther Adv Respir Dis* 17, 17534666231208628
53. Herold, S., *et al.* (2015) Influenza virus-induced lung injury: pathogenesis and implications for treatment. *Eur Respir J* 45, 1463-1478
54. Saunders-Hastings, P.R. and Krewski, D. (2016) Reviewing the History of Pandemic Influenza: Understanding Patterns of Emergence and Transmission. *Pathogens* 5
55. Javanian, M., *et al.* (2021) A brief review of influenza virus infection. *J Med Virol* 93, 4638-4646
56. Kyokha Ameen, Y. (2025) Seasonal Influenza: A Narrative Review of Epidemiology, Clinical Features, and Preventive Strategies. *Cureus* 17, e95336
57. Paules, C. and Subbarao, K. (2017) Influenza. *Lancet* 390, 697-708
58. Jain, S., *et al.* (2009) Hospitalized patients with 2009 H1N1 influenza in the United States, April-June 2009. *N Engl J Med* 361, 1935-1944
59. Dou, D., *et al.* (2018) Influenza A Virus Cell Entry, Replication, Virion Assembly and Movement. *Front Immunol* 9, 1581

60. Benam, K.H., *et al.* (2019) How the Respiratory Epithelium Senses and Reacts to Influenza Virus. *Am J Respir Cell Mol Biol* 60, 259-268
61. Samji, T. (2009) Influenza A: understanding the viral life cycle. *Yale J Biol Med* 82, 153-159
62. Zhang, Y. and Bergelson, J.M. (2005) Adenovirus receptors. *J Virol* 79, 12125-12131
63. Kajon, A.E. (2024) Adenovirus infections: new insights for the clinical laboratory. *J Clin Microbiol* 62, e0083622
64. MacNeil, K.M., *et al.* (2023) Adenoviruses in medicine: innocuous pathogen, predator, or partner. *Trends Mol Med* 29, 4-19
65. Montgomery, J.P., *et al.* (2024) Detection of a Human Adenovirus Outbreak, Including Some Critical Infections, Using Multipathogen Testing at a Large University, September 2022-January 2023. *Open Forum Infect Dis* 11, ofae192
66. Wang, C., *et al.* (2024) An Outbreak of Human Adenovirus Infection Among Children Post COVID-19 Pandemic in Southern China. *J Med Virol* 96, e70139
67. Lynch, J.P., 3rd and Kajon, A.E. (2021) Adenovirus: Epidemiology, Global Spread of Novel Types, and Approach to Treatment. *Semin Respir Crit Care Med* 42, 800-821
68. Schwartz, K.L., *et al.* (2019) Adenovirus-Associated Central Nervous System Disease in Children. *J Pediatr* 205, 130-137
69. Garnett, C.T., *et al.* (2009) Latent species C adenoviruses in human tonsil tissues. *J Virol* 83, 2417-2428
70. Hayashi, S. (2002) Latent adenovirus infection in COPD. *Chest* 121, 183S-187S
71. Greber, U.F. and Flatt, J.W. (2019) Adenovirus Entry: From Infection to Immunity. *Annu Rev Virol* 6, 177-197
72. Bergelson, J.M., *et al.* (1997) Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science* 275, 1320-1323
73. Johansson, C., *et al.* (2007) Adenoviruses use lactoferrin as a bridge for CAR-independent binding to and infection of epithelial cells. *J Virol* 81, 954-963
74. Persson, B.D., *et al.* (2020) Lactoferrin-Hexon Interactions Mediate CAR-Independent Adenovirus Infection of Human Respiratory Cells. *J Virol* 94
75. Stasiak, A.C. and Stehle, T. (2020) Human adenovirus binding to host cell receptors: a structural view. *Med Microbiol Immunol* 209, 325-333
76. Vitalis, T.Z., *et al.* (1996) A model of latent adenovirus 5 infection in the guinea pig (Cavia porcellus). *Am J Respir Cell Mol Biol* 14, 225-231
77. Elliott, W.M., *et al.* (1995) Immunodetection of adenoviral E1A proteins in human lung tissue. *Am J Respir Cell Mol Biol* 12, 642-648
78. Matsuse, T., *et al.* (1992) Latent adenoviral infection in the pathogenesis of chronic airways obstruction. *Am Rev Respir Dis* 146, 177-184



Chapter 2

Unravelling the signaling power of pollutants

Ana L. Manzano-Covarrubias[1,2#], Hong Yan[1#], Minh D.A. Luu[1], Phoeja S. Gadjdjoe[1], Amalia M. Dolga[1,2], Martina Schmidt[1,2*]

[1]Department of Molecular Pharmacology, University of Groningen, The Netherlands.

[2]Groningen Research Institute for Asthma and COPD, GRIAC, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

# These authors contributed equally to this work.

*Correspondence: m.schmidt@rug.nl (M. Schmidt)

Antonius Deusinglaan 1

9713 AV Groningen, The Netherlands

orcid.org/0000-0003-3075-0630

Published in *Trends in Pharmacological Sciences* 2023 Vol. 44 Issue 12 Pages 917-933
https://doi.org/10.1016/j.tips.2023.09.002

ABSTRACT

Exposure to environmental pollutants contributes to diverse pathologies, including pulmonary disease, lower respiratory infections, cancer, and stroke. Pollutants' entry can occur through inhalation, traversing endothelial and epithelial barriers, and crossing the blood-brain barrier, leading to a wide distribution throughout the human body via systemic circulation. Pollutants cause cellular damage by multiple mechanisms encompassing oxidative stress, mitochondrial dysfunction, (neuro)inflammation, and protein instability/proteotoxicity. Sensing pollutants has added a new dimension to disease progression and drug failure. Understanding the molecular pathways and potential receptor binding/signaling that underpin 'sensing' could contribute to ways to combat the detrimental effects of pollutants. We highlight key points of pollutant signaling, crosstalk with receptors acting as drug targets for chronic diseases, and discuss the potential for future therapeutics.

Keywords: (environmental) pollutants, oxidative stress, mitochondria, inflammation, protein stability, GPCR

ENVIRONMENTAL POLLUTANTS: IMPACT ON CELLULAR HOMEOSTASIS

Pollutants are estimated to cause 9 million or 1 in every 6 deaths annually, placing pollution as one of the main causes of death worldwide [1]. Environmental pollutants are very diverse in nature ranging from particulate matter (PM), gases (e.g., cigarette smoke (CS)), and organic compounds (e.g., polycyclic aromatic hydrocarbons (PAHs)) (Box 1 provides a brief overview of the main environmental pollutants). Environmental pollutants enter the human body, organs, and cells through various routes. Pollutants seem to disrupt cellular homeostasis; however, the underlying mechanisms are yet to be fully understood [2, 3] (Figure 1). Recent studies reported that exposure of human airway epithelial (Calu-3) cells to pollutants inhibited the cystic fibrosis transmembrane conductance regulator (CFTR), an anion channel important for mucociliary clearance (**MCC**) (see Glossary) [4] (Figure 2). Other studies in blood brain barrier (**BBB**) models also reported that pollutants decreased protein stability of tight junctions (TJ; for example, zonula occludens-1) and adherence junctions (E-cadherin) (Figure 3) [5]. These findings indicate that environmental pollutants can mediate decline of lung as well as brain function through elevated exacerbation frequency and increased leakage of the BBB, respectively. Pollutants also increase inflammatory load, potentially acting as disease amplifiers via altering the responses of immune cells such as alveolar macrophages [6] and microglia in the brain [7]. Furthermore, recent evidence indicates that pollutants alter mitochondria homeostasis. They change reactive oxygen species (**ROS**) abundance and have the potential to induce metabolic reprogramming and cell death (e.g., **ferroptosis**) [8-12]. Awareness of the increasing effects of pollutants, originally seen as an inevitable environmental challenge, in human health and disease indicates the urgency to further elucidate the "biology" of pollutants. Studies on pollution-induced alterations of molecular pathways that may contribute to disease progression and drug failure could lead to potential future therapeutic avenues. Here, we focus on the "sensing" of pollutants (with a special focus on PM) and related signaling.

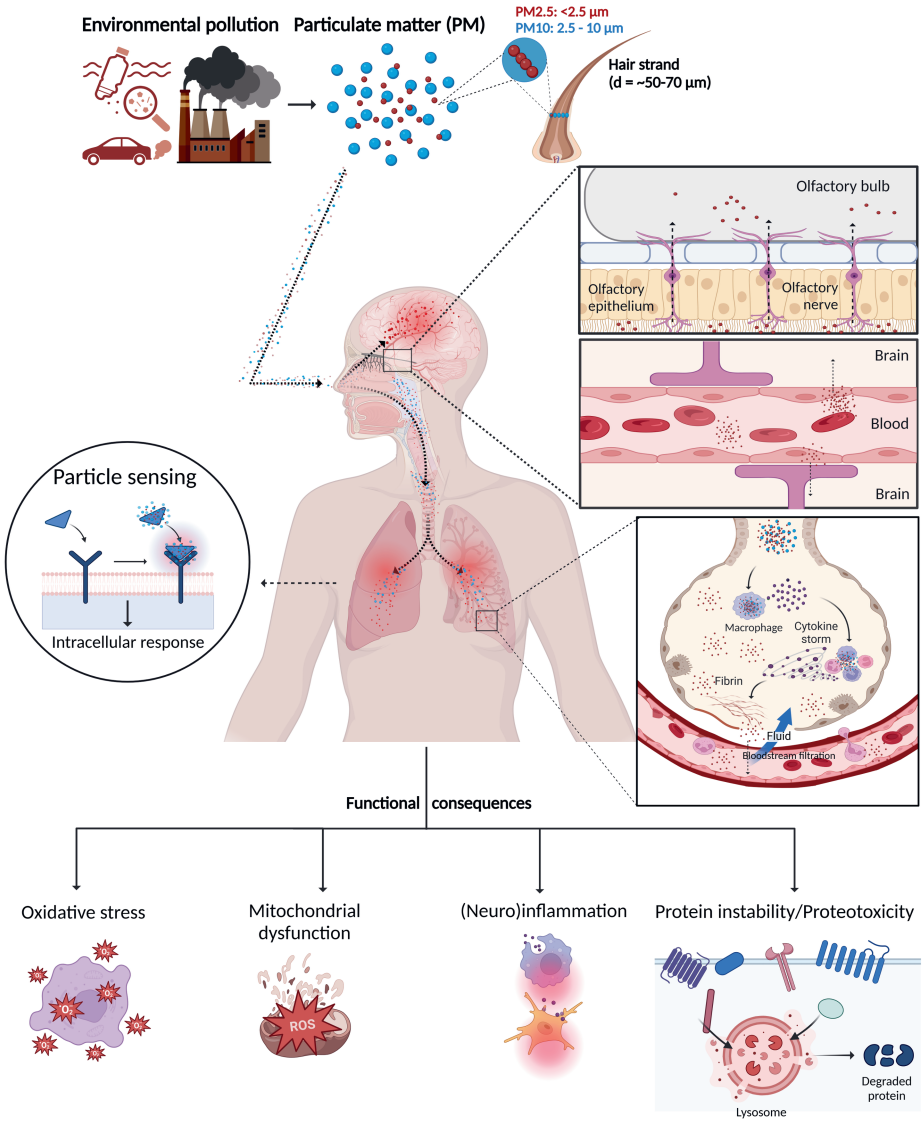


Fig. 1 Environmental pollutant entry and tissue damage mechanisms. Particulate matter (PM) from motor vehicle exhaust, microplastics, and industrial waste make up an important component of environmental pollution. PM is classified according to its diameter. Coarse PM have a diameter of less than 10 µm (PM10) and fine PM (PM2.5) a diameter of less than 2.5 µm. Both can enter the body via inhalation and easily reach the upper respiratory tract. Most PM is cleared as it passes through the airways but some of it can reach the alveoli, damage the lung epithelium and vascular endothelium, and translocate into the bloodstream. Circulating PM2.5 can reach the blood-brain barrier and cross over into the brain. Furthermore, PM can accumulate in the mucosa of the olfactory epithelium and enter olfactory receptor neurons through the cilia, move along the axons, reach the olfactory bulb, and translocate into the brain. In tissues, PM sensing results in cellular damage by increasing reactive oxygen species (ROS) resulting in the induction of oxidative stress, mitochondrial dysfunction, upregulation of pro-inflammatory responses, and protein instability. Figure 1 was created using BioRender (www.biorender.com).

POLLUTANTS AND CELLULAR "SENSING" MECHANISMS

Recent studies indicate that cellular damage caused by PM involves several "classical" receptor signaling pathways (e.g., G protein-coupled receptors (GPCR) and Toll-like receptors (TLRs)) through a plethora of direct and indirect mechanisms [13-15]. It is tempting to speculate that PM bears the potential to positively and/or negatively alter drug action considering several drugs used to treat chronic disease may target such typical signaling pathways [13-15]. Indeed, recent studies indicate that typical signaling mechanisms also targeted by some existing drugs are subject to alterations by PM. For example, PM upregulates extracellular signal-regulated kinase 1 and 2 (ERK1/2), in part acting in concert with cAMP response element-binding protein (CREB) [16, 17] and signal transducer and activator of transcription 1 and 3 (STAT1/3) [18]. In addition, PM seems to downregulate Wnt/β-catenin signaling, which may impact the regulation of embryonic development as well as adult tissue renewal and homeostasis [19]. Interestingly, PM was also reported to upregulate nuclear factor kappa B (NF-kB), a pro-inflammatory signaling molecule, and nuclear factor erythroid 2-related factor (Nrf2) transcription factor, an activator of antioxidant elements, in response to ROS [20], providing a potential link to (neuro)inflammation. Moreover, PM appears to impact cellular calcium and cAMP signaling. Thus, it was shown that PM regulates receptors (e.g., GPCR expression/activity) [21], phosphodiesterases (PDE2A, -3 and -4), and exchange protein directly activated by cAMP (EPAC) [11, 22, 23]. Finally, the potential link between PM exposure, oxidative stress, and alterations in mitochondrial function is becoming increasingly clear (Figures 2 and 3). Several recent studies point to PM-mediated upregulation of lipid peroxidation [8, 16, 24], partially via glutathione peroxidase 4 (GPX4) and ferroptotic cell death mechanisms [10, 16, 25, 26] following ROS and mitochondrial dysfunction induction. Further studies on sensing PM are warranted as additional mechanisms and signalling pathways as well as their potential interconnectivity still need to be defined.

POLLUTANTS AND DISEASE

Chronic obstructive pulmonary disease (**COPD**) and **asthma** represent chronic obstructive inflammatory lung diseases characterized by airway obstruction, chronic inflammation, and airway remodeling; the extent of these features varies based on specific disease (sub)types. Typically, both COPD and asthma patients exhibit signs of airway hyperresponsiveness (**AHR**), whereas **emphysema** is unique to COPD. Exacerbations, episodes of acute worsening of disease symptoms in COPD and asthma, are triggered by allergens, exposure to irritants, viral infections, and environmental pollutants such as CS, nitrogen dioxide (NO_2), PM2.5, ozone (O_3), and carbon monoxide (CO) [27]. Exposure of patients [28, 29] and animals [18, 30] to different PM compositions (details, Table 1) resulted in a decline in lung function (e.g., **FEV1**, **FCV**, **AHR**) and elevated levels of pro-inflammatory cells and mediators, such as eosinophils, interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α), in bronchoalveolar lavage fluid (**BALF**). Interestingly, an *in vitro* model using the mitochondrial-targeted antioxidant MitoQ suggested a central role of mitochondria in mediating PM-induced damage [30].

PM elevates the susceptibility to viral and bacterial infections. This potentially involves TLRs [14], as indicated in patient cohorts [31] and cell-based *in vitro* studies [8, 32]. A549 and human primary epithelial cells exposed to PM showed increased *Streptococcus pneumoniae* bacterial adhesion, a process involving the GPCR for platelet-activating factor, a mediator known to drive pathogen phagocytosis and inflammation [33] (Figure 2). Also, PM has been suggested to act as a pathogen carrier possibly serving as an infection spreading mechanism [32]. Whether PM plays other roles in host-pathogen interactions remains to be investigated.

Similar disease mechanisms driven by PM seem to be operational in Alzheimer's (**AD**) and Parkinson's disease (**PD**) [3, 34-37] (Figures 1 and 3). Studies in patient cohorts [34, 35, 37] and animal models [24, 38, 39], partly confirmed by *in vitro* studies [39-41], revealed that exposure to different PM compositions (details, Table 1) promoted degenerative neurological disease pathology. Observations included aberrant quadruple tau hyperphosphorylation (pTau), increased amyloid-beta (Aβ) plaques, α-synuclein protein (α-Syn) aggregation in nuclei of cerebellar and

olfactory bulb neurons, impaired motor function, increased expression of amyloid precursor protein (APP) and beta secretase 1 (BACE1), and increased oxidation of membrane lipids (e.g., lipid rafts, malondialdehyde (MDA)). Importantly, changes in the expression of several inflammatory (e.g., inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, IL-1β, IL-6) and oxidative stress markers (e.g., heme oxygenase-1 (HO-1), heat shock protein 70 (Hsp70), cytochrome P450 1B1 (Cyp1b1), superoxide dismutase (SOD), MDA) were detected as well. Notably, alterations in mitochondria such as cytochrome c oxidase (COX-IV) and complex I activity, and mitochondrial fusion were linked to neuronal death [41]. These studies clearly point to a close interconnectivity between inflammation, oxidative stress and mitochondrial function, processes sensitive to PM exposure.

POLLUTANTS AND IMMUNE RESPONSES

Exposure to PM activates (alveolar) macrophages, known to act as the first line of defense to clear and protect against pollutants. Their activation leads to PM phagocytosis and pro-inflammatory cytokine release to initiate tissue repair [42, 43] (Box 2 provides a brief overview of tissue repair and extracellular matrix (**ECM**) dynamics). However, PM can cause over-activation of macrophages, a process known to involve TLRs [14, 42], resulting in excessive release of TNF-α, IL-6, monocyte chemoattractant protein-1 (MCP-1), IL-8, and IL-1β [42-44]. Indeed, exposure of macrophage RAW264.7 cells to PM (details, Table 1) increased NF-kB, COX-2 and TLR4 protein expression in a time-dependent manner; these effects could be prevented by the NF-kB inhibitor Bay 11-7821, COX-2 inhibitor Etoricoxib, and TLR4 inhibitor TAK-242 [42]. Furthermore, exposure of RAW264.7 cells and a human acute monocytic leukemia cell line (THP-1) to PM suppressed macrophage phagocytosis [43, 44]. Li *et al.* showed that exposure of THP-1 monocytes to PM reduced macrophage phagocytosis upon interference with autophagosome-lysosome fusion, a process associated with ERK1/2-dependent ROS production [43]. In A549 cells, PM increased levels of the inflammatory cytokines IL-6, IL-8 and TNF-α, which regulated expression of DNA damage marker histone H2AX, senescence markers p16 and p21, senescence-associated β-galactosidase, cyclic GMP-AMP

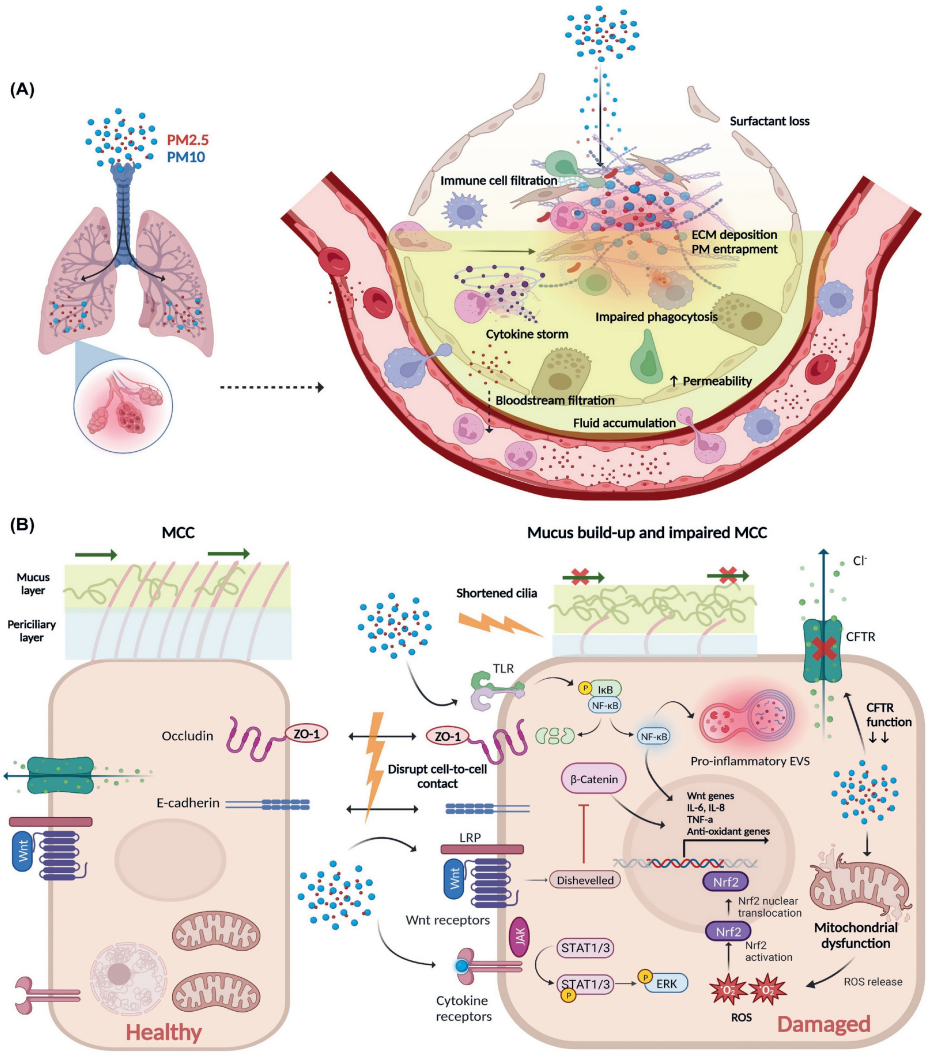


Fig 2. Effects of environmental pollutants in the lung. (A) The lungs act as an entry port for inhaled particulate matter (PM). In the alveoli, PM sensing causes a cytokine storm, excessive mucus production, extracellular matrix (ECM) alterations, immune cell infiltration, loss of surfactant, and impaired phagocytosis and damage to the lung epithelium leading to increased permeability. **(B)** On the cellular level, PM is sensed by yet ill-defined receptors, potentially targets the cystic fibrosis transmembrane conductance regulator (CFTR) and Toll-like receptors (TLR), and leads to the upregulation of pro-inflammatory cytokines such as interleukin (IL)-6, IL-8, and tumor necrosis alpha (TNF-α). These cytokines are packaged into extracellular vesicles (EVs) and transported to the extracellular space further contributing to pro-inflammatory responses. PM also causes an increase in reactive oxygen species (ROS) and activates the antioxidant response involving nuclear factor erythroid 2-related factor (Nrf2) and nuclear factor kappa B (NF-kB). PM lowers the expression of tight and adherens junction proteins such as zonula occludens-1 (ZO-1), E-cadherin, and occludin, leading to higher permeability of the epithelium and endothelial barriers. In ciliated cells of the epithelium, PM causes shortened motile cilia and disrupted ciliary beat frequency, collectively resulting in the accumulation of mucus and impaired mucociliary clearance (MCC). Abbreviations: ERK, extracellular signal-regulated kinase; IkB, inhibitor of NF-kB; JAK, Janus kinase; LRP: Low density lipoprotein *receptor*-related protein; STAT1/3, signal transducer and activator of transcription 1 and 3. Figure 2 was created using BioRender (www.biorender.com).

synthase (cGAS), stimulator of interferon genes (STING), and p-p65; these responses were effectively reduced by the pharmacological inhibitors PF-06928215 (specific to cGAS) and BAY 11-7082 (specific to NF-kB) [45]. These data indicate that PM-induced cellular damage involves inflammation, potentially leading to senescence.

Microglia constitute the main resident innate immune cells in the central nervous system (**CNS**). They serve as neuroprotectors by responding to environmental alterations and are importantly involved in sensing, housekeeping of neuronal well-being, and normal function [46]. The glia-like astrocytes surround the microvessel wall of the BBB with their end-feet and play key roles in immune surveillance and BBB integrity [46] (Figure 3). PM exposure triggers and/or exacerbates pro-inflammatory responses in microglia and increases astrocyte reactivity, thereby potentially amplifying PM-induced neurotoxicity and neurodegeneration [7]. Exposure of primary mouse mixed glia (microglia and astrocytes) to PM increased TNF-α, IL-1β, IL-6, interferon gamma (IFN-γ), and IL-5, which was attenuated by TLR4 silencing. In adult mice, PM led to increased hippocampal expression of TLR4, myeloid differentiation primary response 88 (MyD88)-dependent NF-κB, and TNF-α [47]. Han *et al*. showed that PM exposure of primary mouse microglia mediated a change into an amoeboid morphology with larger, rounder cell bodies and reduced but thickened protrusions [48]. Studies using a human Neuro-Glia-Vascular-on-a-chip (NGV) model of neurodegeneration revealed that PM induced vascular cell-mediated astrocyte activation upon increased expression of glial fibrillary acidic protein (GFAP) and vimentin. In addition, PM exposure stimulated microglial activation and subsequently resulted in neurotoxicity as indicated by elevated H_2O_2/ROS production, accumulation of Aβ and pTau, and increased NF-κB signaling [5]. Other work demonstrated that exposure of co-cultured murine neurons and astrocytes to PM activated astrocytes due to increased levels of IL-1β and IFN-γ, of which the latter is known to recruit and activate microglia [49]. Thus, this considerable body of evidence supports that PM disrupts (alveolar) macrophage, microglia, and astrocyte function. This results in oxidative stress, mitochondrial dysfunction, and protein instability that leads to (neuro)toxicity, inflammation, and tissue damage.

POLLUTANTS AND OXIDATIVE STRESS SENSING: ROS AND MITOCHONDRIA

ROS are highly reactive free radicals produced as by-products of cellular metabolic processes and important for the activation of transcription factors, immune responses, and apoptosis [12, 50] (Figures 1-3). Exposure of bronchial epithelial (BEAS-2B) cells to PM increased mitochondrial ROS subsequently leading to Nrf2 activation to mitigate ROS damage; this protective mechanism was PM dose and exposure time dependent [50]. In line with these findings, ROS induced by PM impaired ATP production, which was due to metabolic reprogramming characterized by dampening of oxidative phosphorylation and elevation of glycolysis to safeguard the ATP generation of the "stressed" mitochondria [9, 51, 52]. Further supporting mitochondrial effects of PM, chronic exposure of Sprague-Dawley rats to PM increased glycolytic enzyme activity (hexokinase, pyruvate kinase) and reduced tricarboxylic acid (TCA) enzyme activity (citric synthase, malate dehydrogenase) in lung slices [9]. Similarly, PM reduced TCA enzyme activity in brain tissue of CB57BL/6J mice [52]. In studies using patient samples and *in vitro* cell models, PM induced damaged mitochondrial DNA (mtDNA) and an alteration in fusion-fission dynamics (involving dynamin-related protein 1 (DRP1), mitochondria fission protein 1 (FIS1), and mitofusin 2) [50, 53, 54]. Collectively, these observations suggest that chronic PM exposure can gradually lead to metabolic reprogramming, which may promote low-grade chronic inflammation and mitochondrial dysfunction.

Oxidative stress in response to PM exposure has been shown to increase MDA levels, decrease SOD, HO-1 [55], and GSH-Px (GPX4 cofactor) activities [56], and reduce the neural proliferation markers neurotropin and brain-derived neurotrophic factor (BDNF) expression [57]. Exposure of human neuroblastoma (SH-SY5Y) cells to PM has been shown to induce ferroptosis, a form of programmed cell death associated with oxidative stress, ROS, and lipid peroxidation [25]. CREB inhibited ferroptotic cell death in non-small lung cancer cells upon binding to GPX4 and inhibition of lipid peroxidation [16]. Likewise, PM reduced GPX4 in mouse neuroblastoma (N2a) cells and subsequently induced ferroptosis, a process mediated by p-CREB and p-ERK [17].

Signals regulating cAMP levels have been implicated in pollutant-mediated effects on oxidative stress, mitochondrial function, and ferroptosis [11, 17, 22]. Pollutants are known to reduce intracellular cAMP levels and cellular nanodomains [58]. For example, exposure of murine precision cut lung slices (PCLS) to CS reduced cAMP levels, probably due to an upregulation of cAMP-hydrolyzing PDE (e.g., PDE3) [23]. In addition, PDE2A has been reported to regulate cAMP levels in mitochondria; it was postulated that protein kinase A (PKA)-dependent phosphorylation of Drp1 protected against mitochondrial-dependent cell death [22]. Because cAMP nanodomains are essential for proper spatiotemporal control of cAMP responses, an alteration in the cAMP nanodomains might disrupt protein-protein complex assembly, ultimately leading to dysfunctions in downstream signaling. In addition to involvement of the canonical cAMP–PKA–CREB pathway in mitochondrial function, studies in mouse hippocampal- neuronal HT22 cells demonstrated that pharmacological inhibition of EPAC1 reduced ferroptosis in a mitochondria-dependent manner [11]. Thus, a potential role for the noncanonical cAMP–EPAC–ERK–CREB-BDNF signaling pathway in PM-induced ferroptosis in neuronal cells can be postulated [57, 59, 60] (Figure 3). Future studies are required to further delineate the impact of PM on signaling nanodomains.

POLLUTANTS AND CILIA FUNCTION

Cilia are specialized microtubule-based structures that extend beyond the cell surface. Based on axoneme structure, two main types of cilia can be distinguished: i) primary or non-motile cilia; nine peripheral doublet microtubules (9+0), and ii) motile cilia; two single microtubules at the center of the nine peripheral doublet microtubule ring (9+2) (Figures 2 and 3). Primary cilia are present throughout the human body; their functions are regulated by receptor ligands (e.g., transforming growth factor beta (TGF-β), Wnt, and Hedgehog) upon elevation of ciliary cAMP. This subsequently leads to the opening of cyclic nucleotide-gated channels to increase calcium in the cytoplasm [61, 62] (Figure 3).

Cilia that line the airway epithelium are motile and rely on calcium and cAMP to drive MCC and ciliary beat frequency (CBF) [23, 63] (Figure 2). Recent studies showed that PM severely impacts the function of CFTR, a regulator closely linked

to MCC and cystic fibrosis [4] (Figures 1 and 2). Acute exposure to PM disordered cilia arrangement of human nasal epithelial cells (HNEpCs) and impacted CBF, an effect accompanied by reductions in both ATP level and mitochondrial membrane potential [64].

As primary cilia seem to protect cortical neurons from environmental stress, it is tempting to hypothesize that patients with ciliopathies might display abnormal brain development [65]. Accumulating evidence indicates that olfactory impairments caused by PM are characterized by disease-specific alterations in neurodegeneration [66]. In line with these findings, PM exposure reduced **ciliogenesis** through c-Jun-mediated induction of small proline-rich protein 3 (SPRP3) expression in keratinocytes and retinal pigment epithelial cells [67]. Other work showed that blockage of primary cilia sensitizes SH-SY5Y cells to mitochondrial stress-induced autophagy caused by respiratory complex inhibitors, confirming the (neuro)protective role of these cilia [68]. Collectively, these studies indicate that PM negatively impacts the function of primary and motile cilia.

POLLUTANTS AND ALTERED CELL-CELL COMMUNICATION: EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) are small membrane-bound vesicles and can be released by various cell types. They are present in many tissues, from blood to nasal secretions and BALF, and have been recognized as the key players in a novel cell-cell communication mechanism [6, 13, 69]. Once released from cells, EVs can be absorbed by neighboring and distant cells, where the contents of the vesicles (**EV-cargo**) are released. Recent evidence indicates that EV content is biologically active and able to modulate inflammation and functional responses [6, 13, 69]. For example, PM exposure dose-dependently promoted the release of macrophage-derived EVs. The produced EVs induced IL-6 and TNF-α release from BEAS-2B cells, suggesting pro-inflammatory effects [6]. In addition, EVs secreted by HBE cells after exposure to PM caused AHR in BALB/c mice [70]. EVs have also been implicated in respiratory infections. Infection of A549 cells with respiratory syncytial virus induced the release of EV cargo such as MCP-1 [71]. Recent research even implicated that exposure to PM could alter the release, composition, and

function of brain-derived EVs [72]. In conjunction with this note, exposure to PM was shown to induce the release of glutaminase-containing EVs from the olfactory bulb and murine microglial line BV2 cells, thereby promoting glutamate generation and subsequent neurotoxicity [73].

EVs also have been suggested as potential biomarkers for pollution exposure. For example, exposure to PAH increased the release of EVs *in vitro* from endothelial cells and *in vivo* in urine from rats [74, 75]. GPCRs also have been reported to be closely intertwined with the production, release, and function of EVs. Considering GPCRs are crucial in cell communication and drive many cellular pathways, it is a possibility that EV-GPCR complexes and/or functional interactions can be useful as biomarkers and potentially serve as therapeutic targets [6, 13].

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Over the past decade(s), our understanding of mechanisms encompassing the "biology" of PM has seen significant advances, increasingly revealing how PM exposure can change cellular signaling, cell-cell communication, drug action, and disease progression. Unequivocally, fully elucidating the effects of PM on human health is a rather complex challenge. Studies focusing on uptake mechanisms and "receptors" potentially involved in PM sensing are still in an infant stage. One step forward in studying the effects of PM exposure should include the introduction of defined PM compositions that closely mimic real-life ecosystems. Moreover, the development of more sophisticated model systems (e.g., *in vivo* models, organoids, and microfluidic devices; for example, to facilitate studying cell-cell communication in a highly advanced manner) will enable the scientific community to compare obtained results.

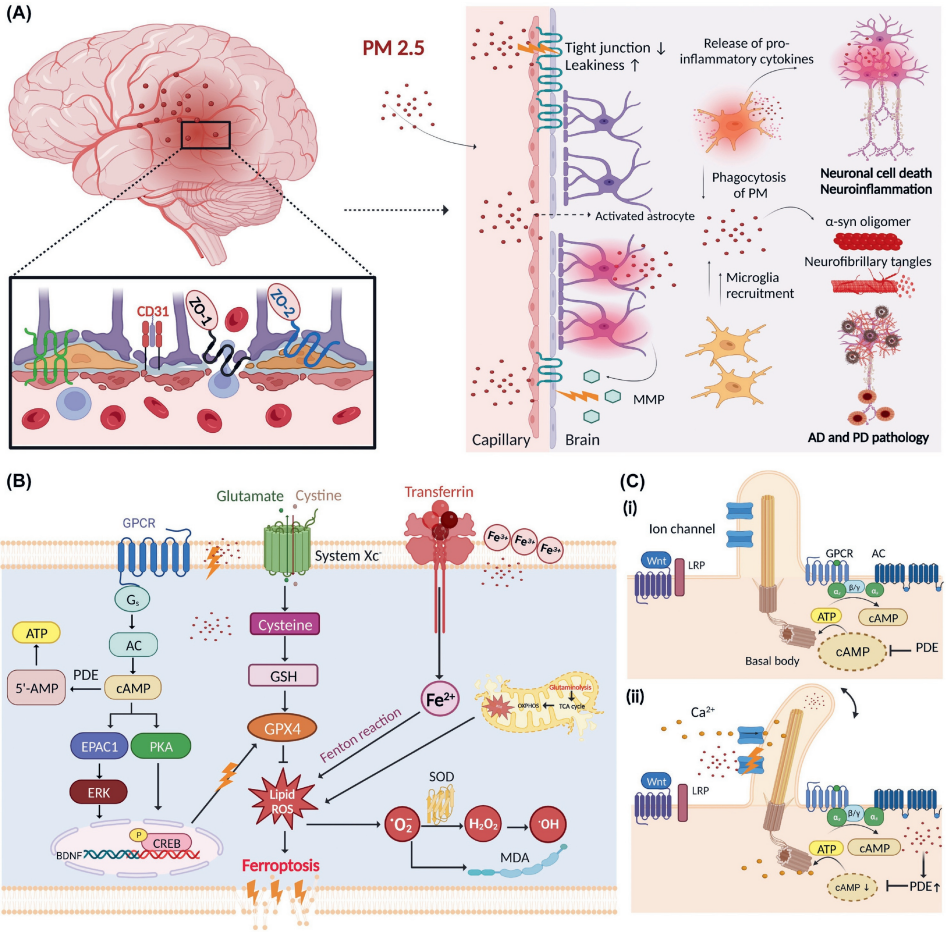


Fig 3. Effects of particulate matter (PM) in the brain. (**A**) PM2.5 can enter the bloodstream, infiltrate the blood-brain barrier, and translocate into brain tissue. In the brain, PM2.5-reactive astrocytes recruit and activate microglia and drive (neuro)inflammation by inducing the production of proinflammatory cytokines (IL-1β, IL-6, IFN-γ). (**B**) Intracellularly, PM leads to iron overload, glutamate accumulation, mitochondrial dysfunction, and down-regulation of extracellular signal-regulated kinase/cAMP response element-binding proteins (ERK/CREB); this then triggers neuronal ferroptosis by excessive reactive oxygen species (ROS) production, malondialdehyde (MDA) accumulation, as well as superoxide dismutase (SOD) and glutathione (GSH) depletion. (**C**) i. Primary cilia regulation involves receptors ligands (such as Wnt), cAMP and calcium: ii. PM disrupts primary ciliary morphology, activates adenylate cyclases (AC) and calcium channels, induces an increase in ciliary cAMP, calcium, and phosphodiesterases (PDE). These events alter cAMP nanodomains, ultimately leading to protein instability/proteotoxicity and disrupted G-protein coupled receptor (GPCR) signaling. Abbreviations: α-Syn, α-synuclein protein; AD, Alzheimer's disease; CD31, platelet endothelial cell adhesion molecules; EPAC1, exchange protein directly activated by cAMP; GPX4, glutathione peroxidase 4; LRP: low density lipoprotein *receptor*-related protein; MMP, matrix metalloproteinases; OXPHOS, oxidative phosphorylation; PD, Parkinson's disease; PKA, protein kinase A; TCA, tricarboxylic acid cycle. Figure 3 was created using BioRender (www.biorender.com).

As described in this review, recent research clearly indicates that PM alters typical cellular signaling cascades, receptor systems (e.g., GPCRs), cilia, mitochondrial function, and EV production to induce cellular damage and drive disease progression. Based on the wide array of tissues, cells, and mechano- as well as signalling transduction pathways affected by PM, future studies aimed at unravelling the cross-talk between these systems in response to PM exposure seem warranted (see Outstanding questions).

In order to advance PM research, proper models (e.g., to study cross-talk) are an absolute necessity. To date, classical 2D cell cultures still constitute the most common *in vitro* models, with the benefit of easy maintenance and reproducibility; however, these models lack the complexity of cellular 3D environments. Indeed, advanced models would benefit our understanding of the effects of PM exposure on complex isolated (multi-)cell systems. For example, air-liquid interface (ALI) cultures of differentiated airway epithelial cells are in contact with nutrient media on the basal side, while in direct contact with air on the apical side, allowing aerosol exposure to pollutants; this accurately mimics *in vivo* conditions in the airways and alveoli [76]. Organoids represent another alternative to closely resemble *in vivo* conditions. These miniature organs are generated *in vitro* in 3D cultures that self-assemble from healthy or diseased (human) donor tissue [19, 77]. Current attempts to improve organoids aim to integrate macrophages and endothelial cells in organoids to study inflammatory and resident cell interactions [77]. Organs-on-chips (OoCs) or multi-organ fluidics are another type of 3D modeling system aiming to culture basic functional units on a micrometer-sized chamber [76, 78, 79]. In contrast to other models, microfluidic chip devices can be designed to recreate interactions between two or more different cell types. For example, they can recreate the functional interplay between lung endothelium and epithelium, or mimic the BBB by co-culturing brain microvasculature endothelium and a mixture of brain cells [5, 76].

One of the most promising recent developments in PM "biology" is the finding that PM induces EVs. In fact, EV release may even serve as a biomarker to monitor pollutant exposure and disease risk/progression [74, 75]. Moreover, these observations have led to a proposed role for EVs in drug delivery to specific organs.

Thus, because PM induces EV release which travel through the human body (complexed with yet to be identified "receptors"), increasing our knowledge about the mechanisms involved in PM-induced cell damage and trafficking abilities may provide a novel approach for drug delivery. In addition, as PM-induced EVs have been shown to contain GPCRs, it is appealing to postulate that the complex biology of GPCRs could be explored to develop advanced treatment strategies [13-15], especially considering GPCRs represent the most promiscuous and promising drug targets in current drug development [15]. Indeed, GPCR modulators are used in the treatment of a multitude of diseases, including those affecting the respiratory, CNS, cardiovascular, and metabolic systems [15]. Intriguingly, there is evidence to suggest that PM changes GPCR abundance and/or signaling properties (e.g., calcium, cAMP, NF-kB, ERK1/2) [21] (Figures 2 and 3). Of particular interest, it has been reported that direct interaction of PM with the beta2-adrenergic receptor leads to its activation and subsequent signaling [21]; this exemplifies the potential impact of PM/EV/GPCR interactions on drug discovery and development, and emphasizes the need for future research using advanced technologies (e.g., cryo-electron microscopy) [15]. Lastly, transfer of well-defined EVs and GPCRs might support future treatment opportunities of chronic disease in the aging population.

Nowadays exposure to PM is most likely impossible to avoid. Therefore, future research should focus primarily on understanding the underlying cellular and molecular mechanisms of PM-induced effects, with particular emphasis on unravelling the highly dynamic systems involved in PM/EV/GPCR biology, and use this to our advantage in targeting the threat of pollution on human health.

HIGHLIGHTS

Pollutants can induce persistent (neuro)inflammation and oxidative stress; this leads to changes in protein (in)stability, protein composition, and protein-protein complexes.

Pollutants cause alterations in receptor abundance and/or signaling (e.g., G protein-coupled receptor (GPCR) signaling) and, as such, dysregulation of a plethora of downstream signaling mechanisms.

Pollutants are "sensed" - through yet ill-defined molecular mechanisms - by a wide-range of effector proteins, including GPCRs and Toll-like receptors, and bear the potential to change various cellular responses involving (classical) calcium and cAMP signaling.

Insights into particulate matter (PM) "biology", and in PM/extracellular vesicles/GPCR dynamics, may open exciting avenues for drug targeting and delivery, potentially leading to novel therapeutic strategies for the treatment of (chronic) diseases.

GLOSSARY

AD: Alzheimer's disease is the most prevalent neurodegenerative disease characterized by Aβ plaques formation accumulation of pTau (neurofibrillary tangles); these processes lead to progressive loss of neuronal function.

Asthma: common chronic respiratory disease characterized by reversible airway obstruction and airway inflammation (infiltration/activation of eosinophils, $CD4^+$ T-lymphocytes and mast cells).

AHR: airway hyperresponsiveness is the enhanced sensitivity and responsiveness of the airways to stimuli that would hardly be effective or ineffective in healthy individuals.

BALF: bronchoalveolar lavage fluid are samples collected from the lining of the lower respiratory tract.

BBB: the blood-brain barrier is a tight defense line composed of multiple cell types, metabolizing enzymes, and transporter proteins that prevent crossing of substances (particles) to enter the brain.

Ciliogenesis: formation of cilia in the cells, a process that begins when basal bodies migrate to cell surfaces, fuse with cellular membranes and axonemal microtubules to form the cilia.

CNS: the central nervous system consists of the brain and spinal cord and is made up of neurons and glial cells.

COPD: chronic obstructive pulmonary disease is characterized by infiltration of $CD8^+$ T-lymphocytes, macrophages, and neutrophils. Typical features include chronic inflammation, remodeling, excess mucus production, emphysema, coughing, and wheezing.

ECM: the extracellular matrix is a highly dynamic, non-cellular, tissue-specific structure consisting of structural proteins and proteoglycans that form complex three-dimensional networks important for cell homeostasis, signal transduction, cell adhesion and migration.

Emphysema: shortness of breath caused by the enlargement of alveolar spaces due to the loss of inner walls of the air sacs.

EV-cargo: content inside membrane-bound vesicles which can be composed of microRNA (miRNA), proteins, lipids, GPCRs, signaling molecules (e.g., calcium, cAMP), nutrients, and even whole organelles (e.g., mitochondria).

FEV1: forced expiratory volume in one second is a parameter used to measure the air exhaled in one second.

Ferroptosis: iron-dependent cell death characterized by accumulation of lipid peroxidases as well as GSH and GPX4 synthesis disruption. GSH is a cofactor to GPX4 that transforms lipid peroxides to lipid alcohols and protects cells from oxidative damage.

FVC: forced vital capacity is the total amount of air exhaled during a forced expiratory volume test.

MCC: mucociliary clearance is the unidirectional and synchronous coordinated movement of motile cilia to remove mucus from airway surfaces.

PD: Parkinson's disease is the second most common neurodegenerative disease characterized by movement dysfunctions (e.g., bradykinesia, rigidity, rest tremor),

(neuro)inflammation, accumulation of misfolded α-Syn, and neuronal degeneration.

ROS: main endogenous producer of ROS are the mitochondria; these organelles regulate programmed cell death, oxidative stress, and energy metabolism by producing ATP via oxidative phosphorylation, the TCA cycle, and glycolysis. To combat ROS generation, mitochondria activate the antioxidant pathway via Nrf2, subsequently leading to fine-tuning of homeostasis, membrane potential, and other events (e.g., apoptosis).

OUTSTANDING QUESTIONS

Can PM "trafficking" be visualized *in vivo* and does it change under pathophysiological conditions?

Can PM composition be standardized in model systems? Does this foster drug development?

Does PM "trafficking" mimic uptake mechanisms of drugs? Can such a strategy lead to more sophisticated drug targeting of drugs already approved for the treatment of chronic disorders?

Does PM "biology" define cilia, mitochondria, and EV composition and function? Can PM targeting regulate metabolic reprogramming?

Can PM "targeting" be used to interfere with ciliogenesis and/or mucus clearance?

What is the level of convergence of PM and GPCR "biology"? Can GPCR targeting lead to modification of PM-induced EVs?

BOX 1: OVERVIEW OF ENVIRONMENTAL POLLUTANTS: COMPOSITION AND UPTAKE MECHANISM

Pollutants that threaten human health and harm ecosystems include particulate matter (PM), (micro)plastics and fibers/textiles, gases (e.g., CS, CO, O_3, NO_2, sulfur dioxide), heavy metals (e.g., vanadium, nickel, lead, manganese), pesticides and organic compounds (e.g., PAHs, endotoxins), industrial chemicals, petroleum, and electronic and radioactive waste [80]. PM is a mixture of micro-sized liquid and solid particles produced by motor engines, photo-oxidation and abrasion of plastic, and industrial activities [1, 80, 81]. PM is categorized into three classes based on its aerodynamic diameter: up to 0.1 μm (PM0.1), 2.5 μm (PM2.5) or 10 μm (PM10) [2].

Following inhalation of PM, nasal MCC removes a significant amount of PM10 from the upper respiratory tract; however, inhaled PM0.1 and PM2.5 accumulate in the olfactory mucosa subsequently facilitating cellular uptake in the lower respiratory tract [2, 82]. PM is phagocytized and/or degraded by (alveolar) macrophages [2]. Free PM alters MCC [2, 30], lipid metabolism [83], causes oxidative stress [84], disrupts the lung endothelial and epithelial barriers, and enters the systemic circulation. Qi *et al.* reported the presence of exogenous fine particles in human cerebrospinal fluid and blood [3]. Thus, PM can reach distant organs [82] such as the liver, heart, kidney, and placenta [85]. Recent studies in Calu-3 cells exposed to CS, urban PM, and DEP reported CFTR dysfunction, which was sensitive to pharmacological intervention [4, 86]. As MCC is an important host defense mechanism against pollutants, its disruption certainly impacts clearance of pollutants, mucus secretion, and ultimately disease progression.

Inhaled particles translocated from the lung to the brain (via the blood) as observed by intratracheal instillation with ^{125}I isotope labeling carbon black particles in BALB/c mice [3, 87]. PM0.1 and PM2.5 cross the BBB via receptor-mediated transcytosis involving apolipoprotein E (ApoE) [88], and following loss of BBB integrity due to systemic inflammation [89]. Indeed, *in vitro* studies using human endothelial cells to model the BBB show that PM2.5 can penetrate the BBB and increase endothelial permeability by decreasing the expression level of TJ proteins, such as ZO-1 and occludens-2 [3, 49], vascular endothelial cadherin, platelet

endothelial cell adhesion molecules (CD31), and occludin [5]. Not surprisingly, evidence of damage to other organs due to PM is gradually accumulating; indeed, damage to the heart, liver and kidney has been described [83, 84, 90, 91], seemingly involving similar mechanisms as in the lung and brain.

BOX 2: OVERVIEW OF POLLUTANTS: TISSUE REPAIR AND ECM

Remodeling of the ECM is important for tissue repair and depends on the balance between matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs). Tissue repair is the restoration of structure and function following injury. It generally encompasses an inflammatory response, a proliferative stage to restore cell composition and structures, and a resolution of repair that also ends the previous stages. Alterations in tissue repair mechanisms have been reported after exposure to PM. Exposure to microfibers significantly reduced both size and number of (human/murine) airway organoids [92]. Similarly, exposure of airway/alveolar-type organoids to DEP also showed a reduction in size and number. RNA sequencing analysis revealed downregulation of the Wnt signaling pathway in epithelial cells, pointing to a possible dysfunctional response in alveolar progenitors in response to DEP [19]. Indeed, exposure of CB57/6J mice to PM2.5 impaired self-renewing of alveolar type II cells and induced differentiation into type I cells [93]. Importantly, pharmacological treatment with MitoQ and Wnt/β-catenin activator CHIR99021 restored Wnt signaling and promoted organoid growth upon DEP exposure [19], further pointing to the importance of oxidative stress responses and subsequent protein (in)stability impaired by pollutants.

Pollutants impact protein (in)stability, leading to ECM remodeling and ECM-related pathologies [94-96]. Acute exposure of guinea pigs to pollutants increased total collagen in BALF, MMP-2, MMP-9, TIMP-1 and TIMP-2 abundance in lung tissue, and lung MMP-2 activity measured by zymography [97], demonstrating remodeling of the ECM. Histological staining of lung sections indicated neutrophils and macrophages infiltration [97]. Similarly, exposure of female ApoE-/- mice to pollutants increased MMP-2 and -9 activity measured by *in situ* zymography, leading to a reduction in TJ and subsequently BBB integrity [98]. In contrast, Wang et al. found that a single intratracheal administration of PM in murine cancer

models increased collagen IV crosslinking leading to a reduction in lymphocyte migration and impaired immune responses [99]. Similarly, exposure of C57BL/6J mice to plastic particles induced the (myo)fibroblast marker α-smooth muscle actin (α-SMA) and the ECM component fibronectin [100]. Thus, the exact mechanisms by which pollutants affect ECM structure, composition, and remodeling are incompletely understood; however, a link between (chronic) pollutant exposure and disease seems to be present. Whether differences in ECM damage in response to PM exposure are due to differences in pollutant size, chemical composition, or administration, and/or rely on the animal model used remains to be further investigated.

ACKNOWLEDGEMENTS

A.L.M.C. is a recipient of a Consejo Nacional de Humanidades, Ciencias y Tecnologías (CONAHCYT) Scholarship (2021-000022-01EXTF-00086). H.Y. is a recipient of a China Scholarship Council (CSC, 202006300052). A.M.D. is the recipient of an Alzheimer Nederland grant (WE.03-2018-04, The Netherlands), Parkinson Fonds (The Netherlands) and a Rosalind Franklin Fellowship co-funded by the European Union and the University of Groningen. MS received support by Alzheimer Nederland grant WE.03-2019-05, M.S. was supported by the Deutsche Forschungsgemeinschaft (IRTG1874DIAMICOM-SP2) and Novartis unrestricted grant 50199468. The authors would like to thank Dedmer Schaafsma for (post)editing the manuscript.

DECLARATION OF INTEREST

All authors declare no conflict of interest.

Table 1: Overview of models exposed to pollutants

Species	Effect	Model	Bio-specimen	Dosing	Measured parameters*	Ref.
Human	Inflammation	SRM 1650b	3D brain model	10 µg/ml for 9 wk	- ↑ Microglia recruitment, astrocyte activity - ↑ IL-1β, IFN-γ, pTau - ↓ ZO-1	[49]
Human	Inflammation	- CSE - SRM 1650b	BEAS-2B cells	- 2% for 48 h - 100 µg/ml for 48 h	- ↑ IL-6, IL-8 - ↑ PD-L1 - ↑ p-ERK1/2, p-STAT1/3 protein	[18]
Human	Inflammation Senescence	SRM 1649b	A549 cells	50 and 100 µg/ml for 48 h	- ↑ G2/M phase arrest - ↑ SA-β-gal-positive cells - ↑ p16, p21, γ-H2AX, cGAS, STING, p-p65, TNF-α, IL-6, IL-8 protein expression	[45]
Human	Inflammation Impaired phagocytosis	PM2.5 from Nanjing, China	THP-1 cells	300-500 µg/ml for 24 h	- ↑ intracellular ROS - ↑ IL-6, CXCL8, TNF-α mRNA - ↓ cell viability, phagocytotic capacity	[44]
Human	Inflammation Extracellular vesicles	PM2.5-0.3 samples from Dunkerque, France	THP-1 cells	12.5 and 50 µg/ml for 24 and 48 h	- ↑ CYP1A1, CYP1B1 - ↑ SOD2 HMOX, NQO1 - ↑ IL-8, IL-1β, IL-6, TNF-α, EVs release	[6]
Human	Infection	PM10 samples from London Underground	A549cells HPNEpC cells	20 µg/ml	- ↑ PAFR-positive cells - ↑ bacterial adhesion - ↑ CFU	[33]
Human	Cilia dysfunction	PM2.5 from Seoul, Korea	NHEKs cells RPE cells	50 µg/ml for 2 and 4 d	- ↑ SPRR3 expression via c-Jun activation leading to ciliogenesis inhibition	[67]

Table 1: Overview of models exposed to pollutants *(continued)*

Species	Effect	Model	Bio-specimen	Dosing	Measured parameters*	Ref.
Human	Cilia dysfunction	DEP	HBE cells	100 µg/ml for 30 and 60 min	- ↑ Ca^{2+}, MMP-1, p-PLCb3	[21]
Human	EVs	SRM 2786	HBE cells	250 µg/ml for 24 h	- ↑ apoptosis, G1/G2 arrest, - ↑let-7i-5p-EVs release →DUSP1 and MAPK activation in recipient cells - ↓ cell number	[70]
Human	Mitochondrial dysfunction	TRAP-UFP	SH-SY5Y cells	10 µg/ml for 24 h	- ↑ mtDNA oxidation - ↓ mitochondrial consumption rate, mtDNAcn	[54]
Human	Mitochondrial dysfunction	PM2.5 samples from Beijing, China	HNEpCs cells	100 µg/ml for 12 h	- ↓ ATP**,** mitochondrial membrane potential	[64]
Human	Mitochondrial dysfunction Oxidative stress	PM2.5 samples from Taiyuan China	SH-SY5Y cells	80 µg/ml for 24 or 72 h	- ↓ basic respiration, ATP levels, typical index of mitochondrial respiration, glycolysis, cell viability, Mn-SOD - ↑ MDA, ROS, apoptosis	[51]
Human	Mitochondrial dysfunction	PM2.5 (2.5-0.18 um) and UFM (<0.18 um) from France	BEAS-2B cells	repeatedly 5 µg/cm^2 for 24, 48 and 72 h	- ↑ oxygen consumption rate, ROS - ↑ Nrf, NQO1, HO-1, GCLM, OPA-1, DRP1	[50]
Human	Mitochondrial dysfunction	PM2.5 samples from Hong Kong	BEAS-2B cells	10 µg/cm^2 for 24 h	- ↓ maximal respiration reversed by 100 nM MitoQ	[30]

Table 1: Overview of models exposed to pollutants *(continued)*

Species	Effect	Model	Bio-specimen	Dosing	Measured parameters*	Ref.
Human	Oxidative stress	PM2.5 samples from Taiyuan, China	SH-SY5Y cells	160 µg/ml for 24 h	- ↓ cell viability - ↓ GSH-PX, SOD, HO-1, Nrf2, SLC7A11, GPX4 - ↑ iron, MDA, lipid ROS	[25]
Human	Oxidative stress Protein instability	SRM 2975	NGV	200 µg/ml for 24 h	- ↑ Aβ accumulation, pTau, H_2O_2/ROS production, neuronal cell death, vascular permeability, GFAP, GM-CSF, and NF-kB	[5]
Human	Oxidative stress Wnt signaling	SRM 2975	A549 cells	200 µg/ml for 24 h	- ↑ total ROS - ↓ β-catenin-target gene Axin2 expression, Wnt activation	[19]
Human	Oxidative stress	Collected PM2.5 from Wuhan China	SH-SY5Y cells	31.25, 62.5, and 125 µg/ml for 24 h	- ↑ iron - ↓ cell viability, TFRC, FTL - ↓ GPX4, FTH, SLC7A11 protein expression	[17]
Human	Phagocytosis	SRM 2786	THP-1 cells	200 µg/ml for 24 h	- ↑ LC3II, SQSTM1, JNK, p38, p-ERK1/2, ROS - ↓ phagocytosis	[43]
Human	Oxidative stress	- TSE from one research cigarette - SRM 1648a	Calu-3 cells	- 10 or 20% TSE - 125 µg/ml for 24 h	- ↑ H2DCFDA - ↓ CFTR activity	[4]
Human	Protein instability Mitochondria dysfunction	PM2.5 from Wuhan, China	α-Syn-HEK293 cells SH-SY5Y cells	10, 50, and 100 µg/ml for 24 h	- ↑ α-Syn preformed fibrils and aggregation - ↓ COX-IV - ↓ bioactivity of mitochondrial complex I - ↓ mitochondria fusion	[39]

Table 1: Overview of models exposed to pollutants *(continued)*

Species	Effect	Model	Bio-specimen	Dosing	Measured parameters*	Ref.
Mouse (female ApoE-/-)	BBB integrity Inflammation	Mixed vehicle emissions	Brain	200 µg /m^3 for 6 h/d, 7 d/wk, for 30 d	- ↑MMP-2/9 activity - ↑IgG extravasation - ↑IL-1β, TNF-α, ICAM-1 - IKKα, IKKβ mRNA - ↓occludin, claudin-5 protein expression	[98]
Mouse (C57BL/6J)	ECM	FPM-samples from Nanjing, Suzhou, and Tieling, China	-Lewis lung cancer cell lines, mouse bone marrow fibroblasts, M2-10B4 Jurkat T cells -Lung	- 10 µg/ml for 48 h - 0.2 mg FPM in 50 µL of sterile physiological saline	- ↑Col IV crosslinking - ↑ECM density - ↑sulfonimine bond formation - ↑lung tumorigenesis by impairing cytotoxic T lymphocytes' migration, PXDN activity	[99]
Mouse (male C57BL/6)	EV	Urban airborne PM2.5	BV2 cells	50 µg/ml for 12 h	- ↑EV release, glutamate generation	[73]
Mouse	Inflammation	PM2.5 samples from Hangzhou China	RAW 264.7 cells	250 µg/ml for 0, 24, 48, and 72 h	- ↑TLR4, NF-κB, COX-2, MCP-1, TNF-α, IL-6, iNOS	[42]
Mouse (C57BL/6)	Inflammation	SRM 1648a	Primary microglia	100 µg/ml for 24 h	- ↑TLR4, NF-κB, IL-6, IL-1β, TNF-α, CCl2, STAT3, STAT6	[48]
Mouse (female BALB/c)	Inflammation Mitochondria dysfunction	PM2.5 samples from Hong Kong, China	Lung, BALF, bronchioles	5 µg/d for 6 wk plus pregnancy and lactation period	- ↑mitochondrial and total ROS - ↑Drp-1 protein levels - ↑tissue elastance and damping, inflammation score, epithelial thickness, Ashcroft score, PAS-positive cells, mean linear intercept, and α-SMA in lung slices	[30]

Table 1: Overview of models exposed to pollutants *(continued)*

Species	Effect	Model	Bio-specimen	Dosing	Measured parameters*	Ref.
					- ↑number of macrophages, eosinophils, and neutrophils in BALF	
Mouse (female C57BL/6J)	Inflammation	TRAP samples from Los Angeles, USA	Brain (hippocampus)	- 300 µg/ml, 5 h/d, 3 d/wk for 10 wk	- ↑ TLR4, MyD88, TNF-α, Nrf2 mRNA - ↓ NF-κB, TRAF6 mRNA, TLR4 and NF-κB responses	[47]
Mouse (female BALB/c)	Inflammation	PM2.5 from Nanjing, China	BALF, lung and liver tissue	50 µg nasal instillation once every 3 days, 6 times in total	- ↑ inflammation and fibrosis score in lung slices, liver injury in liver slices, cell infiltration in BALF	[44]
Mouse	Impaired phagocytosis	PM2.5 from Nanjing, China	RAW264.7 cells	100 µg/ml for 24 h	- ↓ phagocytic capacity	[44]
Mouse (female C57BL/6)	Mitochondria dysfunction	PM2.5 samples from Taiyuan, China	Brain	3 mg/kg every other day for 4 wk	- ↓ ATP, ROS, pTau	[52]
Mouse (male SD)	Metabolic rewriting	PM2.5 samples from Taiyuan, China	Lung	Summer (1.5 mg/kg) and winter groups (1.5, 2.7 mg/kg), once every 3 days for 2 m	- ↓ ATP, citric synthase, MDH, electron transport chain complex I and III - ↑ MDA, pyruvate, lactic acid, lactate dehydrogenase, glucose transporter 1	[9]
Mouse (male C57BL/6J)	Metabolic rewriting	PM2.5 samples from Taiyuan, China	Brain, skeletal muscle, and liver	5 mg/kg body weight, every other day for 4 wk	- ↑ pTau, serum insulin - ↓ p-IRS-1, p-AKT, P-GSK-3β	[38]
Mouse	Oxidative stress	TRAP-PM from Los Angeles, USA	- Brain (male J20-hAPPswe) N2a cells	- 300 µg/m^3 for 5 h/d, 3d/wk, for 10 wk 5, 10 µg/ml for 24 h	- ↑ Aβ, APP, 4-HNE in cerebral cortex - ↑ Aβ, sAPPβ:sAPPα, PS1, NO, 3NT, 4-HNE, MitoSOX	[24]

Table 1: Overview of models exposed to pollutants *(continued)*

Species	Effect	Model	Bio-specimen	Dosing	Measured parameters*	Ref.
Mouse (female C57BL/6)	Oxidative stress	Mixture of diesel and biodiesel particles	Lung	250 or 1000 μg intranasal instillation for 5 d	- ↑static elastance, lung resistive pressure - ↑MMP-12, TNF-α, ROS, MDA, Nrf2, p-NF-kB, HO-1 - ↓MMP-2, SOD-3, CAT, GPx-1/2, Keap-1	[20]
Mouse (female BALB/c)	Oxidative stress	SRM 2975	Brain	100 μg/m^3 for 1 h/d, 5 d/wk, for 8 wk	- ↑HO-1, SOD-2, XBP-1S, TNF-α and pro-BDNF in temporal cortex - ↓neurotrophin-3 and - pro-BDNF in striatum	[57]
Mouse	Oxidative stress	SRM 1650b	HT22 cells	50 μg/ml for 24 h	- ↑HO-1, Hsp70, Cyp1b1, COX-2, iNOS, APP, BACE1	[40]
Mouse (male C57BL/6)	Oxidative stress	PM2.5 samples from Beijing, China	Brain	2.5 mg/kg for 28 d	- ↑IL-1β, IL-6, MDA, Bcl2/Bax ratio, Caspase-3 fragment/pro-caspase-3 - ↓LC3-II, mTOR, PINK1, Parkin	[41]
Mouse	Oxidative stress Mitochondria dysfunction	PM2.5 samples from Wuhan, China	N2a cells	31.25, 62.5, and 125 μg/ml for 24 h	- ↑iron, TFRC, FTL, ROS, NO, MDA, mitochondrial membrane potential - ↓cell viability, GPX4, FTH, SOD, GSH, p-ERK, p-CREB	[17]
Mouse (α-Syn A53T mice)	Protein instability	PM2.5 from Wuhan, China	- Brain - Primary cortical neuron	- 30 μg in 15 μL PBS via intranasal instillation every 2 d for 6 m - 10, 50, and 100 μg/ml for 24 h	- ↑α-Syn pathology - ↓number and density of dopaminergic neurons - ↑α-Syn preformed fibrils and aggregation	[39]

Table 1: Overview of models exposed to pollutants *(continued)*

Species	Effect	Model	Bio-specimen	Dosing	Measured parameters*	Ref.
Mouse (CAG-Epac1-camps FVB/N)	Protein instability Cilia	CS of filter free 3R4F research cigarettes	PCLS	10 cigarettes/d for 4 d	- ↓intracellular cAMP levels and CBF - ↑PDE4D and PDE4B mRNA	[23]
Mouse (C57BL/6J)	Tissue repair	SRM 2975	Lung organoids	200 µg/ml for 7 and 14 d	- ↓in total number and size - upregulation of Nrf2 pathway and Wnt-related genes	[19]
Mouse (female C57BL/6)	Tissue repair	PM2.5 from bio-mass fuels	Lung	6 h/d for 6 m	- ↓body weight, pulmonary compliance, forced pulmonary ventilation, mean number of alveoli and AT2 to AT1 cell transition - ↑mean alveolar linear intercept	[93]
Mouse (female C57BL/6)	Tissue repair	PM2.5 from conventional Chinese wood stove fires	Alveolar organoids	50 µg/ml	- ↓AT2 to AT1 cell transition - ↓claudin 4 - downregulation of Wnt and MAPK pathways - ↑Ki67	[93]
Rat (female SD)	Inflammation	TRAP samples from Los Angeles, USA	Primary mixed glia (microglia and astrocytes)	10 µg/mL for 24 h	- Activated TLR4 and NF-κB	[47]
Rat (male SD)	Oxidative stress	PM2.5	Serum, brain microvessels	44 µg/m^3 for 12 wk	- ↑ROS, TNF-α, IL-6, NOX activity, Nrf2, NQO-1 mRNA - ↓SOD activity	[55]

Table 1: Overview of models exposed to pollutants *(continued)*

Species	Effect	Model	Bio-specimen	Dosing	Measured parameters*	Ref.
Rat	Oxidative stress	PM2.5 samples from Beijing, China	PC12 cells	50 μg/ml for 4 d	- ↑ROS, Bcl2/Bax ratio, Caspase-3 fragment/pro-caspase-3 - ↓cell viability, mitochondrial membrane potential - ↓LC3-II, Atg5, PINK1, Parkin	[41]
Rat (male SD)	Oxidative stress Mitochondria dysfunction	PM2.5 from Tangshan, China	Brain	10 ml/kg every 7 d for 3, 6, and 12 m	- ↑caspase-3, caspase-9, MDA - mitochondrial morphological changes - ↓SOD, GSH-Px	[56]
Guinea pig (female)	Inflammation ECM	Wood smoke	Lung, BALF	Smoke of 60 g of wood for 3 h/d, 24 and 72 h	- ↑TGF-β1, TNF-α, IFN-γ, IL-1β, IL-6, IL-8, MMP-2, MMP-9, TIMP-1, and TIMP-2, total collagen	[97]

* ↓ indicates reduction; ↑ indicates increase

α-Syn-HEK293: α-Syn- expression human embryonic kidney cells; Atg5: autophagy related 5 protein; BDNF: brain-derived neurotrophic factor; CAT: catalase; CCL2: monocyte chemoattractant protein-1; CFU: colony forming units; CSE: cigarette smoke extract; CXCL8: C-X-C motif chemokine ligand 8; CYP1A1: cytochrome p450 1A1; DUSP1: dual specificity protein phosphatase-1; FTH: ferritin heavy chain; FTL: ferritin light chain; GCLM: glutamate-cysteine ligase modifier; GM-CSF: granulocyte-macrophage colony-stimulator factor; H2DCFDA: ROS indicator 2′,7′-Dichlorodihydrofluorescein diacetate; HPNEpC: human primary nasal epithelial cells; ICAM-1: intercellular adhesion molecule-1; IKKα: IkB kinase alpha; IKKβ: IkB kinase beta; Keap-1: Kelch-like ECH-associated protein; MAPK: mitogen-activated protein kinases; MDH: malate dehydrogenase; mtDNAcn: mitochondrial DNA copy number; NHEKs: normal human epidermal keratinocytes; NQO-1: NAD(P)H quinone dehydrogenase-1; OPA-1: optic atrophy-1; PAFR: platelet-activating factor receptor; PAS: periodic acid-Schiff stain; PC12: rat adrenal pheochromocytoma cells; PD-L1: programmed death-ligand; PS1: presenilin-1; PXDN: peroxidase; p-AKT: phosphorylated phosphoinositide-3-kinase; p-ERK: phosphorylated ERK; p-GSK-3β: phosphorylated glycogen synthase kinase-3; p-IRS-1: phosphorylated insulin receptor substrate-1; p-PLCb3: phosphorylated phospholipase C beta-3; p-STAT: phosphorylated STAT; RPE: retinal pigment epithelium; SA-β-gal: senescence-associated beta galactosidase; SH-SY5Y: human neuroblastoma cells; SLC7A11: cystine/glutamate antiporter; SQSTM1: sequestosome-1; SRM: standard reference material; TFRC: transferrin receptor; TIMP: tissue inhibitor metalloproteinase; TRAF6: TNF receptor associated factor; TRAP traffic related air pollution; TSE: tobacco smoke extract; UFM: ultra fine particulate matter; XBP-1S: X-box binding protein 1; 4-HNE: 4-hydroxy-2-nonenal, lipid peroxidation; 3NT: 3-nitro-tyrosine.

REFERENCES

1. Fuller, R., *et al.* (2022) Pollution and health: a progress update. *Lancet Planet. Health* 6, e535-e547
2. Arias-Perez, R.D., *et al.* (2020) Inflammatory effects of particulate matter air pollution. *Environ. Sci. Pollut. Res. Int.* 27, 42390-42404
3. Qi, Y., *et al.* (2022) Passage of exogeneous fine particles from the lung into the brain in humans and animals. *Proc. Natl. Acad. Sci. U. S. A.* 119, e2117083119
4. Nguyen, J.P., *et al.* (2021) Effects of environmental air pollutants on CFTR expression and function in human airway epithelial cells. *Toxicol. In Vitro* 77, 105253
5. Seo, S., *et al.* (2023) Neuro-Glia-Vascular-on-a-Chip system to assess aggravated neurodegeneration via brain endothelial cells upon exposure to diesel exhaust particles. *Adv. Funct. Mater.* 33, 2210123
6. Martin, P.J., *et al.* (2019) Cellular response and extracellular vesicles characterization of human macrophages exposed to fine atmospheric particulate matter. *Environ. Pollut.* 254, 112933
7. Gómez-Budia, M., *et al.* (2020) Glial smog: Interplay between air pollution and astrocyte-microglia interactions. *Neurochem. Int.* 136, 104715
8. Cheng, J., *et al.* (2022) Swine influenza virus triggers ferroptosis in A549 cells to enhance virus replication. *Virol. J.* 19, 104
9. Jin, X., *et al.* (2019) Exposure to ambient fine particles causes abnormal energy metabolism and ATP decrease in lung tissues. *Chemosphere* 224, 29-38
10. Majerníková, N., *et al.* (2021) The potential of ferroptosis-targeting therapies for Alzheimer's disease: From mechanism to transcriptomic analysis. *Front. Aging Neurosci.* 13, 745046
11. Musheshe, N., *et al.* (2022) Pharmacological Inhibition of Epac1 Averts Ferroptosis Cell Death by Preserving Mitochondrial Integrity. *Antioxidants (Basel)* 11, 314
12. Zhang, B., *et al.* (2022) Role of mitochondrial reactive oxygen species in homeostasis regulation. *Redox Rep.* 27, 45-52
13. Bebelman, M.P., *et al.* (2020) The convergence of extracellular vesicle and GPCR biology. *Trends Pharmacol. Sci.* 41, 627-640
14. Li, D. and Wu, M. (2021) Pattern recognition receptors in health and diseases. *Signal Transduct. Target Ther.* 6, 291
15. Yang, D., *et al.* (2021) G protein-coupled receptors: structure- and function-based drug discovery. *Signal Transduct. Target Ther.* 6, 7
16. Wang, Z., *et al.* (2021) CREB stimulates GPX4 transcription to inhibit ferroptosis in lung adenocarcinoma. *Oncol. Rep.* 45, 88
17. Xiong, Q., *et al.* (2022) $PM_{2.5}$ exposure-induced ferroptosis in neuronal cells via inhibiting ERK/CREB pathway. *Environ. Toxicol.* 37, 2201-2213
18. Rui, C., *et al.* (2022) Cigarette smoke or motor vehicle exhaust exposure induces PD-L1 upregulation in lung epithelial cells in COPD model rats. *COPD* 19, 206-215
19. Wu, X., *et al.* (2022) Diesel exhaust particles distort lung epithelial progenitors and their fibroblast niche. *Environ. Pollut.* 305, 119292

20. Cattani-Cavalieri, I., *et al.* (2019) Acute exposure to diesel-biodiesel particulate matter promotes murine lung oxidative stress by Nrf2/HO-1 and inflammation through the NF-kB/TNF-alpha pathways. *Inflammation* 42, 526-537
21. Holme, J.A., *et al.* (2019) Combustion particle-induced changes in calcium homeostasis: A contributing factor to vascular disease? *Cardiovasc. Toxicol.* 19, 198-209
22. Monterisi, S., *et al.* (2017) PDE2A2 regulates mitochondria morphology and apoptotic cell death via local modulation of cAMP/PKA signalling. *eLife* 6, e21374
23. Zuo, H., *et al.* (2018) Cigarette smoke up-regulates PDE3 and PDE4 to decrease cAMP in airway cells. *Br. J. Pharmacol.* 175, 2988-3006
24. Cacciottolo, M., *et al.* (2020) Traffic-related air pollutants (TRAP-PM) promote neuronal amyloidogenesis through oxidative damage to lipid rafts. *Free Radic. Biol. Med.* 147, 242-251
25. Guo, C., *et al.* (2023) Organic extracts in PM2.5 are the major triggers to induce ferroptosis in SH-SY5Y cells. *Ecotoxicol. Environ. Saf.* 249, 114350
26. Zheng, H., *et al.* (2021) Nanoparticle-induced ferroptosis: detection methods, mechanisms and applications. *Nanoscale* 13, 2266-2285
27. Doiron, D., *et al.* (2019) Air pollution, lung function and COPD: results from the population-based UK Biobank study. *Eur. Respir. J.* 54, 1802140
28. Elbarbary, M., *et al.* (2020) Ambient air pollution, lung function and COPD: cross-sectional analysis from the WHO Study of AGEing and adult health wave 1. *BMJ Open Respir. Res.* 7
29. Lu, C., *et al.* (2022) Interaction effect of prenatal and postnatal exposure to ambient air pollution and temperature on childhood asthma. *Environ. Int.* 167, 107456
30. Wang, B., *et al.* (2021) Maternal particulate matter exposure impairs lung health and is associated with mitochondrial damage. *Antioxidants (Basel)* 10, 1029
31. Xu, L., *et al.* (2022) Short-term air pollution exposure and COVID-19 infection in the United States. *Environ. Pollut.* 292, 118369
32. Nor, N.S.M., *et al.* (2021) Particulate matter ($PM_{2.5}$) as a potential SARS-CoV-2 carrier. *Sci. Rep.* 11, 2508
33. Miyashita, L., *et al.* (2022) Underground railway particulate matter and susceptibility to pneumococcal infection. *EBioMedicine* 80, 104063
34. Calderón-Garcidueñas, L., *et al.* (2018) Alzheimer's disease and alpha-synuclein pathology in the olfactory bulbs of infants, children, teens and adults ≤40 years in Metropolitan Mexico City. APOE4 carriers at higher risk of suicide accelerate their olfactory bulb pathology. *Environ. Res.* 166, 348-362
35. Calderón-Garcidueñas, L., *et al.* (2022) Environmentally toxic solid nanoparticles in noradrenergic and dopaminergic nuclei and cerebellum of metropolitan Mexico city children and young adults with neural quadruple misfolded protein pathologies and high exposures to nano particulate matter. *Toxics* 10, 164
36. Costa, L.G., *et al.* (2020) Effects of air pollution on the nervous system and its possible role in neurodevelopmental and neurodegenerative disorders. *Pharmacol. Ther.* 210, 107523

37. Franco, P., *et al.* (2023) Short-term exposure to particulate matter and effects on emergency hospital admissions for Alzheimer's disease and Parkinson's disease: an ecological study from an aged European metropolis. *Air Qual. Atmos. Health* DOI: 10.1007/s11869-023-01359-4 (https://doi.org/10.1007/s11869-023-01359-4)
38. Wei, W., *et al.* (2019) Atmospheric PM2.5 aspiration causes tauopathy by disturbing the insulin signaling pathway. *Ecotoxicol. Environ. Saf.* 169, 301-305
39. Yuan, X., *et al.* (2022) Fine particulate matter triggers alpha-synuclein fibrillization and parkinson-like neurodegeneration. *Mov. Disord.* 37, 1817-1830
40. Milani, C., *et al.* (2018) Early evidence of stress in immortalized neurons exposed to diesel particles: the role of lipid reshaping behind oxidative stress and inflammation. *Toxicology* 409, 63-72
41. Wang, Y., *et al.* (2021) Exposure to PM2.5 aggravates Parkinson's disease via inhibition of autophagy and mitophagy pathway. *Toxicology* 456, 152770
42. Fu, H., *et al.* (2020) PM2.5 exposure induces inflammatory response in macrophages via the TLR4/COX-2/NF-kappaB pathway. *Inflammation* 43, 1948-1958
43. Li, Y., *et al.* (2020) Fine particulate matter inhibits phagocytosis of macrophages by disturbing autophagy. *FASEB J.* 34, 16716-16735
44. Zhao, C., *et al.* (2022) Respiratory exposure to PM2.5 soluble extract induced chronic lung injury by disturbing the phagocytosis function of macrophage. *Environ. Sci. Pollut. Res. Int.* 29, 13983-13997
45. Wang, X., *et al.* (2022) Selenomethionine mitigate PM2.5-induced cellular senescence in the lung via attenuating inflammatory response mediated by cGAS/STING/NF-kappaB pathway. *Ecotoxicol. Environ. Saf.* 247, 114266
46. Knox, E.G., *et al.* (2022) The blood-brain barrier in aging and neurodegeneration. *Mol. Psychiatry* 27, 2659-2673
47. Woodward, N.C., *et al.* (2017) Toll-like receptor 4 in glial inflammatory responses to air pollution in vitro and in vivo. *J Neuroinflammation* 14, 84
48. Han, B., *et al.* (2022) Atmospheric particulate matter aggravates CNS demyelination through involvement of TLR-4/NF-kB signaling and microglial activation. *eLife* 11, e72247
49. Kang, Y.J., *et al.* (2021) An air particulate pollutant induces neuroinflammation and neurodegeneration in human brain models. *Adv. Sci. (Weinh)* 8, e2101251
50. Sotty, J., *et al.* (2020) Mitochondrial alterations triggered by repeated exposure to fine PM2.5-0.18 and quasi-ultrafine PM0.18 fractions of ambient particulate matter. *Environ. Int.* 142, 105830
51. Li, Z., *et al.* (2022) Energy metabolism disorders and oxidative stress in the SH-SY5Y cells following PM2.5 air pollution exposure. *Toxicol. Lett.* 369, 25-33
52. Gao, R., *et al.* (2017) Abnormal energy metabolism and tau phosphorylation in the brains of middle-aged mice in response to atmospheric PM(2.5) exposure. *J. Environ. Sci. (China)* 62, 145-153
53. Guo, L., *et al.* (2022) Aberrant mitochondrial DNA methylation and declined pulmonary function in a population with polycyclic aromatic hydrocarbon composition in particulate matter. *Environ. Res.* 214, 113797

54. Breton, C.V., *et al.* (2019) Effects of air pollution on mitochondrial function, mitochondrial DNA methylation, and mitochondrial peptide expression. *Mitochondrion* 46, 22-29
55. Guan, L., *et al.* (2019) PM2.5 exposure induces systemic inflammation and oxidative stress in an intracranial atherosclerosis rat model. *Environ Toxicol* 34, 530-538
56. Zhang, Q., *et al.* (2018) PM2.5 impairs neurobehavior by oxidative stress and myelin sheaths injury of brain in the rat. *Environ. Pollut.* 242, 994-1001
57. Kim, S.Y., *et al.* (2018) Effects of inhaled particulate matter on the central nervous system in mice. *Neurotoxicology* 67, 169-177
58. Cattani-Cavalieri, I., *et al.* (2020) Nanodomains in cardiopulmonary disorders and the impact of air pollution. *Biochem. Soc. Trans.* 48, 799-811
59. Liu, F., *et al.* (2023) Neurotoxicity of the air-borne particles: From molecular events to human diseases. *J Hazard Mater* 457, 131827
60. Wang, H., *et al.* (2018) cAMP response element-binding protein (CREB): A possible signaling molecule link in the pathophysiology of schizophrenia. *Front. Mol. Neurosci.* 11, 255
61. McConnachie, D.J., *et al.* (2021) Ciliopathies and the Kidney: A Review. *Am. J. Kidney Dis.* 77, 410-419
62. Reisert, J. and Reingruber, J. (2019) Ca(2+)-activated Cl(-) current ensures robust and reliable signal amplification in vertebrate olfactory receptor neurons. *Proc. Natl. Acad. Sci. U. S. A.* 116, 1053-1058
63. Joskova, M., *et al.* (2020) Respiratory cilia as a therapeutic target of phosphodiesterase inhibitors. *Front. Pharmacol.* 11, 609
64. Jia, J., *et al.* (2019) Investigation of the impact of PM2.5 on the ciliary motion of human nasal epithelial cells. *Chemosphere* 233, 309-318
65. Ishii, S., *et al.* (2021) Primary cilia safeguard cortical neurons in neonatal mouse forebrain from environmental stress-induced dendritic degeneration. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2012482118
66. Kanninen, K.M., *et al.* (2020) Olfactory cell cultures to investigate health effects of air pollution exposure: Implications for neurodegeneration. *Neurochem. Int.* 136, 104729
67. Bae, J.E., *et al.* (2019) Fine particulate matter PM2.5 inhibits ciliogenesis by increasing SPRR3 expression via c-Jun activation in RPE cells and skin keratinocytes. *Sci. Rep.* 9, 3994
68. Bae, J.E., *et al.* (2019) Primary cilia mediate mitochondrial stress responses to promote dopamine neuron survival in a Parkinson's disease model. *Cell Death Dis.* 10, 952
69. Kim, H., *et al.* (2021) Spatiotemporal characterization of GPCR activity and function during endosomal trafficking pathway. *Anal. Chem.* 93, 2010-2017
70. Zheng, R., *et al.* (2022) Fine particulate matter induces childhood asthma attacks via extracellular vesicle-packaged Let-7i-5p-Mediated Modulation of the MAPK signaling pathway. *Adv. Sci. (Weinh)* 9, e2102460
71. Chahar, H.S., *et al.* (2018) Respiratory syncytial virus infection changes cargo composition of exosome released from airway epithelial cells. *Sci. Rep.* 8, 387

72. Nicholson, S., *et al.* (2022) Role of brain extracellular vesicles in air pollution-related cognitive impairment and neurodegeneration. *Environ. Res.* 204, 112316
73. Chen, X., *et al.* (2020) Urban airborne PM2.5-activated microglia mediate neurotoxicity through glutaminase-containing extracellular vesicles in olfactory bulb. *Environ. Pollut.* 264, 114716
74. Le Goff, M., *et al.* (2019) PAHs increase the production of extracellular vesicles both in vitro in endothelial cells and in vivo in urines from rats. *Environ. Pollut.* 255(Pt 1), 113171
75. Sundar, I.K., *et al.* (2019) Small RNA-sequence analysis of plasma-derived extracellular vesicle miRNAs in smokers and patients with chronic obstructive pulmonary disease as circulating biomarkers. *J Extracell Vesicles* 8, 1684816
76. Al-Rekabi, Z., *et al.* (2023) Uncovering the cytotoxic effects of air pollution with multi-modal imaging of in vitro respiratory models. *R. Soc. Open Sci.* 10, 221426
77. Ashok, A., *et al.* (2020) Towards manufacturing of human organoids. *Biotechnol. Adv.* 39, 107460
78. Goldsteen, P.A., *et al.* (2020) Advanced modeling of peripheral neuro-effector communication and -plasticity. *Physiology (Bethesda)* 35, 348-357
79. Goldsteen, P.A., *et al.* (2022) Differentiation and on axon-guidance chip culture of human pluripotent stem cell-derived peripheral cholinergic neurons for airway neurobiology studies. *Front. Pharmacol.* 13, 991072
80. Landrigan, P.J., *et al.* (2018) The Lancet Commission on pollution and health. *Lancet* 391, 462-512
81. Eriksen, M., *et al.* (2023) A growing plastic smog, now estimated to be over 170 trillion plastic particles afloat in the world's oceans-Urgent solutions required. *PLoS One* 18, e0281596
82. Ragusa, A., *et al.* (2021) Plasticenta: First evidence of microplastics in human placenta. *Environ. Int.* 146, 106274
83. Cheng, W., *et al.* (2022) Polystyrene microplastics induce hepatotoxicity and disrupt lipid metabolism in the liver organoids. *Sci. Total Environ.* 806, 150328
84. Horvatits, T., *et al.* (2022) Microplastics detected in cirrhotic liver tissue. *EBioMedicine* 82, 104147
85. Bové, H., *et al.* (2019) Ambient black carbon particles reach the fetal side of human placenta. *Nat. Commun.* 10, 3866
86. Hanrahan, J.W., *et al.* (2022) Chronic obstructive pulmonary disease and the modulation of CFTR by acute exposure to cigarette smoke. *Am J Physiol Cell Physiol* 323, C1374-C1392
87. Guo, Z., *et al.* (2021) Biotransformation modulates the penetration of metallic nanomaterials across an artificial blood–brain barrier model. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2105245118
88. Topal, G.R., *et al.* (2020) ApoE-targeting increases the transfer of solid lipid nanoparticles with donepezil cargo across a culture model of the blood-brain barrier. *Pharmaceutics* 13, 38
89. Yang, L., *et al.* (2020) The impact of $PM_{2.5}$ on the host defense of respiratory system. *Front. Cell Dev. Biol.* 8, 91

90. Tanwar, V.*, et al.* (2017) In utero particulate matter exposure produces heart failure, electrical remodeling, and epigenetic changes at adulthood. *J. Am. Heart Assoc.* 6, e005796
91. Yuan, C.S.*, et al.* (2022) Kidney damage induced by repeated fine particulate matter exposure: Effects of different components. *Sci. Total Environ.* 847, 157528
92. van Dijk, F.*, et al.* (2020) Exposure to textile microplastic fibers impairs epithelial growth. *Eur. Respir. J.* 56 (suppl 64), 1972
93. Yu, H.*, et al.* (2022) Impaired AT2 to AT1 cell transition in PM2.5-induced mouse model of chronic obstructive pulmonary disease. *Respir. Res.* 23, 70
94. Frantz, C.*, et al.* (2010) The extracellular matrix at a glance. *J. Cell Sci.* 123, 4195-4200
95. Park, J.H.*, et al.* (2023) Materials and extracellular matrix rigidity highlighted in tissue damages and diseases: Implication for biomaterials design and therapeutic targets. *Bioact. Mater.* 20, 381-403
96. Sorokin, L. (2010) The impact of the extracellular matrix on inflammation. *Nat. Rev. Immunol.* 10, 712-723
97. Ramos, C.*, et al.* (2021) Short-term exposure to wood smoke increases the expression of pro-inflammatory cytokines, gelatinases, and TIMPs in guinea pigs. *Toxics* 9, 227
98. Adivi, A.*, et al.* (2021) Exposure to traffic-generated air pollution promotes alterations in the integrity of the brain microvasculature and inflammation in female ApoE(-/-) mice. *Toxicol. Lett.* 339, 39-50
99. Wang, Z.*, et al.* (2022) Air pollution particles hijack peroxidasin to disrupt immunosurveillance and promote lung cancer. *eLife* 11, e75345
100. Shen, R.*, et al.* (2022) Accumulation of polystyrene microplastics induces liver fibrosis by activating cGAS/STING pathway. *Environ. Pollut.* 300, 118986



Chapter 3

Therapeutic potential of targeting novel signaling pathways in regulating chronic inflammation in obstructive lung disorders

Dan Li[1,2], Ana L. Manzano-Covarrubias[1,2], Kelly B.I. Douglas[1], Karim Rafie[1,2], Martina Schmidt[1,2*,ǂ]

[1]Department of Molecular Pharmacology, University of Groningen, Groningen, The Netherlands.

[2]Groningen Research Institute for Asthma and COPD (GRIAC), University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

*Corresponding author:
Prof. Dr. Martina Schmidt
Antonius Deusinglaan 1
9713 AV Groningen, The Netherlands

ǂORCID:
Martina Schmidt, orcid.org/0000-0003-3075-0630
Karim Rafie, orcid.org/0000-0003-2418-0061

Published in *Pharmacology and Therapeutics* 2026 Vol. 279
https://doi.org/ 10.1016/j.pharmthera.2026.108983

ABSTRACT

Chronic obstructive pulmonary disease (COPD) and asthma are two major obstructive lung disorders characterized by persistent airway inflammation that leads to progressive lung function decline. Although both chronic in nature, the inflammatory profiles that characterize these diseases differ significantly: COPD is predominantly driven by neutrophilic inflammation, whereas allergic asthma, a major subtype of asthma disease, is traditionally associated with eosinophilic and T helper 2 (Th2)-mediated responses. This review explores first the mechanisms underlying chronic inflammation in COPD and asthma, emphasizing thereafter the impact of bacterial and viral infections in exacerbating inflammatory responses and accelerating lung damage. Current therapeutic approaches, including the use of corticosteroids, bronchodilators, and biologics, are evaluated, highlighting their mechanisms of actions and limitations. Finally, the review focuses on novel therapeutic targets that have emerged from recent advances in (airway) inflammation research. The roles of key signaling pathways such as those involving Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), C-X-C motif chemokine receptor 2 (CXCR2), toll-like receptors (TLRs), tumor necrosis factor (TNF) signaling, P2X purinoceptor 4 (P2X4 receptor), and the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome in sustaining chronic inflammation are discussed. Understanding these pathways offers insights into the potential for development of more targeted and effective treatments. By offering a comprehensive overview of both established and (potential) novel approaches, this review aims to promote the identification and development of therapeutic strategies that could revolutionize the options for effective treatment of chronic inflammation in obstructive lung disorders.

Keywords: COPD, asthma, chronic inflammation, exacerbation, anti-inflammatory therapies

1. INTRODUCTION

Chronic obstructive pulmonary disease (COPD) and asthma are the two most prevalent chronic obstructive lung diseases worldwide, both characterized by persistent airflow obstruction and chronic inflammation. However, despite the shared clinical features, emerging research highlights fundamental differences in their underlying pathophysiology [1, 2]. COPD is primarily characterized by chronic airway inflammation, mucus hypersecretion, and alveolar wall destruction, while asthma involves airway inflammation, reversible bronchoconstriction, and airway hyperresponsiveness [3]. Despite some similarities, the cellular and molecular inflammatory mechanisms underlying these diseases differ significantly. In this review, we aim to outline our current understanding of the mechanisms that drive chronic inflammation in COPD and asthma, and discuss the therapeutic potential of targeting novel signaling pathways for the treatment of both diseases.

2. CHRONIC INFLAMMATION IN COPD AND ASTHMA

In healthy lungs, inflammation triggered by foreign particles or irritants is typically acute and self-limiting. Airway epithelial cells, specifically the ciliated and goblet cells lining the respiratory tract, which form a protective barrier via tight junctions, help to restrict airway surface exposure to irritants [4]. These cells support irritant removal through mucociliary clearance mechanisms [5, 6] and the action of resident macrophages on bronchial and alveolar surfaces [7]. Type 2 pneumocytes also secrete surfactant onto the alveolar surface, which facilitates macrophage movement and the collection of foreign debris for clearance [8]. However, when exposure to irritants becomes chronic or exaggerated, the lung's ability to resolve inflammation is impaired, leading to persistent injury and pathological changes as seen in conditions such as COPD and asthma.

2.1 Chronic inflammation in COPD

COPD is primarily associated with chronic bronchitis and emphysema, which both can contribute to irreversible airflow limitation [9]. The inflammatory response in COPD is predominantly driven by persistent exposure to irritants such as cigarette smoke, which triggers a series of pro- and anti-inflammatory immune responses.

Upon irritant exposure, activated or damaged structural lung cells, such as epithelial cells, fibroblasts, endothelial cells, and airway smooth muscle cells, release damage-associated molecular patterns (DAMPs) that engage pattern recognition receptors (PRRs) on these cell types, including toll-like receptors (TLRs) [10]. The interaction between irritants and epithelial cells, together with the activation of fibroblasts, endothelial cells, and airway smooth muscle cells initiates a cascade of chemo/cytokine storm, from both the structural cells and immune cells, such as tumor necrosis factor (TNF), interleukin (IL)-1β, IL-6, IL-12, and chemokines like IL-8, monocyte chemoattractant protein-1 (MCP-1), and chemokine (C-C motif) ligand 5 (CCL5) [9, 11]. These mediators stimulate the recruitment of immune cells to the site of irritant exposure, contributing to the chronic inflammatory environment characteristic of COPD. In addition, the activation of airway smooth muscle cells lead to airway narrowing and fixed airflow limitation—a hallmark of COPD—and the chronic inflammation of the disease [12].

Macrophages play a central role in COPD by releasing cytokines, matrix metalloproteinase (MMP) -2, -9, and -12, as well as chemokines that further promote recruitment of monocytes from the bloodstream into the lung tissue and their differentiation into macrophages. Excessive cytokine release and macrophage recruitment are key drivers of inflammation in COPD, characterized by increased numbers of activated macrophages in bronchoalveolar lavage fluid (BALF) and lung parenchyma of COPD patients [9]. Additionally, chemokines and cytokines orchestrate the recruitment of polymorphonuclear leukocytes (PMNs), such as neutrophil and eosinophils, natural killer (NK) cells, and dendritic cells (DCs) to the sites of tissue damage. Neutrophils are recruited by chemoattractants—substances released from endothelial cells and macrophages that attract leukocytes [13]. Neutrophils are also attracted by extracellular matrix (ECM) breakdown products, such as proline-glycine-proline (PGP), which are generated as a result of ongoing inflammatory activity [14]. Activated neutrophils released proteolytic enzymes—such as neutrophil elastase, cathepsin G, and MMPs—break down the structural components of the alveoli, leading to alveolar wall destruction and the development of emphysema [15]. NK cells have an elusive and controversial role in regulating immune responses in COPD, and further clinical studies are needed to clarify their

function in the disease [16]. DCs play a critical role in bridging innate and adaptive immune responses by presenting antigens to lymphocytes (CD4+ and CD8+ T cells, and B cells) [17].

Another subtype of COPD is marked by elevated eosinophils in blood and lungs (sputum, BALF, tissue) and is often associated with a higher risk of exacerbations [18]. Up to 40% of patients suffering from COPD exhibit eosinophilic airway inflammation [19]. In response to inflammatory signals triggered by environmental or immune-related stimuli, eosinophils are recruited to the lungs. Once activated, they release a variety of chemokines (*e.g.*, CCL5, -11, and -13), cytokines (*e.g.*, IL-2, -3, -4, -5, -10, -12, -13, -16, and -25), and cytotoxic granular products (*e.g.*, major basic protein, eosinophil cationic protein, eosinophil peroxidase, and eosinophil-derived neurotoxin) that amplify airway inflammation and lead to the pathogenesis of eosinophilic COPD [20].

The persistent nature of airway obstruction in COPD is reflected by a consistently reduced post-bronchodilator ratio (typically <0.7) of forced expiratory volume in 1 *s* (FEV1; maximum air a subject can forcibly expel during the first second following maximal inhalation) to forced vital capacity (FVC; the total amount of air that can be forcibly expelled from the lungs after a deep inhalation) ratio (FEV_1/FVC), reflecting irreversible airway structural changes that are driven by chronic inflammatory processes [21-24].

2.2 Chronic inflammation in asthma

Asthma—as an inflammatory disease caused by allergens, irritants, or respiratory infections—is generally characterized by airway hyperresponsiveness, reversible airway obstruction, mucus hyper-production and airway remodeling [1]. Due to the complex pathogenesis of asthma, several asthma phenotypes and endotypes have been described. Two main endotypes of asthma are type 2-high (allergic) asthma and type 2-low (non-allergic) asthma [25].

Allergic asthma is characterized by Type 2 (Th2)-driven inflammation involving Group 2 innate lymphoid cells (ILC2s), eosinophils, $CD4^+$ T cells, mast cells, and B cells [26]. Exposure to allergens such as house dust mites, pollen, or animal dander

triggers airway epithelial cells to release alarmins— (Thymic stromal lymphopoietin (TSLP), IL-33, and IL-25—which activate dendritic cells and ILC2s, initiating adaptive immune responses [27]. These cytokines stimulate IL-4, IL-5, IL-9, and IL-13 production, promoting eosinophil recruitment, mucus secretion, and airway hyperresponsiveness (AHR) [26, 28].

Activated fibroblasts differentiate into myofibroblasts, driving airway wall thickening and fibrosis [29], while airway smooth muscle cells contract excessively, contributing to bronchoconstriction [30]. Mast cells, abundant in asthmatic airways, release histamine, prostaglandins, and leukotrienes, further amplifying inflammation [27]. Th2 cells secrete IL-4, IL-5, and IL-13, stimulating Immunoglobulin E (IgE) production by B cells and activating mast cells and eosinophils, leading to goblet cell hyperplasia, mucus overproduction, and AHR [26]. IgE binding to high affinity FcεRI receptors on mast cells and basophils induces rapid mediator release upon allergen re-exposure [31, 32]. T helper 17 (Th17) cells release IL-17, promoting neutrophil recruitment, while dysregulated regulatory T cells (Tregs) fail to suppress inflammation [27]. Although allergic asthma is marked by persistent inflammation, airway obstruction is typically variable and reversible, with FEV_1/FVC ratios improving spontaneously or after treatment.

Type 2-low (non-allergic) asthma, triggered by irritants or respiratory infections, is defined by the absence of Th2 cytokine blood and sputum signatures, and has been associated with disease onset later in life (typically >40 years of age), use of high dose corticosteroids, and obesity [3]. Non-allergic asthma is less dependent on IgE but can involve neutrophils, macrophages, and Th1 or -17 cells. Th17-mediated pathways lead to the release of IL-17, which further promotes neutrophilic inflammation. This type of inflammation is often observed in more severe or difficult-to-treat asthma, and has been associated with increased levels of cytokines that favor neutrophil recruitment and/or function (e.g., IL-6 and interferon gamma (IFN-γ)) [33]. The most consistently identified pathways in type 2-low asthma patients are related to the inflammasome activation, particularly those involved in IL-1β signaling [3].

COPD and asthma subtypes and corresponding inflammatory biomarkers are summarized in Table 1. The distinct and overlapping immune responses in COPD and asthma are shown in Figure 1.

Table 1. COPD and asthma subtypes and corresponding inflammatory biomarkers

Disease	Subtypes	Biomarkers
COPD	Neutrophilic inflammation in COPD	Increased macrophages and neutrophils, chemokines (IL-8, MCP-1, CCL5), and cytokines (TNF, IL-1β, IL-6, IL-12)
	Eosinophilic inflammation in COPD	Increased eosinophils, chemokines (CCL5, -11, and -13), cytokines (IL-2, -3, -4, -5, -10, -12, -13, -16, and -25), and cytotoxic granular products
Asthma	Th2-high (allergic) asthma	Increased ILC2, eosinophils, Th2 cells, mast cells, IgE, and cytokines (IL-4, -5, -13)
	Th2-low (non-allergic) asthma	Increased neutrophils and macrophages, Th1 or -17 cells, and cytokines (IL-6 and IFN- γ)

2.3 Shared features in COPD and asthma

Despite their distinct inflammatory profiles, COPD and asthma share several clinical and pathological features. Both diseases involve chronic airway inflammation, which contributes to the development of tissue remodeling and persistent symptoms such as coughing, wheezing, and dyspnea [1, 2]. Structural cells of the airways, including epithelial cells and fibroblasts, play key roles in these processes by promoting the chronic inflammatory response through the release of cytokines, chemokines, and other mediators.

Although airway remodeling is a common feature of COPD and asthma, the nature and extent of the structural changes are different. In COPD, remodeling often includes alveolar destruction and enlargement (emphysema) due to the breakdown of ECM by neutrophil-derived proteases and MMPs. In asthma, airway remodeling is characterized by increased airway smooth muscle mass, thickened airway walls (subepithelial fibrosis), and excessive mucus production, driven primarily by eosinophils and Th2 cytokines [11]. Furthermore, both diseases involve the

activation of innate and adaptive immune responses. In COPD, DCs present antigens to CD8+ T cells, which induces the production of pro-inflammatory cytokines such as IFN-γ and TNF [9]. On the other hand, CD4+ T cells play a major role in orchestrating the Th2 response in allergic asthma [1]. In either case, chronic inflammation leads to tissue damage and impaired lung function over time.

Although asthma and COPD differ in their underlying immune pathways, they share critical features of chronic airway inflammation, epithelial barrier dysfunction, and impaired immune regulation, which together set the stage for recurrent exacerbations.

3. EXACERBATIONS IN COPD AND ASTHMA

Episodes of sudden worsening of symptoms are common in chronic obstructive lung diseases and often require a change in treatment. These exacerbations may present differently in COPD and asthma but share common triggers, including viral and/or bacterial infections and exposure to pollutants (*e.g.*, cigarette smoke and airborne particulate matter) or allergens [1, 2]. Bacterial and/or viral infections are intricately linked to both the onset and exacerbation of COPD and asthma. Pathogens are frequently detected in patients hospitalized for exacerbations and are associated with severity, especially in the case of bacterial and viral co-infection in COPD and asthma diseases [34]. Exacerbations can arise due to the acquisition of novel bacterial strains, and viral infection may facilitate subsequent bacterial outgrowth [35, 36]. The complex underlying mechanisms of exacerbations involve disruption of epithelial barrier integrity, various interactions with immune cells, and even changes in gut microbiota composition [35].

The diverse symptoms induced by infections and their consequences for patients with COPD and asthma is heavily dependent on the specific pathogen(s) involved. In the following sections, we provide a detailed outline of the responses induced by the most commonly isolated respiratory pathogens and their implications (Figure 2).

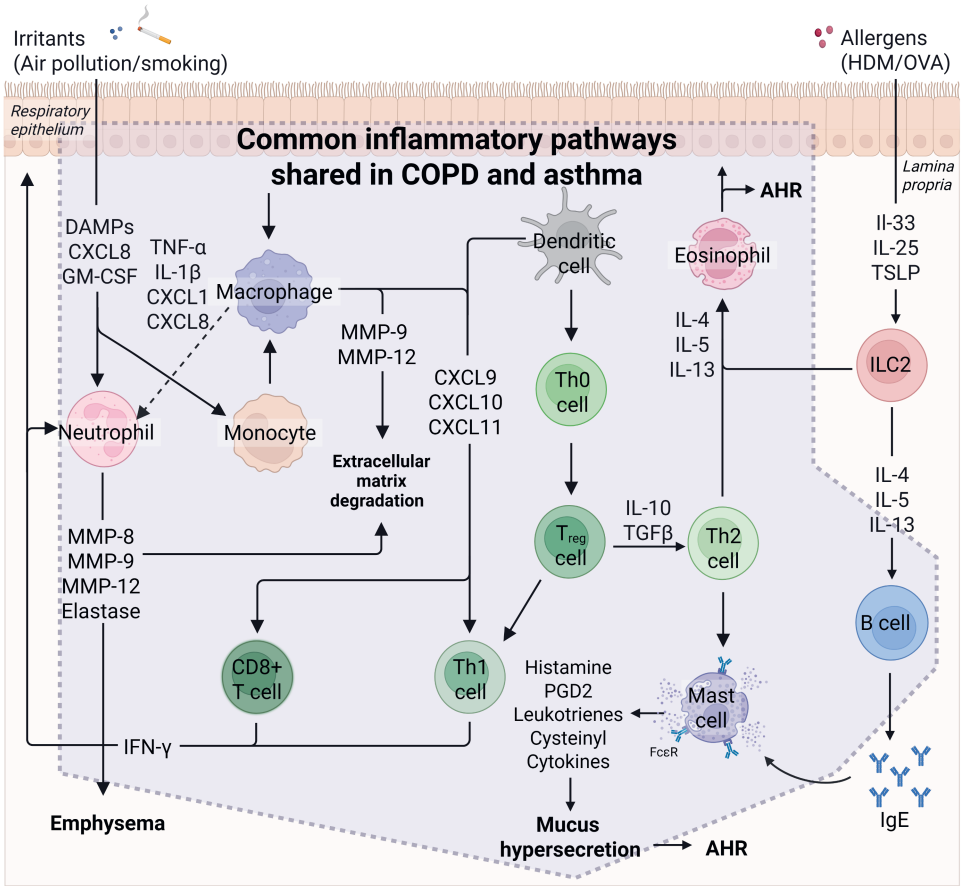


Figure 1. Distinct and overlapping immune responses in COPD and asthma. In COPD, the inflammatory response is mainly driven by neutrophils. The release of MMPs and elastase leads to emphysema. In asthma, the inflammatory response is predominantly mediated by eosinophils and Th2 cells, resulting in the release of interleukins that contribute to the development of airway hyperresponsiveness and reversible airway obstruction. The activation and infiltration of neutrophils, monocytes, macrophages, dendritic cells, eosinophils, T cells, B cells, and mast cells are overlapped in COPD and asthma (created in BioRender). Abbreviations: AHR, airway hyperresponsiveness; CXCL1, C-X-C Motif Chemokine Ligand 1; CXCL8, C-X-C Motif Chemokine Ligand 8; CXCL9, C-X-C Motif Chemokine Ligand 9; CXCL10, C-X-C Motif Chemokine Ligand 10; CXCL11, C-X-C Motif Chemokine Ligand 11; DAMPs, damage-associated molecular patterns; GM-CSF, granulocyte-macrophage colony-stimulating factor; HDM, house dust mite; IFN-γ, interferon gamma; IgE, immunoglobulin E; IL-1β, interleukin-1-beta; IL-4, interleukin-4; IL-5, interleukin-5; IL-10, interleukin-10; IL-13, interleukin-13; IL-25, interleukin-25; IL-33, interleukin-33; ILC2, type 2 innate lymphoid cells; MMP-8, matrix metalloproteinase-8; MMP-9, matrix metalloproteinase-9; MMP-12, matrix metalloproteinase-12; OVA, ovalbumin; PGD2, prostaglandin D2; TGFβ, transforming growth factor beta; TH0 cell, naive T cell; Th1 cell, type 1 T helper cell; Th2 cell, type 2 T helper cell; TNF-α, tumor necrosis factor-alpha; Treg cell, regulatory T cell; TSLP, thymic stromal lymphopoietin.

3.1 Bacterial infections and exacerbations in COPD and asthma

While previously considered sterile, recent technological advances have revealed that the healthy lung has a robust microbiome, of which the composition is altered in COPD and asthma patients compared to healthy individuals [37, 38]. Interestingly, the composition fluctuates with disease severity and during exacerbations with recent evidence suggesting a strong association between (acute) exacerbations (e.g. in COPD and asthma) and the acquisition of novel virulent bacterial strains [39]. The strongest correlations have been demonstrated for *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Moroxella catarrhalis*, and *Streptococcus pneumoniae* [34, 40, 41]. Although COPD and asthma share a very similar microbial ecology, their clinical characteristics and inflammatory profiles are rather distinct [42].

A compromised integrity of the lung epithelial barrier facilitates the colonization of the airways. In a healthy respiratory tract, goblet cells secrete mucus that traps pathogens, which enables clearance through the coordinated movement of ciliated cells [43]. In patients with COPD or asthma, effective mucociliary clearance of pathogens is impaired by mucus hypersecretion [44, 45]. Subsequently, proliferation of bacteria leads to increased bacterial load, reduced diversity, and further colonization, which collectively aggravate airway inflammation and disease progression [46, 47].

Additionally, the disruption of tight junctions in epithelial barrier and increased epithelium permeability facilitates pathogen entry. Subsequent bacterial or viral infections or a combination of both worsen tissue injury and promotes abnormal remodeling of airway wall as well as repair processes [48], thereby exacerbating and prolonging symptoms in patients with COPD and asthma [49, 50].

The pathogenicity of these bacteria, as well as their link to exacerbation severity are related to the induction of a diverse subset of inflammatory responses. These are initiated by the binding of pathogens to epithelial cell surface receptors such as intercellular adhesion molecule 1 (ICAM-1) for viruses, and platelet-activating factor (PAF) and mannose-6 phosphate receptors for bacteria [51]. Upregulation of such receptors on epithelial cells by viral and/or bacterial infections may facilitate

further infection and increase the chance of exacerbations of COPD and asthma. Notably, virus-induced upregulation of ICAM-1 and PAF promotes the adhesion of *Haemophilus influenzae* to (primary) epithelial cells [52]. Interestingly, influenza virus does not upregulate ICAM or PAF receptor expression *in vitro* but rather appears to increase bacterial adherence by a mechanism that involves the production of neuraminidase to release the new virions from the cell surface, which cleaves sialic acid residues, thereby promoting receptor exposure to facilitate bacterial entry [53].

The precise inflammatory response generated against bacteria depends on the recognition of pathogen-associated molecular patterns (PAMPs) by the PRRs expressed on airway epithelial cells [54]. Which bacterial PAMPs are presented is determined by the molecular composition of the pathogen [55]. *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Moroxella catarrhalis* are gram-negative bacteria, while *Streptococcus pneumoniae* is gram-positive. Although both gram-negative and -positive species have a plasma membrane surrounding the cytoplasm, only gram-negative bacteria exhibit a thin peptidoglycan cell wall surrounded by an outer membrane that contains lipopolysaccharide (LPS), a glycolipid that provides structural integrity and strengthens the function of the bacterial membrane [56]. Gram-positive bacteria lack an outer membrane and are enclosed by a thick peptidoglycan cell wall instead [57].

Of the various surface proteins and polysaccharides that can act as PAMPs, LPS stands out as a highly potent inducer of inflammatory responses [58-60]. Owing to its structural diversity, the precise response triggered by LPS varies greatly, which contributes to the complex relationship between lipopolysaccharides and chronic inflammatory lung diseases. As an endotoxin, LPS is primarily released following bacterial lysis and can subsequently associate with environmental particles such as (house) dust and air pollutants (*e.g.*, cigarette smoke) [61]. The ubiquitous presence of LPS in household and occupational environments has been linked to the development of COPD and asthma [62, 63]. Furthermore, bacterial infections may lead to airway injury, airway inflammation, and tissue remodeling, indicating exposure to LPS may be particularly dangerous for already vulnerable patients (*i.e.*, those with existing lung disease) [64].

Acute responses to LPS are initiated following its recognition by TLR4, expressed on immune cells [65], which also requires LPS-binding protein (LBP) and CD14 [66]. Subsequently, TLR4 triggers two (non-distinct) signaling cascades: 1) activation of interferon regulatory factors (IRFs) and the release of type 1 IFNs, which are essential for the innate immune response to bacterial infections, via TIR domain-containing adaptor molecule 1 (TICAM1/TRIF) [67]; 2) the myeloid differentiation primary response 88 (MYD88) protein- and tumor necrosis factor receptor-associated factor 6 (TRAF6) -mediated release of pro-inflammatory cytokines and chemokines [68, 69]. Other notable components of the TLR4 signaling cascade include the inhibitor of nuclear factor-κB kinase (IKK) enzyme complex and mitogen-activated protein kinases (MAPKs), which alter gene expression via the activation of NF-κB and Activator Protein-1 (AP-1), respectively [68].

TNF and CXCL8/IL-8 are two critical mediators released in response to LPS stimulation. TNF is initially generated in its transmembrane form mTNF-α and converted to its soluble form sTNF-α by specific metalloproteinases such as TNF-α-converting enzyme (TACE) [70]. TNF can exert its effects via TNFR 1 and 2, which are expressed on a wide variety of cell types, including immune cells, epithelial cells, and fibroblasts in the lungs [71, 72]. TNFR1 is mainly associated with pro-inflammatory and cell death signaling, whereas TNFR2 is predominantly involved in immunomodulation and cell survival, particularly in Tregs [73, 74]. However, the distinctions between the two receptors or ligands are not absolute: while sTNF can only bind TNFR1, mTNF can activate either receptor and both receptors can induce activation of the NF-κB pathway [75, 76]. In the lungs, TNF has been implicated in inflammation and tissue repair processes, and its increased levels have been linked to COPD and asthma [77]. Interestingly, TNF (through either TNFR) can also induce the release of CXCL8/IL-8, as such amplifying LPS-induced TLR4 signaling [78-80]. CXCL8/IL-8 can bind the CXC-receptors 1 and 2 (CXCR1/2), subsequently activating multiple signaling pathways related to epithelial cell proliferation and differentiation [81]. In addition to stimulating various pathways related to cell proliferation and differentiation, CXCL8/IL-8 promotes the recruitment and activation of neutrophils to sites of infection marked by the presence of LPS [82].

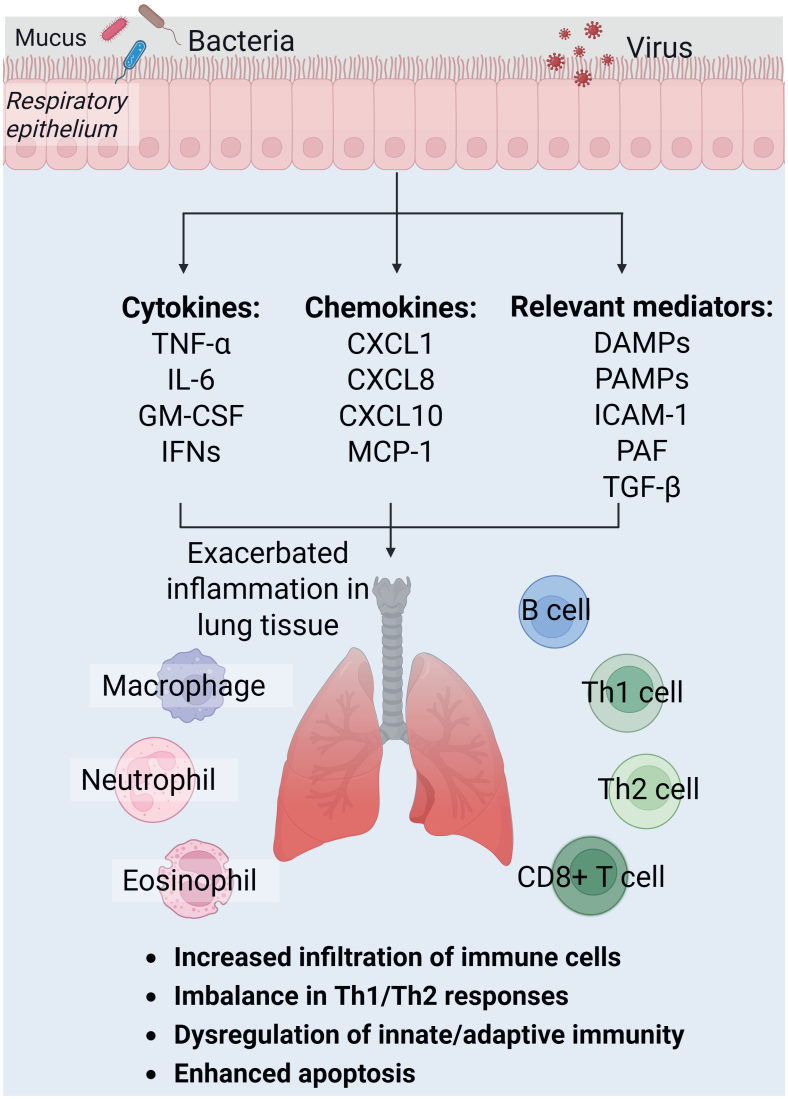


Figure 2. Immune responses related to bacterial and viral respiratory infections. Bacterial and viral infections may lead to the release of cytokines, chemokines, and other inflammatory mediators, which contribute to the activation of immune cells involved in both innate and adaptive immune responses. In addition, the immune responses in COPD and asthma diseases can be exacerbated by bacterial and/or viral infections (created in BioRender). Abbreviations: CXCL1, C-X-C Motif Chemokine Ligand 1; CXCL8, C-X-C Motif Chemokine Ligand 8; CXCL10, C-X-C Motif Chemokine Ligand 10; DAMPs, damage-associated molecular patterns; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICAM-1, Intercellular Adhesion Molecule 1; IFNs, interferons; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; PAF, platelet-activating factor; PAMPs, pathogen-associated molecular patterns; TGF-β, transforming growth factor-beta; TH1 cell, type 1 T helper cell; TH2 cell, type 2 T helper cell; TNF-α, tumor necrosis factor-alpha.

Neutrophils release various mediators that, in addition to eliminating the pathogen, contribute to tissue damage and inflammation. The importance of neutrophilic inflammation in particularly COPD (but also certain subtypes of asthma) underlines the clinical relevance of the complex interplay between bacterial infections and pulmonary diseases [13, 83].

3.2 Virus infections and exacerbations in COPD and asthma

In addition to bacteria, viruses may also induce or worsen exacerbations in chronic obstructive pulmonary diseases [84]. The most common viral culprits in COPD and asthma exacerbations are rhinovirus, influenza, and respiratory syncytial virus (RSV) [85, 86]. Despite the continuous development of vaccines, influenza infections remain the main cause of respiratory infections and are responsible for up to 650,000 deaths each year [87].

An effective immune reaction in response to viral entry requires an intact epithelial and endothelial barrier as well as a controlled innate immune response. Influenza virus readily infects epithelial cells in the upper respiratory tract following attachment to sialic acid residues present on the cell surface. The virally damaged epithelium initiates recruitment of immune cells, including neutrophils, macrophages, and monocytes, to the site of infection. Simultaneously, activation of TLR pathways and detection of viral RNA by the DNA-dependent activator of IFN regulatory factors (DAI) drive the cells to apoptosis and necroptosis, thereby increasing the release of inflammatory IL-1β and IL-8 that subsequently mediate polarization of resident immune cells toward a pro-inflammatory phenotype [88].

The virus-induced immune response in COPD patients and asthmatics causes a hyperinflammatory state that primes the epithelial barrier to further damage of tight junctions; this facilitates viral infection of the alveolar epithelium and causes lung injury that could, for instance, result in the development of acute respiratory distress syndrome (ARDS) [89]. The continued activation of TLRs, RIG-I-like receptors (RLR) and C-type lectin receptors induces the production and release of IFNs, cytokines and chemoattractants [90], including CCL2, which culminates in the recruitment of circulating monocytes and macrophage polarization towards the M1 phenotype [87]. Monocytes and macrophages are essential for the resolution of

infections by inducing cell death through the activation of Fas ligand and the TNF receptor apoptosis-inducing ligand (TRAIL) [91]. Phagocytic activity of macrophages is also important for the clearance of viral particles. This is evidenced by a study in mice showing that alveolar macrophage-depleted mice infected with H1N1 influenza virus had a higher percentage of dead cells and debris in BALF, lower oxygen levels, as well as a higher mortality rate and viral load compared to wildtype mice [92].

The release of pro-inflammatory cytokines TNF, IL-1β, IL-6, and IL-12 from epithelial cells are equally important for the activation of the innate and later the adaptive immune response in both COPD and asthma diseases. However, the release of cytokines accompanied by the cytopathic damage caused by the virus act synergistically with existing inflammatory conditions and, as such, contribute to a cytokine storm, worsening of lung pathology, and exacerbations. Targeting inflammation during viral infections drastically improves clinical outcomes. Preclinical studies in mice suggest that combined therapy of TNF inhibitors with the antiviral drug oseltamivir effectively reduces cytokines and chemokines, such as IL-1β, IL-6, CCL2, CCL5, and CXCL10, as well as viral load [93]. These findings might have implications for the treatment of COPD and asthma patients suffering from viral infections to help reduce the risk of exacerbations. Interestingly, findings suggest that epithelial cells are active participants in immune regulation, capable of storing "memory-like" information through epigenetic mechanisms [94]. However, further validation is needed to determine the therapeutic relevance of the epigenetic training response induced by rhinovirus infections. Activation of the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome is another key component of the anti-viral response. The NLRP3 inflammasome is a multiprotein complex that plays a key role in the innate immune response, particularly under conditions of inflammation [95].

It is part of the broader family of NOD-like receptors (NLRs), which are responsible for detecting harmful stimuli (*e.g.*, pathogens or cellular stress) and triggering an immune response [96]. Its deletion has been associated with a disrupted barrier function that makes the lung more susceptible to secondary bacterial infections [97]. However, as it is induced by IL-1β and IL-18, inflammasome activation has also

been linked to hyperinflammation in neutrophilic asthma and acute exacerbations in COPD [98]. Additionally, chronic viral infections, including those that are latent, may contribute to exacerbations and worsen disease severity in COPD, as the virus can persist undetected in the airways during stable phases of the disease; this represents a challenge for the treatment of viral respiratory infections and related COPD exacerbations [99].

4. CURRENT THERAPEUTIC STRATEGIES FOR THE TREATMENT OF INFLAMMATION IN COPD AND ASTHMA

4.1 Current therapeutic strategies for COPD

As outlined in GOLD 2024, the management of COPD prioritizes reducing symptoms and future risks, with the goal to improve lung function, prevent disease progression, and ultimately enhance the quality of life for those affected by the condition [21]. The management of COPD includes smoking cessation, vaccination, active lifestyle and exercise, pharmacotherapy, self-management education, and managing comorbidities. From a clinical standpoint, pharmacotherapy plays a central role in the control of COPD symptoms. Currently, the main pharmacological therapeutic interventions include bronchodilators, anti-inflammatory drugs, and combination therapy [21] (Table 2, Figure 3).

Bronchodilators play a central role in the management of COPD by (acutely) decreasing airflow obstruction and dyspnea (through induction of airway smooth muscle relaxation), and improving quality of life (Qol) [100]. Short-acting bronchodilators are recommended either as stand-alone treatment for patients only experiencing occasional symptoms or as a supplemental rescue therapy for those already on maintenance treatment. Long-acting bronchodilator therapy is the cornerstone treatment for most patients with COPD [101-103]. The two main classes of inhaled long-acting bronchodilators are long-acting β2-adrenergic agonists (LABAs) and long-acting anticholinergics (LAMAs). LABAs promote airway smooth muscle relaxation by stimulating β2-adrenergic receptors, which increases cyclic 3′,5′-adenosine monophosphate (cAMP) production and leads to muscle relaxation. Rather than inducing relaxation, LAMAs interfere with bronchoconstriction by

blocking the M3 muscarinic acetylcholine receptor, thereby preventing M3-mediated intracellular increases of Ca2+, which would otherwise trigger airway smooth muscle contraction [104, 105]. Initial therapy for patients at low risk of acute COPD exacerbations may consist of either short-acting or long-acting bronchodilator monotherapy [101]. Recently, the combination of LABA and LAMA has been recommended in symptomatic patients who do not respond satisfactorily to monotherapy, and are at risk of severe exacerbations. In cases where dual therapy (LABA/LAMA) fails to control COPD, treatment may be extended to triple therapy involving ICS/LABA/LAMA [102].

Airway inflammation plays a crucial role in the pathogenesis of COPD and represents a critical therapeutic target [106]. ICS constitute the most commonly used class of anti-inflammatory drugs for the management of COPD [107]. ICS can help to alleviate symptoms, reduce exacerbations, and improve lung function in COPD patients by interfering with the production of inflammatory mediators from both immune cells and structural cells and suppressing the activity of immune cells involved in the inflammatory response [108].

Exacerbations of COPD are generally characterized by increased breathlessness, coughing, and sputum production. Therapeutic management of these exacerbations primarily aims to relieve symptoms, improve lung function, and prevent further health deterioration [109]. In COPD patients with a history of asthma or those experiencing frequent exacerbations with blood eosinophil counts exceeding 300 cells/µl, initial maintenance therapy typically involves the combination of ICS and long-acting bronchodilators, including LABAs and LAMAs [102]. Preclinical research showed decreased sensitivity to the β_2-adrenoceptor agonist—salbutamol —after cigarette smoke (CS) or a combination of CS and influenza A virus [110]. Short-acting inhaled bronchodilators, including ipratropium, are used to reduce breathlessness during acute exacerbations of COPD (AECOPD) by reducing dynamic pulmonary hyperinflation [21]. Bronchodilators are most commonly administered via inhalation to ensure targeted delivery to the lungs [111], but can also be delivered orally (e.g., tablet) as well as via nebulization or injection. Oral or intravenous corticosteroids are prescribed to reduce airway inflammation and suppress the immune responses in severe cases

with significant (acute) distress. Systemic corticosteroids help to speed up the recovery process and reduce the severity of symptoms [112]. There is some evidence to suggest that ICS may be as effective as systemically administered corticosteroids in managing COPD exacerbations but with less side effects [113].

Table 2. Current approved treatments for inflammation in COPD and asthma

Treatments	COPD	Asthma
Bronchodilators	Long-acting β2-adrenergic agonists (LABAs) Long-acting anticholinergics (LAMAs)	LABAs LAMAs
Anti-inflammatory medications	Inhaled corticosteroids (ICS)	ICS
Antibiotics	Amoxicillin-clavulanate Doxycycline Azithromycin	
Antiviral medications		
Vaccinations	RSV vaccination Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccination Influenza vaccination COVID-19 vaccination	
Monoclonal antibodies	Dupilumab	Omalizumab Mepolizumab Benralizumab Reslizumab Dupilumab Tezepelumab
Phosphodiesterase 4 (PDE4) Inhibitors	Roflumilast	Roflumilast

Antibiotics can be prescribed in cases of suspected or confirmed bacterial infections during a COPD exacerbation. Respiratory tract infections, particularly those caused by bacteria such as *Haemophilus influenzae* or *Streptococcus pneumoniae*, can trigger, prolong, and/or worsen symptoms, which usually can be effectively counteracted by common antibiotics (*e.g.*, amoxicillin-clavulanate, doxycycline, and azithromycin) [86, 112]. However, there is a growing concern about the risk of developing antibiotic resistance with frequent or inappropriate use. Therefore, antibiotics should be used judiciously, only when there is strong suspicion/evidence of bacterial infection. In addition, antiviral drugs can also be prescribed in combination with (an)other agent(s) to target multiple pathways of the pathogen and/or host to improve therapeutic efficacy [114] (Table 2).

Vaccinations against both bacterial and viral infection, for instance, tuberculosis and influenza can be used for the prevention of infection-induced exacerbation of COPD as well as asthma [115, 116]. Viral vaccines play a crucial role in reducing exacerbations and improving QoL in patients with COPD, where viral infections—particularly influenza, RSV, and SARS-CoV-2—are common triggers of acute disease worsening [117, 118] (Table 2). Annual influenza vaccination has been consistently shown to lower the incidence of exacerbations, hospitalizations, and mortality, while also contributing to better health-related QoL by minimizing the burden of seasonal respiratory illness [119]. Similarly, pneumococcal vaccination, though targeting bacterial pathogens, helps prevent secondary infections that often follow viral insults [120]. Recently approved RSV vaccines for older adults show promise in preventing RSV-associated lower respiratory tract infections, which may help reduce COPD exacerbations, although COPD-specific outcomes are still under investigation [121, 122]. Coronavirus disease 2019 (COVID-19) vaccination has also significantly reduced virus-induced exacerbations and severe outcomes in COPD patients, improving physical activity and psychosocial well-being [123]. While no rhinovirus vaccine is currently available, emerging research on repeated rhinovirus exposure suggests future potential in targeting this common exacerbation trigger. Overall, vaccination against key respiratory viruses remains a fundamental strategy in COPD management, effectively reducing exacerbation frequency and severity while enhancing long-term stability and QoL (Table 2).

Recently approved monoclonal antibody—dupilumab—marketed as Dupixent, is applied as an add-on maintenance treatment for adults with inadequately controlled COPD and an eosinophilic phenotype. It is the first targeted therapy approved for this specific COPD population, which inhibits the signaling of IL-4 and IL-13 pathways, driving the type 2 inflammation underlying COPD [124].

4.2 Current therapeutic strategies for asthma

In a manner similar to COPD, the management of asthma primarily focuses on controlling symptoms, reducing exacerbations, and improving overall lung function and QoL [125]. Although Reddel et al. suggested that asthma management should be personalized through an ongoing process of clinical assessment, treatment adjustment, and review of the executive summary of the GINA Strategy Report 2021, there are some common medications used for asthma symptom control and risk reduction, categorized as controllers, relievers, and add-on therapies. Controllers are long-term medications that have been developed to reduce symptoms and prevent exacerbations; these include ICS, LABAs, LAMAs, and biologics such as omalizumab (anti-IgE) [2, 126]. Examples of relievers are low dose ICS-formoterol or short-acting β2 agonist (SABA) such as albuterol, which contain rapid-acting bronchodilators. Add-on therapies such as LAMA combined with ICS-formoterol or ICS-LABA (triple therapy) are predominantly intended for individuals experiencing difficult-to-treat or severe asthma with exacerbations. Assessment of the clinical and inflammatory phenotype is necessary to guide the selection of add-on treatment(s) [2].

Monoclonal antibody therapy is also used for controlling COPD and asthma. Omalizumab, as a recombinant humanized monoclonal antibody, functions by inhibiting the binding of IgE to its receptors located on mast cells and basophils. This effectively impedes the release of various allergic response mediators, including those responsible for the induction of bronchospasms (*e.g.*, histamine and specific leukotrienes). Thus, the intervention leads to a reduction in allergic inflammation and serves as a preventive measure against asthma exacerbations [127, 128]. In addition, studies of monoclonal antibodies targeting IL-5 or its

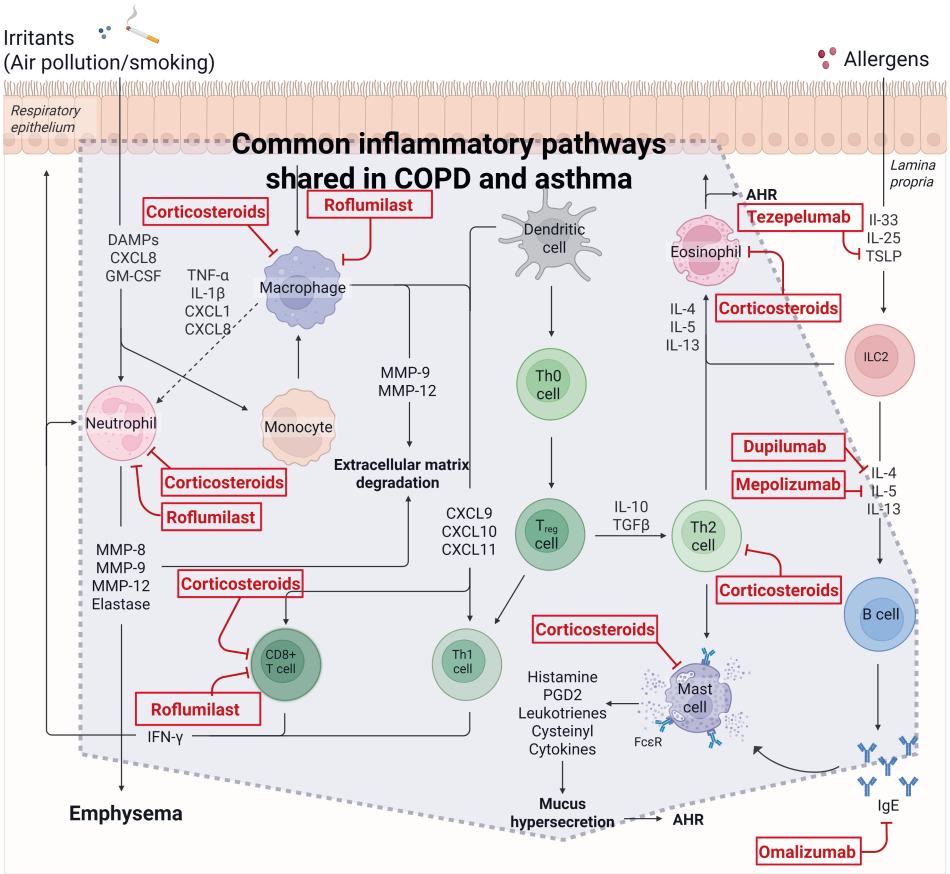


Figure 3. Current pharmacotherapeutic options for the management of chronic inflammation in COPD and asthma. Current treatments of chronic inflammation in COPD and asthma include the use of corticosteroids, monoclonal antibody therapy, and specific molecular inhibitors. Corticosteroids have main effects on reducing airway inflammation, and monoclonal antibody therapy such as omalizumab, dupilumab, and mepolizumab are antibodies used targeting on specific immunoglobulins or cytokines to reduce airway inflammation (created in BioRender). Abbreviations: AHR, airway hyperresponsiveness; CXCL1, C-X-C Motif Chemokine Ligand 1; CXCL8, C-X-C Motif Chemokine Ligand 8; CXCL9, C-X-C Motif Chemokine Ligand 9; CXCL10, C-X-C Motif Chemokine Ligand 10; CXCL11, C-X-C Motif Chemokine Ligand 11; DAMPs, damage-associated molecular patterns; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon gamma; IgE, immunoglobulin E; ILC2, type 2 innate lymphoid cells; IL-1β, interleukin-1-beta; IL-4, interleukin-4; IL-5, interleukin-5; IL-10, interleukin-10; IL-13, interleukin-13; IL-25, interleukin-25; IL-33, interleukin-33; MMP-8, matrix metalloproteinase-8; MMP-9, matrix metalloproteinase-9; MMP-12, matrix metalloproteinase-12; PGD2, prostaglandin D2; TGFβ, transforming growth factor beta; Th0 cell, naive T cell; Th1 cell, type 1 T helper cell; Th2 cell, type 2 T helper cell; TNF-α, tumor necrosis factor-alpha; Treg cell, regulatory T cell; TSLP, thymic stromal lymphopoietin.

receptor (IL-5R) suggest anti-IL5 therapies reduce asthma exacerbations and improve lung function in carefully selected (*i.e.*, type 2-high) asthmatics. Indeed, current anti-IL-5 therapies, including mepolizumab, benralizumab, and reslizumab, show long-term safety and efficacy in patients with severe eosinophilic asthma [85, 129]. IL-4 and IL-13 share the IL-4 receptor (IL-4RII), which consists of a heterodimer composed of IL-4Rα and IL-13Rα1 [130]. Similarly to anti-IL5 therapy, treatment with the anti-IL-4/IL-13 monoclonal antibody dupilumab has shown therapeutic efficacy in the management of Th2 severe asthma [131, 132]. Notably, IL-4, IL-5 and IL-13 are regulated by the transcription factor GATA-binding protein 3 (GATA3), and inhalation of GATA3 DNAzyme (SB010) diminishes both the early and late responses to inhaled allergens in individuals diagnosed with mild asthma [133]. However, further exploration regarding specificity of the antibodies is needed to better block IL-4 and IL-13 function in COPD and asthma.

Tezepelumab has been approved by the Food and Drug Administration (FDA) for the treatment of severe, uncontrolled asthma, regardless of the blood eosinophil count. Its mechanism of action involves blocking TSLP, potentially leading to reduced exacerbations and improved lung function, asthma control, and quality of life [134, 135].

The use of phosphodiesterase 4 (PDE4) inhibitors to treat COPD and asthma can be traced back to the 1980s in situations where patients failed to sufficiently respond to other bronchodilators [136]. By catalyzing the hydrolysis of cAMP, PDE4 plays a crucial role in modulating the release and activity of pro- and/or anti-inflammatory mediators as well as regulating airway smooth muscle tone. Inhibition of PDE4 has shown promise in reducing exacerbations and improving lung function in both COPD and asthma patients [137, 138]. Roflumilast is currently the only PDE4 inhibitor approved for the treatment of a subset of patients with severe COPD. In extensive clinical trials, roflumilast significantly improved lung function and reduced the frequency of exacerbations in these patients, particularly when used in conjunction with long-acting bronchodilators [139].

4.3 Differences in treatment strategies in COPD and asthma

Although COPD and asthma share overlapping clinical features such as airflow limitation and episodic breathlessness, their treatment strategies differ substantially due to distinct underlying pathophysiological mechanisms. COPD features chronic airway inflammation and remodeling leading to fixed airflow limitation, with bronchodilation serving as the principal treatment objective. Long-acting bronchodilators, either LABAs or LAMAs, are first-line treatments that improve airflow and reduce symptoms [102]. The use of ICS in COPD is more selective, typically reserved for patients with frequent exacerbations or elevated blood eosinophil counts, as routine corticosteroid use carries an increased risk of pneumonia [107]. Additional agents such as PDE4 inhibitors or macrolide antibiotics may be considered for their anti-inflammatory properties in patients with severe or refractory disease [86]. In contrast, airway inflammation is primarily eosinophilic and Th2-mediated in asthma, making ICS the cornerstone of both symptom control and exacerbation prevention [126]. Long-acting bronchodilators, including LABAs, are added when symptoms persist [2], whereas leukotriene receptor antagonists and biologic therapies targeting IgE or interleukin pathways (e.g., anti-IgE, anti–IL-5, anti–IL-4Rα) are reserved for severe or refractory cases [129, 131]. Asthma management follows a stepwise approach, with therapy adjusted according to disease control and exacerbation frequency.

4.4 Current therapeutic gaps targeting endotypes/sub-phenotypes

Current therapeutic gaps in COPD and asthma stem largely from the incomplete understanding and targeting of endotypes (biologically defined disease mechanisms) and sub-phenotypes (clinically distinct patient subsets). Despite significant progress in precision medicine, there remain challenges in matching the right therapy to the right endotype.

In COPD, unlike asthma, validated endotypes are not yet clinically actionable. Current therapies still focus on symptom control rather than targeting underlying molecular drivers. Some evidence suggests inflammatory or immune subtypes—such as eosinophilic COPD and exacerbations of COPD—may respond differently to corticosteroids or biologics, but data remain inconclusive [140, 141].

In asthma, not like COPD, the classical division into Th2-high and Th2-low endotypes has led to targeted biologics for Th2-high disease—such as anti-IgE, anti-IL5, and anti-IL4Rα therapies—but Th2-low asthma remains poorly addressed. Novel inflammatory pathways involving Th1 and Th17 cytokines are emerging, yet therapies for these mechanisms are still under development [142]. Furthermore, there is considerable overlap and plasticity between endotypes, meaning patients can shift between inflammatory states, complicating long-term treatment strategies [143]. Efforts to refine asthma sub-phenotypes—based on clinical, molecular, and transcriptomic profiles—have advanced, but heterogeneity persists, especially among pediatric and non-Th2 asthma populations. Studies underscore the need for integrating multi-omics data to develop more granular sub-phenotypes [144, 145]. Despite these insights, biomarkers that reliably guide therapy selection remain limited.

Bridging these gaps requires moving from phenotype-based classification toward integrative molecular models, validated through longitudinal and multi-omic studies. This will be crucial for developing therapies that target mechanisms rather than symptoms—bringing COPD and asthma management fully into the precision medicine era.

5. NOVEL POTENTIAL THERAPEUTIC TARGETS FOR TREATING COPD AND ASTHMA: AN OUTLOOK

Despite advances in current treatment paradigms, many patients with asthma and COPD remain symptomatic or experience frequent exacerbations, reflecting the heterogeneous inflammatory mechanisms underlying these diseases. Conventional therapies such as corticosteroids and bronchodilators provide symptomatic relief but often fail to modify disease progression. Consequently, there is a growing need to develop novel and more targeted therapeutic strategies. Our understanding of the pathophysiologies of COPD and asthma has significantly progressed since the introduction of conventional bronchodilators and anti-inflammatory agents, and various novel therapeutic targets that may drive critical disease features have been identified. In the following sections, we highlight these

signaling pathways/mediators and discuss how their modulation could have potential for the treatment of COPD and asthma (Tables 3 and 4, Figure 4).

Immunomodulatory drugs and biologics, which selectively target immune cells, inflammatory mediators and/or associated signaling pathways, are being studied and developed in an attempt to specifically modulate immune responses and effectively reduce inflammation in COPD and asthma patients [3, 134, 146, 147]. In addition, pro-resolution pathways offer an advanced therapeutic approach for treating inflammatory responses in COPD and asthma. Pro-resolution pathways are biological mechanisms that actively resolve inflammation. Unlike traditional anti-inflammatory approaches (like corticosteroids), which inhibit the production of inflammatory mediators, pro-resolution pathways restore tissue homeostasis by promoting clearance of inflammatory cells (e.g., neutrophils, eosinophils) through apoptosis and efferocytosis and stimulating tissue repair. Key mediators included as specialized pro-resolving mediators (SPMs) are lipoxins A4 (LXA4), resolvins (E-series and D-series), protectins, maresins, which are derived from polyunsaturated fatty acids (like omega-3 fatty acids) [148]. Recent studies have highlighted the role of SPMs in modulating pro-inflammatory host responses and promoting the activation of inflammation-resolution pathways in respiratory inflammatory diseases [149-152].

Novel anti-eosinophilic approaches are increasingly being used to manage Th2 inflammation and disease exacerbations. Such strategies include the use of monoclonal antibodies (*e.g.*, to block IgE, IL-5, IL-13, TSLP, or IL-33) and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) antagonists; the latter prevent the interaction between PGD2 and CRTH2, thereby inhibiting the pro-inflammatory and contractile effects of PGD2 [153]. IL-33 and TSLP are released during airway epithelial injury and act as alarmins. Because of their critical roles in the activation of mast cells, eosinophils, and ILC2s, inhibition of these cytokines may hold therapeutic promise. Current clinical data suggest that both anti-IL-33 and -TSLP antibodies exhibit an acceptable safety profile in lung patients with COPD or asthma, although further clinical validation is warranted [134]. Additionally, IL-33, but not TSLP, was shown necessary for exacerbations caused by influenza virus infection in mice, which further supports its potential as

a therapeutic target in chronic obstructive diseases, particularly during periods of viral infection [154]. However, recent clinical trials have suggested that IL-25, IL-33, and TSLP blockade either individually or combined could represent a promising therapeutic strategy for COPD [155-157]. Additionally, fevipiprant, a PGD2 receptor antagonist, reduces sputum eosinophils and improves lung function in asthma patients [85, 158].

Table 3. Novel potential therapeutic targets for treating COPD

Strategies	Targets	Therapy development stages
Immunomodulatory drugs	CRTH2 antagonists	Clinical trials (Phase IIa)
	Doxycycline	Randomised placebo-controlled trial
	MMPs inhibitors (MMP-1, MMP-9, and MMP-12)	Clinical trials (Phase IIa)
	TNF inhibitors (etanercept, infliximab, and golimumab)	Clinical trials (Phase II)
	P2X4 receptor antagonists	Preclinical research
Monoclonal antibodies	IL-25 monoclonal antibody	Preclinical research
	IL-33 monoclonal antibody	Clinical trials (Phase III)
	TSLP monoclonal antibody	Clinical trials (Phase III)
	Anti-IL-1β antibody	Clinical trials (Phase II)
Regulation of oxidative stress	Antioxidants	Preclinical research
	Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) inhibitors	Preclinical research
	Peroxidase inhibitors	Preclinical research
	Nrf2 activators	Preclinical research

Th2-low or non-Th2 disease can be neutrophilic in nature and targeted by macrolides, CXCR2 antagonists, antibodies to block IL-1, TNF, IL-17, or IL-23, inflammasome inhibitors (such as MCC950/CRID3), as well as selective p38 MAPK or PDE4 inhibitors [159]. Elevated levels of multiple chemokines, including CXCL1/ growth-regulated alpha protein (GRO-α), CXCL5/ epithelial-derived neutrophil-

activating peptide 78 (ENA-78), and CXCL8/IL-8, are observed in patients diagnosed with COPD, severe asthma, or smoking-induced asthma. These chemokines act as attractants for neutrophils by binding to the shared chemokine receptor CXCR2, which is expressed on both neutrophils and monocytes [133]. Studies with an oral CXCR2 antagonist showed attenuated sputum neutrophil counts and improvement in lung function [160]. Although anti-IL1β was proven mostly unsuccessful in patients with COPD or severe asthma, its application has shown potential effect against COPD or asthma exacerbations [161-164]. The macrolide clarithromycin was found to moderately attenuate airway hyperresponsiveness, but did not produce improvements in other secondary outcomes [165]. In a recent pre-clinical model of acute CS exposure, treatment with the monoclonal antibody CSL311—targeting the common β*c* cytokine receptor and blocking signaling by GM-CSF, IL-5, and IL-3—significantly reduced lung inflammation and injury, via attenuation of macrophage, neutrophil, and eosinophil recruitment [166]. Another preclinical research showed that IL-17A from innate lymphocytes (NK and γδ T-cells)—not classical Th17 cells—is a critical mediator of cigarette smoke-induced macrophage accumulation and inflammation. Therefore, targeting these non-conventional IL-17A sources could represent a novel therapeutic approach to dampen macrophage-driven pathology and chronic inflammation in COPD [167].

In recent years, strategies targeting specific molecular pathways implicated in COPD and asthma such as the inhibition of MMPs by doxycycline and the modulation of oxidative stress pathways have been reported [168, 169]. MMPs are enzymes involved in the breakdown of extracellular matrix components. In COPD, excessive MMP activity contributes to alveolar destruction (emphysema), fibrosis, and airway remodeling [170]. In asthma, MMPs are implicated in airway thickening, basement membrane damage, and mucus hypersecretion [169, 171]. Several MMPs, such as MMP-1, MMP-9, and MMP-12, are reported to be possible therapeutic targets in COPD and asthma [172, 173].

Table 4. Novel potential therapeutic targets for treating asthma

Strategies	Targets	Therapy development stages
Immunomodulatory drugs	CRTH2 antagonists	Clinical trials (Phase II)
	PGD2 antagonists (fevipiprant)	Clinical trials (Phase III)
	CXCR2 antagonists (macrolides)	Clinical trials (Phase II)
	Inflammasome inhibitors (MCC950/CRID3)	Clinical trials (Phase II)
	TNF inhibitors (etanercept, infliximab, and golimumab)	Clinical trials (Phase II)
	P2X4 receptor antagonists	Preclinical research
Monoclonal antibodies	IL-33 monoclonal antibody	Clinical trials (Phase IIa)
	TSLP monoclonal antibody	Clinical trials (Phase III)
	Anti-IL-1β antibody	Clinical trials (Phase II)
	Anti-TNF antibody	Clinical trials (Phase II)
	Anti-IL-17 antibody	Clinical trials (Phase II)
	Anti-IL-23 antibody	Clinical trials (Phase IIa)
Antibiotics	Clarithromycin	Clinical trials (Phase III)
Regulation of oxidative stress	Antioxidants	Observational and early-stage trials
	Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) inhibitors	Preclinical research and Clinical trials (Phase I)
	Peroxidase inhibitors	Preclinical to early translational stage
	Nrf2 activators	Preclinical research

MMPs can be activated by oxidative stress, which is a key pathological mechanism driving inflammation and tissue remodeling in both COPD and asthma [174]. Oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense system, leading to molecular damage and amplification of inflammatory cascades. In airway diseases, oxidative stress is primarily generated by activated inflammatory cells such as neutrophils, macrophages, and eosinophils, as well as structural airway cells exposed to

environmental insults like cigarette smoke and pollutants. In COPD, oxidative stress plays a dominant role by promoting chronic neutrophilic inflammation, tissue destruction, and corticosteroid resistance. In asthma, oxidative stress contributes to airway hyperresponsiveness and mucus hypersecretion by activating redox-sensitive transcription factors such as NF-κB and AP-1, which upregulate pro-inflammatory cytokines and adhesion molecules. The interplay between oxidative stress and immune dysregulation is central to both diseases. Several common strategies on reducing oxidative stress include antioxidants, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) inhibitors, peroxidase inhibitors, and nuclear factor erythroid 2–related factor 2 (Nrf2) activators [170, 175-179]. NOX are a family of enzymes that generate ROS. The NOX family includes several isoforms (NOX1–NOX5, DUOX1, and DUOX2) that are expressed in airway epithelial cells, macrophages, neutrophils, and smooth muscle cells [180]. These enzymes are major sources of ROS, which regulate inflammatory signaling pathways such as NF-κB, MAPK, and AP-1, leading to cytokine release and airway remodeling [181]. A recent study identified NOXO1, a cytosolic subunit of NOX1/NOX3 complexes, as a potential therapeutic target for emphysema; its inhibition attenuated airspace enlargement and inflammation in COPD models [182]. Besides NOX inhibition, pharmacological activation of Nrf2 has shown significant promise on oxidative stress derived persistent airway inflammation and neutrophil infiltration in COPD. Preclinical studies demonstrate that Nrf2 activators reduce airway inflammation, neutrophil infiltration, and mucus hypersecretion while protecting against cigarette smoke–induced oxidative injury [183-185]. However, more data from clinical trials is needed to validate the therapeutic functions of these strategies on treatment for COPD and asthma.

As mentioned, PDE4 inhibitors can be used to treat features of COPD and asthma. Their beneficial effects include the consequential inhibition of TNF signaling, which constitutes an active part of the inflammatory response [186]. TNF, a pleiotropic cytokine, exerts a variety of effects, including cell growth regulation, inflammation, and immunomodulation. Considering the accumulating body of (pre)clinical evidence to support a pathobiological role of TNF in COPD and asthma,

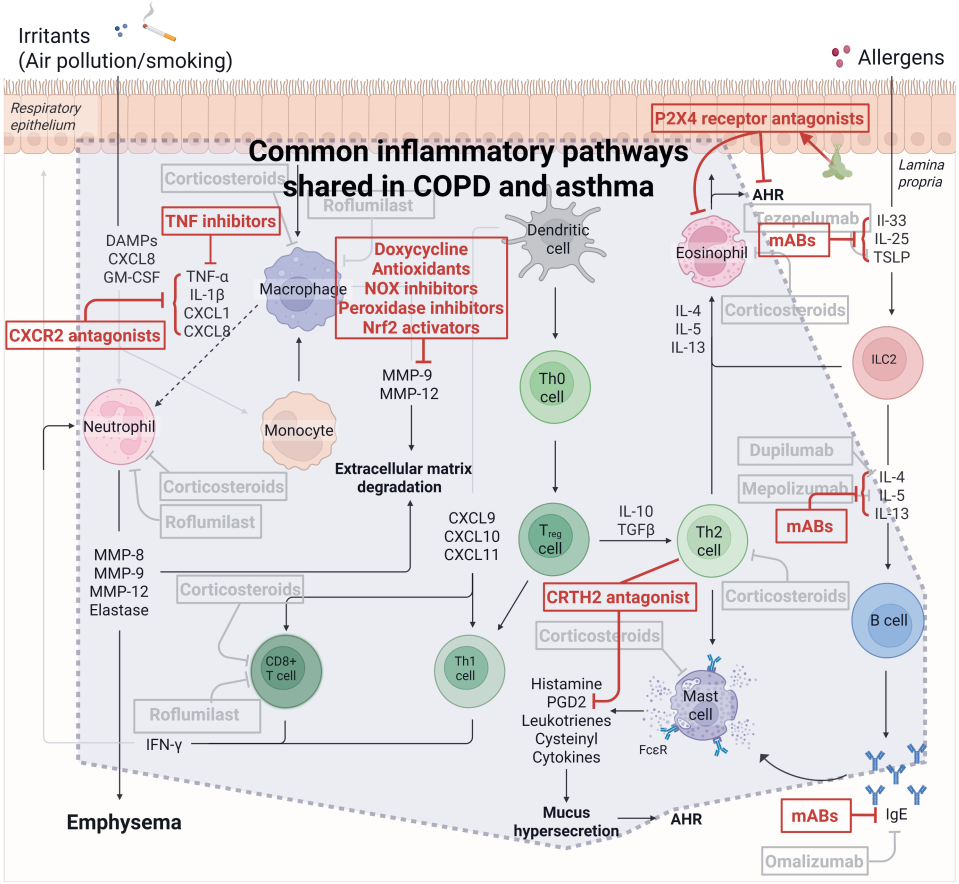


Figure 4. Novel therapeutic strategies for the treatment of chronic inflammation in COPD and asthma. Several promising novel therapeutic approaches to target chronic inflammation in COPD and/or asthma include (highlighted in red): 1) CXCR2 antagonists to block the release of cytokines and chemokines, 2) TNF inhibitors to interfere with pro-inflammatory TNF signaling pathways, 3) CRTH2 antagonists to inhibit the effects of PGD2, 4) P2X4 receptor antagonists to suppress P2X4-mediated activation of eosinophils and induction of AHR, 5) specific monoclonal antibody (mABs) therapies to block the secretion of cytokines and immunoglobulins, 6) doxycycline to inhibit specific MMPs and prevent unwanted ECM degradation (created in BioRender). Abbreviations: AHR, airway hyperresponsiveness; CRTH2, chemoattractant receptor-homologous molecule on T helper type 2 cells; CXCL1, C-X-C Motif Chemokine Ligand 1; CXCL8, C-X-C Motif Chemokine Ligand 8; CXCL9, C-X-C Motif Chemokine Ligand 9; CXCL10, C-X-C Motif Chemokine Ligand 10; CXCL11, C-X-C Motif Chemokine Ligand 11; CXCR2, C-X-C chemokine receptor 2; DAMPs, damage-associated molecular patterns; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon gamma; IgE, immunoglobulin E; ILC2, type 2 innate lymphoid cells; IL-1β, interleukin-1-beta; IL-4, interleukin-4; IL-5, interleukin-5; IL-10, interleukin-10; IL-13, interleukin-13; IL-25, interleukin-25; IL-33, interleukin-33; MMP-8, matrix metalloproteinase-8; MMP-9, matrix metalloproteinase-9; MMP-12, matrix metalloproteinase-12; P2X4, P2X purinoceptor 4; PGD2, prostaglandin D2; mABs, monoclonal antibodies; TGFβ, transforming growth factor beta; Th0 cell, naive T cell; Th1 cell, type 1 T helper cell; Th2 cell, type 2 T helper cell; TNF, tumor necrosis factor; TNF-α, tumor necrosis factor-alpha; Treg cell, regulatory T cell; TSLP, thymic stromal lymphopoietin.

TNF inhibitors such as etanercept, infliximab, and golimumab, have been explored as potential therapeutic management agents in COPD and asthma [187-190]. Recent studies showed that TNF knockdown interfered with disease progression in a rat model of COPD by inhibiting MAPK signaling and increasing the expression of Suppressor of cytokine signaling 3/ TNF receptor-associated factor (SOCS3/TRAF1) [191]. Positive clinical outcomes of treatment with TNF inhibitors such as improved lung function and relief of asthma exacerbations have been reported in a relatively small subgroup of patients with severe asthma [164, 192, 193]. In contrast, several randomized controlled trials revealed these inhibitors were generally ineffective in COPD or asthma with potential side effects [77], although one observational study indicated an association between the use of TNF inhibitors and reduced COPD hospitalizations in patients treated for rheumatoid arthritis [194]. *In vitro* organoid and co-culture studies demonstrated that TNF enhances alveolar type II (AT2) cell proliferation through an IL-1/TNFα-NF-κB signaling axis, thereby promoting lung regeneration and alveolar barrier function restoration [195, 196]. While the development of targeted therapies that modulate TNF signaling holds promise, further research is needed to validate its efficacy and safety in patients with COPD.

The P2X purinoceptor 4 (P2X4) receptor is an adenosine triphosphate (ATP)-gated ion channel that belongs to the P2X family of purinergic receptors. It is expressed in various cell types involved in inflammation (*e.g.*, epithelial cells, macrophages, dendritic cells, and T cells) and plays a role in regulating immune responses [197, 198]. During tissue injury or cellular stress, common in chronic lung diseases, ATP is released in large amounts. This extracellular ATP activates P2X4 receptors and leads to the opening of the ATP-gated ion channels, which in turn allows the influx of Ca^{2+} and Na^{+}. The activation of these receptors can trigger downstream signaling pathways that promote the production of pro-inflammatory cytokines, including IL-1β and TNF-α, which are key mediators of (chronic) airway inflammation in both COPD and asthma [199-201]. In chronic lung diseases, dysregulated P2X4 activity can contribute to persistent inflammation [202]. The P2X4 receptor is associated with lung surfactant exocytosis and alveolar fluid transport in airway epithelium [203], indicating its relevance in airway homeostasis. Elevated expression of the P2X4 receptor has been reported in asthmatics (BALF, blood monocytes and

eosinophils) and a mouse model of asthma [201]. In the latter, P2X4 antagonists efficiently alleviated eosinophilia, inflammation, mucus production, and cellular infiltration into the airways [201, 204]. Interestingly, P2X4-deficient mice also seem less prone to Th2 responses; the same was observed in mice that had received an adoptive transfer of P2X4-deficient bone marrow cells [201]. Collectively, blocking P2X4 activity may represent a promising therapeutic strategy to reduce inflammation and prevent tissue damage in asthma and other chronic lung disorders.

6. CONCLUSIONS AND PERSPECTIVES

Chronic inflammation is a major driver of progressive lung damage and increased disease severity in obstructive lung disorders such as COPD, asthma, and respiratory infection-related exacerbations. Although the underlying inflammatory pathways are complex, recent advances in our understanding of immune regulation and signaling offer promising opportunities for therapeutic intervention. In addition to interference on the receptor level, monoclonal antibodies directed against specific cytokines provide a more straightforward approach to mitigate chronic airway inflammation.

Future research focusing on the identification and validation of novel therapeutic targets within inflammation-related signaling pathways that drive the onset and progression of chronic obstructive lung diseases is warranted. Such efforts have the potential to advance the development of more personalized and effective therapies that align with the unique pathological profiles of individual patients.

ABBREVIATIONS

AECOPD	Acute exacerbations of COPD
AHR	Airway hyperresponsiveness
AP-1	Activator Protein-1
ARDS	Acute respiratory distress syndrome
AT2 cell	Alveolar type II cell
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
cAMP	Cyclic 3′,5′-adenosine monophosphate
CCL11	Chemokine (C-C motif) ligand 11
CCL13	Chemokine (C-C motif) ligand 13
CCL5	Chemokine (C-C motif) ligand 5
CCL2	Chemokine (C-C motif) ligand 2
CD4+	Cluster of differentiation 4
CD8+	Cluster of differentiation 8
CD14	Cluster of differentiation 14
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
CRID3	Cytokine Release Inhibitory Drug 3
CRTH2	Chemoattractant receptor-homologous molecule on T helper type 2 cells
CS	Cigarette smoke
CXCL1	C-X-C motif chemokine ligand 1
CXCL8	C-X-C motif chemokine ligand 8
CXCL9	C-X-C motif chemokine ligand 9
CXCL10	C-X-C motif chemokine ligand 10
CXCL11	C-X-C motif chemokine ligand 11
CXCR2	C-X-C motif chemokine receptor 2
DAI	DNA-dependent activator of IFN regulatory factors
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
ECM	Extracellular matrix
ENA-78	Epithelial-derived neutrophil-activating peptide 78
FcεRI	High affinity IgE receptor
FDA	Food and Drug Administration
FEV1	Forced Expiratory Volume in 1 second
FVC	Forced vital capacity
GATA3	GATA-binding protein 3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRO-α	Growth-regulated alpha protein
HDM	House dust mite
ICAM-1	Intercellular adhesion molecule 1
ICS	Inhaled corticosteroids
IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IKK	Inhibitor of nuclear factor-κB kinase
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-16	Interleukin-16
IL-17	Interleukin-17
IL-18	Interleukin-18
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-23	Interleukin-23
IL-25	Interleukin-25
IL-3	Interleukin-3
IL-33	Interleukin-33
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-9	Interleukin-9
IL-10	Interleukin-10
ILC2	Group 2 innate lymphoid cells
IRFs	Interferon regulatory factors
LABAs	Long-acting β2-adrenergic agonists
LAMAs	Long-acting anticholinergics
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LXA4	Lipoxins A4
MAPKs	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein-1
MMP-12	Matrix metalloproteinase-12
MMP-2	Matrix metalloproteinase-2
MMP-9	Matrix metalloproteinase-9
MYD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells

NLRP3 NOD-, LRR-, and pyrin domain-containing protein 3
NLRs NOD-like receptors
NOX Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase
Nrf2 Nuclear factor erythroid 2–related factor 2
OVA Ovalbumin
P2X4 receptor P2X purinoceptor 4
PAF Platelet-activating factor
PAMPs Pathogen-associated molecular patterns
PDE4 Phosphodiesterase 4
PGD2 Prostaglandin D2
PGP Proline-glycine-proline
PMNs Polymorphonuclear leukocytes
PRRs Pattern recognition receptors
QoL Quality of life
RLR RIG-I-like receptors
ROS Reactive oxygen species
RSV Respiratory syncytial virus
SABA Short-acting β2 agonist
SARS-CoV-2 Severe acute respiratory syndrome coronavirus 2
SOCS3 Suppressor of cytokine signaling 3
SPMs Specialized pro-resolving mediators
TACE TNF-α-converting enzyme
TGFβ Transforming growth factor beta
Th0 Naive T cells
Th1 T helper type 1 cell
Th17 T helper 17 cell
Th2 T helper type 2 cell
TICAM1 Toll/interleukin-1 receptor (TIR) domain-containing adaptor molecule 1
Treg Regulatory T cells
TLRs Toll-like receptors
TNF Tumor necrosis factor
TRAF1 TNF receptor-associated factor 1
TRAF6 Tumor necrosis factor receptor-associated factor 6
TRAIL TNF receptor apoptosis-inducing ligand
TRIF TIR domain-containing adaptor-inducing interferon-β
TSLP Thymic stromal lymphopoietin

AUTHOR CONTRIBUTIONS

Dan Li: Writing – review & editing, Writing – original draft, Project administration, Investigation, Conceptualization. Ana L. Manzano-Covarrubias: Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. Kelly B.I. Douglas: Writing – original draft, Data curation, Conceptualization. Karim Rafie: Writing – review & editing, Investigation. Martina Schmidt: Writing – review & editing, Writing – original draft, Visualization, Resources, Funding acquisition, Conceptualization.

FUNDING

D.L. is a recipient of a China Scholarship Council (201906350225) and supported by Groningen Research Institute of Pharmacy (GRIP) from the Graduate School of Science and Engineering of the University of Groningen. A.L.M.C. is a recipient of a Consejo Nacional de Humanidades, Ciencias y Tecnologías (CONAHCYT) Scholarship (2021-000022-01EXTF-00086). K.R. is a recipient of a ZonMW Off Road grant (04510242410069). M.S. received support by Alzheimer Nederland grant WE.03-2019-05, M.S. was supported by the Deutsche Forschungsgemeinschaft (IRTG1874DIAMICOM-SP2) and Novartis unrestricted grant 50199468.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

The authors would like to thank Dedmer Schaafsma for (post)editing the manuscript.

REFERENCES

1. Agustí, A., *et al.* (2023) Global Initiative for Chronic Obstructive Lung Disease 2023 Report: GOLD Executive Summary. *American Journal of Respiratory and Critical Care Medicine*
2. Reddel, H.K., *et al.* (2022) Global Initiative for Asthma Strategy 2021 Executive Summary and Rationale for Key Changes. *American Journal of Respiratory and Critical Care Medicine*
3. Hammad, H. and Lambrecht, B.N. (2021) The basic immunology of asthma. *Cell*
4. Hulbert, W.C., *et al.* (1981) Airway permeability to horseradish-peroxidase in guinea-pigs - the repair phase after injury by cigarette-smoke. *American Review of Respiratory Disease*
5. Drannik, A.G., *et al.* (2004) Impact of cigarette smoke on clearance and inflammation after Pseudomonas aeruginosa infection. *American Journal of Respiratory and Critical Care Medicine*
6. Knowles, M.R. and Boucher, R.C. (2002) Mucus clearance as a primary innate defense mechanism for mammalian airways. *Journal of Clinical Investigation*
7. Fels, A.O.S. and Cohn, Z.A. (1986) The alveolar macrophage. *Journal of Applied Physiology*
8. Macklin, C.C. (1955) Pulmonary sumps, dust accumulations, alveolar fluid and lymph vessels. *Acta Anat*
9. Barnes, P.J. (2016) Inflammatory mechanisms in patients with chronic obstructive pulmonary disease. *Journal of Allergy and Clinical Immunology*
10. Pouwels, S.D., *et al.* (2014) DAMPs activating innate and adaptive immune responses in COPD. *Mucosal Immunology*
11. Hogg, J.C. and Timens, W. (2009) The Pathology of Chronic Obstructive Pulmonary Disease. *Annual Review of Pathology-Mechanisms of Disease*
12. Yan, F.R., *et al.* (2018) Roles of airway smooth muscle dysfunction in chronic obstructive pulmonary disease. *Journal of Translational Medicine*
13. Jasper, A.E., *et al.* (2019) Understanding the role of neutrophils in chronic inflammatory airway disease. *F1000Research*
14. Patel, D.F. and Snelgrove, R.J. (2018) The multifaceted roles of the matrikine Pro-Gly-Pro in pulmonary health and disease. *European Respiratory Review*
15. Trivedi, A., *et al.* (2021) Orchestration of Neutrophil Extracellular Traps (Nets), a Unique Innate Immune Function during Chronic Obstructive Pulmonary Disease (COPD) Development. *Biomedicines*
16. Rao, Y.F., *et al.* (2021) NK Cells in the Pathogenesis of Chronic Obstructive Pulmonary Disease. *Frontiers in Immunology*
17. Xuan, S.R., *et al.* (2024) The implication of dendritic cells in lung diseases: Immunological role of toll-like receptor 4. *Genes & Diseases*
18. Bafadhel, M., *et al.* (2017) Eosinophils in COPD: just another biomarker? *Lancet Respiratory Medicine*

19. Saha, S. and Brightling, C.E. (2006) Eosinophilic airway inflammation in COPD. *International journal of chronic obstructive pulmonary disease*
20. David, B.*, et al.* (2021) Eosinophilic inflammation in COPD: from an inflammatory marker to a treatable trait. *Thorax*
21. Global Initiative for Chronic Obstructive Lung, D. (2024) Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: 2024 report. https://goldcopd.org
22. Johannessen, A.*, et al.* (2006) Post-bronchodilator spirometry reference values in adults and implications for disease management. *American Journal of Respiratory and Critical Care Medicine*
23. Langan, R.C. and Goodbred, A.J. (2020) Office Spirometry: Indications and Interpretation. *American Family Physician*
24. Matarese, A.*, et al.* (2019) Why is chronic obstructive pulmonary disease linked to atrial fibrillation? A systematic overview of the underlying mechanisms. *International Journal of Cardiology*
25. Akar-Ghibril, N.*, et al.* (2020) Allergic Endotypes and Phenotypes of Asthma. *Journal of Allergy and Clinical Immunology-in Practice*
26. Fahy, J.V. (2015) Type 2 inflammation in asthma - present in most, absent in many. *Nature Reviews Immunology*
27. Murdoch, J.R. and Lloyd, C.M. (2010) Chronic inflammation and asthma. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis*
28. Dong, B.*, et al.* (2021) Exosomes from human umbilical cord mesenchymal stem cells attenuate the inflammation of severe steroid-resistant asthma by reshaping macrophage polarization. *Stem Cell Research & Therapy*
29. Thiam, F.*, et al.* (2023) Understanding fibroblast-immune cell interactions via co-culture models and their role in asthma pathogenesis. *Frontiers in Immunology*
30. Doeing, D.C. and Solway, J. (2013) Airway smooth muscle in the pathophysiology and treatment of asthma. *Journal of Applied Physiology*
31. Schroeder, H.W. and Cavacini, L. (2010) Structure and function of immunoglobulins. *Journal of Allergy and Clinical Immunology*
32. Shamji, M.H.*, et al.* (2021) The role of allergen-specific IgE, IgG and IgA in allergic disease. *Allergy*
33. Hinks, T.S.C.*, et al.* (2021) Treatment options in type-2 low asthma. *European Respiratory Journal*
34. Wark, P.A.B.*, et al.* (2013) Viral and bacterial infection in acute asthma and chronic obstructive pulmonary disease increases the risk of readmission. *Respirology*
35. Mallia, P.*, et al.* (2012) Rhinovirus Infection Induces Degradation of Antimicrobial Peptides and Secondary Bacterial Infection in Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory and Critical Care Medicine*
36. Wilkinson, T.M.A.*, et al.* (2006) Effect of interactions between lower airway bacterial and rhinoviral infection in exacerbations of COPD. *Chest*

37. Barcik, W., *et al.* (2020) The Role of Lung and Gut Microbiota in the Pathology of Asthma. *Immunity*
38. Love, M.E. and Proud, D. (2022) Respiratory Viral and Bacterial Exacerbations of COPD-The Role of the Airway Epithelium. *Cells*
39. MacLeod, M., *et al.* (2021) Chronic obstructive pulmonary disease exacerbation fundamentals: Diagnosis, treatment, prevention and disease impact. *Respirology*
40. Iikura, M., *et al.* (2015) The Importance of Bacterial and Viral Infections Associated with Adult Asthma Exacerbations in Clinical Practice. *Plos One*
41. Sethi, S., *et al.* (2002) New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. *New England Journal of Medicine*
42. Ghebre, M.A., *et al.* (2018) Biological exacerbation clusters demonstrate asthma and chronic obstructive pulmonary disease overlap with distinct mediator and microbiome profiles. *Journal of Allergy and Clinical Immunology*
43. Whitsett, J.A. (2018) Airway Epithelial Differentiation and Mucociliary Clearance. *Annals of the American Thoracic Society*
44. Crisford, H., *et al.* (2021) Neutrophils in asthma: the good, the bad and the bacteria. *Thorax*
45. Weeks, J.R., *et al.* (2021) The Role of Non-Typeable Haemophilus influenzae Biofilms in Chronic Obstructive Pulmonary Disease. *Frontiers in Cellular and Infection Microbiology*
46. Hilty, M., *et al.* (2010) Disordered Microbial Communities in Asthmatic Airways. *Plos One*
47. Sze, M.A., *et al.* (2012) The Lung Tissue Microbiome in Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory and Critical Care Medicine*
48. Ganesan, S., *et al.* (2013) Barrier function of airway tract epithelium. *Tissue Barriers*
49. Brightling, C. and Greening, N. (2019) Airway inflammation in COPD: progress to precision medicine. *European Respiratory Journal*
50. Edwards, M.R., *et al.* (2012) The microbiology of asthma. *Nature Reviews Microbiology*
51. Invernizzi, R., *et al.* (2020) Respiratory microbiome and epithelial interactions shape immunity in the lungs. *Immunology*
52. Avadhanula, V., *et al.* (2006) Respiratory viruses augment the adhesion of bacterial pathogens to respiratory epithelium in a viral species- and cell type-dependent manner. *Journal of Virology*
53. McCullers, J.A. (2014) The co-pathogenesis of influenza viruses with bacteria in the lung. *Nature Reviews Microbiology*
54. Leiva-Juárez, M.M., *et al.* (2018) Lung epithelial cells: therapeutically inducible effectors of antimicrobial defense. *Mucosal Immunology*
55. Mitchell, J.A., *et al.* (2007) Critical role of toll-like receptors and nucleotide oligomerisation domain in the regulation of health and disease. *Journal of Endocrinology*
56. Nikaido, H. (2003) Molecular basis of bacterial outer membrane permeability revisited. *Microbiology and Molecular Biology Reviews*
57. Silhavy, T.J., *et al.* (2010) The Bacterial Cell Envelope. *Cold Spring Harbor Perspectives in Biology*

58. Hassan, F., *et al.* (2012) Moraxella catarrhalis Activates Murine Macrophages through Multiple Toll Like Receptors and Has Reduced Clearance in Lungs from TLR4 Mutant Mice. *Plos One*
59. Lam, J.S., *et al.* (2011) Genetic and functional diversity of Pseudomonas aeruginosa lipopolysaccharide. *Frontiers in Microbiology*
60. Schweda, E.K.H., *et al.* (2007) Expression and structural diversity Haemophilus influenzae:: Implication of the lipopolysaccharide of in virulence. *International Journal of Medical Microbiology*
61. Shamsollahi, H.R., *et al.* (2019) Environmental exposure to endotoxin and its health outcomes: A systematic review. *Ecotoxicology and Environmental Safety*
62. Douwes, J., *et al.* (2003) Bioaerosol health effects and exposure assessment: Progress and prospects. *Annals of Occupational Hygiene*
63. Michel, O. (2003) Role of lipopolysaccharide (LPS) in asthma and other pulmonary conditions. *Journal of Endotoxin Research*
64. Malaviya, R., *et al.* (2017) Anti-TNFα therapy in inflammatory lung diseases. *Pharmacology & Therapeutics*
65. Raetz, C.R.H. and Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annual Review of Biochemistry*
66. Tsukamoto, H., *et al.* (2018) Lipopolysaccharide (LPS)-binding protein stimulates CD14-dependent Toll-like receptor 4 internalization and LPS-induced TBK1-IKKE-IRF3 axis activation. *Journal of Biological Chemistry*
67. Tanimura, N., *et al.* (2008) Roles for LPS-dependent interaction and relocation of TLR4 and TRAM in TRIF-signaling. *Biochemical and Biophysical Research Communications*
68. Li, X.L., *et al.* (2022) Sorafenib inhibits LPS-induced inflammation by regulating Lyn-MAPK-NF-kB/AP-1 pathway and TLR4 expression. *Cell Death Discovery*
69. Verstak, B., *et al.* (2009) MyD88 Adapter-like (Mal)/TIRAP Interaction with TRAF6 Is Critical for TLR2-and TLR4-mediated NF-κB Proinflammatory Responses. *Journal of Biological Chemistry*
70. Horiuchi, T., *et al.* (2010) Transmembrane TNF-α: structure, function and interaction with anti-TNF agents. *Rheumatology*
71. Gough, P. and Myles, I.A. (2020) Tumor Necrosis Factor Receptors: Pleiotropic Signaling Complexes and Their Differential Effects. *Frontiers in Immunology*
72. Wajant, H. and Siegmund, D. (2019) TNFR1 and TNFR2 in the Control of the Life and Death Balance of Macrophages. *Frontiers in Cell and Developmental Biology*
73. Ortí-Casañ, N., *et al.* (2022) A TNF receptor 2 agonist ameliorates neuropathology and improves cognition in an Alzheimer's disease mouse model. *Proceedings of the National Academy of Sciences of the United States of America*
74. Yang, S.J., *et al.* (2018) Role of TNF-TNF Receptor 2 Signal in Regulatory T Cells and Its Therapeutic Implications. *Frontiers in Immunology*
75. Dolga, A.M., *et al.* (2008) TNF-α-mediates neuroprotection against glutamate-induced excitotoxicity via NF-κB-dependent up-regulation of KCa2.2 channels. *Journal of Neurochemistry*

76. Fischer, R. and Maier, O. (2015) Interrelation of Oxidative Stress and Inflammation in Neurodegenerative Disease: Role of TNF. *Oxidative Medicine and Cellular Longevity*
77. Matera, M.G.*, et al.* (2010) TNF-α inhibitors in asthma and COPD: We must not throw the baby out with the bath water. *Pulmonary Pharmacology & Therapeutics*
78. Gong, K.*, et al.* (2021) Tumor necrosis factor In lung cancer: Complex roles in biology and resistance to treatment. *Neoplasia*
79. He, W.*, et al.* (2013) LPS induces IL-8 expression through TLR4, MyD88, NF-kappaB and MAPK pathways in human dental pulp stem cells. *International Endodontic Journal*
80. Saperstein, S.*, et al.* (2009) IL-1β Augments TNF-α-Mediated Inflammatory Responses from Lung Epithelial Cells. *Journal of Interferon and Cytokine Research*
81. Liu, Q.*, et al.* (2016) The CXCL8-CXCR1/2 pathways in cancer. *Cytokine & Growth Factor Reviews*
82. Mukaida, N. (2003) Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases. *American Journal of Physiology-Lung Cellular and Molecular Physiology*
83. Jatakanon, A.*, et al.* (1999) Neutrophilic inflammation in severe persistent asthma. *American Journal of Respiratory and Critical Care Medicine*
84. Cookson, W.*, et al.* (2022) A Pandemic Lesson for Global Lung Diseases: Exacerbations Are Preventable. *American Journal of Respiratory and Critical Care Medicine*
85. Miller, R.L.*, et al.* (2021) Advances in asthma: New understandings of asthma's natural history, risk factors, underlying mechanisms, and clinical management. *Journal of Allergy and Clinical Immunology*
86. Viniol, C. and Vogelmeier, C.F. (2018) Exacerbations of COPD. *European Respiratory Review*
87. Li, H.N.*, et al.* (2023) Diverse roles of lung macrophages in the immune response to influenza A virus. *Frontiers in Microbiology*
88. Thapa, R.J.*, et al.* (2016) DAI Senses Influenza A Virus Genomic RNA and Activates RIPK3-Dependent Cell Death. *Cell Host & Microbe*
89. Linfield, D.T.*, et al.* (2021) Airway tight junctions as targets of viral infections. *Tissue Barriers*
90. Zhao, X.Y.*, et al.* (2020) Activation of C-Type Lectin Receptor and (RIG)-I-Like Receptors Contributes to Proinflammatory Response in Middle East Respiratory Syndrome Coronavirus-Infected Macrophages. *Journal of Infectious Diseases*
91. Ellis, G.T.*, et al.* (2015) TRAIL+ monocytes and monocyte-related cells cause lung damage and thereby increase susceptibility to influenza-Streptococcus pneumoniae coinfection. *Embo Reports*
92. Schneider, C.*, et al.* (2014) Alveolar Macrophages Are Essential for Protection from Respiratory Failure and Associated Morbidity following Influenza Virus Infection. *Plos Pathogens*
93. Pandey, P.*, et al.* (2023) Therapeutic Targeting of Inflammation and Virus Simultaneously Ameliorates Influenza Pneumonia and Protects from Morbidity and Mortality. *Viruses-Basel*

94. Risha, M.A., *et al.* (2024) Epigenetic training of human bronchial epithelium cells by repeated rhinovirus infections. *Allergy*
95. Blevins, H.M., *et al.* (2022) The NLRP3 Inflammasome Pathway: A Review of Mechanisms and Inhibitors for the Treatment of Inflammatory Diseases. *Frontiers in Aging Neuroscience*
96. Fu, J.N. and Wu, H. (2023) Structural Mechanisms of NLRP3 Inflammasome Assembly and Activation. *Annual Review of Immunology*
97. Klomp, M., *et al.* (2021) From virus to inflammation, how influenza promotes lung damage. *Journal of Leukocyte Biology*
98. Panek, I., *et al.* (2023) Inflammasome signalling pathway in the regulation of inflammation - its involvement in the development and exacerbation of asthma and chronic obstructive pulmonary disease. *Postepy Dermatologii I Alergologii*
99. Ritchie, A.I. and Wedzicha, J.A. (2020) Definition, Causes, Pathogenesis, and Consequences of Chronic Obstructive Pulmonary Disease Exacerbations. *Clinics in Chest Medicine*
100. Mahler, D.A., *et al.* (2014) Dual bronchodilation with QVA149 reduces patient-reported dyspnoea in COPD: the BLAZE study. *European Respiratory Journal*
101. Anzueto, A. and Miravitlles, M. (2018) The Role of Fixed-Dose Dual Bronchodilator Therapy in Treating COPD. *American Journal of Medicine*
102. Skolnik, N.S., *et al.* (2020) Current evidence for COPD management with dual long-acting muscarinic antagonist/long-acting β2-agonist bronchodilators. *Postgraduate Medicine*
103. Williams, D.M. and Rubin, B.K. (2018) Clinical Pharmacology of Bronchodilator Medications. *Respiratory Care*
104. Billington, C.K., *et al.* (2013) cAMP regulation of airway smooth muscle function. *Pulmonary Pharmacology & Therapeutics*
105. Gosens, R., *et al.* (2006) Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respiratory Research*
106. Yadav, A.K., *et al.* (2023) Current Perspectives on Biological Therapy for COPD. *Copd-Journal of Chronic Obstructive Pulmonary Disease*
107. Cazzola, M., *et al.* (2023) Novel Anti-Inflammatory Approaches to COPD. *International Journal of Chronic Obstructive Pulmonary Disease*
108. Lea, S., *et al.* (2023) How inhaled corticosteroids target inflammation in COPD. *European Respiratory Review*
109. Vogelmeier, C.F., *et al.* (2020) Goals of COPD treatment: Focus on symptoms and exacerbations. *Respiratory Medicine*
110. Donovan, C., *et al.* (2016) Influenza A virus infection and cigarette smoke impair bronchodilator responsiveness to β-adrenoceptor agonists in mouse lung. *Clinical Science*
111. Halpin, D.M.G., *et al.* (2017) Impact and prevention of severe exacerbations of COPD: a review of the evidence. *International Journal of Chronic Obstructive Pulmonary Disease*
112. Ko, F.W., *et al.* (2016) Acute exacerbation of COPD. *Respirology*

113. Papadopoulou, E., *et al.* (2024) Inhaled versus systemic corticosteroids for acute exacerbations of COPD: a systematic review and meta-analysis. *European Respiratory Review*
114. Batool, S., *et al.* (2023) Influenza Treatment: Limitations of Antiviral Therapy and Advantages of Drug Combination Therapy. *Microorganisms*
115. Schrager, L.K., *et al.* (2020) The status of tuberculosis vaccine development. *Lancet Infectious Diseases*
116. Sekiya, T., *et al.* (2021) Selecting and Using the Appropriate Influenza Vaccine for Each Individual. *Viruses-Basel*
117. Debbag, R., *et al.* (2025) The Impact of Vaccination on COVID-19, Influenza, and Respiratory Syncytial Virus-Related Outcomes: A Narrative Review. *Infectious Diseases and Therapy*
118. Kwok, W.C., *et al.* (2025) Vaccination in Chronic Obstructive Pulmonary Disease. *Vaccines*
119. Cheng, Y.Y., *et al.* (2020) Effects of influenza vaccination on the risk of cardiovascular and respiratory diseases and all-cause mortality. *Ageing Research Reviews*
120. Majumder, S., *et al.* (2024) A bacterial vesicle-based pneumococcal vaccine against influenza-mediated secondary Streptococcus pneumoniae pulmonary infection. *Mucosal Immunology*
121. Tanriover, M.D., *et al.* (2025) Respiratory syncytial virus (RSV) infections in adults: Current trends and recommendations for prevention - a global challenge from a local perspective. *Human Vaccines & Immunotherapeutics*
122. Wiseman, D.J., *et al.* (2024) Respiratory Syncytial Virus-related Community Chronic Obstructive Pulmonary Disease Exacerbations and Novel Diagnostics A Binational Prospective Cohort Study. *American Journal of Respiratory and Critical Care Medicine*
123. Fekete, M., *et al.* (2023) COVID-19 vaccination coverage in patients with chronic obstructive pulmonary disease - A cross-sectional study in Hungary. *Vaccine*
124. Bhatt, S.P., *et al.* (2024) Dupilumab for COPD with Blood Eosinophil Evidence of Type 2 Inflammation. *New England Journal of Medicine*
125. Chung, K.F., *et al.* (2014) International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *European Respiratory Journal*
126. Global Initiative for, A. (2024) Global strategy for asthma management and prevention.
127. Namazy, J.A., *et al.* (2020) Pregnancy outcomes in the omalizumab pregnancy registry and a disease-matched comparator cohort. *Journal of Allergy and Clinical Immunology*
128. Thomson, N.C. and Chaudhuri, R. (2012) Omalizumab: Clinical Use for the Management of Asthma. *Clinical Medicine Insights-Circulatory Respiratory and Pulmonary Medicine*
129. Farne, H.A., *et al.* (2022) Anti-IL-5 therapies for asthma. *Cochrane Database of Systematic Reviews*
130. McCormick, S.M. and Heller, N.M. (2015) Commentary: IL-4 and IL-13 receptors and signaling. *Cytokine*

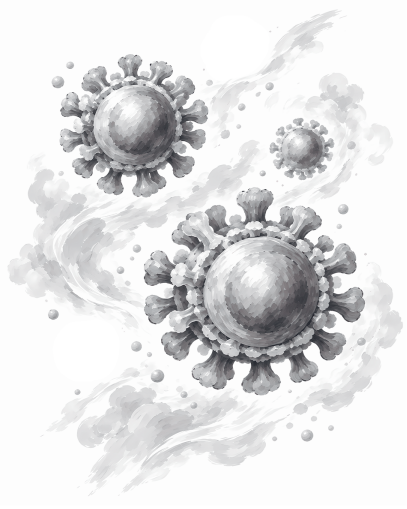
131. Portacci, A., *et al.* (2023) Severe asthma clinical remission after biologic treatment with anti-IL4/IL13: A real-life experience. *Respiratory Medicine*
132. Wenzel, S., *et al.* (2016) Dupilumab efficacy and safety in adults with uncontrolled persistent asthma despite use of medium-to-high-dose inhaled corticosteroids plus a long-acting β2 agonist: a randomised double-blind placebo-controlled pivotal phase 2b dose-ranging trial. *Lancet*
133. Barnes, P.J. (2015) Therapeutic approaches to asthma-chronic obstructive pulmonary disease overlap syndromes. *Journal of Allergy and Clinical Immunology*
134. Calderon, A.A., *et al.* (2023) Targeting interleukin-33 and thymic stromal lymphopoietin pathways for novel pulmonary therapeutics in asthma and COPD. *European Respiratory Review*
135. Menzies-Gow, A., *et al.* (2021) Tezepelumab in Adults and Adolescents with Severe, Uncontrolled Asthma. *New England Journal of Medicine*
136. Vignola, A.M. (2004) PDE4 inhibitors in COPD - a more selective approach to treatment. *Respiratory Medicine*
137. Li, G., *et al.* (2023) Advances in the development of phosphodiesterase-4 inhibitors. *European Journal of Medicinal Chemistry*
138. Spina, D. (2008) PDE4 inhibitors: current status. *British Journal of Pharmacology*
139. Phillips, J.E. (2020) Inhaled Phosphodiesterase 4 (PDE4) Inhibitors for Inflammatory Respiratory Diseases. *Frontiers in Pharmacology*
140. Bhatt, S.P., *et al.* (2023) Phenotypes, Etiotypes, and Endotypes of Exacerbations of Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory and Critical Care Medicine*
141. Garudadri, S. and Woodruff, P.G. (2018) Targeting Chronic Obstructive Pulmonary Disease Phenotypes, Endotypes, and Biomarkers. *Annals of the American Thoracic Society*
142. Polu, P.R. and Bikki, V.K. (2025) Asthma endotypes in flux: integrating type 1 and type 2 inflammation for biological therapy advancement. *Journal of Asthma*
143. Ray, A., *et al.* (2020) Are we meeting the promise of endotypes and precision medicine in asthma? *Physiological Reviews*
144. Conrad, L.A., *et al.* (2021) Defining pediatric asthma: phenotypes to endotypes and beyond. *Pediatric Research*
145. Fuhlbrigge, A.L. and Sharma, S. (2025) Unraveling the heterogeneity of asthma: Decoding subtypes of asthma. *Journal of Allergy and Clinical Immunology*
146. Barnes, P.J. (2018) Targeting cytokines to treat asthma and chronic obstructive pulmonary disease. *Nature Reviews Immunology*
147. Caramori, G., *et al.* (2016) COPD immunopathology. *Seminars in Immunopathology*
148. Bozinovski, S., *et al.* (2014) Targeting pro-resolution pathways to combat chronic inflammation in COPD. *Journal of Thoracic Disease*
149. Distefano, A., *et al.* (2024) Fish Oil Containing Pro-Resolving Mediators Enhances the Antioxidant System and Ameliorates LPS-Induced Inflammation in Human Bronchial Epithelial Cells. *Pharmaceuticals*

150. Finney, L.J., *et al.* (2025) Select Airway Specialized Proresolving Mediators Are Associated with Recovery from Nonviral Chronic Obstructive Pulmonary Disease Exacerbations. *American Journal of Respiratory and Critical Care Medicine*
151. Kytikova, O., *et al.* (2019) Pro-Resolving Lipid Mediators in the Pathophysiology of Asthma. *Medicina-Lithuania*
152. Ortega, A., *et al.* (2025) Specialized Pro-Resolving Lipid Mediators in Pulmonary Diseases: Molecular and Therapeutic Implications. *Molecules*
153. Kupczyk, M. and Kuna, P. (2017) Targeting the PGD2/CRTH2/DP1 Signaling Pathway in Asthma and Allergic Disease: Current Status and Future Perspectives. *Drugs*
154. Ravanetti, L., *et al.* (2019) IL-33 drives influenza-induced asthma exacerbations by halting innate and adaptive antiviral immunity. *Journal of Allergy and Clinical Immunology*
155. An, G., *et al.* (2020) Combined blockade of IL-25, IL-33 and TSLP mediates amplified inhibition of airway inflammation and remodelling in a murine model of asthma. *Respirology*
156. Yousuf, A., *et al.* (2019) T2 Biologics for Chronic Obstructive Pulmonary Disease. *Journal of Allergy and Clinical Immunology-in Practice*
157. Yousuf, A.J., *et al.* (2022) Astegolimab, an anti-ST2, in chronic obstructive pulmonary disease (COPD-ST2OP): a phase 2a, placebo-controlled trial. *Lancet Respiratory Medicine*
158. Brightling, C.E., *et al.* (2021) Effectiveness of fevipiprant in reducing exacerbations in patients with severe asthma (LUSTER-1 and LUSTER-2): two phase 3 randomised controlled trials. *Lancet Respiratory Medicine*
159. Khalil, B.A., *et al.* (2023) Role of inflammasome in severe, steroid-resistant asthma. *Current research in immunology*
160. Holz, O., *et al.* (2010) SCH527123, a novel CXCR2 antagonist, inhibits ozone-induced neutrophilia in healthy subjects. *European Respiratory Journal*
161. Dentener, M.A., *et al.* (2008) Effect of infliximab on local and systemic inflammation in chronic obstructive pulmonary disease: A pilot study. *Respiration*
162. Rennard, S.I., *et al.* (2007) The safety and efficacy of infliximab in moderate to severe chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine*
163. Rogliani, P., *et al.* (2015) Canakinumab for the treatment of chronic obstructive pulmonary disease. *Pulmonary Pharmacology & Therapeutics*
164. Wenzel, S.E., *et al.* (2009) A Randomized, Double-blind, Placebo-controlled Study of Tumor Necrosis Factor-α Blockade in Severe Persistent Asthma. *American Journal of Respiratory and Critical Care Medicine*
165. Sutherland, E.R., *et al.* (2010) A trial of clarithromycin for the treatment of suboptimally controlled asthma. *Journal of Allergy and Clinical Immunology*
166. Fung, N.H., *et al.* (2022) Targeting the human βc receptor inhibits inflammatory myeloid cells and lung injury caused by acute cigarette smoke exposure. *Respirology*

167. Bozinovski, S., *et al.* (2015) Innate cellular sources of interleukin-17A regulate macrophage accumulation in cigarette-smoke-induced lung inflammation in mice. *Clinical Science*
168. Cabral-Pacheco, G.A., *et al.* (2020) The Roles of Matrix Metalloproteinases and Their Inhibitors in Human Diseases. *International Journal of Molecular Sciences*
169. Lee, K.S., *et al.* (2004) Doxycycline reduces airway inflammation and hyperresponsiveness in a murine model of toluene diisocyanate-induced asthma. *Journal of Allergy and Clinical Immunology*
170. Barnes, P.J. (2022) Oxidative Stress in Chronic Obstructive Pulmonary Disease. *Antioxidants*
171. Dekkers, B.G.J., *et al.* (2021) Basement membranes in obstructive pulmonary diseases. *Matrix biology plus*
172. Dahl, R., *et al.* (2012) Effects of an oral MMP-9 and-12 inhibitor, AZD1236, on biomarkers in moderate/severe COPD: A randomised controlled trial. *Pulmonary Pharmacology & Therapeutics*
173. Li, K., *et al.* (2020) The past, present and future perspectives of matrix metalloproteinase inhibitors. *Pharmacology & Therapeutics*
174. Yao, H.W., *et al.* (2010) Extracellular superoxide dismutase protects against pulmonary emphysema by attenuating oxidative fragmentation of ECM. *Proceedings of the National Academy of Sciences of the United States of America*
175. Bernardo, I., *et al.* (2015) Targeting oxidant-dependent mechanisms for the treatment of COPD and its comorbidities. *Pharmacology & Therapeutics*
176. Churg, A., *et al.* (2012) Late Intervention with a Myeloperoxidase Inhibitor Stops Progression of Experimental Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory and Critical Care Medicine*
177. Elbatreek, M.H., *et al.* (2021) NOX Inhibitors: From Bench to Naxibs to Bedside. In *Reactive Oxygen Species* (Schmidt, H., *et al.*, eds), Springer-Verlag Berlin
178. Oostwoud, L.C., *et al.* (2016) Apocynin and ebselen reduce influenza A virus-induced lung inflammation in cigarette smoke-exposed mice. *Scientific Reports*
179. Wise, R.A., *et al.* (2016) Lack of Effect of Oral Sulforaphane Administration on Nrf2 Expression in COPD: A Randomized, Double-Blind, Placebo Controlled Trial. *Plos One*
180. Leto, T.L. and Geiszt, M. (2006) Role of Nox family NADPH oxidases in host defense. *Antioxidants & Redox Signaling*
181. Lee, I.T. and Yang, C.M. (2012) Role of NADPH oxidase/ROS in pro-inflammatory mediators-induced airway and pulmonary diseases. *Biochemical Pharmacology*
182. Seimetz, M., *et al.* (2020) NADPH oxidase subunit NOXO1 is a target for emphysema treatment in COPD. *Nature Metabolism*
183. Chan, S.M.H., *et al.* (2023) Inhibition of oxidative stress by apocynin attenuated chronic obstructive pulmonary disease progression and vascular injury by cigarette smoke exposure. *British Journal of Pharmacology*
184. Lee, J., *et al.* (2021) An Update on the Role of Nrf2 in Respiratory Disease: Molecular Mechanisms and Therapeutic Approaches. *International Journal of Molecular Sciences*

185. Mizumura, K., *et al.* (2020) Role of Nrf2 in the pathogenesis of respiratory diseases. *Respiratory Investigation*
186. Jin, J., *et al.* (2023) PDE4 Inhibitors: Profiling Hits through the Multitude of Structural Classes. *International Journal of Molecular Sciences*
187. Baker, K.E., *et al.* (2014) Novel drug targets for asthma and COPD: Lessons learned from in vitro and in vivo models. *Pulmonary Pharmacology & Therapeutics*
188. Berry, M.A., *et al.* (2006) Evidence of a role of tumor necrosis factor α in refractory asthma. *New England Journal of Medicine*
189. Durham, A.L., *et al.* (2016) Targeted anti-inflammatory therapeutics in asthma and chronic obstructive lung disease. *Translational Research*
190. Holgate, S.T., *et al.* (2011) Efficacy and safety of etanercept in moderate-to-severe asthma: a randomised, controlled trial. *European Respiratory Journal*
191. Feng, Q., *et al.* (2021) Blocking tumor necrosis factor-α delays progression of chronic obstructive pulmonary disease in rats through inhibiting MAPK signaling pathway and activating SOCS3/TRAF1. *Experimental and Therapeutic Medicine*
192. Barnes, P.J. (2012) Severe asthma: Advances in current management and future therapy. *Journal of Allergy and Clinical Immunology*
193. Rogliani, P., *et al.* (2020) Severe Asthma and Biological Therapy: When, Which, and for Whom. *Pulmonary Therapy*
194. Suissa, S., *et al.* (2008) TNF-α antagonists and the prevention of hospitalisation for chronic obstructive pulmonary disease. *Pulmonary Pharmacology & Therapeutics*
195. Cakarova, L., *et al.* (2009) Macrophage Tumor Necrosis Factor-α Induces Epithelial Expression of Granulocyte-Macrophage Colony-stimulating Factor Impact on Alveolar Epithelial Repair. *American Journal of Respiratory and Critical Care Medicine*
196. Katsura, H., *et al.* (2019) IL-1 and TNFα Contribute to the Inflammatory Niche to Enhance Alveolar Regeneration. *Stem Cell Reports*
197. Obara, K., *et al.* (2022) Effects of NP-1815-PX, a P2X4 Receptor Antagonist, on Contractions in Guinea Pig Tracheal and Bronchial Smooth Muscles. *Biological & Pharmaceutical Bulletin*
198. Paalme, V., *et al.* (2019) Human Peripheral Blood Eosinophils Express High Level of the Purinergic Receptor P2X4. *Frontiers in Immunology*
199. Bonvini, S.J., *et al.* (2020) Novel airway smooth muscle-mast cell interactions and a role for the TRPV4-ATP axis in non-atopic asthma. *European Respiratory Journal*
200. Tu, Y.M., *et al.* (2017) A high concentration of fatty acids induces TNF-α as well as NO release mediated by the P2X4 receptor, and the protective effects of puerarin in RAW264.7 cells. *Food & Function*
201. Zech, A., *et al.* (2016) P2rx4 deficiency in mice alleviates allergen-induced airway inflammation. *Oncotarget*
202. Suurväli, J., *et al.* (2017) P2X4: A fast and sensitive purinergic receptor. *Biomedical Journal*
203. Miklavc, P., *et al.* (2013) A new role for P2X4 receptors as modulators of lung surfactant secretion. *Frontiers in Cellular Neuroscience*

204. Chen, H.X., *et al.* (2016) Effect of P2X4R on airway inflammation and airway remodeling in allergic airway challenge in mice. *Molecular Medicine Reports*



Chapter 4

Respiratory viruses induce ferroptosis-like features in lung epithelial cells

Ana L. Manzano-Covarrubias[1,2], Alessandra Tosato[1], Christina HT J. Mol-van der Veen[1], Philip Ahmadyar[1], Jai Walia[1], Manon E. Woest, Jacqueline De Vries-Idema[3], Anke Huckriede[3], Amalia M. Dolga[1], Martina Schmidt[1, 2*], Karim Rafie[1,2*]

[1]Department of Molecular Pharmacology, Groningen Research Institute of Pharmacy, Faculty of Science and Engineering, University of Groningen, The Netherlands

[2]Groningen Research Institute for Asthma and COPD (GRIAC), University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

[3]Department of Medical Microbiology & Infection Prevention, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

*Correspondence to:
Karim Rafie
Antonius Deusinglaan 1
9713 AV Groningen, The Netherlands
k.rafie@rug.nl

Martina Schmidt
Antonius Deusinglaan 1
9713 AV Groningen, The Netherlands
m.schmidt@rug.nl

Published in *Microbiology Spectrum* 2026 Vol. 14 Issue 10
https://doi.org/10.1128/spectrum.01957-26

ABSTRACT

During infection, viruses can modulate various regulated cell death (RCD) mechanisms to evade host immunity and enhance replication. However, less is known about how viruses alter the recently discovered RCD ferroptosis, which is characterized by an iron-dependent accumulation of lipid peroxidation and mitochondrial fragmentation. Influenza A virus (IAV) H1N1 and human adenovirus type C5 (HAdV-C5) are two common causes of respiratory infections of the upper and lower respiratory tract and can lead to severe illness. While IAV has been shown to induce ferroptosis to support its replication, less is known whether this is also true for HAdV-C5. Here we perform a comparative study investigating ferroptosis features during IAV H1N1 and HAdV-C5 infections using alveolar (A549) and bronchial (BEAS-2B) epithelial cells. Our data reveal that HAdV-C5, similar to H1N1, induces lipid peroxidation in a time-dependent manner, which is partially suppressed in the presence of the ferroptosis inhibitor ferrostatin-1 (Fer-1). Strikingly, HAdV-C5 infections do not lead to changes in ferritin protein levels, which is responsible for iron storage. Furthermore, both H1N1 and HAdV-C5 infections trigger a profound mitochondrial network remodeling in A549, BEAS-2B, and primary human epithelial cells, comparable to that induced by the ferroptosis inducer RSL3, with effects varying by cell type and infection stage. These findings suggest that HAdV-C5, just like H1N1, can activate ferroptosis-like processes, highlighting a potential role for lipid peroxidation and mitochondrial alterations in adenoviral infection.

Keywords: Influenza A virus, human adenovirus C5, ferroptosis, mitochondria, lipid peroxidation, iron homeostasis

1. INTRODUCTION

Regulated cell death (RCD) is an important host defense mechanism during viral infections restricting viral spreading and promoting viral clearance. It is also known that viruses can modulate RCD mechanisms to their benefit to promote replication and evade the immune system response [1-3]. Different types of RCD, including apoptosis, pyroptosis, and necroptosis have been extensively studied in the context of viral infection, however, much less is known about ferroptosis, an iron-dependent form of RCD [4-6]. Ferroptosis is characterized by several distinct hallmarks. First, the iron-dependent increase of lipid peroxidation, which results from a dysfunctional System Xc- (Xc-)/Glutathione Peroxidase 4 (GPX4) antioxidant pathway, ultimately reducing the levels of the antioxidant glutathione (GSH) [7]. Second, mitochondrial fragmentation, which is characterized by the loss of cristae as well as a smaller and more compact mitochondrial morphology [4, 7, 8]. Although, the role of mitochondria in ferroptosis is not well defined, a growing body of evidence indicates that they actively participate in ferroptosis by regulating mitochondrial reactive oxygen species (mtROS) production, iron and lipid metabolism, and redox balancing in the cell [9-11]. Further, recent studies reported that viral infections can disrupt these mitochondria-linked pathways to modulate ferroptosis [5, 6, 12, 13]. However, little information is available regarding whether virus-induced ferroptosis hallmarks are responsible for any changes in mitochondrial morphology.

Influenza A virus (IAV) is an RNA virus with a negative sense, single stranded, segmented genome that recognizes and binds to sialic acids widely present on the surface of nasal and bronchial epithelial cells [14]. IAV causes annual epidemics of acute respiratory infections and was the cause of four major pandemics [15]. Being one of the few viruses in which ferroptosis has been studied, evidence shows that influenza virus modulates the iron metabolism and GPX4 antioxidant axes to induce ferroptosis for its own benefit [16]. It was reported that swine influenza virus upregulates transferrin and transferrin receptor expression to promote intracellular iron accumulation and downregulates GPX4 expression, leading to lipid peroxide production and accumulation in alveolar epithelial A549 cells [17].

Similarly, H1N1 IAV increased ferrous and total intracellular iron and caused the mitochondria of infected cells to become smaller, with less cristae and compacted mitochondrial membranes in MLE-12 mouse lung epithelial cells [18]. Furthermore, H1N1 IAV uses hemagglutinin to form ferritin-nuclear receptor coactivator 4 (NCOA4) condensates, promoting ferritinophagy to increase free intracellular iron [19] and uses transferrin receptors to enter the host to enhance its replication [20].

Human adenoviruses (HAdV) are a large group of non-enveloped, double-stranded DNA viruses most of which use the coxsackievirus and adenovirus receptor (CAR) protein present in epithelial tight junctions for viral attachment and entry [21, 22]. With over 110 types classified into seven species (A-G), HAdVs are widely spread, and are known to cause severe disease in immunocompromised patients [23, 24]. HAdV regulation of apoptosis, necrosis, and autophagy have been widely studied [25], but whether ferroptosis is a common RCD mechanism across HAdVs remains unclear. HAdV species C (HAdV-C) account for up to 10% of pediatric and up to 7% of adult respiratory infections and have been known to cause epidemics in congregate living spaces [26, 27]. HAdV-C5, is among the most common HAdVs associated with human disease, especially in immunocompromised patients [28], but whether or not it also causes ferroptosis-like features has not yet been reported. The adenovirus early region 1A gene has been reported to inhibit the expression of ferritin and sensitizing infected cells to oxidative stress [29]. Recent research on fowl adenovirus serotype 4, a virus that causes hydropericardium syndrome and liver damage in poultry, reported on the induction of ferroptosis features including elevated levels of malondialdehyde (MDA), an end product of lipid peroxidation, as well as reduced levels of GSH, reduced GPX4 activity, and a reduced expression of ferritin heavy chain (FTH1) in a chicken hepatocellular carcinoma cell line (LMH) [30]. Similar findings have been reported for HAdV D36, which caused an increase of MDA in liver tissue of embryonated chicken eggs [31] and a decrease in mitochondrial mass in a dose-dependent manner in infected human skeletal muscle cells [32].

In general, as mentioned above, research with focus on the interaction between ferroptosis and virus infections is limited. Therefore, we investigated in this study

whether HAdV-C5 infections induce ferroptosis-like features in human bronchial (BEAS-2B), alveolar (A549) lung epithelial cell lines, and primary human airway epithelial cells. As IAV are among the few viruses in which ferroptosis signaling mechanisms have been studied [6, 16, 17, 19, 20, 33], we used the H1N1 influenza virus as the model virus to determine whether HAdV-C5 is capable of activating this form of RCD. Our findings indicate that HAdV-C5 causes an increase in lipid peroxidation seemingly leaving the expression of iron storage protein ferritin unaffected. Importantly, we demonstrate changes in the mitochondrial network in lung epithelial cells caused by both H1N1 and HAdV-C5 infection.

2. MATERIALS AND METHODS

2.1 Cell culture and treatment

Adenocarcinomic human alveolar epithelial (A549) cells (ATCC CCL-185) and human bronchial epithelial (BEAS-2B) cells (ATCC CRL-3588) were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium with GlutaMAX™ Supplement (RPMI 1640 Medium, GlutaMAX™ Supplement; Cat. #61870044; Gibco, Thermo Fisher Scientific, Netherlands) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HI-FBS, Hyclone, USA) and 2% (v/v) of antibiotics (Penicillin-Streptomycin 5000 U/mL, Cat. #11528876, Gibco, Thermo Fisher Scientific, Netherlands) and kept in a humidified incubator at 37 °C and 5% (v/v) CO_2. Cells were detached with trypsin-EDTA (Cat#L0930, Biowest) and seeded in appropriate cell culture plates. During treatment cells were maintained in starvation media (RPMI-1640 medium with GlutaMAX™ Supplement with 1% (v/v) HI-FBS and 2% (v/v) antibiotics) [34, 35].

RAS-selective lethal small molecule 3 (RSL3, #S8155, Selleckchem, USA) at a concentration of 10 μM for A549 and 250 nM for BEAS-2B was used to trigger ferroptosis, unless otherwise indicated. Ferrostatin-1 (Fer-1; #SML0583, Sigma-Aldrich) was used at 5 μM as a ferroptosis inhibitor [19, 36].

Staurosporine (Cat. #15495139, Thermo Scientific Alfa Aesar, The Netherlands) was used at a concentration of 50 nM for A549 cells and at 5 nM for BEAS-2B cells as apoptosis inducer. Pan-caspase inhibitors Q-VD-Oph (#1135695-98-5, MP

Biomedicals, USA) and Z-VAD-FMK (Cat. #S7023, Selleckchem, The Netherlands) were used at a concentration of 10 μM and caspase-3 inhibitor Z-DEVD-FMK (Cat. #FMK004, R&D Systems, The Netherlands) was used at concentrations of 10, 50, and 100 μM.

2.2 Human airway epithelial cells

Primary human airway epithelial cells (hAECs) were obtained from healthy lung transplant donors *post-mortem* within 1-8 h after lung donation. Donor selection followed the Eurotransplant guidelines, requiring the absence of primary lung diseases such as asthma or COPD, and limiting smoking history to no more than 20 pack years. Tissue was collected in Krebs–Henseleit-buffer (composition in mM: 117.5 NaCl, 5.6 KCl, 1.18 MgSO4, 2.5 CaCl2, 1.28 NaH2PO4, 25 NaHCO3 and 5.5 glucose) [37]. Airway epithelial cells were isolated by enzymatic digestion then scraped from residual tracheal and main stem bronchial tissue, and cultured as previously described [38, 39]. Briefly, cells were grown in pre-coated flasks (fibronectin (10 ug/mL), bovine type I collagen (30 ug/mL) and bovine serum albumin (10 ug/mL) in phosphate buffered saline (PBS)) [40] and cultured in Keratinocyte Serum Free Medium (KSFM) medium with Bovine Pituitary Extract and Epidermal Growth Factor (Cat. #17005042, Invitrogen), supplemented with isoproterenol 1 μM (#I6504, Sigma-Aldrich) and maintained at 37 °C and 5% (v/v) CO_2 until confluency. During experiments, submerged cells were cultured in bronchial epithelial cell growth medium (BEGM, Cat. #CC-3170, Lonza) and treated with 1 μM of RSL3 or infected with viruses as described below.

Human lung tissue was obtained from lung transplant donors in strict adherence to the Research Code 138 of the UMCG, as stated on https:// umcgresearch.org/w/research-code-umcg as well as national ethical 139 and professional guidelines, including the Code of Conduct for Health Research and Declaration of 140 Helsinki. The use of left-over lung tissue in this study was not subject to the Medical Research Human 141 Subjects Act in the Netherlands, as confirmed by a statement of the Medical Ethical Committee of the 142 UMCG, and therefore exempt from consent according to national laws (Dutch laws: Medical Treatment 143 Agreement Act (WGBO) art 458 / GDPR art 9/ UAVG art 24). Human

lung tissue was acquired from 144 extra tissue left over after lung surgeries, which exceed the amount needed for clinical care purposes. 145 All samples and clinical information were blinded before experiments.

2.3 Viruses and infection

Influenza virus strain A/Puerto Rico/8/34 (H1N1/PR8) was kindly provided by Prof. Dr. Anke Huckriede (Department of Medical Microbiology, University of Groningen. Netherlands). The virus was propagated in embryonated chicken eggs, purified by sucrose density gradient centrifugation, and titrated on MDCK cells as described [41]. Human adenovirus type 5 (HAdV-C5) was purchased from The Gene Vector Core at the Baylor College of Medicine. Virus stocks were prepared by amplification in A549 cells followed by titre determination by plaque assay in A549 cells [42].

At 80% confluence, cells were infected with H1N1 or HAdV-C5 at multiplicity of infection of 1.0 (MOI 1) in serum-free medium (RPMI-1640 medium with GlutaMAX™ Supplement with 2% (v/v) antibiotics for A549 and BEAS-2B and BEGM medium for hAECs) and incubated for one hour with H1N1 and two hours with HAdV-C5 to allow the adsorption of the virus, then cells were washed three times with PBS and starvation medium was added. Infected cells were incubated for the desired time in a humidified incubator at 37 °C and 5% (v/v) CO_2.

2.4 Immunofluorescence staining of viral proteins

A549 and BEAS-2B cells were seeded at 20,000 cells/well onto 48-well plates and infected with influenza virus (H1N1/PR8) virus as described above. After 0, 4, 6, 8, 18 and 24 hours post infection (hpi) cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) solution at room temperature (RT) for 15 min. After washing three more times with PBS, cells were incubated overnight at 4 °C in blocking buffer (0.1% (w/v) saponine, 1% (w/v) bovine serum albumin in PBS). On the following day, cells were stained with primary antibody against influenza A nucleoprotein (NP) (Cat. #MAB8257, Millipore, 1:750 in blocking buffer) for 1 h at RT. Following incubation cells were washed three times with blocking buffer and then incubated with Alexa Fluor 568-conjugated donkey anti-mouse IgG (H+L) secondary antibody (Cat. #A10037, Invitrogen) for 45 min at RT [43].

A549 and BEAS-2B cells were seeded at 20,000 cells/well onto 48-well plates and infected with HAdV-C5 virus as described above. After 0, 24, 48, 72, and 96 hpi cells were washed with PBS, fixed with ice-cold methanol, and incubated at -20 °C for 10 min. Methanol was removed and primary antibody against adenovirus hexon protein (Cat. #MAB8052, Millipore, 1:500 in PBS) was added and incubated for 45 min at RT. After incubation cells were washed three times with PBS and incubated with Alexa Fluor 568-conjugated donkey anti-mouse IgG (H+L) secondary antibody (Cat. #A10037, Invitrogen) for 30 min at RT [44].

After incubation with secondary antibody, cells were washed three times with PBS and nuclei were stained with DAPI (Cat. #ab104139, Abcam). Fluorescence was detected on a Nikon Inverted Research Fluorescence Microscope ECLIPSE Ti2-E at constant exposure of 100 ms. At least 5 images of each well were captured at a nominal magnification of 20x. H1N1 and HAdV-C5 infected cells were quantified by counting nucleoprotein positive (NP+) cells and hexon protein positive (Hexon+) cells, respectively, and DAPI positive (DAPI+) cells using ImageJ [45]. Results are shown as percentage of NP+/DAPI+ or Hexon+/DAPI+ cells.

2.5 Cell morphology

After the desired time post infection or 24 hour treatment with RSL3, cells were washed with PBS and fixed with 4% PFA solution at RT for 15 min. Cells were washed three more times with PBS and imaged using a Nikon Eclipse Ti-S microscope at a nominal magnification of 10x.

2.6 Cell metabolic activity assay

Cell metabolic activity was measured using the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (#M5655, Sigma-Aldrich, Netherlands). A549 cells were seeded at 9,000 cells/well and BEAS-2B cells at 10,000 cells/well onto a 96-well plate. After treatment time, MTT solution was added at a final concentration of 0.5 mg/mL and incubated for 1 h at 37 °C and 5% (v/v) CO_2. The MTT solution was completely removed and to dissolve the formed formazan 70 µL dimethyl sulfoxide (DMSO) was added and incubated for 45 min at 37 °C at a constant shaking rate of 120 rpm. Lastly, absorbance was measured at wavelengths 570 and 630 nm using a Synergy™ H1 Multi-Mode Reader (BioTek, USA) [46].

2.7 Cell death assay

Cell death was determined by staining the cells with propidium iodide (PI) and Hoechst 33258. A549 and BEAS-2B cells were seeded at 20,000 cells/well onto 48-well plates and infected with influenza virus (H1N1/PR8), HAdV-C5 virus, or treated with RSL3 as described above. At the indicated times post infection, PI (PI; Cat. #V13242; Fisher Scientific, Landsmeer, the Netherlands) and Hoechst 33258 (Cat. #H1398; Invitrogen, the Netherlands) were added to each well at a final concentration of 1 µM and 1 µg/mL, respectively. Cells were incubated at 37 °C and 5% (v/v) CO_2 for 10 min and then imaged using a Nikon Inverted Research Fluorescence Microscope ECLIPSE Ti2-E at a nominal magnification of 20x. The number of PI+ and Hoechst+ cells was quantified using Fiji [47]. The average number of $Hoechst^+$ cells in the control group was calculated ($\overline{Hoechst^+_{Control}}$), then for each image the percentage of cell death relative to control was calculated the following way:

$$\frac{(\overline{Hoechst^+_{Control}} - Hoechst^+_{Treatment}) + PI^+_{Treatment}}{\overline{Hoechst^+_{Control}}}$$

Ten images per condition were analyzed.

2.8 Lipid peroxidation

Lipid peroxidation was determined using BODIPY 581/591 C11 Lipid Peroxidation Sensor (Cat. #D3861, Fisher Scientific, Landsmeer, the Netherlands) [46, 48, 49]. A549 and BEAS-2B cells were seeded at 20,000 cells/well onto 48-well plates and infected with IAV (H1N1/PR8) or HAdV-C5 or treated with RSL3 as described above. RSL3-treated samples served as a positive control and were analyzed only after 24 h treatment, which was performed concurrently with both H1N1 and HAdV-C5 samples at 24 hpi; therefore, the RSL3 data presented is the same for both viruses. At the indicated times post infection, BODIPY 581/591 C11 and Hoechst 33258 dye were added to each well at a final concentration of 1.5 µM and 1 µg/mL, respectively. Cells were incubated at 37 °C and 5% (v/v) CO_2 for 15 min and then imaged using a Nikon Inverted Research Fluorescence Microscope ECLIPSE Ti2-E at a nominal magnification of 20x. Ten images per condition per biological replicate were taken and analyzed using Fiji [47].

2.9 Malondialdehyde assay

Malondialdehyde (MDA) was measured as a marker of lipid peroxidation by the thiobarbituric acid reactive substances (TBARS) assay [46, 50]. A549 cells were seeded at 275,000 cells/well and BEAS-2B cells at 320,000 cells/well onto a 6-well plate. Infection was done as described above. Fer-1 treatment was added one hour prior to infection and removed at 0 hpi. RSL3 treatment was added at the same time of infection. At the indicated times post-infection cells were washed three times with PBS and collected by scraping in ice-cold water. Cells were lysed by sonication and freeze-thaw cycles for a total of three times. 50 µL of cell lysate was mixed with 100 µL ice-cold 10% trichloroacetic acid (TCA, Cat.#1.00807, Merck) and incubated for 15 min on ice. Samples were centrifuged for 15 min at 5,000x g at 4 °C and 100 µL of supernatant was mixed with an equal volume of 0.75% 2-thiobarbituric acid (TBA, Cat. #T550, Merck). An MDA (1,1,3,3-tetramethoxypropane, Cat.#108383, Merck) standard curve (0-500 µM) was prepared the same way. Samples and standards were immediately boiled at 95 °C for 15 min then allowed to cool to RT. Fluorescence was measured at $\lambda_{Ex}/\lambda_{Em}$ = 533/559 nm using a BioRad CFX96 Real-Time PCR System. Total protein concentration of samples was measured using the Pierce BCA Protein Assay Kit (Cat. #23335, Thermo Scientific). MDA concentrations in samples were calculated from the MDA standard curve and normalized by their respective total protein concentration.

2.10 Western blotting

A549 cells were seeded at 275,000 cells/well and BEAS-2B cells at 320,000 cells/well onto a 6-well plate. Infection was done as described above. Samples were collected at the indicated times post-infection in lysis buffer (0.25 M D-Manitol, 0.05 M Tris-Base, 1 mM EDTA, 1mM EGTA, pH 7.8 and a mixture of protease inhibitors: 1 mM Na3VO4, 1 mM NaF, 10 µg/mL leupetin, 10 µg/mL pepstatin A, 10 µg/mL apoprotein and 1 mM DTT). Total protein was quantified by Pierce BCA Protein Assay Kit (Cat. #23335, Thermo Scientific). Equal amounts of protein were loaded and separated by 12% SDS polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and blocked with 5% milk. Subsequently, membranes were incubated overnight at 4 °C with primary antibodies against ferritin light chain (FTL) (1:1000, Cat. #ab69090, Abcam) and GAPDH (1:3000, Cat. # sc-47724, Santa Cruz Technology)

in 1% milk. Membranes were washed thoroughly and incubated with horse-radish peroxidase-conjugated secondary antibodies (anti-rabbit IgG, 1:3000; anti-mouse IgG, 1:3000, respectively) at RT for 1 h. Antibody-labeled proteins were detected using a Western Lightning ECL-plus Chemiluminescent Substrate detection kit (Cat. #50-904-9326, FisherScientific). Band intensity was quantified using Image Lab Software Version 6.1 (https://www.bio-rad.com/en-nl/product/image-lab-software?ID=KRE6P5E8Z). Expression of ferritin was normalized to GAPDH.

2.11 Mitochondrial staining and morphology analysis

A549 and BEAS-2B cells were seeded at 40,000 cells/well onto glass coverslips and hAECs were seeded at 65,000 cells/well onto coated coverslips (fibronectin (10 ug/mL), bovine type I collagen (30 ug/mL) and bovine serum albumin (10 ug/mL) in PBS [40]) in a 24-well plate followed by the virus infections as outlined above. Mitochondria were labeled by incubating the cells with 100 nM MitoTracker™ Red FM (Cat. #M22425 Invitrogen, Oregon City, OR, USA) for 30 min. Following incubation time, cells were washed three times with PBS and fixed with 4% PFA solution at RT for 15 min. After washing three more times with PBS, coverslips were mounted on glass slides using mounting media with DAPI (Cat. #ab104139, Abcam). At least ten images per condition were acquired using a Nikon Inverted Research Fluorescence Microscope ECLIPSE Ti2-E at a nominal magnification of 60x and analyzed using ImageJ software.

Mitochondrial footprint was analyzed using the *MiNA* plugin (https://github.com/StuartLab/MiNA/blob/master/README.md) for Fiji [47, 51]. At least ten single cell images were analyzed for each condition. Branch length per mitochondrion, branches per mitochondrion, branch junctions per mitochondrion and mean form factor were measured from binarized images using the *Mitochondria Analyzer* plugin (https://github.com/AhsenChaudhry/Mitochondria-Analyzer) [47, 52]. Optimal threshold parameters were first determined and maintained for subsequent analysis for all images.

2.12 Statistical analysis

GraphPad Prism software (version 8.4.2, GraphPad Software Inc., La Jolla, CA, USA) was used to create all graphs and analyze data. All data are expressed as mean ± standard deviation (SD) for all experiments repeated as indicated in the figure legends. Analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons or by Tukey's post hoc multiple comparisons test. Significance is indicated by: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, or otherwise not significant (n.s.).

3. RESULTS

3.1 H1N1-induced cell death of epithelial cells leads to increases in lipid peroxidation and ferritin expression

It was previously demonstrated that influenza virus induces ferroptosis in A549 cells [17, 19]. We evaluated whether the H1N1/PR8 strain causes ferroptosis in both alveolar (A549) and bronchial (BEAS-2B) epithelial cells. We first assessed infection dynamics by infecting cells with H1N1/PR8 at a multiplicity of infection (MOI) of 1 and monitored nucleoprotein (NP) expression at 0, 4, 6, 8, 18 and 24 hours post infection (hpi) by immunostaining (supplementary Fig. 1). In A549 cells, NP-positive (NP+) cells reached 84.7% by 18 hpi and 93.5% by 24 hpi (Fig. 1A and 1B). In contrast, BEAS-2B cells showed faster NP production, with 58.9% cells already being NP+ at 4 hpi and peak infection levels (>90%) by 6 hpi (Fig. 1C and 1D).

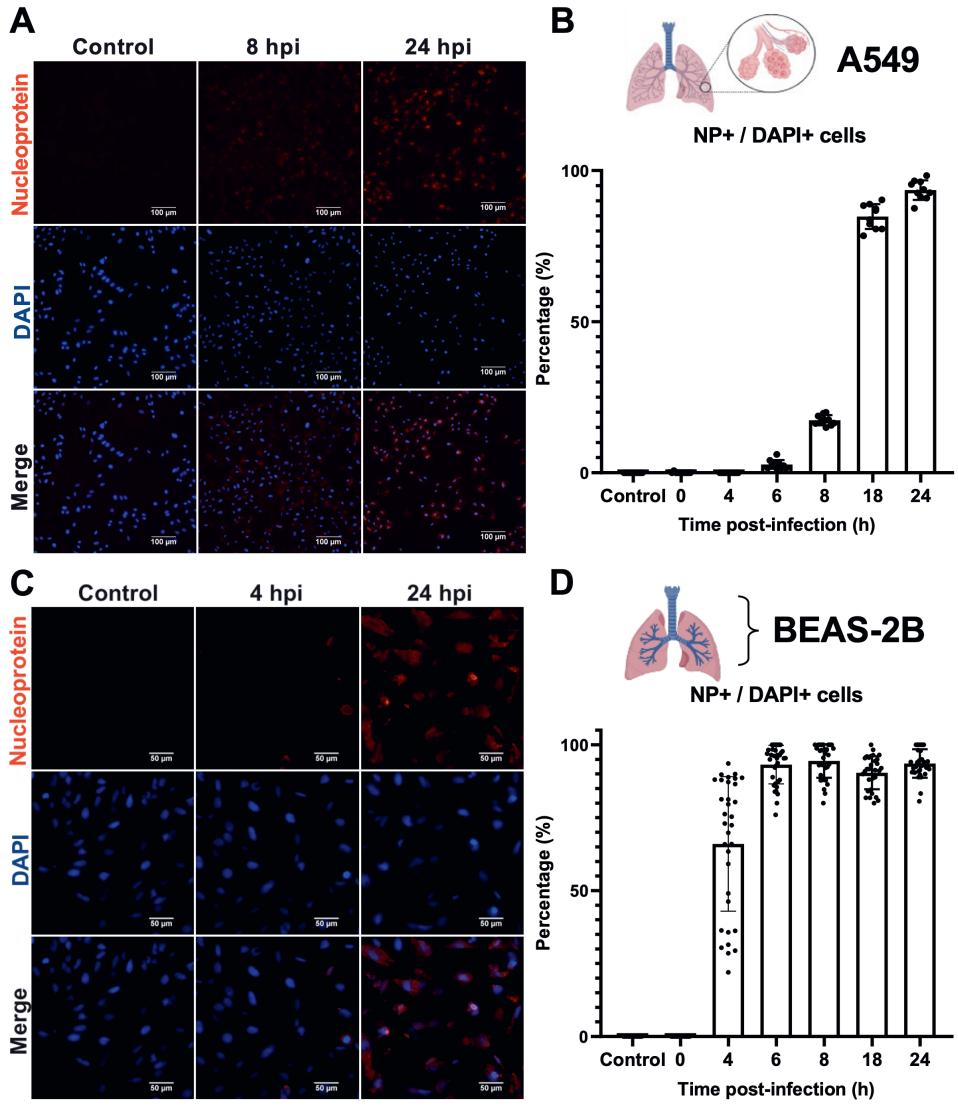


Fig. 1. H1N1 replication in lung epithelial cells. Fluorescence images of (A) A549 and (C) BEAS-2B cells infected with H1N1/PR8 influenza virus at a MOI = 1, fixed and stained at the indicated time post infection taken at a nominal magnification of 20x. Staining was performed with an anti-influenza nucleoprotein (NP) antibody and counterstaining with DAPI. Red: NP positive cells; Blue: nucleus (scale bar = 100 µm for A549 cells and 50 µm for BEAS-2B cells). Quantitative analysis of NP positive and DAPI positive (B) A549 and (D) BEAS-2B cells was done on at least 5 images per condition per biological replicate using ImageJ. Results are shown as percentage of $NP^+/DAPI^+$ cells (mean ± SD).

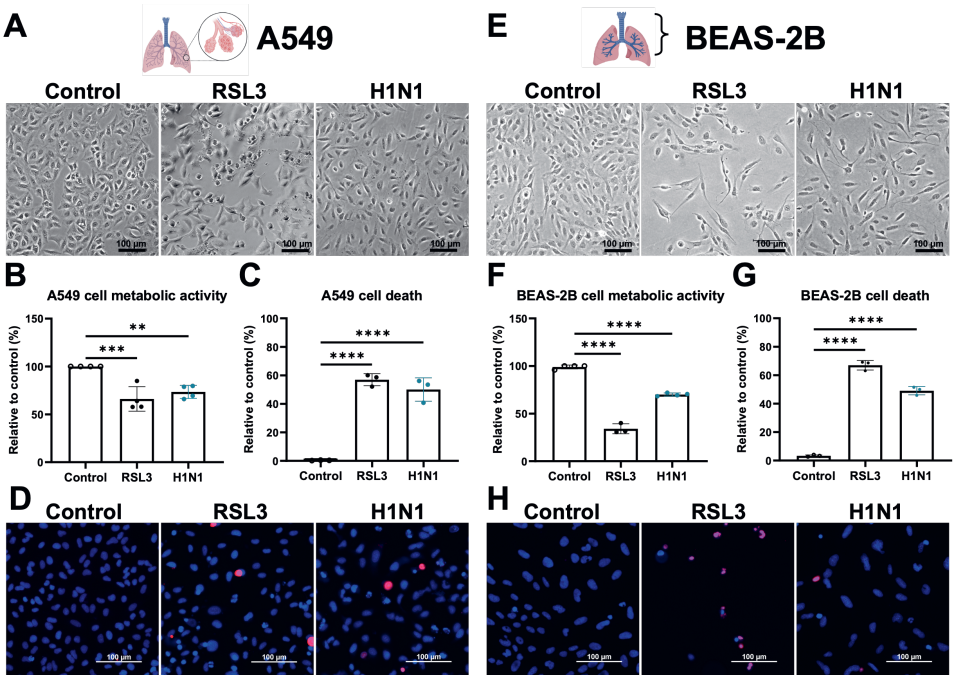


Fig. 2. H1N1 causes cell death in lung epithelial cells. Representative brightfield microscope images of (A) A549 and (E) BEAS-2B cells infected with H1N1/PR8 influenza virus at a MOI = 1 at 24 hpi or treated with RSL3 (A549: 10 µM; BEAS-2B: 250 nM) (scale bar = 100 µm). MTT assay as measurement of cell metabolic activity of viable cells (B) A549 and (F) BEAS-2B cells. Cell metabolic activity of the control group was defined as 100% activity. Each dot represents a biological replicate with at least 6 technical replicates per condition. Results are shown as percentage relative to the control (mean ± SD). Cell death of (C) A549 and (G) BEAS-2B quantification by PI and Hoechst double staining and representative images (D: A549 and H: BEAS-2B) taken at a nominal magnification of 20x. The number of dead cells was quantified according to the equation stated in the methods section. In short, the total number of dead cells is calculated as the sum of PI+ cells and the difference between the average number of DAPI+ cells in the control and the number of DAPI+ cells in the treatment, divided by the average number of DAPI+ cells in the control. Red: PI positive, dead cells; Blue: nucleus (Scale bar = 100 µm). Each dot represents the average of ten images per condition. Results are shown as percentage of dead cells relative to number of nuclei present in the control group (mean ± SD). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Results are of at least three biological replicates.

Since both cell lines showed maximal infection by 24 hpi, we continued to use this time point for subsequent experiments, with RSL3, a known ferroptosis inducer [53, 54], used as a positive control. In conjunction with increased NP production, morphological changes, and a reduction in cell number were observed in both H1N1-infected and RSL3-treated cultures for both A549 (Fig. 2A) and BEAS-2B (Fig. 2E) cells. To investigate whether virus infection leads to changes in the metabolic activity, we performed MTT assays, which showed a significant reduction in cell metabolic activity in H1N1-infected A549 (73.48% ± 6.83%) (Fig. 2B) and BEAS-2B (70.03% ± 1.62%) cells (Fig. 2F) compared to their respective controls. Given that the MTT assay measures cell metabolic activity and only indirectly provides insight into cell viability, we sought to quantify cell death by using propidium iodide (PI) and Hoechst 33258 staining. These results confirmed significant cell death in both A549 (50.1% ± 8.19%) (Fig. 2C and 2D) and BEAS-2B (49.1% ± 2.94%) (Fig. 2G and 2H) cells following H1N1 infection, which correlates with our findings in the MTT assay. For comparison, RSL3 treatment induced cell death levels of 56.95% ± 4.21% in A549 and 66.98% ± 3.32% in BEAS-2B cells.

A central feature of ferroptosis is the excessive and uncontrolled peroxidation of polyunsaturated fatty acids (PUFAs) present in cell membranes, a process that precedes and contributes to cell death [55, 56]. To assess this in our model, we measured lipid peroxidation in H1N1-infected cells at 24 hpi by staining A549 (Fig. 3A) and BEAS-2B (Fig. 3B) cells with the fluorescent probe BODIPY™ 581/591 C11. Oxidation of the polyunsaturated butadienyl portion of the dye causes a fluorescence emission shift from red (~590 nm) to green (~510), allowing for the quantification of oxidized lipids to non-oxidized lipids. The ratio of green to red fluorescence intensity then allows for the quantification of lipid peroxidation (Fig. 3C).

In A549 cells, H1N1 infection increased the ratio of green to red signal (2.1 ± 0.1 fold) compared to the control group. As expected, the RSL3 treatment caused a more pronounced increase (2.7 ± 1.1 fold) in lipid peroxidation (Fig. 3C, left). Similarly, in BEAS-2B cells, H1N1 infection increased the ratio of green to red signal (1.4 ± 0.1 fold) compared to the control, while RSL3 treatment caused a marked increase (4.8 ± 2.2 fold) (Fig. 3C, right). To confirm whether the increased lipid

peroxidation may be linked to ferroptosis, the ferroptosis inhibitor Ferrostatin-1 (Fer-1) was used to pre-treat cells for one hour prior to infection or RSL3 treatment followed by the determination of malondialdehyde (MDA) as a measure of lipid peroxidation [57] (Fig. 3D). In A549 cells, MDA levels significantly increased in H1N1-infected (1.9 ± 0.4 fold) and RSL3-treated groups (3.2 ± 0.3 fold) compared to the control condition (Fig. 3E, left). In BEAS-2B cells, RSL3 treatment induced a similar increase in MDA levels (3.4 ± 1.2 fold) and H1N1 infection caused a lower but significant increase (1.7 ± 0.3 fold) (Fig. 3E, right). As expected, Fer-1 pre-treatment prevented the increase of MDA induced by RSL3 in both cell lines (Fig. 3E). Infected BEAS-2B and A549 cells pre-treated with Fer-1 also showed a lower level of MDA with 1.3 ± 0.4 fold and 1.4 ± 0.3 fold compared to untreated infected cells (1.7 ± 0.3 and 1.9 ± 0.4 fold), respectively.

The excess oxidation of PUFAs is driven by labile, ferrous iron (Fe^{2+}) participating in the Fenton reaction, indicating that processes involved in the import, export, and storage of iron may be tightly regulated to prevent lipid peroxidation [55]. Ferritin, the major iron storage protein in cells (Fig. 3D), is known to play an important role in ferroptosis and in the replication of H1N1 [19], therefore, we next determined whether ferritin expression levels were altered in H1N1-infected cells. In A549, ferritin expression levels significantly increased (6.0 ± 1.5 fold) in H1N1-infected cells (Fig. 3F), in a similar trend to the RSL3-treated group (13.2 ± 4.2 fold) relative to the control. In BEAS-2B cells infected with H1N1, ferritin expression levels significantly increased (10.2 ± 3.7 fold) compared to the control.

This increase in ferritin expression levels of infected cells confirms a disruption of intracellular iron homeostasis and suggests a compensating mechanism as a response to an increase in intracellular labile iron. Consistent with previous reports [17, 19], our results indicate H1N1 influenza virus infection causes ferroptosis features, as evidenced by increased lipid peroxidation, MDA levels and ferritin protein expression in A549 and BEAS-2B cells.

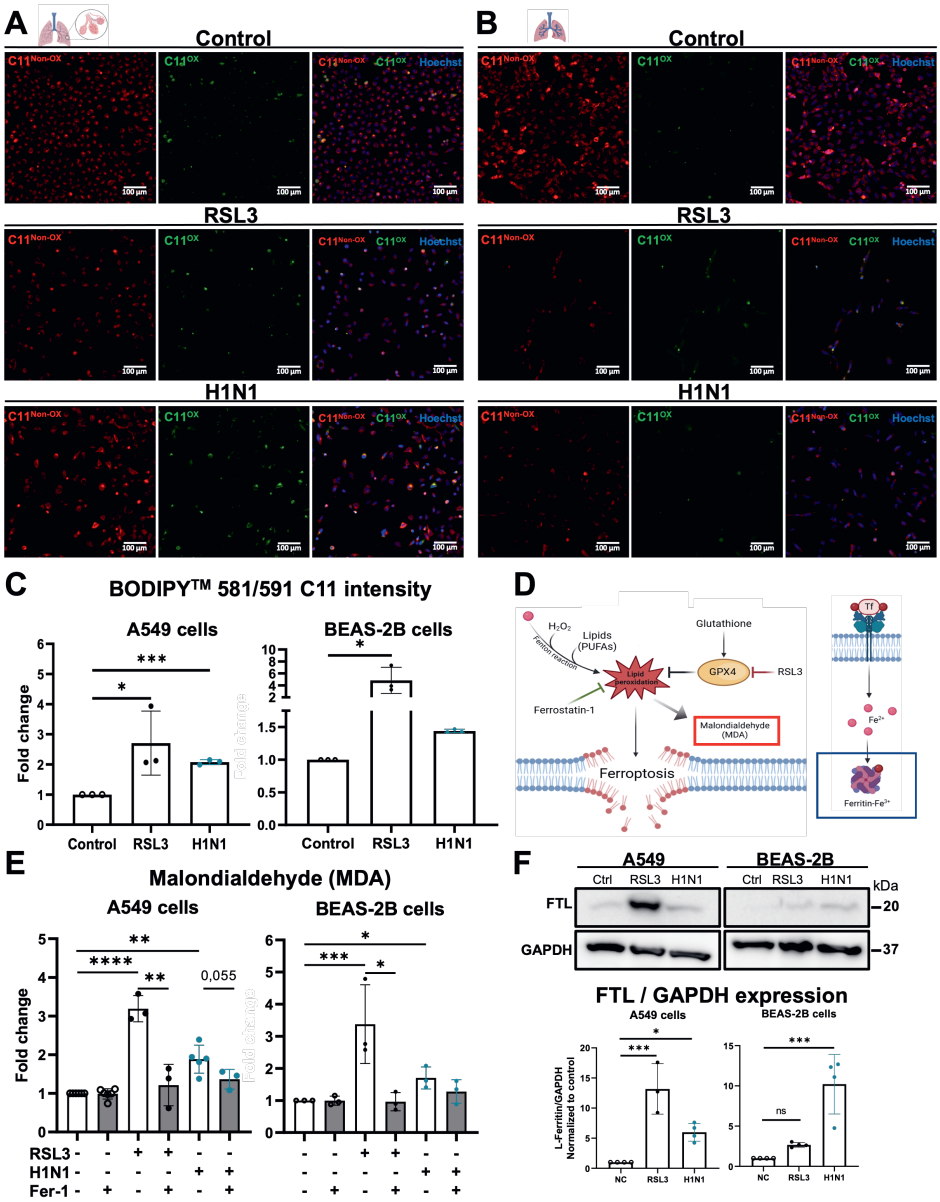


Fig. 3. Influenza virus H1N1 causes increased levels of lipid peroxidation and FTL expression in A549 and BEAS-2B cells. Fluorescence images taken at a nominal magnification of 20x of (A) A549 and (B) BEAS-2B cells treated with RSL3 (A549: 10 µM and BEAS-2B: 250 nM) or infected with H1N1/PR8 influenza virus at MOI 1 stained with BODIPY™ 581/591 C11 at 24 hpi and counterstained with Hoechst 33258. Red: non-oxidized lipids ($C11^{Non\text{-}Ox}$); Green: oxidized lipids ($C11^{Ox}$) Blue: nucleus (scale bar = 100 µm). Quantitative analysis of mean integrated fluorescence intensity of $C11^{Ox}$ /$C11^{Non\text{-}Ox}$ ratio was measured for both (C) A549 and BEAS-2B cells (mean ± SD). Each dot represents the quantification of one biological replicate. At least 10 images per condition per biological replicate were analyzed. $C11^{Ox}$ /$C11^{Non\text{-}Ox}$ ratio of

Fig. 3. Influenza virus H1N1 causes increased levels of lipid peroxidation and FTL expression in A549 and BEAS-2B cells. *(continued)* control was defined as 100%. (D) Schematic representation of iron participation in Fenton reaction leading to oxidation of polyunsaturated fatty acids (PUFAs), the production of malondialdehyde (MDA), and representation of iron transport into the cell by transferrin receptor (Tf), as well as the subsequent storage of intracellular iron in ferritin. (E) MDA levels of A549 and BEAS-2B cell lysates collected at 24 hpi with influenza H1N1/PR8 and treatment with RSL3 (A549:10 μM and BEAS-2B: 250 nM) and pre-treatment with Fer-1 (5 μM). MDA concentrations were normalized to the corresponding protein concentration and are presented as fold changes to the control. (F) Representative blot showing the relative expression of FTL in A549 and BEAS-2B cells infected with influenza H1N1/PR8 at an MOI = 1 or treated with RSL3 (A549: 30 μM and BEAS-2B: 300 nM). Cell lysates were harvested at 24 hpi for Western blot analysis. Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test. *$p < 0.05$, **$p < 0.01$ ***$p < 0.001$. Data are represented as mean ± SD of at least three independent biological replicates.

3.2 Human adenovirus type C5 causes cell death at longer times of incubation

Next, we investigated whether ferroptosis features are also triggered by human adenovirus type C5 (HAdV-C5). To assess infection dynamics and to capture potential early infection events, cells were infected with HAdV-C5 at an MOI of 1 and the expression of hexon, a major structural protein found in the capsid of HAdVs, was monitored as a marker of infection. Since HAdV structural proteins can take over 36 hpi to become detectable [58], we stained for hexon protein at 24, 48, 72 and 96 hpi (supplementary Fig. 2).

In A549 cells, it took over 48 hpi for most cells to become hexon$^+$, with nearly all cells (93.48% ± 4.75%) being hexon$^+$ by 72 hpi, whereas at 96 hpi only few nuclei were visible, indicating a substantial cytopathic effect took place (Fig. 4A). In contrast, in BEAS-2B cells, it took over 72 hpi for the majority of cells (96.81% ± 4.36%) to become hexon$^+$, indicating a slower infection rate in bronchial epithelial cells (Fig. 4D).

At 48 hpi, morphological changes consistent with the cytopathic effect of HAdVs such as cell rounding and swelling, were observed in A549 (Fig. 5A, left). In contrast, comparable morphological changes were observed in BEAS-2B cells only at 72 hpi (Fig. 5A, right). PI staining revealed an increasing number of PI$^+$ cells over time in A549, but not in BEAS-2B cells (Fig. 5B). Consistent with these findings, cell metabolic activity in A549 cells was significantly reduced (59.6% ± 6.1%) by 72 hpi (Fig. 5C). Quantification of PI$^+$ cells showed a significant increase in cell death at 48 hpi (23.30% ± 7.83%), with a further increase at 72 hpi (40.77% ± 13.94%) (Fig. 5D).

In BEAS-2B cells, metabolic activity remained unaffected up to 72 hpi, and PI quantification showed only a small increase in cell death at 72 hpi (18.0% ± 1.7%) (Fig. 5E and 5F). Importantly, the cell death caused by HAdV-C5 at 72 hpi was not prevented by apoptosis inhibitors Z-DEVD-FMK, Z-VAD-FMK and Q-VD-Oph in A549 and BEAS-2B cells (supplementary Figure 4), suggesting that although apoptosis is known to be a RCD mechanism induced by HAdV-C5 infection [2, 25], other forms of RCD, such as ferroptosis, are involved.

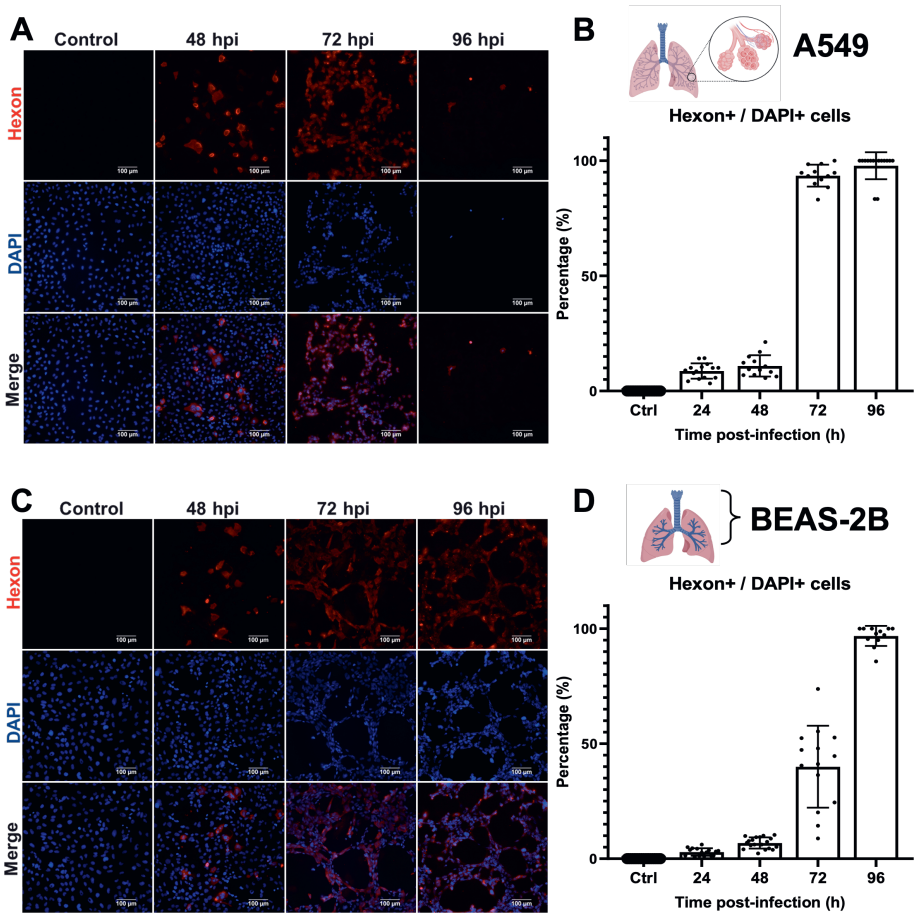


Fig. 4. HAdV-C5 replication rate in lung epithelial cells. Fluorescence images taken at a nominal magnification of 20x of (A) A549 and (C) BEAS-2B cells infected with HAdV-C5 virus at a MOI = 1 fixed and stained at 48, 72 and 96 hpi with anti-hexon antibody and counterstained with DAPI. Red: hexon positive cells; Blue: nucleus (scale bar = 100 µm). Quantitative analysis of hexon positive and DAPI positive (B) A549 and (D) BEAS-2B cells was done on at least 10 images per condition using ImageJ. Results are shown as percentage of Hexon+/DAPI+ cells (mean ± SD).

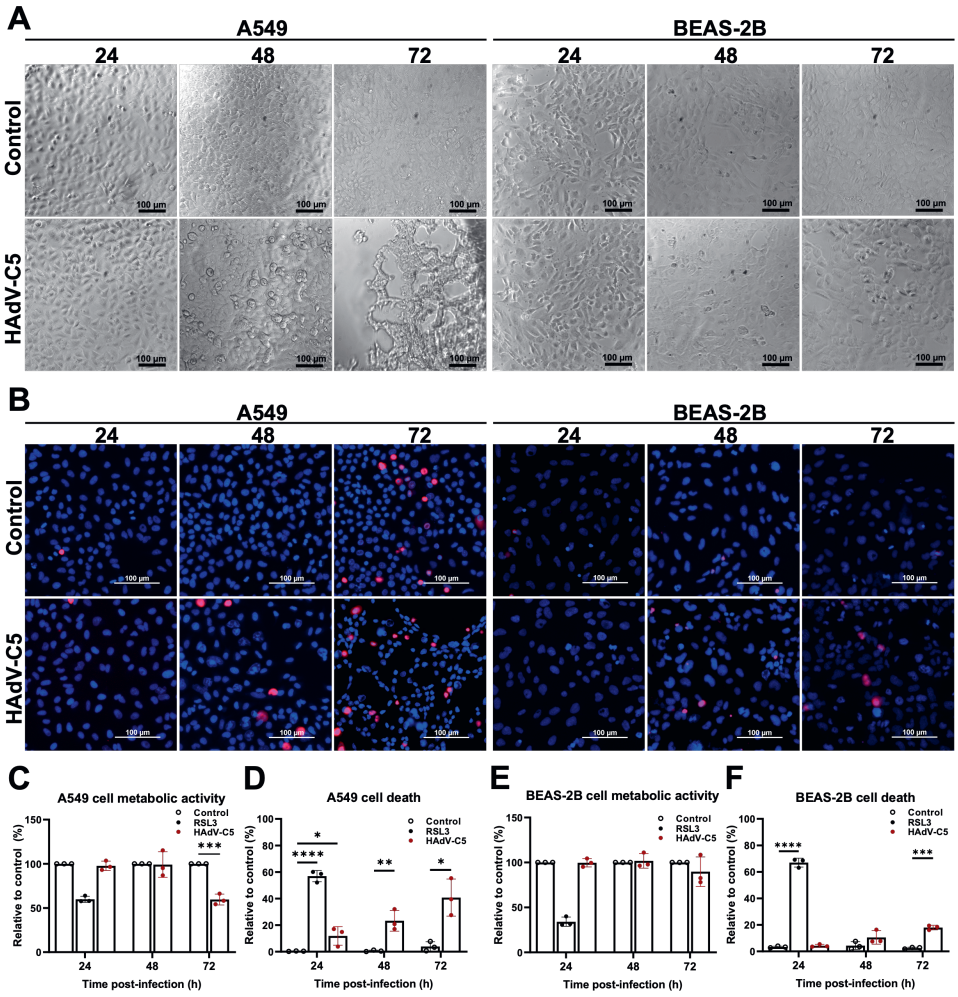


Fig. 5. HAdV-C5-induced cell death has an earlier onset in A549 cells compared to BEAS-2B cells. (A) Representative brightfield microscope images of A549 and BEAS-2B cells infected with HAdV-C5 virus at a MOI = 1 at 24, 48, and 72 hpi (scale bar = 100 µm). (B) Representative fluorescence images of PI and Hoechst double staining taken at a nominal magnification of 20x. Red: PI positive, dead cells; Blue: nucleus (Scale bar = 100 µm). MTT assay as measurement of cell metabolic activity of viable cells of (C) A549 and (E) BEAS-2B cells. Cell metabolic activity of the control group was defined as 100% activity. Each dot represents a biological replicate with at least 6 technical replicates per condition. Results are shown as percentage relative to the control (mean ± SD). Cell death of (D) A549 and (F) BEAS-2B as measured by PI staining. RSL3 at 24 h was introduced as a single time point positive control. The number of dead cells was quantified according to the equation stated in the methods section. In short, the total number of dead cells is calculated as the sum of PI+ cells and the difference between the average number of DAPI+ cells in the control and the number of DAPI+ cells in the treatment, divided by the average number of DAPI+ cells in the control. Each dot represents the average of ten images per condition (mean ± SD). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns: non-significant. Results are of at least three biological replicates.

Next, lipid peroxidation was measured by staining with BODIPY™ 581/591 C11 following infection with HAdV-C5 at 24, 48 and 72 hpi in A549 (Fig. 6A) and BEAS-2B (Fig. 6B) cells. RSL3 treatment at 24 h served as a positive control (same as for H1N1 as the RSL3 and HAdV-C5 samples were prepared in the same experiment). Quantification of green to red fluorescence intensity ratios showed a time-dependent increase of oxidized lipids in A549 cells, with a significant change at 72 hpi (1.9 ± 0.4 fold) compared with the respective 72 h control. In contrast, no change in the ratio of oxidized to non-oxidized lipids was observed in BEAS-2B cells at any of the time points measured (Fig. 6D).

Based on these findings, which indicate a higher susceptibility of A549 cells to HAdV-C5 infection, and the absence of observable changes in cell viability or lipid peroxidation in BEAS-2B cells, follow-up experiments were performed only on A549 cells. To further confirm the increase in lipid peroxidation, MDA levels were measured and assessed for sensitivity to the ferroptosis inhibitor Fer-1. MDA levels were significantly increased as early as 48 hpi (2.2 ± 0.6 fold) and remained significantly higher at 72 hpi (1.3 ± 0.2 fold) compared with their respective controls (Fig. 6D). Pre-treatment with Fer-1 significantly reduced the amount of MDA compared to non-Fer-1 pre-treated samples at 72 hpi in both control (0.7 ± 0.06 fold vs 1) and HAdV-C5 infected (0.8 ± 0.03 vs. 1.3 ± 0.2 fold) cells [19, 36]. Lastly, contrary to H1N1 infections of A549, the expression of FTL was found to be lower compared to control conditions, however, did not significantly change between sampled time points, only showing a significant decrease at 72 h (Fig. 6E).

Overall, our data indicate that HAdV-C5 induces a marked increase in lipid peroxidation at later times of infection, which correlate with observable changes in cell morphology and hexon expression. However, the increase of MDA was only partially reduced by Fer-1, and no changes in FTL expression was detected, suggesting an iron-independent increase in lipid peroxidation.

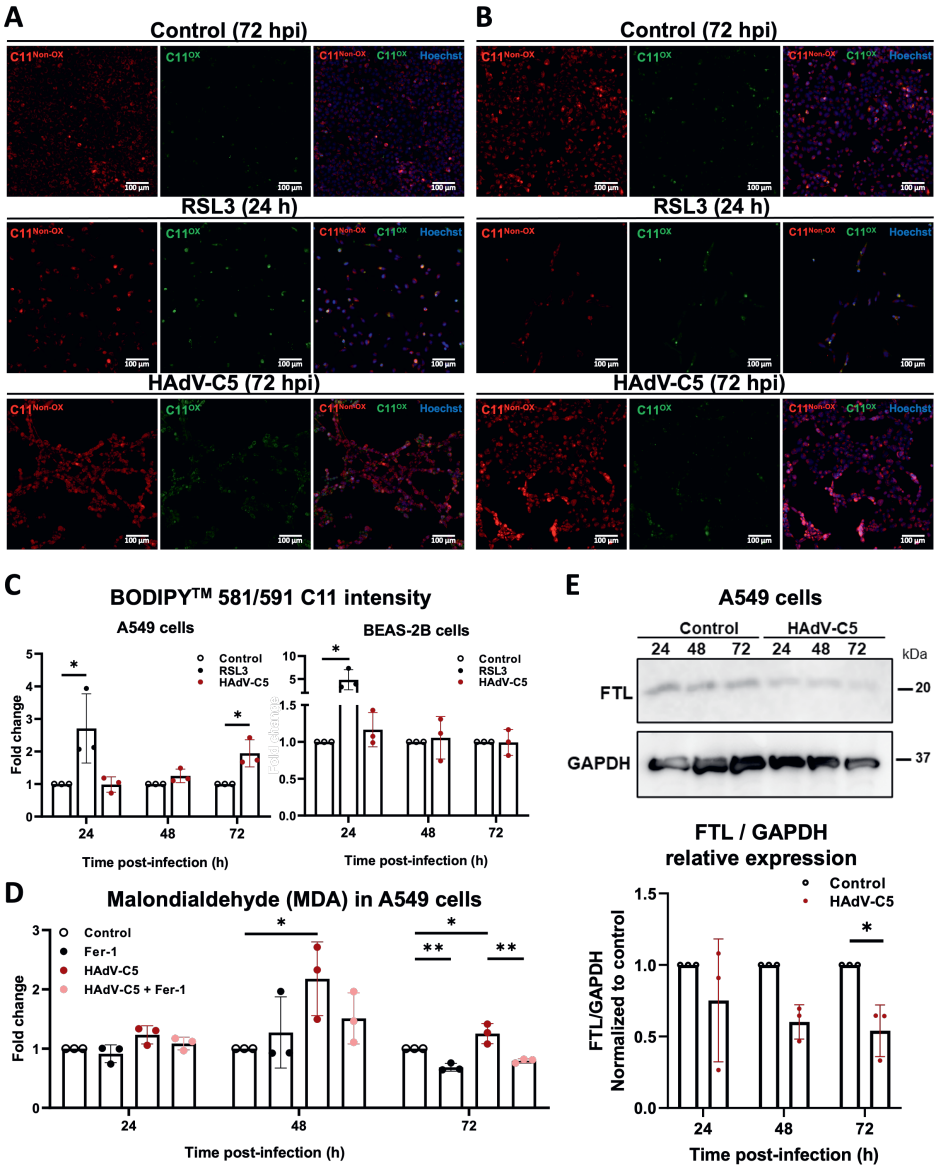


Fig. 6. HAdV-C5-induced lipid peroxidation in A549 cells is time-dependent. Representative fluorescence images taken at a nominal magnification of 20x of (A) A549 and (B) BEAS-2B cells treated with RSL3 (A549:10 μM and BEAS-2B: 250 nM) for 24 h or infected with HAdV-C5 at a MOI = 1 stained with BODIPY™ 581/591 C11 at 72 hpi and counterstained with Hoechst 33258. RSL3 positive control treatment was performed concurrently with the 24 hpi H1N1 and HAdV-C5 experiments, therefore the same data to that presented in the H1N1 panel is reproduced here for comparison. Red: non-oxidized lipids ($C11^{Non\text{-}Ox}$); Green: oxidized lipids ($C11^{Ox}$) Blue: nucleus (scale bar = 100 μm). (C) Quantitative analysis of mean integrated fluorescence intensity of $C11^{Ox}$ /$C11^{Non\text{-}Ox}$ ratio was measured for both A549 and BEAS-2B cells (mean ± SD) at 24, 48 and 72 hpi. Each dot represents the quantification of one biological replicate.

Fig. 6. HAdV-C5-induced lipid peroxidation in A549 cells is time-dependent. *(continued)* At least 10 images per condition per biological replicate were analyzed. $C11^{Ox}/C11^{Non\text{-}Ox}$ ratio of control was defined as 100%. Statistical analysis was performed using one-way ANOVA followed by Dunnett's comparisons test. (D) MDA levels of A549 cell lysates collected at 24, 48 and 72 hpi with HAdV-C5 and treatment with RSL3 (10 μM) and pre-treatment with Fer-1 (5 μM). MDA concentration was normalized to the corresponding protein concentration and are presented as fold changes compared to the control. (E) Representative blot showing the relative expression of FTL in A549 cells infected with HAdV-C5 at an MOI = 1 or treated with RSL3 (30 μM). Cell lysates were harvested at 24 h, 48 h, and 72 h hpi for Western blot analysis. Data are represented as mean ± SD of at least three independent biological experiments with three technical replicates. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc multiple comparisons test. *$p < 0.05$, **$p < 0.01$.

3.3 H1N1 and HAdV-C5 infections cause alterations to the mitochondrial network in bronchial epithelial cells

Mitochondria regulate lipid metabolism, iron transport, and reactive oxygen species (ROS) production and thus play an active role in triggering ferroptotic cell death [10]. When exposed to ferroptosis inducers, such as RSL3, the mitochondrial outer membrane ruptures, causing the mitochondria to appear compacted and fragmented [4, 10]. Viruses are known to manipulate mitochondrial dynamics and morphology to benefit their own replication and survival [59, 60]. Therefore, we aimed to determine the effect of H1N1 and HAdV-C5 infection on the mitochondrial network and changes in morphology.

Morphological changes to mitochondria have been observed in H1N1 infected A549 cells at 24 hpi [18, 61]. To recapitulate this phenotype, we stained cells with MitoTracker™ Red FM and visualized the mitochondrial network with fluorescence microscopy images, which were binarized using the *Mitochondria Analyzer* plugin [47, 52] for the quantification of mitochondria morphology and the *MiNA* plugin [47, 51] for the quantification of the footprint (area) occupied by mitochondria. We observed that mitochondria formed long, tubular-like structures in control A549 cells (Fig. 7A). In comparison, mitochondria in the RSL3-treated groups appeared fragmented and dot-like (Fig. 7A), observations confirmed by the significant decrease in mitochondrial footprint (14.4 ± 8.9 μm² vs. 24.8 ± 6.8 μm²), branch length per mitochondrion (0.76 ± 0.2 μm vs.1.2 ± 0.2 μm), number of branches per mitochondrion (1.2 a.u. ± 0.1 vs. 1.3 ± 0.1 a.u), junctions per mitochondrion (0.07 ± 0.04 a.u.vs. 0.1 ± 0.06 a.u.), and mean form factor (1.5 ± 0.1 a.u. vs.1.8 ± 0.1 a.u.) indicating less and smaller, rounder mitochondria (Fig. 7B). In contrast, only small changes of mitochondrial network structure could be seen in H1N1 and HAdV-C5-

infected A549 cells with more doughnut-shaped mitochondria appearing compared to the control. However, quantification confirmed no significant changes in mitochondrial footprint and morphology parameters, such as branch length and junctions per mitochondrion and mean form factor, caused by the viruses at 24 hpi (Fig. 7B).

Interestingly, BEAS-2B cells infected with H1N1 had mitochondria that appeared shorter and doughnut-shaped (Fig. 7C). Indeed, quantification of mitochondrial footprint and morphology parameters showed a significant decrease in mitochondrial footprint (20 ± 6 μm^2 vs. 27 ± 12 μm^2), branch length per mitochondrion (1.3 ± 0.4 μm vs. 1.5 ± 0.3 μm), and mean form factor (1.9 ± 0.3 a.u. vs. 2.1 ± 0.3 a.u.) indicating rounder mitochondria with shorter branches compared to controls (Fig. 7D). Similarly, BEAS-2B cells infected with HAdV-C5 exhibited shorter mitochondrial branches (Fig. 7C), which was reflected by the significant reduction in mitochondrial footprint (15 ± 7 μm^2 vs. 27 ± 12 μm^2), branch length per mitochondrion (1.2 ± 0.2 μm vs. 1.5 ± 0.3 μm), junctions per mitochondrion (0.09 ± 0.03 a.u. vs. 0.12 ± 0.06 a.u.) and mean form factor (1.9 ± 0.1 a.u. vs. 2.1 ± 0.3 a.u) compared to controls (Fig. 7D). Profound changes could be seen in the RSL3 treated cells which appeared fragmented and dot-like. These observations were confirmed by the significant reduction in mitochondrial footprint (13.6 ± 10.7 μm^2 vs. 27 ± 12 μm^2), branch length per mitochondrion (0.95 ± 0.5 μm vs. 1.5 ± 0.3 μm), branches per mitochondrion (1.2 ± 0.1 a.u. vs. 1.2 ± 0.1 a.u.), junctions per mitochondrion (0.08 ± 0.07 a.u. vs. 0.12 ± 0.06 a.u.), and mean form factor (1.7 ± 0.4 a.u. vs. 2.1 ± 0.3 a.u) compared to controls (Fig. 7D).

Since the changes caused by HAdV-C5 infection were observed only at later times of infection, we stained the mitochondria of A549 infected cells at 48, 60 and 72 hpi. Images showed a clear reduction of tubular-like structures and an increase in fragmented, dot-like mitochondria that could be observed in the HAdV-C5 infected cells at 48 hpi compared to its control group. By 72 hpi, only a few mitochondria could be observed in the infected cells, all of which appeared dot-like and compacted compared to the control cells at the same time point. The progressive deterioration in the mitochondria morphology (supplementary Fig. 3), coincided with time points in which we observed the increase in lipid peroxidation and cell

death. However, accurate quantification of mitochondrial footprint and morphology parameters was not possible due to the mitochondrial signal spanning over multiple imaging layers [52].

Overall, our results indicate that H1N1 and HAdV-C5 infection decrease mitochondrial footprint in BEAS-2B cells and cause detrimental changes to mitochondrial morphology. The level of mitochondrial alterations in these cells was comparable to the damage caused by RSL3.

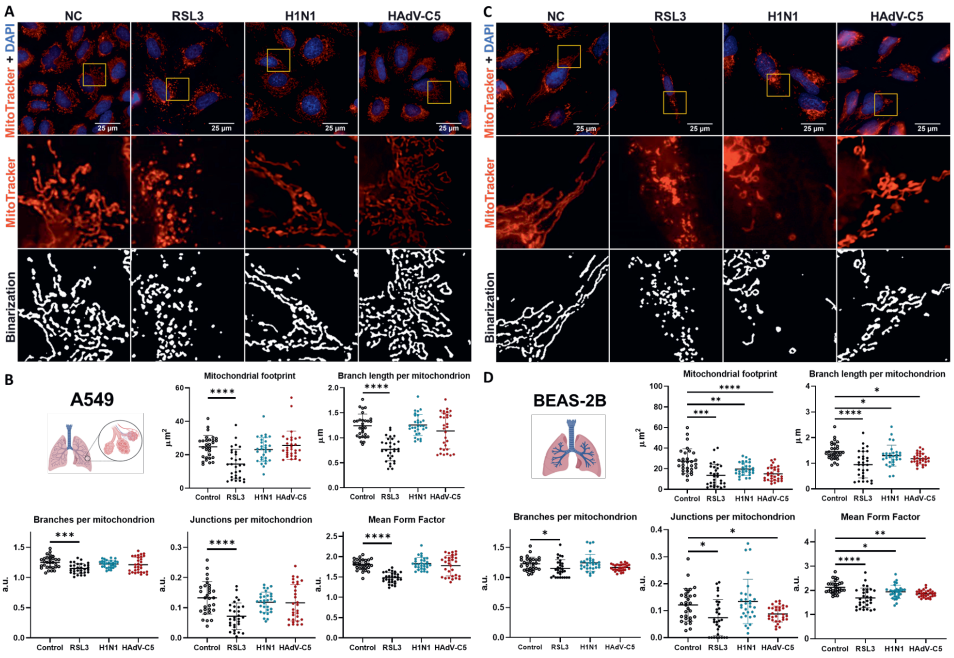


Fig. 7. Influenza H1N1 and HAdV-C5 infections cause alterations in mitochondrial morphology in BEAS-2B but not A549 cells. MitoTracker™ Red FM fluorescence images of (A) A549 and (C) BEAS-2B cells at a nominal magnification of 60X after treatment with RSL3 (A549: 10 µM; BEAS-2B: 250 nM) for 24 h or infection with influenza H1N1/PR8 or HAdV-C5 at a MOI = 1 at 24 hpi Red: mitochondria; Blue: nucleus (scale bar = 25 µm). Enlarged images of boxed area are shown for MitoTracker™ and after binarization using the *Mitochondria Analyzer* plugin [47, 52]. Parameters of mitochondrial network analysis for (B) A549 and (D) BEAS-2B cells. Mitochondrial footprint of at least 10 single cells was analyzed for each condition (mean ± SD). Branch length per mitochondrion, branches per mitochondrion, junctions per mitochondrion and mean form factor of at least 10 whole images per condition was determined (mean ± SD). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Results are of at least three biological replicates.

3.4 H1N1 and HAdV-C5 infections cause alterations to the mitochondrial network in primary human airway epithelial cells

To be able to translate our findings to human disease conditions, we infected primary human airway epithelial cells (hAECs) with H1N1 and HAdV-C5 at an MOI of 1 or treated with RSL3. At 24 hpi, we stained the cells with MitoTracker™ Red FM and analyzed mitochondrial morphology. In control cells we observed that mitochondria formed both tubular and donut-like structures. In contrast, mitochondria in the RSL3-treated groups showed a broader range of morphologies, including shorter more condensed mitochondria as well as longer and expanded structures (Fig. 8A). Quantitative analysis confirmed a significant increase in mitochondrial footprint in the RSL3-treated cells (19.3 ± 7.2 μm^2 vs. 43.0 ± 37.8 μm^2) with no changes in the branch length per mitochondrion (0.71 ± 0.07 µm vs.0.67 ± 0.13 µm), number of branches per mitochondrion (1.1 ± 0.03 a.u. vs. 1.1 ± 0.04 a.u), junctions per mitochondrion (0.05 ± 0.01 a.u. vs. 0.05 ± 0.02 a.u.), or mean form factor (1.5 ± 0.04 a.u. vs.1.5 ± 0.08 a.u.).

In contrast to RSL3 treatment, mitochondrial footprint was not altered in hAECs infected H1N1 or HAdV-C5 (19.3 ± 7.2 μm^2 vs. 21.1 ± 7.23 μm^2 and 20.07 ± 8.41 μm^2, respectively). Interestingly, mitochondrial network in both H1N1 and HAdV-C5 infected cells appeared more branched and expanded compared to the control group and the findings in cell lines. Indeed, results indicate a significant increase in the branch length per mitochondrion (0.71 ± 0.07 µm vs.0.84 ± 0.22 µm and 0.84 ± 0.18 µm), number of branches per mitochondrion (1.1 ± 0.03 a.u. vs. 1.14 ± 0.07 a.u and 1.14 ± 0.05 a.u), junctions per mitochondrion (0.05 ± 0.01 a.u.vs. 0.07 ± 0.04 a.u. and 0.07 ± 0.03 a.u), and mean form factor (1.5 ± 0.04 a.u. vs.1.56 ± 0.16 a.u. and 1.55 ± 0.11 a.u.). Taken together, our results demonstrate that H1N1 and HAdV-C5 infections cause remodeling of the mitochondrial network in hAECs towards a more interconnected and branched network, with only small increase of the mitochondrial footprint.

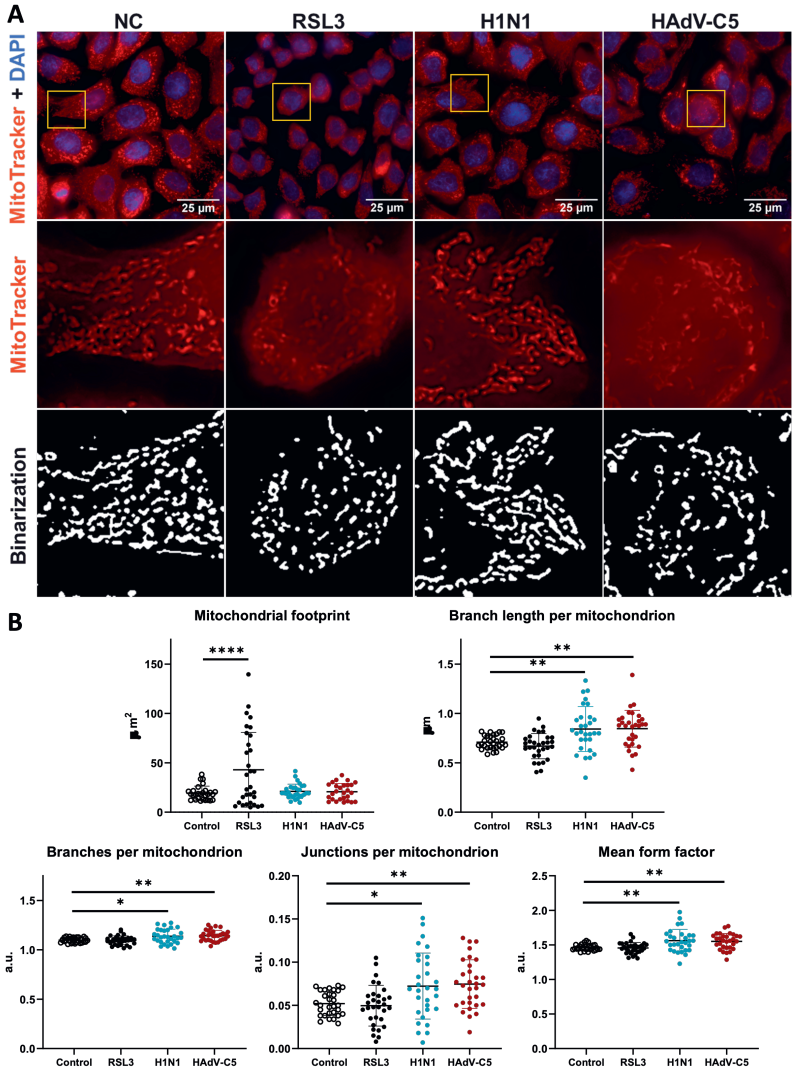


Fig. 8. Influenza H1N1 and HAdV-C5 infections cause alterations in mitochondrial morphology in primary human airway epithelial cells. (A) MitoTracker™ Red FM fluorescence images of hAECs at a nominal magnification of 60X after treatment with RSL3 (1 µM) for 24 h or infection with influenza H1N1/PR8 or HAdV-C5 at a MOI = 1 at 24 hpi Red: mitochondria; Blue: nucleus (scale bar = 25 µm). Enlarged images of boxed area are shown for MitoTracker™ and after binarization using the Mitochondria Analyzer plugin [43, 48]. (B) Parameters of mitochondrial network analysis. Mitochondrial footprint of at least 10 single cells was analyzed for each condition (mean ± SD). Branch length per mitochondrion, branches per mitochondrion, junctions per mitochondrion and mean form factor of at least 10 whole images per condition was determined (mean ± SD). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test. *p < 0.05, **p < 0.01, ****p < 0.0001. Results are of three biological replicates.

4. DISCUSSION

In this study we investigated ferroptosis-like hallmarks induced by H1N1 and HAdV-C5 infections in BEAS-2B (bronchial) and A549 (alveolar) epithelial cells. We provide evidence that influenza A virus (H1N1) induces lipid peroxidation and disrupts iron metabolism (Fig. 3), two key hallmarks of ferroptosis, positioning our findings in line with earlier reports [17, 19]. Further studies reported a downregulation of ferritin heavy chain (FTH1) expression in A549 cells infected with H1N1/PR8 at a low MOI (0.1) [19], whereas our data shows an increase in the expression of ferritin light chain (FTL) in both A549 and BEAS-2B cells following infection with H1N1/PR8 at a higher MOI 1. Proteomics data of normal human bronchial epithelial (NHBE) cells infected with different strains of IAV at an MOI 2 indicate that the relative expression levels of FTH1 fluctuate in a time-dependent manner [62]. Indeed, clinical studies showed, using direct quantification methods of serum ferritin level, that high ferritin levels are linked to a more severe infection and can be used as a marker for severe complications and poor patient outcomes [63, 64]. Furthermore, the upregulation in FTL was comparable to that seen in RSL3-treated groups, suggesting a potential compensatory response, likely resulting from the upregulated expression of transferrin and transferrin receptor that has been previously reported in H1N1-infected A549 cells [17].

We further demonstrate, for the first time, that HAdV-C5 infection also induces lipid peroxidation in A549 cells at later times of infection (48 and 72 hpi) but not at early time points of infection (24 hpi) (Fig. 6) as indicated by the quantification of hexon protein expression (Fig. 4). These findings indicate that lipid peroxidation is a late-stage event in HAdV-C5 infection, rather than an early response. Strikingly, we did not see an increase of lipid peroxidation in BEAS-2B cells within the time frame of our experiments. However, given HAdV-C5 replication kinetics and onset of cell death in BEAS-2B cells are different compared to A549 cells, it may indicate that cellular pathways may be regulated differently eliciting an RCD pathway independent of ferroptosis-like hallmarks. Another explanation is that HAdVs elicit different innate and inflammatory responses during infection in different airway epithelial cells [65, 66], some of which may suppress lipid peroxidation cascades.

Interestingly, previous studies, performed in NHI3T3 fibroblasts, have shown that expression of the adenovirus early region 1A (E1A) gene can block the expression of ferritin in response to oxidative stress [29], further showing differential regulation of ferroptosis-like hallmarks in different cell types. We observed a trend towards a decrease in the expression of FTL in A549 cells infected with HAdV-C5. However, whether this trend reflects E1A-mediated suppression of ferritin or the increase in lipid peroxidation observed is unrelated to iron metabolism remains to be clarified.

Beyond lipid peroxidation and ferritin expression, we also explored virus-induced alterations in mitochondrial footprint and morphology. Mitochondria actively contribute to the onset of cysteine-deprivation-induced ferroptosis through glutathione depletion and consequently the generation of lipid peroxides [11]. Erastin-induced ferroptosis dramatically changes mitochondria morphology, causing an increase of mtROS, leading to loss of mitochondrial membrane potential [67]. The influenza A virus PB1-F2 protein is known to induce mitophagy by translocating to mitochondria and mediating its degradation by binding to LC3 to promote degradation and elimination of mitochondrial antiviral signaling proteins (MAVS) to impair innate immunity [68-70]. Here, we show a decrease in mitochondrial content and morphology in BEAS-2B cells, but not in A549 cells, following H1N1 infection at 24 hpi (Fig.7). This cell-type-specific effect may be caused by the distinct pattern of sialic acids on bronchial epithelial cells, which promote H1N1 uptake, [14, 71] and therefore faster infection in BEAS-2B cells compared to A549 cells (Fig. 1). Furthermore, a study on mitochondrial morphology changes in H1N1 infected A549 cells also found distinct fragmentation patterns under starvation conditions, confirming the findings presented in the present study [61].

Strikingly, the number of studies done on mitochondrial network changes during IAV or HAdV-C5 infections is sparse and limited to model cell lines, also utilized in this study. To the best of our knowledge, this is the first study showing the changes in mitochondrial network in primary hAECs infected with IAV and HAdV-C5, adding an important finding to the respiratory virus research field. It needs to be pointed out that studies on primary airway and nasal epithelial cells have been

performed, showing alterations to mitochondrial function [72-74], however, have not investigated changes in the mitochondrial network structure. Our findings reveal an increase in mitochondrial network parameters (Fig. 8), interestingly, contrary to what our findings in model cell lines (Fig. 7). A previous study done by Pila-Castellano *et al.* reported an elongation of the mitochondrial network, in line with our findings in hAECs. Interestingly, these changes were only seen in infected cells maintained under regular, non-starvation, media conditions differing from our findings in A549 cells, which were maintained in 1% FBS during the duration of the experiment. Coinciding, with these findings Pila-Castellano *et al.* also reported a reduction in DRP1 localization to mitochondria, whereas OPA1 localization was increased [61], which offers a molecular insight into why these changes were observed. Due to the lack of systematic exploration of mitochondrial network morphology during viral infections, we cannot currently suggest a clear underlying mechanism that explains why hAECs, under starvation conditions, show a mitochondrial fusion phenotype and will thus need to be addressed in future studies. Strikingly, treatment of hAECs with RSL3 leads to a marked increase in mitochondrial footprint, whereas the other mitochondrial network parameters were unchanged. This is in line with previously published work on hAECs that showed no measurable changes in mitochondrial volume upon treatment with a higher concentration of RSL3 (10 μM) [75].

Adenoviruses are also known to modulate mitochondrial homeostasis to enhance virus replication [76-78]. The viral E1B-19k protein localizes to mitochondria, where it binds to the pro-apoptotic proteins Bax and Bak to prevent apoptosis [77, 79-82]. Recently, the viral minor capsid protein VI was shown to localize to mitochondria during late stages of infection and promote, via its membrane-lytic function, the release of mitochondrial DNA (mtDNA) and heat shock protein 60 (HSP60) into the cytosol [83], both of which are important for maintaining mitochondrial integrity [84]. We observed a decrease in mitochondrial footprint and changes in mitochondrial morphology in BEAS-2B cells infected with HAdV-C5 at early times of infection (24 hpi) that could not be seen in A549 cells at the same time point (Fig. 7). Further suggesting that adenovirus-induced disruption of mitochondrial homeostasis is both cell type- and time-dependent and is likely also connected to

the lytic function of protein VI [83]. Indeed, the drastic changes in mitochondrial morphology at later time points post HAdV-C5 infection prevented reliable quantification of changes in mitochondrial network, as diffusion of the MitoTracker signal did not allow for accurate determination of the parameters, which is a known limitation of the software as specified by the developer [52].

Recent research shows that mitochondrial integrity is regulated by the localization of cyclic GMP-AMP (cGAMP) synthase (cGAS) to the outer mitochondrial membrane where it associates with dynamin-related protein 1 (DRP1), one of the known key regulators of mitochondrial fission, and facilitates its oligomerization to control excessive ROS production and limit lipid peroxidation, thereby inhibiting ferroptosis [85]. Importantly, cGAS, a cytosolic DNA sensor that following its binding to DNA catalyzes the synthesis of second messenger cGAMP to activate stimulator of interferon genes (STING) and the downstream production of interferons [86], was found to be significantly increased in cancer cells, including A549 cells [85]. The higher cGAS expression in A549 cells may explain why we only observed smaller changes in the mitochondrial network in H1N1- and HAdV-C5-infected cells at 24 hpi. In addition, levels of DRP1 were shown to be differentially altered during IAV infection at early and late stages of infection [61, 87]. Interestingly, it was further shown that increasing cyclic adenosine monophosphate (cAMP) levels activate protein kinase A (PKA), which is subsequently translocated to the outer mitochondrial membrane via A-kinase anchoring protein (AKAP), where it can phosphorylate DRP1 thereby inhibiting its function [88]. Importantly, it was previously shown that mitochondrial integrity and function is linked to cAMP levels and signaling in lung epithelial cells [35, 89]. Indeed, other studies showed that inhibition of either exchange protein activated by cAMP 1 (EPAC1) or phosphodiesterase 4 (PDE4) reduces IAV replication efficacy, suggesting a potential link between cAMP levels [90, 91], mitochondrial function and morphology, and viral replication. Strikingly, it was shown that HAdV infections can hijack cAMP signaling via E1A-mediated mimicry of AKAP functionality and binding of PKA, suggesting a similar mechanism as for IAV [92]. However, our knowledge on HAdV induced changes in DRP1 or other mitochondrial integrity regulators is limited. Therefore, future studies should

address how viruses alter cAMP-linked signaling and its impact on mitochondrial function.

5. CONCLUSIONS

In conclusion, we confirm previous findings regarding the role of ferroptosis during H1N1 infection by demonstrating increased lipid peroxidation, elevated MDA levels, and altered FTL expression in two different lung epithelial cell types. Importantly, we provide novel evidence that HAdV-C5 infection induces lipid peroxidation and MDA accumulation at later stages of infection, a process that is prevented by pre-treatment with the ferroptosis inhibitor Fer-1 only at advanced stages of infection and is not accompanied by significant changes in FTL expression. Additionally, we show for the first time that both H1N1 and HAdV-C5 infections lead to significant changes in mitochondrial content and morphology in bronchial (BEAS-2B) epithelial cells at early times of infection comparable to mitochondrial changes caused by RSL3. Contrastingly, in alveolar A549 cells significant mitochondrial changes were observed only at later times of infection with HAdV-C5, whereas H1N1 induced changes recapitulate phenotypes that were seen before [61]. In addition, our observations of changes in the mitochondrial network in primary human lung epithelial cells (hAECs) add novel findings to the field of respiratory virus and mitochondrial research, which offer the basis for future research investigating mitochondrial dynamics during viral infections. Taken together our comparative study of H1N1 and HAdV-C5 induced ferroptosis-like hallmarks, offer a more extended and detailed understanding of how lipid peroxidation, iron homeostasis, and mitochondrial morphology are altered. However, whether the cell death caused by them is ferroptosis, or a blend of various regulated cell death pathways will require further investigation.

ABBREVIATIONS

AKAP	A-kinase anchoring protein
cAMP	cyclic AMP
cGAS	cyclic GMP-AMP synthase
cGAMP	cyclic GMP-AMP
DMSO	dimethyl sulfoxide
DRP1	dynamin-related protein 1
EPAC	exchange protein activated by cAMP 1
E1A	adenovirus early region 1A
FTH1	ferritin heavy chain
FTL	ferritin light chain
GPX4	glutathione peroxidase 4
GSH	glutathione
HAdV	human adenovirus
HAdV-C5	human adenovirus type C5
hAECs	human airway epithelial cells
hpi	hours post infection
HSP60	heat shock protein 60
IAV	influenza A virus
MAVS	mitochondrial antiviral signaling proteins
MDA	malondialdehyde
MOI	multiplicity of infection
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
mtROS	mitochondrial ROS
mtDNA	mitocondrial DNA
NCOA4	nuclear receptor coactivator 4
NP	nucleoprotein
PBS	phosphate buffered saline
PDE4	phosphodiesterase 4
PFA	paraformaldehyde
PI	propidium iodide
PKA	protein kinase A
PUFA	polyunsaturated fatty acid
pVI	viral minor capsid protein VI
RCD	regulated cell death
RIPK1	receptor interacting protein kinase 1
ROS	reactive oxygen species
RT	room temperature
STING	stimulator of interferon genes
TBA	2-thiobarbituric acid
TBARS	thiobarbituric acid reactive substances assay
TCA	trichloroacetic acid

ACKNOWLEDGMENTS

A.L.M.C is a recipient of a Secretaría de Ciencia, Humanidades, Tecnología e Innovación (SECIHTI) scholarship (2021-000022-01EXTF-00086). A.M.D. is the recipient of an Alzheimer Nederland grant (WE.03-2018-04, The Netherlands), Parkinson Fonds (The Netherlands), and a Rosalind Franklin Fellowship co-funded by the European Union and the University of Groningen. M.S. received support by Alzheimer Nederland grant WE.03-2019-05 and is supported by the Deutsche Forschungsgemeinschaft (IRTG1874DIAMICOM-SP2) and Novartis unrestricted grant 50199468. K.R. is supported by a ZonMW Off Road Grant (04510242410069) and a NWO XS Grant (OCENW.XS26.1.159).

AUTHOR CONTRIBUTIONS

A.L.M.C., C.H.T.J. M.v.d.V, A.T., and P. A., performed experiments. A. L. M. C. and J. W. analysed data. J. d. V. I. and A. H. supplied H1N1/PR8 and guided experimental set-up. A. L. M. C., A. M. D., M. S., and K. R. planned experiments and A. L. M. C., M. S., and K.R. conceptualized the story. A. L. M. C., M. S., and K.R. wrote the manuscript with input from all authors.

DECLARATION OF COMPETING INTERESTS

The authors declare no competing interests.

REFERENCES

1. Rex, D.A.B., *et al.* (2022) Revisiting Regulated Cell Death Responses in Viral Infections. *Int J Mol Sci* 23, 7023
2. Rao, L., *et al.* (1992) The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. *Proc Natl Acad Sci U S A* 89, 7742-7746
3. Takizawa, T., *et al.* (1993) Induction of programmed cell death (apoptosis) by influenza virus infection in tissue culture cells. *J Gen Virol* 74 (Pt 11), 2347-2355
4. Dixon, S.J., *et al.* (2012) Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell* 149, 1060-1072
5. Zhao, X., *et al.* (2025) Ferroptosis, from the virus point of view: opportunities and challenges. *Crit Rev Microbiol* 51, 246-263
6. Niu, S., *et al.* (2025) A comprehension and systematic insight into the interaction between ferroptosis and virus infection: The implications of mechanisms and strategies. *Virulence* 16, 2532806
7. Dixon, S.J. and Stockwell, B.R. (2019) The Hallmarks of Ferroptosis. *Annual Review of Cancer Biology* 3, 35-54
8. Yagoda, N., *et al.* (2007) RAS-RAF-MEK-dependent oxidative cell death involving voltage-dependent anion channels. *Nature* 447, 864-868
9. Feng, F., *et al.* (2024) Mitochondria-mediated Ferroptosis in Diseases Therapy: From Molecular Mechanisms to Implications. *Aging Dis* 15, 714-738
10. Javadov, S. (2022) Mitochondria and ferroptosis. *Curr Opin Physiol* 25
11. Gao, M., *et al.* (2019) Role of Mitochondria in Ferroptosis. *Mol Cell* 73, 354-363 e353
12. Wang, J., *et al.* (2023) The role of ferroptosis in virus infections. *Front Microbiol* 14, 1279655
13. Wang, M.P., *et al.* (2022) Ferroptosis in viral infection: the unexplored possibility. *Acta Pharmacol Sin* 43, 1905-1915
14. Shinya, K., *et al.* (2006) Avian flu: influenza virus receptors in the human airway. *Nature* 440, 435-436
15. Saunders-Hastings, P.R. and Krewski, D. (2016) Reviewing the History of Pandemic Influenza: Understanding Patterns of Emergence and Transmission. *Pathogens* 5, 66
16. Letafati, A., *et al.* (2024) Unveiling the intersection: ferroptosis in influenza virus infection. *Virol J* 21, 185
17. Cheng, J., *et al.* (2022) Swine influenza virus triggers ferroptosis in A549 cells to enhance virus replication. *Virol J* 19, 104
18. Huang, J., *et al.* (2023) Network pharmacology and experimental validation of Maxing Shigan decoction in the treatment of influenza virus-induced ferroptosis. *Chin J Nat Med* 21, 775-788
19. Ouyang, A., *et al.* (2024) The Hemagglutinin of Influenza A Virus Induces Ferroptosis to Facilitate Viral Replication. *Adv Sci (Weinh)* 11, e2404365
20. Mazel-Sanchez, B., *et al.* (2023) Influenza A virus exploits transferrin receptor recycling to enter host cells. *Proc Natl Acad Sci U S A* 120, e2214936120
21. Zhang, Y. and Bergelson, J.M. (2005) Adenovirus receptors. *J Virol* 79, 12125-12131

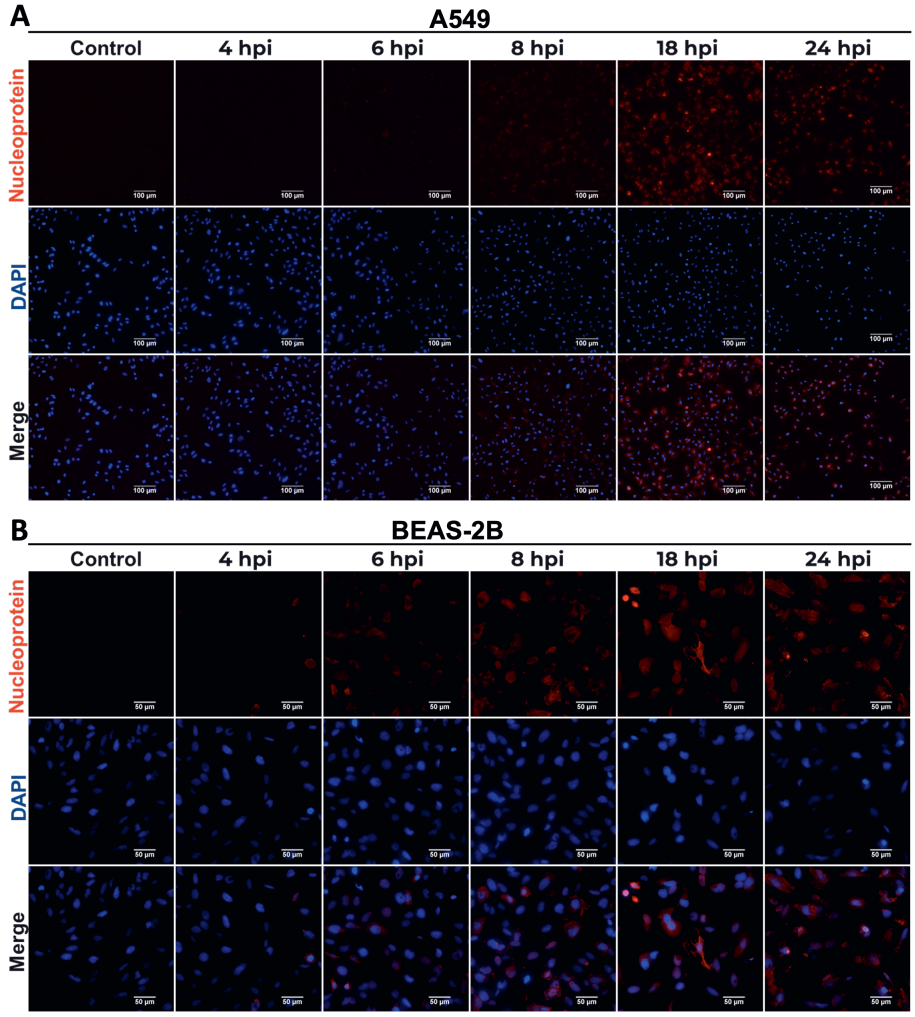
22. Excoffon, K.J., *et al.* (2010) Isoform-specific regulation and localization of the coxsackie and adenovirus receptor in human airway epithelia. *PLoS One* 5, e9909
23. Wold, W. and Ison, M. (2013) Adenoviruses, p 1732–1767. *Fields virology* 2
24. Group, H.W. http://hadvwg.gmu.edu
25. Braithwaite, A.W. and Russell, I.A. (2001) Induction of cell death by adenoviruses. *Apoptosis* 6, 359-370
26. Lynch, J.P., 3rd and Kajon, A.E. (2016) Adenovirus: Epidemiology, Global Spread of Novel Serotypes, and Advances in Treatment and Prevention. *Semin Respir Crit Care Med* 37, 586-602
27. MacNeil, K.M., *et al.* (2023) Adenoviruses in medicine: innocuous pathogen, predator, or partner. *Trends Mol Med* 29, 4-19
28. Lion, T. (2014) Adenovirus infections in immunocompetent and immunocompromised patients. *Clin Microbiol Rev* 27, 441-462
29. Orino, K., *et al.* (1999) Adenovirus E1A blocks oxidant-dependent ferritin induction and sensitizes cells to pro-oxidant cytotoxicity. *FEBS Lett* 461, 334-338
30. Dong, W., *et al.* (2024) FAdV-4-induced ferroptosis affects fat metabolism in LMH cells. *Vet Microbiol* 293, 110068
31. Pogorzelska, A., *et al.* (2024) Impact of Human Adenovirus 36 on Embryonated Chicken Eggs: Insights into Growth Mechanisms. *Int J Mol Sci* 25, 2341
32. Wang, Z.Q., *et al.* (2010) Human adenovirus 36 decreases fatty acid oxidation and increases de novo lipogenesis in primary cultured human skeletal muscle cells by promoting Cidec/FSP27 expression. *Int J Obes (Lond)* 34, 1355-1364
33. Liu, C., *et al.* (2023) H1N1 influenza virus infection through NRF2-KEAP1-GCLC pathway induces ferroptosis in nasal mucosal epithelial cells. *Free Radic Biol Med* 204, 226-242
34. Jansen, S.R., *et al.* (2016) Epac1 links prostaglandin E2 to beta-catenin-dependent transcription during epithelial-to-mesenchymal transition. *Oncotarget* 7, 46354-46370
35. Cattani-Cavalieri, I., *et al.* (2024) Diesel exhaust particles alter mitochondrial bioenergetics and cAMP producing capacity in human bronchial epithelial cells. *Front Toxicol* 6, 1412864
36. Pei, Z., *et al.* (2022) Inhibition of ferroptosis and iron accumulation alleviates pulmonary fibrosis in a bleomycin model. *Redox Biol* 57, 102509
37. Kistemaker, L.E., *et al.* (2015) Tiotropium attenuates IL-13-induced goblet cell metaplasia of human airway epithelial cells. *Thorax* 70, 668-676
38. Rahmawati, S.F., *et al.* (2022) Function-specific IL-17A and dexamethasone interactions in primary human airway epithelial cells. *Sci Rep* 12, 11110
39. van Wetering, S., *et al.* (2000) Regulation of secretory leukocyte proteinase inhibitor (SLPI) production by human bronchial epithelial cells: increase of cell-associated SLPI by neutrophil elastase. *J Investig Med* 48, 359-366
40. Zuo, H., *et al.* (2020) A-Kinase Anchoring Proteins Diminish TGF-beta(1)/Cigarette Smoke-Induced Epithelial-To-Mesenchymal Transition. *Cells* 9
41. Audouy, S.A., *et al.* (2011) Development of a dried influenza whole inactivated virus vaccine for pulmonary immunization. *Vaccine* 29, 4345-4352

42. Lefkovits, I. and Cosenza, H. (1979) 19 - Assay for Plaque-Forming Cells. In *Immunological Methods* (Lefkovits, I. and Pernis, B., eds), pp. 277-285, Academic Press
43. Yang, F.*, et al.* (2021) Discovery of a Novel Specific Inhibitor Targeting Influenza A Virus Nucleoprotein with Pleiotropic Inhibitory Effects on Various Steps of the Viral Life Cycle. *J Virol* 95
44. Danskog, K.*, et al.* (2025) Lactoferricin enables adenovirus infection of human skeletal muscle cells. *Npj Viruses* 3, 62
45. Schneider, C.A.*, et al.* (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9, 671-675
46. Zhang, L.*, et al.* (2025) Regulation of calcium signaling prevents neuronal death mediated by NIST DEP in xenoferroptotic cell death conditions. *J Hazard Mater* 488, 137374
47. Schindelin, J.*, et al.* (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9, 676-682
48. Yan, H.*, et al.* (2025) Exposure to diesel exhaust particles accelerated neuronal cell death in xenoferroptosis-susceptible cells. *Journal of Environmental Sciences*
49. Murray, M.B.*, et al.* (2023) Protocol for detection of ferroptosis in cultured cells. *STAR Protoc* 4, 102457
50. Aguilar Diaz De Leon, J. and Borges, C.R. (2020) Evaluation of Oxidative Stress in Biological Samples Using the Thiobarbituric Acid Reactive Substances Assay. *J Vis Exp*
51. Valente, A.J.*, et al.* (2017) A simple ImageJ macro tool for analyzing mitochondrial network morphology in mammalian cell culture. *Acta Histochem* 119, 315-326
52. Chaudhry, A.*, et al.* (2020) A pipeline for multidimensional confocal analysis of mitochondrial morphology, function, and dynamics in pancreatic beta-cells. *Am J Physiol Endocrinol Metab* 318, E87-E101
53. Yang, W.S.*, et al.* (2014) Regulation of ferroptotic cancer cell death by GPX4. *Cell* 156, 317-331
54. Yang, W.S. and Stockwell, B.R. (2008) Synthetic lethal screening identifies compounds activating iron-dependent, nonapoptotic cell death in oncogenic-RAS-harboring cancer cells. *Chem Biol* 15, 234-245
55. Zheng, Y.*, et al.* (2024) Emerging mechanisms of lipid peroxidation in regulated cell death and its physiological implications. *Cell Death Dis* 15, 859
56. Wiernicki, B.*, et al.* (2020) Excessive phospholipid peroxidation distinguishes ferroptosis from other cell death modes including pyroptosis. *Cell Death Dis* 11, 922
57. Tsikas, D. (2017) Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. *Anal Biochem* 524, 13-30
58. Crisostomo, L.*, et al.* (2019) Temporal dynamics of adenovirus 5 gene expression in normal human cells. *PLoS One* 14, e0211192
59. Elesela, S. and Lukacs, N.W. (2021) Role of Mitochondria in Viral Infections. *Life (Basel)* 11, 232
60. Cervantes-Silva, M.P.*, et al.* (2021) Alterations in mitochondrial morphology as a key driver of immunity and host defence. *EMBO Rep* 22, e53086

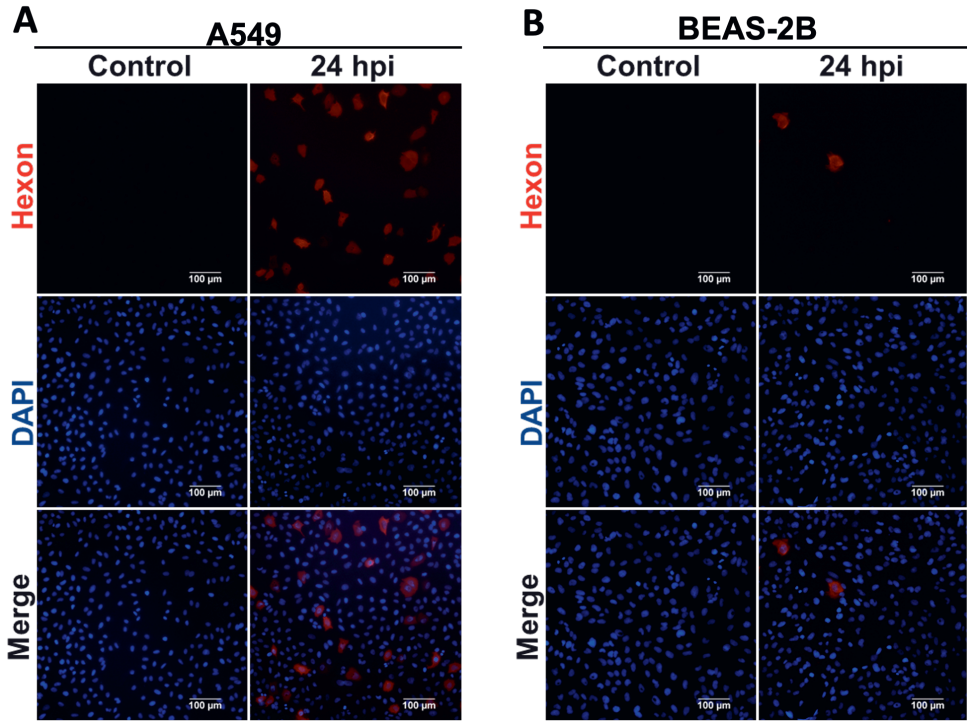
61. Pila-Castellanos, I., *et al.* (2021) Mitochondrial morphodynamics alteration induced by influenza virus infection as a new antiviral strategy. *PLoS Pathog* 17, e1009340
62. Haas, K.M., *et al.* (2023) Proteomic and genetic analyses of influenza A viruses identify pan-viral host targets. *Nat Commun* 14, 6030
63. Wang, H., *et al.* (2020) Iron status is linked to disease severity after avian influenza virus H7N9 infection. *Asia Pac J Clin Nutr* 29, 593-602
64. Lalueza, A., *et al.* (2020) Elevation of serum ferritin levels for predicting a poor outcome in hospitalized patients with influenza infection. *Clin Microbiol Infect* 26, 1557 e1559-1557 e1515
65. Lejeune, N., *et al.* (2021) Infection of Bronchial Epithelial Cells by the Human Adenoviruses A12, B3, and C2 Differently Regulates the Innate Antiviral Effector APOBEC3B. *J Virol* 95, e0241320
66. Qi, L., *et al.* (2020) Adenovirus 7 Induces Interlukin-6 Expression in Human Airway Epithelial Cells via p38/NF-kappaB Signaling Pathway. *Front Immunol* 11, 551413
67. Musheshe, N., *et al.* (2022) Pharmacological Inhibition of Epac1 Averts Ferroptosis Cell Death by Preserving Mitochondrial Integrity. *Antioxidants (Basel)* 11, 314
68. Wang, R., *et al.* (2021) Influenza A virus protein PB1-F2 impairs innate immunity by inducing mitophagy. *Autophagy* 17, 496-511
69. Varga, Z.T., *et al.* (2012) Influenza virus protein PB1-F2 inhibits the induction of type I interferon by binding to MAVS and decreasing mitochondrial membrane potential. *J Virol* 86, 8359-8366
70. Yoshizumi, T., *et al.* (2014) Influenza A virus protein PB1-F2 translocates into mitochondria via Tom40 channels and impairs innate immunity. *Nat Commun* 5, 4713
71. Matrosovich, M.N., *et al.* (2004) Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *Proc Natl Acad Sci U S A* 101, 4620-4624
72. Kim, S., *et al.* (2015) Mitochondrial reactive oxygen species modulate innate immune response to influenza A virus in human nasal epithelium. *Antiviral Res* 119, 78-83
73. Kroeker, A.L., *et al.* (2013) Influenza A infection of primary human airway epithelial cells up-regulates proteins related to purine metabolism and ubiquitin-related signaling. *J Proteome Res* 12, 3139-3151
74. Kazer, S.W., *et al.* (2024) Primary nasal influenza infection rewires tissue-scale memory response dynamics. *Immunity* 57, 1955-1974 e1958
75. Yamada, K., *et al.* (2024) Compartmentalized mitochondrial ferroptosis converges with optineurin-mediated mitophagy to impact airway epithelial cell phenotypes and asthma outcomes. *Nat Commun* 15, 5818
76. Anand, S.K. and Tikoo, S.K. (2013) Viruses as modulators of mitochondrial functions. *Adv Virol* 2013, 738794
77. Lomonosova, E., *et al.* (2005) Mitochondrial localization of p53 during adenovirus infection and regulation of its activity by E1B-19K. *Oncogene* 24, 6796-6808
78. Perez, D. and White, E. (2000) TNF-alpha signals apoptosis through a bid-dependent conformational change in Bax that is inhibited by E1B 19K. *Mol Cell* 6, 53-63
79. Han, J., *et al.* (1998) Interaction of E1B 19K with Bax is required to block Bax-induced loss of mitochondrial membrane potential and apoptosis. *Oncogene* 17, 2993-3005

80. Subramanian, T., *et al.* (2015) Interaction of cellular proteins with BCL-xL targeted to cytoplasmic inclusion bodies in adenovirus infected cells. *Virology* 483, 21-31
81. Subramanian, T., *et al.* (2006) Genetic identification of adenovirus type 5 genes that influence viral spread. *J Virol* 80, 2000-2012
82. Jung, M.S., *et al.* (2004) Bcl-xL and E1B-19K proteins inhibit p53-induced irreversible growth arrest and senescence by preventing reactive oxygen species-dependent p38 activation. *J Biol Chem* 279, 17765-17771
83. Schubert, E., *et al.* (2024) Complex regulation of mitochondrial signaling by human adenovirus minor capsid protein VI. *J Virol* 98, e0035624
84. Singh, M.K., *et al.* (2024) Molecular Chaperonin HSP60: Current Understanding and Future Prospects. *Int J Mol Sci* 25, 5483
85. Qiu, S., *et al.* (2023) Mitochondria-localized cGAS suppresses ferroptosis to promote cancer progression. *Cell Res* 33, 299-311
86. Chen, Q., *et al.* (2016) Regulation and function of the cGAS-STING pathway of cytosolic DNA sensing. *Nat Immunol* 17, 1142-1149
87. Park, H.S., *et al.* (2018) Swine Influenza Virus Induces RIPK1/DRP1-Mediated Interleukin-1 Beta Production. *Viruses* 10
88. Merrill, R.A. and Strack, S. (2014) Mitochondria: a kinase anchoring protein 1, a signaling platform for mitochondrial form and function. *Int J Biochem Cell Biol* 48, 92-96
89. Zuo, H., *et al.* (2018) Cigarette smoke up-regulates PDE3 and PDE4 to decrease cAMP in airway cells. *Br J Pharmacol* 175, 2988-3006
90. Foret-Lucas, C., *et al.* (2023) EPAC1 Pharmacological Inhibition with AM-001 Prevents SARS-CoV-2 and Influenza A Virus Replication in Cells. *Viruses* 15
91. Yang, X., *et al.* (2023) Artesunate inhibits PDE4 leading to intracellular cAMP accumulation, reduced ERK/MAPK signaling, and blockade of influenza A virus vRNP nuclear export. *Antiviral Res* 215, 105635
92. King, C.R., *et al.* (2018) Mimicry of Cellular A Kinase-Anchoring Proteins Is a Conserved and Critical Function of E1A across Various Human Adenovirus Species. *J Virol* 92

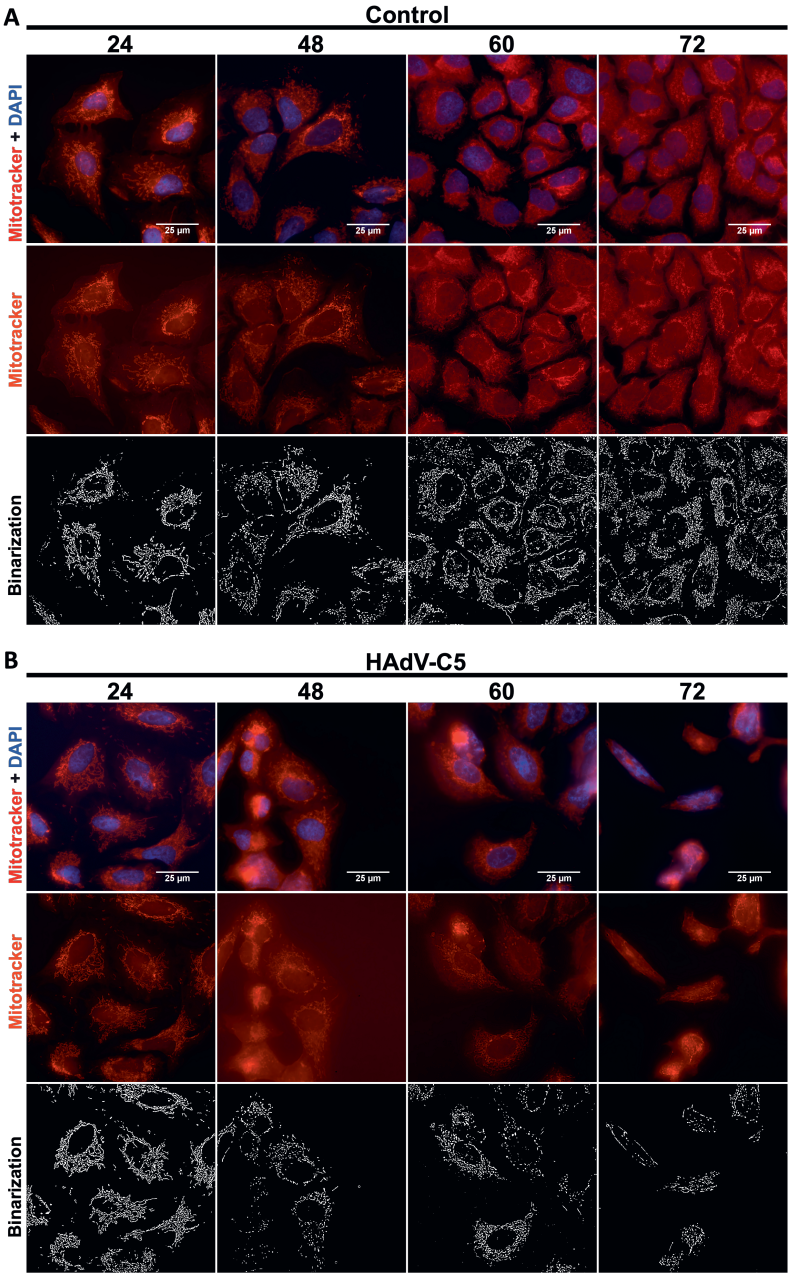
SUPPLEMENTARY FIGURES



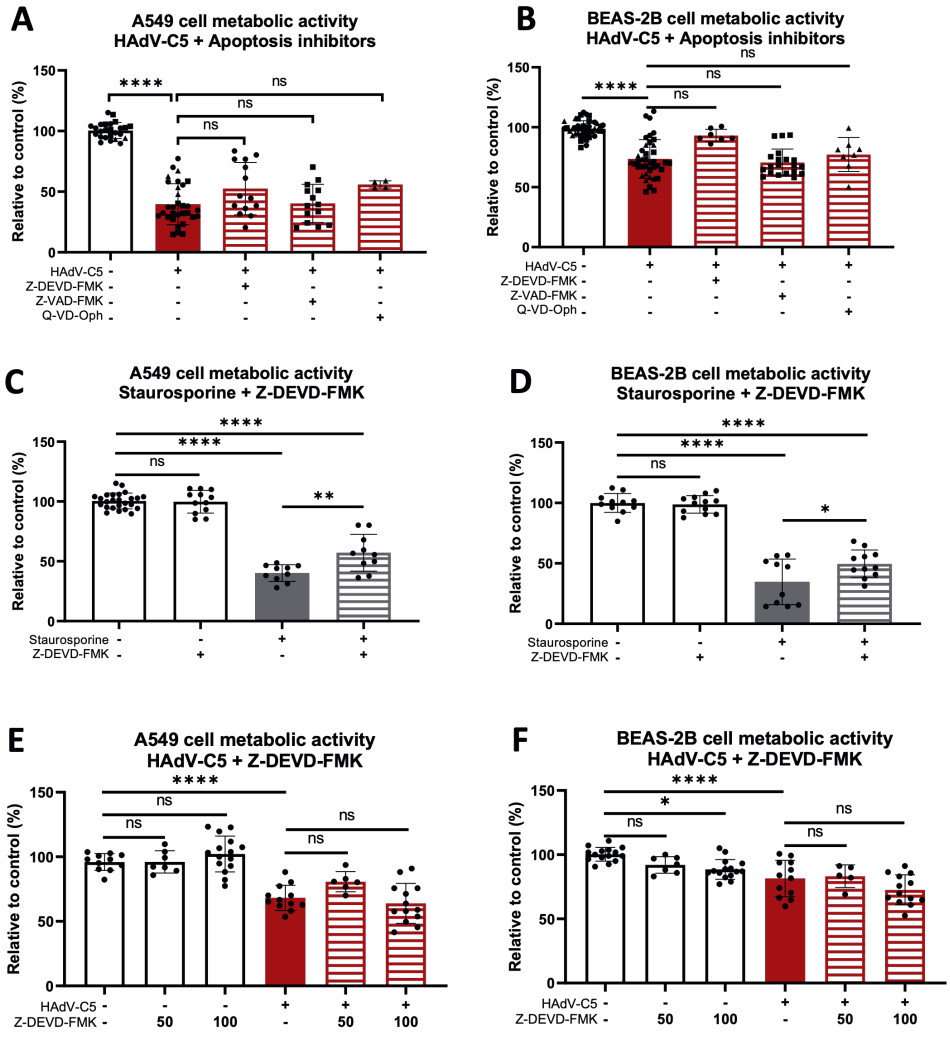
Supplementary Fig. 1. H1N1 replication rate in lung epithelial cells. Fluorescence images taken at a nominal magnification of 20x of (A) A549 and (B) BEAS-2B cells infected with H1N1/PR8 influenza virus at a MOI 1 fixed and stained at 4, 6, 8, 18 and 24 hpi with anti-influenza nucleoprotein (NP) antibody and counterstained with DAPI. Red: NP positive cells; Blue: nucleus (scale bar = 100 µm for A549 cells and 50 µm for BEAS-2B cells).



Supplementary Fig. 2. HAdV-C5 replication rate in lung epithelial cells. Fluorescence images taken at a nominal magnification of 20x of (A) A549 and BEAS-2B cells infected with HAdV-C5 virus at a MOI 1 fixed and stained at 24 hpi with anti-hexon antibody and counterstained with DAPI. Red: hexon positive cells; Blue: nucleus (scale bar = 100 µm).



Supplementary Fig. 3. HAdV-C5 infection causes changes to mitochondria in A549 cells at later states of infection. MitoTracker™ Red FM fluorescence images taken at a nominal magnification of 60X of A549 cells (A) control and (B) after infection with HAdV-C5 at an MOI 1 at 24, 48, 60 and 72 hpi Red: mitochondria; Blue: nucleus (scale bar = 25 µm).



Supplementary Figure 4. Apoptosis inhibitors do not rescue cell death caused by HAdV-C5 in alveolar epithelial (A549) and bronchial epithelial (BEAS-2B) cells. MTT assay as measurement of cell viability. (A) A549 and (B) BEAS-2B cells at 72 hpi with HAdV-C5 (MOI = 1) and co-treated with Z-DEVD-FMK (10 µM) Z-VAD-FMK (10 µM) or Q-VD-Oph (10 µM). (C) A549 and (D) BEAS-2B cells after 72 h treatment with staurosporine (50 nM for A549, 5 nM for BEAS-2B) and co-treated with Z-DEVD-FMK (10 µM). (E) A549 and (F) BEAS-2B at 72 hpi with HAdV-C5 (MOI = 1) and co-treated with 50 or 100 µM Z-DEVD-FMK. Cell viability of the control groups were defined as 100% viability. Results are shown as percentage relative to the control (mean ± SD of technical replicates). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparisons test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. *50 µM Z-DEVD-FMK +/- HAdV represents two biological replicates, other results are of at least three biological replicates.



Chapter 5

General discussion

The airway epithelium is a crucial barrier between the body and the external environment; it protects against pathogens and environmental pollutants. Beyond its physical barrier function, the airway epithelium actively plays a role in coordinating immune responses to external stimuli [1]. In this thesis, I focused on particulate matter (PM) and respiratory viruses as stressors of the airway epithelium. Specifically, I investigated the cellular damaged caused by these external stressors, with particular attention to oxidative stress, mitochondrial dysfunction, and inflammation. I show that influenza A virus (IAV) H1N1 and human adenovirus type C5 (HAV-C5) induce ferroptosis features in bronchial and alveolar epithelial cell lines, including alterations in iron homeostasis, mitochondrial network morphology and lipid peroxidation. Importantly, I demonstrate that both IAV H1N1 and HAdV-C5 profoundly alter the mitochondrial network in primary human airway epithelial cells, adding translational relevance to these findings. In this chapter, I provide a general discussion of the findings presented in this thesis and highlight mitochondria as a key vulnerable target of PM-and virus- induced ferroptosis.

In **Chapter 2** I reviewed the impact of environmental pollutants, with special focus on particulate matter (PM), on the airways as the main point of entrance. I explored how pollutants are sensed via Toll-like receptors (TLRs) and G-coupled proteins and their effect on inflammation and proteotoxicity, cilia function, tissue repair and cell-cell communication, ultimately leading to a detrimental effect on epithelial barriers.

The disruption of epithelial barrier integrity sets the stage for the development of respiratory diseases, including chronic obstructive pulmonary disease (COPD) and asthma [1, 2], on which I focused in **Chapter 3**. I reviewed their distinct chronic inflammatory profiles and how these create a microenvironment that renders the airway epithelium particularly susceptible to external insults. Because infections are a common cause of exacerbation in both COPD and asthma, I highlighted how viral activation of pattern recognition receptors (PRRs) and the cytopathic effect of viruses synergistically contribute to epithelial tight junction disruption, hyperinflammation, and cell death.

FERROPTOSIS AS CELL DEATH MECHANISM AND DRIVER OF INFLAMMATION

The work presented in this thesis delves into ferroptosis as a significant cause of cell death in lung disease. In **Chapter 2** I establish pollutants as triggers of oxidative stress and mitochondrial dysfunction, two key hallmarks of ferroptosis. In **Chapter 4**, I investigated whether ferroptosis-like features were induced in lung epithelial cells during viral infection. Using influenza A virus (IAV) as a reference, I compared ferroptosis-like features induced by human adenovirus type C5 (HAdV-C5) in an alveolar type II epithelial cell line (A549) and a bronchial epithelial cell line (BEAS-2B). I found IAV increased lipid peroxidation and ferritin expression in both cell lines, supporting previous research that IAV activates ferroptosis as an infection mechanism [3, 4]. In the case of HAdV-C5, it caused lipid peroxidation in the alveolar type II epithelial cell line in advanced stages of infection. Further research is needed to clarify the molecular mechanisms by which HAdV-C5 induces lipid peroxidation. Such studies should also consider the role of the HAdV E1A protein, which has been reported to block ferritin transcription [5]. Interestingly, in agreement with these observations and studies on other adenovirus types, HAdV-C5 downregulated ferritin expression [6], which might suggest HAdV-C5 alters iron homeostasis in the cell to sensitize infected cells to oxidative stress and increase the accumulation of reactive oxygen species (ROS) (**Figure 1**).

Persistent adenovirus infections have been linked to the development of COPD [7], in which the integration of E1A gene into the host genome is responsible for enhancing inflammatory responses in the host cells [8] and inducing airway remodeling [9]. By definition, persistent adenovirus infections generally go unnoticed. However, they may act as an important primer for aggravated inflammatory responses upon following a "second hit". Indeed, the inflammatory response of guinea pigs exposed to a single acute exposure to cigarette smoke was higher in those with persistent adenovirus 5 infection compared to those that were uninfected [10]. In **Chapter 3** I review the molecular mechanisms of inflammation in COPD and asthma and how viruses exacerbate and worsen these diseases. Therefore, the findings in **Chapter 4** which show that HAdV-C5 induces the accumulation of lipid peroxides- a hallmark of ferroptosis- lay the groundwork for future research.

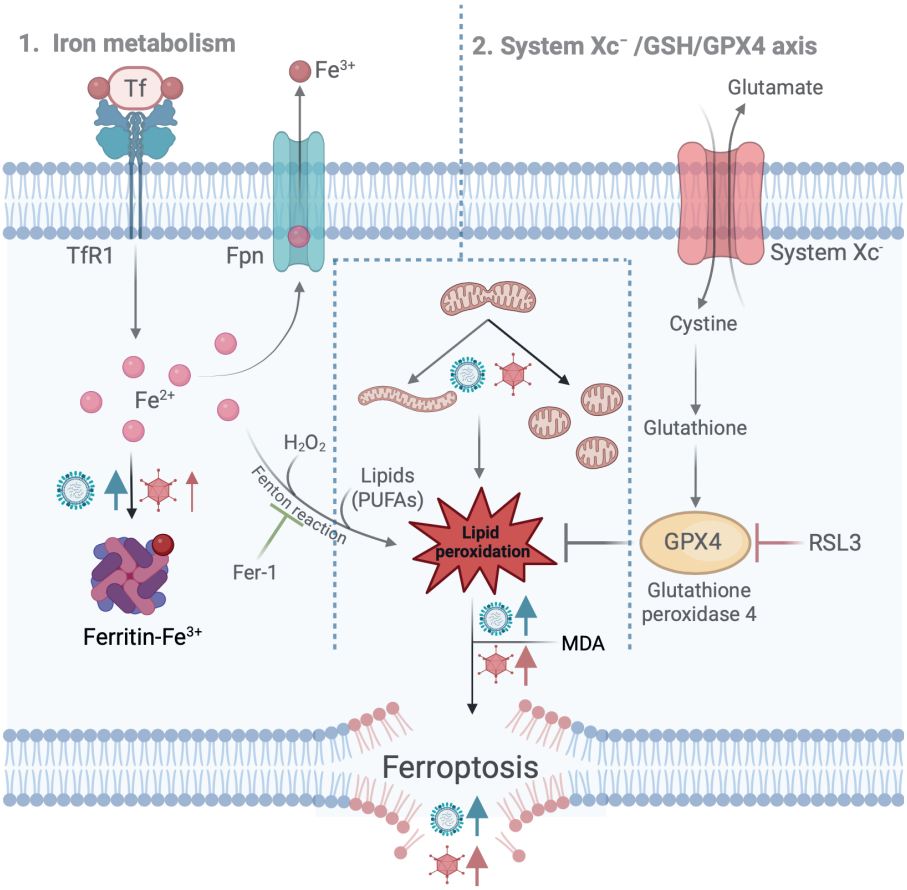


Figure 1. Virus-induced ferroptosis. Mechanisms by which influenza A virus (IAV) H1N1 (blue arrows) and human adenovirus type C5 (HAdV-C5) (red arrows) induce ferroptosis. Abbreviations: Fer-1, ferrostatin-1; Fpn, ferroportin; GPX4, glutathione peroxidase 4; GSH, glutathione; MDA, malondialdehyde; PUFAs, polyunsaturated fatty acids; RSL3, RAS-selective lethal 3; Tf, transferrin; TfR1, transferrin receptor 1. Figure 1 was created using BioRender (www.biorender.com).

MITOCHONDRIA AS CENTRAL HUBS OF FERROPTOSIS AND CELL DAMAGE

Mitochondria, popularly known as the powerhouse of the cell, are double membrane bound organelles, with an outer membrane that is in direct contact with the cytosol and an inner membrane that folds into the matrix (cristae) [11]. The cristae provide a high surface area for the production of ATP through oxidative phosphorylation by the electron transport chain. In the matrix, the tricarboxylic acid enzymes generate the necessary reducing agents and metabolic intermediates [11]. During these processes, considerable amounts of the highly reactive superoxide anion (O_2^-) are generated. Superoxide is quickly converted into hydrogen peroxide, which is more stable and can diffuse across mitochondrial membranes into the cytosol, where it is subsequently neutralized [12]. When mitochondrial reactive oxygen species (mtROS) production surpasses the cellular redox capacity, it may lead to glutathione (GSH) depletion and downstream lipid peroxidation, leading to ferroptosis. Therefore, mitochondria play a central role in ferroptosis.

Mitochondria are highly dynamic organelles that continuously undergo fusion and fission. Fusion merges mitochondria into longer, interconnected networks, whereas fission splits them into smaller fragments or individual mitochondria, thereby allowing them to respond to external stimuli by changing their size, shape and cristae distribution. Mitochondrial dynamics are determined by four large GTPases, optical atrophy 1 (OPA1), mitofusin 1 and 2 (MFN1/2) and dynamin-related protein 1 (DRP1). OPA1, MFN1 and MFN2 are all located in the mitochondrial membranes and drive fusion while DRP1 is located in the cytosol and translocates to the outer mitochondrial membrane (OMM) to trigger fission [11]. Maintaining balance between fission and fusion is essential for cellular health. Dysregulation of either of these opposing processes has been linked to numerous diseases, including lung diseases and infection [12-14]. In **Chapter 4**, I report virus-induced changes in the mitochondrial network of bronchial and alveolar epithelial cell lines following infection with IAV H1N1 and HAdV-C5. The observed changes in branch length, mean form factor and footprint, suggest disruption of mitochondrial homeostasis. Importantly, profound alterations were observed in primary human airway epithelial cells, demonstrating that virus-induced

mitochondrial remodeling is an early feature of IAV H1N1 and HAdV-C5 infection in a physiologically relevant model (**Figure 1**).

Indeed, alterations in mitochondrial dynamics have been reported in cells infected with IAV. Yoshizumi *et al.* found that translocation of IAV protein PB1-F2 to the mitochondria membrane causes fission and results in fragmented mitochondrial morphology [15]. In line with those findings, I report marked changes toward fragmentation of the mitochondrial network in IAV-infected BEAS-2B bronchial epithelial cells and, similarly, a trend towards fragmentation in IAV-infected A549 alveolar epithelial cells. Interestingly, in IAV-infected primary human airway epithelial cells I observed marked changes toward mitochondrial elongation, similar to the findings reported by Pila-Castellanos *et al* [16]. Research on mitochondrial network morphology during IAV infection remains limited. Therefore, I cannot offer a clear explanation of the molecular mechanisms responsible for the different morphologies that I observed. Nevertheless, results indicate that IAV-induced alterations in mitochondrial morphodynamics are cell type-specific and appear to be more evident between immortalized cell lines and primary cells. Consistent with this observation, previous transcriptomic and proteomic studies have reported distinct responses to IAV across different cells [17, 18]. Additionally, I provide supporting evidence of IAV-induced mitochondrial fragmentation under low serum culture conditions.

Similarly, I showed that HAdV-C5 causes alterations to the mitochondrial network of infected cells. Modulation of mitochondrial function is important for adenovirus infection [19]. E1B-19K, an adenovirus protein well known for its ability to bind to Bax and Bak to prevent apoptosis, localizes to the mitochondria during early stages of infection [20, 21]. Recent research by Schubert *et al.* indicates that adenovirus additionally modulates mitochondrial integrity during late stages of infection by localizing its minor capsid protein VI to mitochondrial membranes, causing the release of mitochondrial DNA (mtDNA) and proteins into the cytosol [22]. Interestingly, Schubert *et al.* found that E1B-19K protein counteracted this release, suggesting that adenovirus-induced disruption of mitochondrial homeostasis is controlled by spatiotemporal expression of both E1B-19K and protein VI [22]. In the

HAdV-C5-infected cell models I used, I report alterations to the mitochondrial network that worsens as infection progresses, which may support that mitochondrial disruption by HAdV-C5 occurs predominantly during late stages of infection.

DRP1-cGAS/STING AND cAMP SIGNALING AS REGULATORS OF MITOCHONDRIAL HEALTH

Modulation of mitochondrial morphodynamics by Drp1 is directly linked to acceleration of ferroptosis by promoting lipid peroxidation of mitochondrial membranes [23]. Growing evidence suggest that Drp1 depletion preserves mitochondrial integrity and diminishes the detrimental effects of RSL3- and erastin-induced ferroptotic cell death [24]. To execute its function, Drp1 must be translocated from the cytosol and anchor in the OMM to induce mitochondrial fragmentation. Four mitochondrial adaptor proteins are largely responsible for anchoring Drp1: mitochondrial fission factor (Mff), mitochondrial fission 1 protein (Fis1), and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49 and MiD51, respectively) [25]. Recent research by Qiu *et al.* indicates that cyclic GMP-AMP synthase (cGAS) can also act as an adaptor protein by translocating to the OMM where it facilitates DRP1 oligomerization [26]. Interestingly, the authors observed that mitochondrial cGAS inhibits lipid peroxidation in cancer cells, a process dependent on the interaction with Drp1 and its subsequent oligomerization. cGAS is a cytosolic sensor of viral and bacterial DNA that activates innate immune responses through the production of cyclic GMP-AMP (cGAMP) production and subsequent activation of stimulator of interferon genes (STING). Together with reports of reduced Drp1 levels during IAV infection [16], these findings suggest that further research into the relationship between cGAS, Drp1 and mitochondria morphodynamics during virus-induced ferroptosis is warranted.

As mentioned above, Drp1 must translocate from the cytosol to the OMM. This translocation is a process that depends on the phosphorylation of serine 616 (S616) to activate Drp1 or of serine 637 (S637) to deactivate it [27]. Protein kinase A (PKA), through the phosphorylation of S637, inhibits the activity of Drp1 to promote mitochondrial elongation by unopposed fusion [28]. Activation of PKA depends on

second messenger cyclic AMP (cAMP), which, interestingly, has been linked to mitochondrial integrity regulation in lung epithelial cells under oxidative stress induced by diesel exhaust particles (DEP) [29]. Interestingly, preliminary data from ongoing work in our group indicate profound changes in cAMP levels and differential expression of cAMP effector genes in infected cells. Notably, studies have reported that inhibition of exchange proteins directly activated by cAMP (EPAC) impairs viral replication of respiratory viruses, including IAV H1N1 and HAdV-C5, evidencing an important link to cAMP levels [30, 31]. Furthermore, recent work from our group showed that inhibition of EPAC1 prevented DEP-induced accelerated cell death under ferroptotic conditions by reducing mitochondrial ROS production and lipid peroxidation [32, 33]. These findings strongly suggest a link between cAMP signalling, mitochondrial homeostasis and ferroptosis. Whether cAMP signaling, mitochondria morphodynamics, and viral infection are also linked remain to be elucidated.

FUTURE PERSPECTIVES

This thesis provides important groundwork for research aiming to further understand the molecular mechanisms of ferroptosis during HAdV-C5 infection. Additional knowledge regarding the expression of proteins involved in the different ferroptosis pathways, including glutathione peroxidase 4 (GPX4), transferrin (Tf), transferrin receptor (TfR1), nuclear receptor coactivator 4 (NCOA4), and the System Xc^- subunits SLC3A2 and SLC7A11, is needed. I provide evidence that IAV H1N1 and HAdV-C5 profoundly alter mitochondrial network morphology, and together with ongoing research in our group, our evidence suggests that cAMP modulation impacts oxidative stress and mitochondrial function during viral infection to modulate ferroptosis. Future research should focus on clarifying the molecular mechanisms by which these viruses induce such alterations, for example by using pharmacological tools to regulate cAMP effector proteins and evaluating their effect on mitochondrial morphodynamics and function during infection.

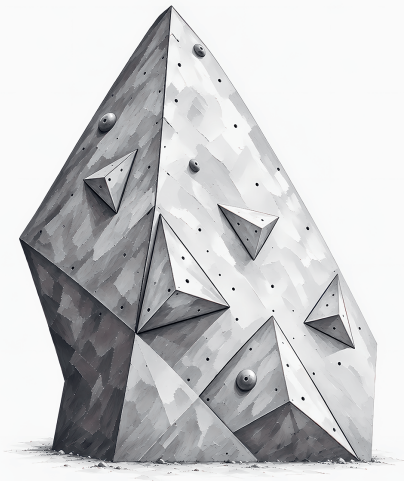
Furthermore, ferroptosis is known to contribute to severe tissue damage and fatal respiratory infections [34, 35], but underlying mechanisms remain unknown. Growing research indicates that ferroptosis is involved in airway inflammatory

diseases and contributes to chronic airway inflammation and oxidative stress [36]. While low levels of ROS can be beneficial to the cell by functioning as signaling molecules to induce adaptive responses, high levels of ROS are detrimental [37]. Under these circumstances, ROS-induced mitochondrial damage seems to mark the point of no return to ferroptotic cell death [24]. Notably, there is a knowledge gap regarding the combined effect of respiratory infections and sub-threshold triggers of ferroptosis, such as environmental pollutants or underlying chronic inflammation profiles. The importance of such "double hits" has primarily been studied in the context of neurotoxicity, where accelerated cell death was observed following exposure to low levels of air pollution in combination with low levels of RSL3 [38]. The accelerated cell death induced by the double hits, also known as xenoferroptosis, is driven by an additional stressor that amplifies the production of ROS and lipid peroxidation in cells with pre-existing, sub-threshold ferroptotic stimulus [39]. As the lungs are the primary point of entry for air pollutants and respiratory viruses, future research should aim to characterize the combined effect of IAV and HAdV-C5 infection with PM in lung epithelial cells and whether they cause xenoferroptosis.

REFERENCES

1. Russell, R.J., *et al.* (2024) The airway epithelium: an orchestrator of inflammation, a key structural barrier and a therapeutic target in severe asthma. *Eur Respir J* 63
2. Carlier, F.M., *et al.* (2021) Epithelial Barrier Dysfunction in Chronic Respiratory Diseases. *Front Physiol* 12, 691227
3. Cheng, J., *et al.* (2022) Swine influenza virus triggers ferroptosis in A549 cells to enhance virus replication. *Virol J* 19, 104
4. Ouyang, A., *et al.* (2024) The Hemagglutinin of Influenza A Virus Induces Ferroptosis to Facilitate Viral Replication. *Adv Sci (Weinh)* 11, e2404365
5. Orino, K., *et al.* (1999) Adenovirus E1A blocks oxidant-dependent ferritin induction and sensitizes cells to pro-oxidant cytotoxicity. *FEBS Lett* 461, 334-338
6. Dong, W., *et al.* (2024) FAdV-4-induced ferroptosis affects fat metabolism in LMH cells. *Vet Microbiol* 293, 110068
7. Matsuse, T., *et al.* (1992) Latent adenoviral infection in the pathogenesis of chronic airways obstruction. *Am Rev Respir Dis* 146, 177-184
8. Hayashi, S. (2002) Latent adenovirus infection in COPD. *Chest* 121, 183S-187S
9. Ogawa, E., *et al.* (2004) Latent adenoviral infection induces production of growth factors relevant to airway remodeling in COPD. *Am J Physiol Lung Cell Mol Physiol* 286, L189-197
10. Vitalis, T.Z., *et al.* (1998) The effect of latent adenovirus 5 infection on cigarette smoke-induced lung inflammation. *Eur Respir J* 11, 664-669
11. Chakrabarty, R.P. and Chandel, N.S. (2022) Beyond ATP, new roles of mitochondria. *Biochem (Lond)* 44, 2-8
12. Harrington, J.S., *et al.* (2023) Mitochondria in health, disease, and aging. *Physiol Rev* 103, 2349-2422
13. Duan, X., *et al.* (2025) The Essential Role of Mitochondrial Dynamics in Viral Infections. *Int J Mol Sci* 26
14. Lerner, C.A., *et al.* (2016) Mitochondrial redox system, dynamics, and dysfunction in lung inflammaging and COPD. *Int J Biochem Cell Biol* 81, 294-306
15. Yoshizumi, T., *et al.* (2014) Influenza A virus protein PB1-F2 translocates into mitochondria via Tom40 channels and impairs innate immunity. *Nat Commun* 5, 4713
16. Pila-Castellanos, I., *et al.* (2021) Mitochondrial morphodynamics alteration induced by influenza virus infection as a new antiviral strategy. *PLoS Pathog* 17, e1009340
17. Bertrams, W., *et al.* (2022) Transcriptomic comparison of primary human lung cells with lung tissue samples and the human A549 lung cell line highlights cell type specific responses during infections with influenza A virus. *Sci Rep* 12, 20608
18. Kroeker, A.L., *et al.* (2012) Response of primary human airway epithelial cells to influenza infection: a quantitative proteomic study. *J Proteome Res* 11, 4132-4146
19. Anand, S.K. and Tikoo, S.K. (2013) Viruses as modulators of mitochondrial functions. *Adv Virol* 2013, 738794
20. Han, J., *et al.* (1998) Interaction of E1B 19K with Bax is required to block Bax-induced loss of mitochondrial membrane potential and apoptosis. *Oncogene* 17, 2993-3005
21. Lomonosova, E., *et al.* (2005) Mitochondrial localization of p53 during adenovirus infection and regulation of its activity by E1B-19K. *Oncogene* 24, 6796-6808

22. Schubert, E., *et al.* (2024) Complex regulation of mitochondrial signaling by human adenovirus minor capsid protein VI. *J Virol* 98, e0035624
23. Pedrera, L., *et al.* (2025) Ferroptosis triggers mitochondrial fragmentation via Drp1 activation. *Cell Death Dis* 16, 40
24. Tang, S., *et al.* (2024) Drp1 depletion protects against ferroptotic cell death by preserving mitochondrial integrity and redox homeostasis. *Cell Death Dis* 15, 626
25. Zerihun, M., *et al.* (2023) The Drp1-Mediated Mitochondrial Fission Protein Interactome as an Emerging Core Player in Mitochondrial Dynamics and Cardiovascular Disease Therapy. *Int J Mol Sci* 24
26. Qiu, S., *et al.* (2023) Mitochondria-localized cGAS suppresses ferroptosis to promote cancer progression. *Cell Res* 33, 299-311
27. Merrill, R.A. and Strack, S. (2014) Mitochondria: a kinase anchoring protein 1, a signaling platform for mitochondrial form and function. *Int J Biochem Cell Biol* 48, 92-96
28. Chang, C.R. and Blackstone, C. (2007) Cyclic AMP-dependent protein kinase phosphorylation of Drp1 regulates its GTPase activity and mitochondrial morphology. *J Biol Chem* 282, 21583-21587
29. Cattani-Cavalieri, I., *et al.* (2024) Diesel exhaust particles alter mitochondrial bioenergetics and cAMP producing capacity in human bronchial epithelial cells. *Front Toxicol* 6, 1412864
30. Choi, E.J., *et al.* (2021) Broad Impact of Exchange Protein Directly Activated by cAMP 2 (EPAC2) on Respiratory Viral Infections. *Viruses* 13
31. Foret-Lucas, C., *et al.* (2023) EPAC1 Pharmacological Inhibition with AM-001 Prevents SARS-CoV-2 and Influenza A Virus Replication in Cells. *Viruses* 15
32. Yan, H., *et al.* (2026) Inhibition of EPAC1 Prevents Neuronal Death Mediated by Diesel Exhaust Particles in Ferroptotic Cell Death Conditions. *Antioxidants (Basel)* 15
33. Musheshe, N., *et al.* (2022) Pharmacological Inhibition of Epac1 Averts Ferroptosis Cell Death by Preserving Mitochondrial Integrity. *Antioxidants (Basel)* 11
34. Habib, H.M., *et al.* (2021) The role of iron in the pathogenesis of COVID-19 and possible treatment with lactoferrin and other iron chelators. *Biomed Pharmacother* 136, 111228
35. Qiu, B., *et al.* (2024) Fatal COVID-19 pulmonary disease involves ferroptosis. *Nat Commun* 15, 3816
36. Lin, Z., *et al.* (2022) The link between ferroptosis and airway inflammatory diseases: A novel target for treatment. *Front Mol Biosci* 9, 985571
37. Ristow, M. and Schmeisser, K. (2014) Mitohormesis: Promoting Health and Lifespan by Increased Levels of Reactive Oxygen Species (ROS). *Dose Response* 12, 288-341
38. Yan, H., *et al.* (2026) Exposure to diesel exhaust particles accelerated neuronal cell death in xenoferroptosis-susceptible cells. *J Environ Sci (China)* 163, 22-36
39. Mitchell-Garcia, T., *et al.* (2026) Xenoferroptosis, a double-hit challenge for regulated cell death. *Ferroptosis Oxid Stress* 2



Appendices

Summary

Nederlandse samenvatting

Resumen en español

Acknowledgements/Agradecimientos

Curriculum vitae

List of publications

SUMMARY

Respiratory diseases are among the leading causes of death worldwide. Two of the most common, chronic obstructive pulmonary disease (COPD) and asthma, are non-curable diseases that make breathing difficult and have a major impact on quality of life. Patients often experience periods in which their symptoms suddenly become worse, known as exacerbations. These episodes may lead to hospitalization, faster disease progression and permanent loss of lung function. Two of the most common triggers of exacerbations are respiratory viral infections and exposure to environmental pollutants, especially particulate matter (PM) present in air pollution.

The airway epithelium forms the first line of defense against inhaled PM and pathogens. In healthy lungs, the epithelium acts a physical barrier, promotes mucus clearance, and supports immune responses and tissue repair. However, in COPD and asthma, the epithelium is already weakened. As a result, exposure to pollutants or viruses triggers an exaggerated cellular response leading to chronic inflammation, oxidative stress, and damage to lung tissue. In addition, recent research has highlighted ferroptosis, a form of regulated cell death associated with oxidative stress by an imbalance in iron metabolism, as a potential mechanism underlying respiratory disease.

This thesis explored how environmental pollutants and respiratory viruses contribute to epithelial damage in chronic respiratory diseases. By combining current knowledge from the literature with experimental studies, the molecular pathways underlying oxidative stress, inflammation and ferroptosis were investigated, providing insights into how environmental and infectious stressors affect the airway epithelium.

In **Chapter 2**, environmental pollutants and their effects on cells were reviewed. This chapter describes how PM enters the body and penetrates deep into the lungs, where it can act as an important stressor and cause damage by activating cellular receptors and signaling cascades. Cells appear to “sense” pollutants, resulting in oxidative stress, mitochondrial dysfunction, inflammatory signaling and protein instability. These changes weaken the epithelial barrier integrity, impair ciliary

function and disrupt communication between epithelial and immune cells. As a result, the lungs become less able to remove inhaled particles and pathogens while persistent inflammation contributes to progression of chronic lung diseases.

In **Chapter 3**, the focus was on chronic inflammation in obstructive lung disorders. Inflammation is a natural response that protects the body against injury and infection, but it becomes harmful when it persists. In COPD and asthma, unresolved inflammation leads to continuous tissue damage, impaired tissue repair and progressive loss of lung function. This chapter reviews the complex interaction between epithelial cells, immune cells and inflammatory mediators. Respiratory viral infections are highlighted as important triggers of exacerbations as they stimulate inflammatory responses that can become excessive in patients with pre-existing chronic airway inflammation. In addition, pollutant-induced epithelial damage and virus-induced inflammation can amplify each other, creating a cycle of injury that becomes increasingly difficult to resolve and leaves the lungs a little more vulnerable than before.

Ferroptosis is a regulated form of cell death characterized by iron overload, excessive lipid peroxidation, oxidative stress and mitochondrial dysfunction. Although increasing evidence links ferroptosis to infectious diseases, its role during respiratory viral infections remains less explored. In **Chapter 4**, ferroptosis was investigated during respiratory viral infections, with the main aim of characterizing features of ferroptosis following viral infection of the airway epithelium. Two clinically relevant respiratory viruses were compared: influenza A virus H1N1 (IAV) and human adenovirus type C5 (HAdV-C5). While IAV has previously been associated with ferroptosis, much less is known about HAdV-C5. Using both bronchial and alveolar epithelial cells provided a better understanding of virus-induced epithelial damage and allowed comparison of responses in different regions of the airway epithelium.

The findings demonstrate that both viruses alter pathways involved in ferroptosis. Specifically, infection altered ferritin, an iron-storage protein important for maintaining intracellular iron levels. Lipid peroxidation, a hallmark of ferroptosis that damages membranes and promotes cell death was markedly increased

following infection with both viruses. In addition, profound alterations in mitochondrial network morphology were observed. Importantly, not all characteristics of ferroptosis were activated to the same extent during infection, rather IAV and HAdV-C5 induced ferroptosis features in a cell-and time-dependent manner. Furthermore, the use of primary human airway epithelial cells adds translational value and clinical relevance of these findings.

In conclusion, the findings presented in this thesis advance our understanding of the molecular mechanisms through which air pollution and respiratory viruses exacerbate chronic respiratory diseases. Lastly, identifying ferroptosis-associated mechanisms during viral infection of the airway epithelium sets a foundation for future studies aimed at using pharmacological tools to preserve airway epithelium integrity.

NEDERLANDSE SAMENVATTING

Luchtwegaandoeningen behoren wereldwijd tot de belangrijkste doodsoorzaken. Twee van de meest voorkomende aandoeningen, chronische obstructieve longziekte (COPD “chronic obstructive pulmonary disease”) en astma, zijn niet-geneesbare aandoeningen die het ademen bemoeilijken en een grote impact hebben op de kwaliteit van leven. Patiënten ervaren vaak perioden waarin hun klachten plotseling verergeren, ook wel exacerbaties genoemd. Deze episoden kunnen leiden tot ziekenhuisopname, een versnelde ziekteprogressie en permanent verlies van longfunctie. Twee van de meest voorkomende triggers van exacerbaties zijn virale infecties en blootstelling aan omgevingsverontreinigende stoffen, met name fijnstof (PM, “particulate matter”) dat aanwezig is in luchtverontreiniging.

Het luchtwegepitheel vormt de eerste verdedigingslinie tegen ingeademd PM en pathogenen. In gezonde longen fungeert het epitheel als een fysieke barrière, het bevordert de verwijdering van slijm en ondersteunt immuunreacties en weefselherstel. Bij COPD en astma is het epitheel echter al verzwakt. Hierdoor kan blootstelling aan verontreinigende stoffen of virussen een versterkte cellulaire respons veroorzaken, wat leidt tot chronische ontsteking, oxidatieve stress en schade aan het longweefsel. Daarnaast heeft recent onderzoek ferroptosis naar voren gebracht als een mogelijk mechanisme dat ten grondslag ligt aan luchtwegaandoeningen. Ferroptosis is een vorm van gereguleerde celdood die voornamelijk wordt geassocieerd met oxidatieve stress als gevolg van een verstoorde ijzerhuishouding.

Dit proefschrift onderzocht hoe omgevingsverontreinigende stoffen en respiratoire virussen bijdragen aan schade aan het epitheel bij chronische luchtwegaandoeningen. Door bestaande kennis uit de literatuur te combineren met experimentele studies werden de moleculaire mechanismen die ten grondslag liggen aan oxidatieve stress, ontsteking en ferroptosis onderzocht. Hiermee werd inzicht verkregen in de manier waarop omgevings- en infectieuze stressoren het luchtwegepitheel beïnvloeden.

Hoofdstuk 2 biedt een overzicht van omgevingsverontreinigende stoffen en hun effecten op cellen. Dit hoofdstuk beschrijft hoe PM het lichaam binnendringt en diep in de longen kan doordringen, waar het als een belangrijke stressor kan optreden en schade kan veroorzaken door cellulaire receptoren en signaalroutes te activeren. Cellen lijken in staat te zijn verontreinigende stoffen “waar te nemen”, wat leidt tot oxidatieve stress, mitochondriale disfunctie, inflammatoire signalering en instabiliteit van eiwitten. Deze veranderingen verzwakken de integriteit van de epitheliale barrière, verminderen de functie van de trilharen en verstoren de communicatie tussen epitheelcellen en immuuncellen. Hierdoor zijn de longen minder goed in staat om ingeademde deeltjes en pathogenen te verwijderen, terwijl aanhoudende ontsteking bijdraagt aan de progressie van chronische longaandoeningen.

In **Hoofdstuk 3** lag de focus op chronische ontsteking bij obstructieve longaandoeningen. Ontsteking is een natuurlijke reactie die het lichaam beschermt tegen acute beschadiging en infectie, maar wordt schadelijk wanneer deze chronisch aanhoudt. Bij COPD en astma leidt aanhoudende ontsteking tot voortdurende weefselschade, verminderd weefselherstel en progressief verlies van longfunctie. Dit hoofdstuk beschrijft de complexe interactie tussen epitheelcellen, immuuncellen en inflammatoire mediatoren. Respiratoire virale infecties worden hierbij benadrukt als belangrijke triggers van exacerbaties, omdat zij inflammatoire reacties stimuleren die excessief kunnen worden bij patiënten met reeds bestaande chronische luchtwegontsteking. Daarnaast kunnen epitheliale schade veroorzaakt door verontreinigende stoffen en door virussen geïnduceerde ontsteking elkaar versterken, waardoor een vicieuze cirkel van schade ontstaat die steeds moeilijker te herstellen is en de longen steeds kwetsbaarder maakt.

Ferroptosis is een vorm van gereguleerde celdood die wordt gekenmerkt door een overmaat aan ijzer, verhoogde lipideperoxidatie, oxidatieve stress en mitochondriale disfunctie. Hoewel steeds meer bewijs een verband laat zien tussen ferroptosis en infectieziekten, is de rol ervan tijdens respiratoire virale infecties nog relatief weinig onderzocht. In **Hoofdstuk 4** werd ferroptosis tijdens respiratoire virale infecties onderzocht, met als belangrijkste doel het karakteriseren van kenmerken van ferroptosis na virale infectie van het

luchtwegepitheel. Twee klinisch relevante respiratoire virussen werden met elkaar vergeleken: influenza A-virus H1N1 (IAV) en humaan adenovirus type C5 (HAdV-C5). Hoewel IAV eerder in verband is gebracht met ferroptosis, is veel minder bekend over HAdV-C5. Het gebruik van zowel (primaire) bronchiale als alveolaire epitheelcellen gaf meer inzicht in virus-geïnduceerde epitheliale schade en maakte het mogelijk om reacties in verschillende delen van het luchtwegepitheel met elkaar te vergelijken.

De bevindingen laten zien dat beide virussen routes die betrokken zijn bij ferroptosis beïnvloeden. Zo veranderde infectie de hoeveelheid en/of eigenschappen van ferritine, een eiwit dat ijzer opslaat en belangrijk is voor het handhaven van intracellulaire ijzerniveaus. Lipideperoxidatie, een kenmerk van ferroptosis dat membranen beschadigt en celdood bevordert, was sterk verhoogd na infectie met beide virussen. Daarnaast werden duidelijke veranderingen in de morfologie van het mitochondriale netwerk waargenomen. Belangrijk is dat niet alle kenmerken van ferroptosis in dezelfde mate werden geactiveerd tijdens infectie. IAV en HAdV-C5 induceerden verschillende kenmerken van ferroptosis op een cel- en tijdsafhankelijke manier. Bovendien draagt het gebruik van primaire humane luchtwegepitheelcellen bij aan de translationele waarde en klinische relevantie van deze bevindingen.

Concluderend dragen de bevindingen uit dit proefschrift bij aan een beter begrip van de moleculaire mechanismen waarmee luchtverontreiniging en respiratoire virussen chronische luchtwegaandoeningen kunnen verergeren. Daarnaast vormt het identificeren van ferroptosis-gerelateerde mechanismen tijdens virale infectie van het luchtwegepitheel een basis voor toekomstig onderzoek naar farmacologische strategieën om de integriteit van het luchtwegepitheel te behouden.

RESUMEN EN ESPAÑOL

Las enfermedades respiratorias se encuentran entre las principales causas de muerte a nivel mundial. Dos de las más comunes, la enfermedad pulmonar obstructiva crónica (EPOC) y el asma, son enfermedades crónicas sin cura que dificultan la respiración y tienen un gran impacto en la calidad de vida. Los pacientes suelen experimentar períodos en los que sus síntomas empeoran repentinamente, conocidos como exacerbaciones. Estos episodios pueden provocar hospitalización, una progresión más rápida de la enfermedad y una pérdida permanente de la función pulmonar. Dos de los desencadenantes más frecuentes de las exacerbaciones son las infecciones virales respiratorias y la exposición a contaminantes ambientales, especialmente a las partículas en suspensión (PM, por sus siglas en inglés) presentes en la contaminación atmosférica.

El epitelio de las vías respiratorias constituye la primera línea de defensa frente a las partículas inhaladas y los patógenos. En los pulmones sanos, el epitelio actúa como una barrera física, favorece la eliminación del moco y contribuye a las respuestas inmunitarias y a la reparación tisular. Sin embargo, en la EPOC y el asma, el epitelio ya se encuentra debilitado. Como consecuencia, la exposición a contaminantes o virus puede desencadenar una respuesta celular exacerbada que conduce a inflamación crónica, estrés oxidativo y daño del tejido pulmonar. Además, investigaciones recientes han señalado la ferroptosis, una forma de muerte celular regulada asociada al estrés oxidativo derivado de un desequilibrio en el metabolismo del hierro, como un posible mecanismo implicado en las enfermedades respiratorias.

Esta tesis investigó cómo los contaminantes ambientales y los virus respiratorios contribuyen al daño epitelial en las enfermedades respiratorias crónicas. Mediante la combinación de conocimientos actuales de la literatura con estudios experimentales, se investigaron las vías moleculares subyacentes al estrés oxidativo, la inflamación y la ferroptosis, proporcionando información sobre cómo los factores de estrés ambientales e infecciosos afectan al epitelio de las vías respiratorias.

En el **Capítulo 2** se revisaron los contaminantes ambientales y sus efectos sobre las células. Este capítulo describe cómo las partículas en suspensión entran en el organismo y penetran profundamente en los pulmones, donde pueden actuar como un factor importante de estrés y causar daño mediante la activación de receptores celulares y cascadas de señalización. Las células parecen ser capaces de "detectar" los contaminantes, lo que provoca estrés oxidativo, disfunción mitocondrial, señalización inflamatoria e inestabilidad de las proteínas. Estos cambios debilitan la integridad de la barrera epitelial, deterioran la función ciliar y alteran la comunicación entre las células epiteliales y las células inmunitarias. Como consecuencia, los pulmones son menos capaces de eliminar las partículas inhaladas y los patógenos, mientras que la inflamación persistente contribuye a la progresión de las enfermedades pulmonares crónicas.

En el **Capítulo 3**, el enfoque se centró en la inflamación crónica en las enfermedades pulmonares obstructivas. La inflamación es una respuesta natural que protege al organismo frente a lesiones e infecciones, pero se vuelve perjudicial cuando persiste. En la EPOC y el asma, la inflamación no resuelta provoca daño tisular continuo, una reparación tisular deficiente y una pérdida progresiva de la función pulmonar. Este capítulo revisa la compleja interacción entre las células epiteliales, las células inmunitarias y los mediadores inflamatorios. Las infecciones virales respiratorias se destacan como importantes desencadenantes de las exacerbaciones, ya que estimulan respuestas inflamatorias que pueden volverse excesivas en pacientes con inflamación crónica preexistente de las vías respiratorias. Además, el daño epitelial inducido por contaminantes y la inflamación inducida por virus pueden amplificarse mutuamente, creando un ciclo de lesión que se vuelve cada vez más difícil de resolver y que deja a los pulmones progresivamente más vulnerables.

La ferroptosis es una forma de muerte celular regulada caracterizada por una sobrecarga de hierro, acumulación de peróxidos lipídicos, alto estrés oxidativo y disfunción mitocondrial. Aunque cada vez existe más evidencia que relaciona la ferroptosis con las enfermedades infecciosas, su importancia durante las infecciones virales respiratorias sigue estando relativamente poco estudiada. En el **Capítulo 4** se investigó la ferroptosis con el objetivo principal de identificar

características distintivas de la ferroptosis tras una infección viral del epitelio de las vías respiratorias. Se compararon dos virus respiratorios clínicamente relevantes: el virus de la influenza A H1N1 (IAV) y el adenovirus humano tipo C5 (HAdV-C5). Aunque previamente se ha relacionado al IAV con la ferroptosis, se sabe mucho menos sobre el HAdV-C5. El uso de células epiteliales tanto bronquiales como alveolares permitió comprender mejor el daño epitelial inducido por los virus y comparar las respuestas en diferentes regiones del epitelio de las vías respiratorias.

Los resultados demuestran que ambos virus alteran vías implicadas en la ferroptosis. En particular, la infección alteró la ferritina, una proteína de almacenamiento de hierro importante para mantener los niveles intracelulares de hierro. La peroxidación lipídica, una característica distintiva de la ferroptosis que daña las membranas y favorece la muerte celular, aumentó considerablemente tras la infección con ambos virus. Además, se observaron alteraciones profundas en la morfología de la red mitocondrial. Es importante destacar que no todas las características de la ferroptosis se activaron en la misma medida durante la infección. Por el contrario, IAV y HAdV-C5 indujeron características de ferroptosis de manera dependiente del tipo celular y del tiempo de infección. Asimismo, el uso de células epiteliales primarias humanas de las vías respiratorias aporta valor traslacional y relevancia clínica a estos hallazgos.

En conclusión, los hallazgos presentados en esta tesis contribuyen a una mejor comprensión de los mecanismos moleculares mediante los cuales la contaminación atmosférica y los virus respiratorios pueden exacerbar las enfermedades respiratorias crónicas. Además, la identificación de mecanismos asociados a la ferroptosis durante la infección viral del epitelio de las vías respiratorias proporciona una base para futuros estudios orientados al desarrollo de estrategias farmacológicas destinadas a preservar la integridad del epitelio de las vías respiratorias.

ACKNOWLEDGEMENTS/AGRADECIMIENTOS

The work presented in this thesis is the product of countless lessons learned. Throughout these four and a half years, I have been fortunate to receive invaluable support from my promotors, colleagues, friends, and family. Here, I would like to thank them.

First, I would like to express my gratitude to my promotors, **Martina**, **Karim** and **Amalia**. Thank you for the opportunity to join your research groups and for your mentorship.

Martina, thank you for all the guidance and encouragement throughout my PhD. We first met in early March 2020, and by the time we finished writing our proposal, we were under strict quarantine. We did not have the smoothest start, but we did not give up, and with your support and our joint determination, I finally joined the Molecular Pharmacology Department in April 2022. Since then, no year has gone by without a beautiful card on my birthday. Thank you for your timely feedback on all my work, for the scientific discussions and challenges, and for encouraging me to celebrate the small achievements. I have always admired how quickly you find solutions to every challenge, and how you turn difficult situations into actionable plans. Your practical and decisive approach was key to the completion of this project. A special thank you for your understanding and support during the unexpected health challenges I faced. Wishing you continued success and many more cycling trips!

Karim, you joined my project after a year and a half, and I could not have been happier to have you on my team. Your expertise in virology was exactly what my project had been missing. I admire your honest and calm demeanor, and your contagious enthusiasm for virology, which none of your students have been able to escape- including me! I will greatly miss the scientific and non-scientific discussions, the stimulating group meetings, and the fun group outings. Thank you for your words of encouragement when it felt like the PhD was getting the best of me, for introducing me to the world of adenovirus research, and for supporting me in attending virus-related conferences. I wish you great success in all that is to come.

Amalia, I always appreciated your interest in the progress of my project and the casual moments of discussion, even in the elevator or during late nights in the office. Every time we talked, you offered new ideas and insights that made my project better. Thank you for your curiosity, your thoughtful suggestions, and for taking the time to discuss my work. I wish you continued success in your career.

I would like to thank the members of the assessment committee, **Prof. K. Haslinger**, **Prof. J.M. van Dijl**, and **Prof. A. Ehrhardt**, for taking the time to review my thesis. I greatly appreciate your contribution to this important milestone in my academic journey.

To **Prof. dr. A. L. W. Huckriede** and **Jacqueline de Vries Idema**, thank you for welcoming me into your laboratory and for all your support with the virus work, especially at the beginning of my PhD.

I would like to thank the **Secretaría de Ciencia, Humanidades, Tecnología e Innovación (SECIHTI**, formerly CONACYT) for the financial support.

I am deeply grateful to have been part of the **Molecular Pharmacology Department**. My time in MF would not have been the same without all the wonderful people who are part of it.

To the pillars of MF:

Reinoud, thank you for all the inspiring chats and for your guidance throughout my PhD, I truly appreciate it. **Melissa**, you are always smiling and enthusiastic about helping everyone, and I am grateful for all your support in the lab and with experiments. **Manon**, I really appreciate all the support you provided when I was working with the primary cells. **Hoeke**, your constructive feedback on my presentations and suggestions on experimental setups really made a difference, thank you. **Barbro**, thank you for so kindly providing me with the tools to learn R. **Carolina** and **Marina**, whenever I had a doubt about lab work, you always had an answer and were happy to help. Thank you for your patience and for all the work you do in the lab. **Jai**, you joined my project as a side quest, and now we are co-authors! I am happy you are back in MF **Annet** and **Roel**, thank you for all your

support over the years and for making sure things in the department always run smoothly.

To my amazing officemates:

I was lucky enough to be in two different offices, in the first, **Luke**, **Kim** and **Xinhui** helped me navigate the first half of my PhD. So many things were new to me, and all your help and advice were priceless. Thank you for all your patience, your warm hellos every morning, and your words of encouragement. I am extremely happy to see you all thriving in your new positions.

Toward the end of my second year, I moved offices, and the support of my new officemates was invaluable during the second half of my PhD. **Xiaoyu**, my third unofficial paranymph, we met even before you started your PhD, and I was so happy when you joined the lab. It was lovely to have such a sweet and smart colleague who was also doing virus-related research in the department. You always manage to keep a calm, relaxing atmosphere, and I am glad we shared an office. Our trip after the adenovirus conference was super fun, and I hope we can travel together again sometime. **Rocío**, eres una superhumana. Admiro todo lo que haces y que, encima, lo haces siempre con una gran sonrisa. Gracias por tus consejos y por permitirme desahogarme cuando más lo necesitaba; fuiste un soporte enorme. Estoy segura de que seguirás triunfando en lo que sea que venga, como científica, mamá, costurera, panadera y todo lo que te propongas. **Carli**, mevrouw, you always manage to find a way to laugh even at the most absurd events. I wish you great success in Antwerp and hope we eventually manage to meet for our annual dinner event. **Xuming** and **Rick**, although we only shared the office for a short time, I really enjoyed our chats. Thank you for being so friendly and for all your help. I wish you both the very best in your careers!

To the students I had the pleasure of working with during my PhD:

Alessandra, **Donia**, **Renske, Lisanne,** and **Phillip**, thank you for your contributions to my project. I enjoyed working with you and learned a lot from each of you. I wish you all great success in your future endeavors.

To the KAR lab:

At the beginning of my PhD, there was no space in MF to discuss my research with other virus enthusiasts. That is why I am especially grateful to the current and former members of the **KAR lab.** Friday morning meetings were always a friendly and welcoming space to share ideas and have nice discussions, thank you for all.

To my fellow PhDs:

Teresa, soy tu fan de aventuras de acampar. Admiro tu perseverancia y la forma en que le sacas la vuelta a los momentos difíciles. Aunque nunca compartimos proyectos, disfruté el tiempo que pasamos fuera del laboratorio, especialmente nuestras sesiones de bouldering. **Leshan**, I will not forget the meeting we went to together and the moments we shared, from cheering each other on during our presentations to watching series together. I got to know you better during those days, and I will always cherish them. **Chiara**, you are a very kind and caring person. Thank you for coming to visit me while I was recovering; it meant a lot. **Tingting**, you are an amazing knowledge people, thank you for all your help. **Luis**, gracias por tu ayuda con los reactivos y por ser siempre alguien con quien contar para desahogarnos de las penas del PhD. **Xiaopeng**, thank you for all your help with R and for helping me make sense of the data. **Chuan,** although we only worked together for a short time, I really enjoyed discussing experimental setups with you, I wish you the best. **Amanda**, it was a joy collaborating with you on the binder project, I wish we had had more time. To **Carol**, **Qiwen**, **Shilei**, **Maria**, **Saskia**, **Polina**, **Dyan**, **Jeremy**, **Henry**, **João**, **Wenyu**, **Mingxi**, and **Ercan**, thank you for the (very necessary) coffee sessions in the therapy room. It was a pleasure to share this journey with you, and I wish you all success in the rest of your PhDs.

To those whose friendship went beyond the lab:

Sara and **Nad'ka**, from day one you made me feel like I belonged in the MF family and that I had made the right choice. We only shared half of our PhDs, but the best was still to come. We have shared trips, birthdays, and many things in between, and you have stood by my side during some very challenging times I have faced. **Sara**, I am constantly in awe of how sweet, kind and caring you are. I feel lucky to call you my friend. **Nad'ka**, you are such a strong and thoughtful person. Thank you for

taking a genuine interest in my life stories, for visiting me every day while I was recovering and for welcoming me into your home and trusting me with Murkita. I am looking forward to everything the future brings us, and I cannot wait to see you both achieve all the wonderful things you deserve.

Hong and **Dan**, I feel so lucky that our paths crossed during our PhDs. **Hong**, working alongside you in the lab made the days much more fun, even when experiments seemed to go on forever. I cherish the time we spent writing together and the time you taught me how to cook eggs Chinese-style. **Dan**, I have always admired your determination and assertiveness. Sharing my first Dutch Lung Congress with you made it even more special. Going to the Christmas Market with both of you is one of my favorite memories. I am so happy we have stayed in touch over the years. Reuniting in Croatia was especially wonderful, and I am glad that we continue to be part of each other's special moments.

To my incredible paranymphs:

Alejandro and **Stephanie**, you have been there not only for the good moments, but also through some of the hardest ones, and I will always be grateful for your care, patience, and, above all, your friendship. I could not have asked for two better people to stand by me on this important day.

Mi querido **Ale**, tú fuiste la primera persona en MF que "conocí". Desde el primer correo supe que podría contar contigo, y no me equivoqué. En cuanto llegué a Groningen, sin más ni más, me invitaste a formar parte de tu familia elegida en este país, y por ello siempre te estaré infinitamente agradecida. Eres una persona talentosísima (chingona, pues), y sé que siempre habrá mucha luz alumbrando tu camino. Es un placer ser parte de tu vida y verte enfrentar cada giro inesperado con una sonrisa y mucha determinación. Espero que en nuestro futuro nos queden muchos brunches, viajes y aventuras, y que nunca nos falten los chismecitos ni la abundancia.

Steph, amiga. Llegaste a mi vida *tan brillante como un trozo de sol cortando la mañana lluviosa (Ángeles Mastretta)*. Es increíble (pero no sorprendente) cómo, en tan poco tiempo, te convertiste en una de mis mejores amigas de la vida. Definitivamente,

subirme a ese tren a Utrecht fue una gran decisión. Gracias por las visitas al hospital, por cuidarme cuando más lo necesité y hasta por salvarme de mí misma. Admiro el poder que tienes de envolver en cariño delicado todo lo que te rodea. Siempre guardaré en mi corazón las caminatas en el Noorderplantsoen y las tardes de tecitos mientras hablábamos de la vida. Gracias por tu amistad y por siempre estar. Nos deseo toda una vida de caminatas juntas, dondequiera que nos lleve la vida.

Success in a PhD depends not only on what happens in the lab, but also on the support and reality checks that others bring into one's life. During my time in Groningen, I had the blessing of meeting many such people, people who now make it very difficult to imagine a life outside of this city. I would like to thank them as well:

Mis queridos Chiquis, futuros habitantes de la Comuna Flor: **Rubisol**, ya no me sorprendo cada vez que llegas con un nuevo talento ni cada vez que nos ganas en los juegos de mesa. Gracias por hostear las mejores fiestas con baile y karaoke, y por siempre jalar a todos los planes. **Rodri**, no olvidaré aquella tarde en que la vida nos volvió a reencontrar, unos diez años después, en el lugar menos esperado. Te deseo mucho éxito en Japón y espero que la vida nos vuelva a cruzar (¡Japón 2027!). **Emil**, el parrillero oficial del grupo, sin ti ninguno de los asados habría sido posible. **Anel**, tú siempre traes los mejores peinados y la risa más contagiosa. Gracias a todos (incluida **Steph**) por siempre hacerle segunda a todos los planes: conciertos, hikes en Italia, hacer una piñata, hornear pan de muerto y hasta planear una vida en Flor. Gracias por convertir los momentos en recuerdos inolvidables y por hacer que la vida lejos de casa se sienta un poquito más como estar en ella. Ustedes son un hogar.

Tiago, **Kim**, **Jose, Erika**, **Kevin**, and **Sem**, I cannot remember exactly how or when we met, but now I cannot imagine what life in Groningen would be like without you. From lunches and Saturday runs to summers filled with birthday celebrations, you have become such an important part of my life here. The way you all show up for one another is something to admire, and I am grateful to be a part of it. Thank you for making Groningen such an amazing experience, I hope to continue sharing life with you.

PB, gracias por todo tu apoyo durante el último año, por mostrarme Ámsterdam y por enseñarme nuevas palabras en español. Espero que hagamos tiempo para mucho más.

Lili, **Alex**, **Rosario**, **Aaron**, **Dinorah**, **Diego**, y con quienes me encontré cuando apenas empezaba a navegar la extrañeza de este país: gracias por todas las risas y por las reuniones que cada vez parecen más un banquete profesional que una reunión. A ustedes y al resto de la banda mexicana, les deseo éxito en todo lo que venga.

Lastly, I would like to thank the people who have been part of my life long before the PhD and who, in many ways, made all this possible.

A mis **amigos**: gracias, porque no importa cuánto tiempo pase ni la distancia que haya de por medio, siempre encuentro en ustedes un lugar al que pertenezco.

A mi **familia**, que me ha dado todas las herramientas para llegar hasta aquí.

Mami, entre más pasan los años, más agradezco todo lo que me has enseñado y la forma en la que, aun en la distancia, nunca has dejado de acompañarme y apoyarme. **Pao**, dichosa yo que, desde el día en que naciste, no he caminado ni un solo día sola. Eres más fuerte de lo que crees y más valiente de lo que a veces te permites ver. Admiro que siempre entregas todo tu corazón a lo que te apasiona. Gracias por ser la mejor hermana. **Chris**, I can't help still calling you my baby brother, but I am constantly amazed by how much you have grown. I am super excited to make new memories with you and Lolis this winter. **Dad**, gracias por haber sido el mejor papá mientras crecía. Gracias por todo lo que me diste y por haber sido una parte tan importante de quien soy hoy.

Gracias por ser mi familia y siempre creer en mí. Los amo sin medida, y aunque a veces se sienta que la distancia nunca termina, sé que algún día la vida nos volverá a juntar.

Y, por último, quiero agradecer a **Ana Lau** chiquita, en todos los universos, porque nunca deja de soñar.

CURRICULUM VITAE

Ana Laura Manzano Covarrubias was born on 26th of August of 1992 in Heroica Puebla de Zaragoza, México. In 2011, she enrolled in the Clinical Biochemistry Bachelor's program at Universidad de las Américas, Puebla. During her bachelor's studies, she was awarded a scholarship to undertake an internship under the supervision of Prof. Steven de Jong in the Oncology Department in the University Medical Center Groningen in the Netherlands, where she investigated biomarkers of ovarian cancer. She completed her bachelor's studies summer of 2015, graduating *cum laude*.

In the fall of the same year, she moved to Sheffield, England to pursue a master's degree in Molecular Medicine at the University of Sheffield. During her master's studies, Ana Laura joined the group of Prof. Andrew Grierson at the Sheffield Institute for Translational Neuroscience (SITraN). There, she investigated the oligogenic basis of motor neuron diseases using a zebrafish model. Her work placed her among the top students of her cohort, and she graduated with Distinction.

In 2017, after completing her master's degree, Ana Laura returned to her hometown. Following a short internship at the Instituto Nacional de Medicina Genómica, she rejoined her alma mater as a research technician in the Molecular Biology group of Prof. José Daniel Lozada-Ramírez.

In 2021, with the support of Prof. Martina Schmidt, she wrote a PhD proposal that was awarded a doctoral fellowship by the Consejo Nacional de Ciencia y Tecnología (CONACYT, now SECIHTI). In the spring of 2022, Ana Laura started her PhD at the Department of Molecular Pharmacology at the University of Groningen under the supervision of Prof. Martina Schmidt, Prof. Amalia Dolga and Dr. Karim Rafie. Her research focused on the detrimental effects of air pollution and respiratory viruses in the upper and lower airway epithelium. The work presented in this thesis is the culmination of those studies and will be defended in the winter of 2026.

LIST OF PUBLICATIONS

Manzano-Covarrubias, A.L. Tosato, A., Mol-van der Veen, C.H.T.J., Ahmadyar, P., et al. (2026) Respiratory viruses induce ferroptosis-like features in lung epithelial cells. Microbiol Spectr14:e01957-26.

Manzano-Covarrubias, A.L., Yan, H., Luu, M.D.A., Gadjdjoe, P.S., Dolga, A., Schmidt M. (2023) Unravelling the signaling power of pollutants. Trends Pharmacol Sci. 44(12), 917-933

Li, D., **Manzano-Covarrubias, A.L.**, et al. (2026) Therapeutic potential of targeting novel signalling pathways in regulating chronic inflammation in obstructive lung disorders. Pharmacol Ther 279, 108983

Yan, H., Zhang, L., **Manzano-Covarrubias, A.L**., et al. (2026) Inhibition of EPAC1 prevents neuronal death mediated by diesel exhaust particles in ferroptotic cell death conditions. Antioxidants 15, 566

Zhang, L., Yan, H., Rahman, M.S., Mol-van der Veen, C.H.T.J., **Manzano-Covarrubias, A.L.**, et al. (2025) Regulation of calcium signaling prevents neuronal death mediated by NIST DEP in xenoferroptotic cell death conditions. J Hazard Mater 488, 137374

Cattani-Cavalieri, I., Trombetta-Lima, M., Yan, H., **Manzano-Covarrubias, A.L.**, et al. (2024) Diesel exhaust particles alter mitochondrial bioenergetics and cAMP

