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Pulmonary lipid homeostasis in cigarette smoke-associated lung diseases

Thèse

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Résumé

Introduction. Les effets du tabagisme demeurent problématiques dans notre société. Les mécanismes initiateurs de la réponse immunitaire pulmonaire induits par la fumée de cigarette sont peu caractérisés. Un des phénomènes dominants en contexte tabagique est l'augmentation de la taille des macrophages alvéolaires. Ce changement phénotypique se distingue par une accumulation intracellulaire de lipides suggérant que le transport lipidique pulmonaire des macrophages alvéolaires est modifié en contexte tabagique. Le transport lipidique pulmonaire est composé de plusieurs étapes, dont la capture, le remaniement et l'export de lipides par les macrophages alvéolaires. Les impacts du tabagisme sur le transport lipidique pulmonaire sont actuellement inconnus.

Hypothèse. Le tabagisme altère le transport lipidique pulmonaire. Objectifs : Chapitre 1) Investiguer l'impact de l'exposition à la fumée de cigarette sur le transport lipidique pulmonaire dans un modèle murin et chez l'humain ainsi qu'évaluer l'impact d'une thérapie d'augmentation des *high-density lipoproteins* (HDLs) dans un modèle murin. Chapitre 2) Investiguer les effets d'une thérapie avec un agoniste du récepteur nucléaire *liver X receptor* (LXR) activant l'export lipidique dans un modèle murin. Chapitre 3) Caractériser les répercussions d'une carence alimentaire sur la santé pulmonaire et sur la réponse pulmonaire en contexte tabagique dans un modèle murin.

Méthodes. 1. Le transcriptome pulmonaire de souris exposées à la fumée de cigarette et de sujets non-fumeurs, fumeurs et ex-fumeurs a été étudié. La capacité d'efflux de cholestérol a été mesurée dans le sérum et dans le lavage bronchoalvéolaire (LBA) de souris fumeuses et non fumeuses. Une HDL recombinante, le MDCO-216, a été administrée à des souris fumeuses et non-fumeuses et leurs réponses immunitaires, leurs fonctions pulmonaires et leurs compositions corporelles ont été analysées.

2. Un agoniste du LXR, le T0901317, a été administré à des souris en contexte tabagique. Le transcriptome pulmonaire relié au transport lipidique, la réponse immunitaire pulmonaire et du macrophage alvéolaire ainsi que les impacts sur le surfactant pulmonaire ont été investigués.

3. Des souris fumeuses et non fumeuses ont été nourries avec des diètes déficientes en méthionine (MD), choline (CD) et méthionine et choline (MCD) et leurs fonctions pulmonaires, leurs réponses immunitaires pulmonaires et leurs expressions géniques pulmonaires ont été caractérisées.

Résultats. Chapitre 1. L'expression des gènes impliqués dans le transport lipidique pulmonaire murin et humain est modifiée en contexte tabagique. La capacité du sérum et du LBA à promouvoir la sortie de cholestérol est augmentée après une seule exposition à la fumée de cigarette. L'administration du MDCO-216 réduit la réponse inflammatoire pulmonaire et la taille des macrophages alvéolaires dans un modèle d'exposition aiguë à la fumée de cigarette. Le MDCO-216 semble protéger les fonctions pulmonaires et induit une augmentation de la quantité de masse maigre chez les souris fumeuses.

Chapitre 2. L'agoniste du LXR augmente l'expression des gènes de transport lipidique pulmonaire, cependant il exacerbe la réponse immunitaire pulmonaire en contexte tabagique. Les macrophages alvéolaires ont aussi un phénotype inflammatoire exacerbé et ont davantage de stress au réticulum endoplasmique lorsqu'ils sont traités en contexte tabagique. L'activation de LXR mène à une réduction des niveaux de surfactant pulmonaire.

Chapitre 3. La diète MCD altère les fonctions pulmonaires en induisant un profil restrictif pulmonaire et abolit la réponse immunitaire pulmonaire à la fumée de cigarette. En histologie, ces souris nourries avec la diète MCD n'ont toutefois aucun foyer fibrotique pulmonaire. L'expression génique de plusieurs gènes associés à la matrice extracellulaire et les niveaux de surfactant pulmonaire sont réduits chez les souris nourries avec la diète MCD. Les phénotypes pulmonaires de la diète MCD sont toutefois réversibles après un retour d'une semaine sur la diète contrôle. La diète CD induit un profil pulmonaire de type emphysémateux et la diète MD mène à un profil restrictif.

Conclusions. Ces travaux démontrent que le transport lipidique pulmonaire a un rôle majeur en contexte tabagique et qu'il est modulé rapidement. La thérapie d'augmentation des HDLs, avec le MDCO-216, propose une nouvelle voie de traitement envisageable pour les ex-fumeurs. La thérapie ciblant LXR suggère qu'il pourrait y avoir des effets délétères chez les sujets fumeurs actifs. Les carences alimentaires en méthionine et en choline démontrent d'importants changements sur la

physiologie pulmonaire. Ce tout nouveau domaine de recherche, le nutri-respiratoire, requiert davantage d'études afin de mieux comprendre l'impact d'une mauvaise nutrition sur la santé pulmonaire.

Abstract

Introduction. Cigarette smoking remains a major problem in our society. While a lot of cigarette smoke impacts are actually known, few data are available on initiating mechanisms involved in the pulmonary immune response to cigarette smoke. One of the most intriguing phenomena under cigarette smoke exposure conditions is the presence of enlarged alveolar macrophages. This phenotypic change is characterized by an intracellular lipid accumulation which may be a sign of inadequate lipid export by alveolar macrophages induced by cigarette smoking. Pulmonary lipid transport begins with lipid capture, lipid reorganization and lipid droplet formation followed by lipid export by alveolar macrophages. Cigarette smoke impacts on these steps are actually unknown.

Hypothesis. Cigarette smoking alters pulmonary lipid transport. Objectives: Chapter 1) To investigate the effect of cigarette smoke exposure on pulmonary lipid transport in cigarette smoke-exposed mice and in healthy controls, smokers and former smokers. To investigate the impact of high-density lipoprotein (HDLs) therapeutic potential in cigarette smoke-exposed mice. Chapter 2) To investigate, in mice, the therapeutic potential of an agonist activating the nuclear receptor liver X receptor (LXR) involved in the transcription of lipid export genes. Chapter 3) To explore, in mice, if a dietary deficiency alters the pulmonary health and the pulmonary response to cigarette smoke.

Methods. 1. The pulmonary transcriptome of cigarette smoke-exposed mice and healthy controls, smokers and former smokers was assessed. Cholesterol efflux capacity of serum and bronchoalveolar lavage (BAL) was measured in unexposed and cigarette smoke-exposed mice. MDCO-216, a recombinant HDL, was administered to unexposed and cigarette smoke-exposed mice and analyzed their pulmonary immune response, lung functions and body composition.

2. T0901317, an LXR agonist, was systemically given to mice under cigarette smoke exposure conditions. Pulmonary genes associated with lipid transport, lungs and alveolar macrophage immune pulmonary response to cigarette smoke and the impact of T0901317 on the pulmonary surfactant were assessed.

3. Unexposed and cigarette smoke-exposed mice were fed with methionine deficient (MD), choline deficient (CD) or methionine and choline deficient (MCD) diet. Diets impact on lung functions, pulmonary immune response to cigarette smoke and pulmonary transcriptome were characterized.

Results. Chapter 1. Cigarette smoking altered the expression of pulmonary lipid transport genes in mice and in humans. Serum and BALF cholesterol efflux capacities were increased following a two-hour cigarette smoke exposure. MDCO-216 dampened the pulmonary inflammatory response and reduced the size of alveolar macrophages in our acute cigarette smoke exposure model. MDCO-216 also seemed to be beneficial to lung functions and induced an increase in lean mass in cigarette smoke-exposed treated mice.

Chapter 2. T0901317 treatments led to an increase in the expression of pulmonary lipid transport genes. However, it also induced an exacerbated pulmonary immune response during cigarette smoking. Cigarette smoke-exposed treated-alveolar macrophages displayed an exacerbated inflammatory phenotype and showed an augmented endoplasmic reticulum stress. Furthermore, LXR activation led to pulmonary surfactant depletion under cigarette smoke exposure conditions.

Chapter 3. The MCD diet altered lung function displaying a restrictive profile and almost abolished the pulmonary immune response to cigarette smoke. Lung histology showed no signs of fibrosis, a phenotype usually associated with restrictive pulmonary functions. MCD diet led to a dramatic change in the pulmonary expression of extracellular matrix genes and also reduced pulmonary surfactant levels. Nevertheless, these pulmonary phenotypes were reversible within a week when mice were re-fed a control diet. Interestingly, the CD diet induced an emphysema-like profile, while MD diet showed similar pulmonary functions to the MCD diet.

Conclusions. The present thesis adds major data to an underestimated field of research and demonstrates the importance of pulmonary lipid transport, especially during cigarette smoking. Recombinant HDL therapy with MDCO-216 may be a new opportunity to overcome adverse effects of cigarette smoking, while activating LXR seems rather deleterious. Nutrient deficiencies, such as methionine and choline led to unprecedented impacts on the pulmonary health and on the pulmonary response to cigarette smoke. This completely new field of research, “nutri-respiratory”, requires additional studies to fully decipher the impact of unhealthy nutrition on the respiratory system.

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List of abbreviations and symbols

4-HNE: 4-hydroxynonenal
AAT1: alpha-1 antitrypsin
ACAT1: acyl-CoA:cholesterol acyltransferase 1
APCs: antigen-presenting cells
Aβ: amyloid beta
ABCA1: ATP-Binding Cassette subfamily A member 1
ABCA3: ATP-Binding Cassette subfamily A member 3
ABCG1: ATP-Binding Cassette subfamily G member 1
Apo: apolipoprotein
ApoA-1: apolipoprotein A-1
ApoC2: apolipoprotein C-2
ApoE: apolipoprotein E
ATGL: adipose triglyceride lipase
ATP: adenosine triphosphate
ATS: American thoracic society
BALF: bronchoalveolar lavage
cAMP: cyclic adenosine monophosphate
CAT: chronic obstructive pulmonary disease assessment test
CCL2: C-C motif chemokine ligand 2
CCL7: C-C motif chemokine ligand 7
CD36: cluster of differentiation 36
CETP: cholesterol ester transfer protein
COPD: chronic obstructive pulmonary disease
CRD: carbohydrate recognition domain
CRP: C reactive protein
CT: computed tomography
CXCL5: C-X-C motif chemokine motif 5
DAMPs: danger-associated molecular patterns
DC: dendritic cell
DIP: desquamative interstitial pneumonia
DNA: deoxyribonucleic acid
DPPC: dipalmitoyl phosphatidylcholine
EGFR: epidermal growth factor receptor
ER: endoplasmic reticulum
FEV_1: forced expiratory volume in one second
FVC: forced vital capacity
G-CSF: granulocyte-colony stimulating factor
GM-CSF: granulocyte-macrophage colony-stimulating factor
HDLc: high-density lipoprotein cholesterol
HDLs: high-density lipoproteins
HDM: house dust mite
ICAM-1: intracellular adhesion molecule-1
IDLs: intermediate density lipoproteins
IDOL: inducible degrader of the low-density lipoprotein receptor

IFN-γ: interferon gamma
IL-1: interleukin 1
IL-1α: interleukin 1 alpha
ILDs: interstitial lung diseases
IPF: idiopathic pulmonary fibrosis
IVUS: intravascular ultrasound
kDa: kilodaltons
LCAT: lecithin-cholesterol acyltransferase
LDLr: low-density lipoprotein receptor
LDLs: low-density lipoproteins
LPS: lipopolysaccharide
LXRα,β: liver X receptor alpha and beta
MAA: malondialdehyde-aldehyde
MARCO: macrophage receptor with collagenous structure
MAPK: mitogen-activated protein kinase
MCD: methionine choline deficient
MCP-1: monocyte chemoattractant protein 1
MIP1α: macrophage inflammatory protein 1 alpha
MIP1β: macrophage inflammatory protein 1 beta
MIP1γ: macrophage inflammatory protein 1 gamma
MIP2: macrophage inflammatory protein 2
MIP3β: macrophage inflammatory protein 3
MMP9: matrix metalloproteinase 9
MMP12: matrix metalloproteinase 12
mMRC: Modified British medical research council
MPO: myeloperoxidase
mRNA: messenger ribonucleic acid
MSR1: macrophage scavenger receptor 1
NAFLD: non-alcoholic fatty liver disease
NETs: neutrophil extracellular traps
NK: natural killer
NNK: nicotine-derived nitrosaminoketone
NOS: nitric oxide synthase
NRDS: newborn respiratory distress syndrome
NSCLC: non-small-cell lung carcinoma
NTHi: nontypeable *Haemophilus influenza*
NrF2: nuclear erythroid-related factor 2
O_2: oxygen
PAP: pulmonary alveolar proteinosis
PAMPs: pathogen-associated molecular patterns
PC: phosphatidylcholine
PE: phosphatidylethanolamine
PIK3CA: phosphoinositide-3-kinase catalytic alpha polypeptides
PLCH: pulmonary Langerhans cell histiocytosis
PLIN2: perilipin 2
PLTP: phospholipid transfer protein
PON1: paraxonase 1

PPARα: peroxisome proliferator-activated receptor alpha
PPARβ/δ: peroxisome proliferator-activated receptor beta and delta
PPARγ: peroxisome proliferator-activated receptor gamma
PRRs: pattern recognition receptors
RB-ILD: bronchiolitis-associated interstitial lung disease
RCT: reverse cholesterol transport
ROS: reactive oxygen species
RXR: retinoid X receptor
SREBP: sterol regulatory element binding proteins
SNPs: single nucleotide polymorphisms
SP-A: surfactant protein A
SP-B: surfactant protein B
SP-C: surfactant protein C
SP-D: surfactant protein D
SR-B1: scavenger receptor class B type 1
TAMs: tumour-associated macrophages
TGF-β: transforming growth factor beta
TLRs: Toll-like receptors
TLTs: tertiary lymphoid tissues
TNF: tumor necrosis factor
US: United States
VCAM-1: vascular cell adhesion molecule
VLDLs: very low-density lipoproteins
WHO: World Health Organization

Dedication

To my parents and people who were told to change career.

Without music, life would be a blank to me.

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Foreword

The results presented in this thesis were generated by me, under the supervision of Dr. Mathieu Morissette (supervisor) and Dr. Benoit Arsenault (co-supervisor). An introduction on how cigarette smoke alters systemic and pulmonary health is presented, focusing on the alveolar macrophage and its unique foamy phenotype during cigarette smoke exposure, followed by a description of lipid transport mechanisms used by alveolar macrophages. Several actual and under development reverse cholesterol transport therapies are also introduced. To conclude, this thesis explores the role of nutrition on pulmonary health.

A total of two studies were published and one submitted. Chapter 1 (published results) focuses on how cigarette smoke interacts with pulmonary lipid transport and if we can promote it via HDL supplementation therapy. Chapter 2 (published results) investigates if the activation of LXR, a nuclear receptor, can increase lipid export mechanisms. Chapter 3 (submitted results) explores the impact of a dietary deficiency on the pulmonary health and the pulmonary response to cigarette smoke. Please refer to the beginning of each chapter for additional descriptions.

Introduction

Although cigarette smoking has declined since the 1960s, the number of smokers worldwide remains impressive [1] and continues to have an important impact on socioeconomic, environmental, and health outcomes. Exposure to tobacco smoke is known to be a major risk factor for the development of many systemic and pulmonary diseases, such as lung cancer, interstitial lung diseases and chronic obstructive pulmonary disease (COPD). In this section, the general effects of cigarette smoking will be introduced followed by a description of lung diseases directly associated with cigarette smoke exposure.

Cigarette smoking and socioeconomic outcomes

Worldwide, the number of smokers is approximately 1.1 billion, 80% of which live in low- or middle-income countries. Cigarette smoking is a key societal problem, causing the death of 7 million people each year. More than 6 million of these deaths are directly associated with cigarette smoking, while the remaining 890 000 deaths are due to second-hand smoke exposure [2]. To summarize these statistics, worldwide cigarette smoking kills every 4.5 seconds.

Roughly 5.2 million Canadians aged 12 or older were daily or occasional smokers, representing 16.9% of the Canadian population in 2016. There was a higher number of smokers among men (19.4%) compared to women (14.5%). Men between 20 and 34 years of age had the highest proportion of smokers, reaching as high as one in four men [3]. The latter statistic is a sign that cigarette smoking still affects young people despite its well-known negative health effects.

Although the prevalence of smoking has decreased since the 1990s in young and adult Quebecers, one in six Quebecers are still current smokers, which represents approximately 1.4 million people. Studies conducted in 2013-2014 showed that 13 000 deaths each year can be attributed to tobacco use in Quebec alone [4].

While the number of smokers is higher in the male Canadian population, in the United States (US) the risk of death in women due to cigarette smoking is increasing and is close to be equal to the risk of men. This is mainly due to a lower number of female smokers during the 1940-50s compared to men. However, the number of women who smoked increased after World War II, resulting in a delay between the negative impact of cigarette smoke between men and women [5]. Studies characterizing the health of female smokers were scarce in the 1960s, mostly because they were not included in the first prospective epidemiological studies [6-8]. It also appears that the risk of lung cancer due to cigarette smoking among men has plateaued in the 1980s while the risk is actually increasing in women. This may be because women also have a harder time quitting compared to men [5]. Besides the effects on mortality rates, cigarette smoking also has important economic implications.

The economic impact of tobacco smoking is significant. Each year, more than 1.4 trillion US dollars is spent worldwide in the health system, for sick days, or due to premature deaths [9]. In the US, approximately $289 billion is spent in the health system because of cigarette smoking [1]. Developing countries are also fighting against the use of tobacco; [10] however, the legislative controls are inappropriate compared to developed countries. This is in part due to the fact that governments in developing countries often have other priorities, such as high infant mortality or communicable diseases and due to the arrival of transnational tobacco companies [10]. One in five smokers, roughly 226 million people, is found in low-income countries [11]. The World Health Organization (WHO) has reported that some households spent up to 10% of their revenue on tobacco products suggesting that additional strategies must be deployed in low-income countries to overcome the harmful aspects of cigarette smoking [11]. Overall, cigarette smoking is a complex and deleterious phenomenon that has substantial economic and societal effects.

Cigarette smoking and general impacts on environment and health

The anthropogenic effects on the environment are a hot topic of the 21st century. The WHO has stated, more than 10 billion cigarettes are disposed of in the environment every day [12]. It was reported that 30-40% of coastal and urban garbage is composed of cigarette butts [12]. On average, cigarette butts

still had more than 60% of their initial mass after two years of decomposition [13] and it is known that cigarette butts are toxic to microbes [14], insects [15], fish [13] and mammals [16]. The emission of cigarette smoke also releases toxicants and greenhouse gases into the atmosphere [12]. To summarize, cigarette smoking has clear impacts on the environment.

Cigarette smoke has deleterious effects on human health. It is composed of more than 7000 toxic compounds, 250 of them are known to be harmful while 50 are known carcinogens [2] [1]. Of importance, acetaldehyde, acrolein, benzene, 1,3-butadiene, carbon monoxide, formaldehyde and polycyclic aromatic hydrocarbons, such as nicotine-derived nitroasminoketone (NNK) and benzo(a)pyrene are found in cigarette smoke [17, 18]. It is widely accepted that these chemicals are the main reason why cigarette smoking is a major risk factor of cardiovascular diseases, cancer and chronic diseases.

Exposure to tobacco smoke is associated with cardiovascular diseases [1]. The Surgeon General in 1983 stated that cigarette smoking accelerates atherosclerosis, a disease characterized by thicker and irregular artery walls, mostly due to the deposition of cholesterol. Tobacco smoke also precipitates thrombosis, hemorrhage and vasoconstriction, which may lead to a narrowing of the arteries resulting in ischemia (less blood and oxygen reaching the heart muscle) and vascular occlusion [1].

Cigarette smoking is also a risk factor for type 2 diabetes. It was shown that smoking is associated with abdominal obesity [19-22], even though smokers tend to be thinner than non-smokers, a well-established risk factor for insulin resistance and the development of diabetes. Smoking also leads to increased inflammatory markers in the blood, oxidative stress and endothelial dysfunction, key contributing factors linked to diabetes. Lastly, nicotine on its own may lead to a reduced release of insulin due to the presence of neuronal nicotinic acetylcholine receptors on beta islet cells, responsible for the regulation of secretion of insulin [1].

Cigarette smoking is also associated with autoimmune diseases like rheumatoid arthritis, reproductive complications, such as congenital malformation, ectopic pregnancy and male sexual function, ocular diseases like age-related macular degeneration, dental diseases, as well as a variety of cancers, such

as liver, pancreas, colorectal, kidney, etc. [1]. However, the most important effects of cigarette smoking are on the pulmonary health and the development of lung disease.

Cigarette smoking and associated lung diseases

Since cigarette smoke is inhaled, it is primarily associated with lung disease. Cigarette smoking is a major risk factor for oropharynx, larynx, trachea, bronchus and lung cancer [1]. Smoking can also lead to interstitial lung diseases (ILDs) and COPD [23, 24]. Among other pulmonary diseases, cigarette smoking increases the risk of pulmonary infections due to bacteria or virus [1].

Cigarette smoking and lung cancer

Lung cancer is the leading cause of cancer death and it is the deadliest cancer among all cancers [25] [26], killing more Americans than prostate, breast and colon cancers combined in the US [25]. In 2012, close to 1.6 million deaths worldwide were due to lung cancer [27]. Interestingly, half of the new cases of lung cancer are diagnosed in developing countries [25]. Approximately 90% of all lung cancers are caused by cigarette smoke [17], whereas occupational and environmental exposures to asbestos, radon, arsenic and polyaromatic hydrocarbons from air pollution are also important determinants of lung cancer [28-30]. Smokers have a striking 30-fold increase chance of developing lung cancer compared to non-smokers [31-33]. In addition, half of lung cancer patients die within 1 year after diagnosis and the survival rate after 5 years is approximately 18% [34].

Lung cancers are divided into different two main subtypes: small-cell lung carcinoma and non-small-cell lung carcinoma (NSCLC). They represent 15% and 85% of all lung cancers respectively [35] and are usually diagnosed by histology or genome analysis. NSCLC is further classified into three different subtypes: adenocarcinoma, squamous cell carcinoma and large-cell carcinoma [36]. Adenocarcinoma is the most common type of lung cancer representing around 40-50% of lung cancer cases and usually develops in glandular structures [37]. This type of cancer is mostly found in the periphery of the lung [38]. Squamous cell carcinoma represents 30% of NSCLCs [37]. This type of NSCLC is associated with lung squamous cells and is highly correlated with cigarette smoking [39]. Large cell carcinomas

represent the remaining 10-15% of lung cancers [37]. This type of NSCLC is diagnosed when the two other types cannot be confirmed and it is mostly found in central parts of the lung near lymph nodes [36]. The latter type is also highly associated with cigarette smoking [40]. Several different mechanisms have been linked to the development of lung cancer, the most important being direct mutagenesis induced by carcinogens found in cigarette smoke.

In 1961, it was described that smokers had more premalignant lesions in their respiratory epithelium compared to non-smokers [41]. This finding was named the field cancerization effect, which associates tobacco smoke and its capacity to induce mutagenesis of the lung epithelium [42]. One well-accepted mechanism leading to lung cancer is the covalent binding of carcinogens found in cigarette smoke, such as NNK, to deoxyribonucleic acid (DNA) [17, 43, 44] [1]. In the presence of carcinogens, the system is usually detoxified via cytochrome P450 enzymes, glutathione S-transferases, UDP-glucuronosyl transferases and sulfotranferases, etc. [1]. As an example, cytochrome P450 enzymes oxidize carcinogens, making them more soluble and easier to excrete from the body. It is now known that some intermediate compounds that react with these enzymes render them more electrophilic, which gives them the ability to bind DNA [17]. These newly formed compounds are called DNA-adducts and are crucial to carcinogenesis [1, 45]. Cells have different systems in place for DNA repair in order to remove DNA-adducts [46, 47]. However, some DNA-adducts are able to evade DNA repair mechanisms. This may lead to the addition of an incorrect nucleotide during DNA replication resulting in a permanent mutation in the DNA sequence [17]. For example, if the mutation occurs in an oncogene, a gene that has the potential to cause cancer, such as *KRAS* or *p53*, it could consequently affect key pathways involved in cellular growth and proliferation ultimately causing cancerous cell replication [43]. Other mechanisms, such as inflammation [48] and genetic susceptibility [28, 49, 50] are also associated with lung cancer.

More recently, human genome analysis has led us to a better understanding of lung cancer. It is now known that carriers of mutated p53 genes are three times more likely to develop lung cancer [51]. For instance, multiple mutations are now associated with adenocarcinomas. In order of importance, unknown mutations (40%), Kirsten rat sarcoma 2 viral oncogene homology (*KRAS*) (30%), epidermal growth factor receptor (*EGFR)* (15%), anaplastic lymphoma kinase (5%), *BRAF* are the most common mutations associated with lung adenocarcinomas [37]. In comparison, few squamous cell carcinomas

are due to unknown mutations (20%). Phosphoinositide-3-kniase catalytic alpha polypeptides (PIK3CA) amplifications (35%), fibroblast growth factor receptor 1 (20%), PIK3CA mutations (15%), phosphatase and tensin homolog (10%) are the most common oncogenic drivers of squamous cell carcinoma [37]. These genes are involved in many pathways (such as extracellular regulated kinase, mitogen-activated protein kinase (MAPK), mammalian target of rapamycin, etc.) leading to uncontrolled cell growth, proliferation, and survival. These findings led to the development of personalized biomarker testing but also targeted therapeutic options for patients with certain lung cancers. These therapies mostly use inhibitors or monoclonal antibodies which block these upregulated pathways.

Different treatment options are available for patients diagnosed with NSCLC. If the tumour is resectable, surgeons could remove a lobe or a section of the lung in order to get rid of the tumour [52]. This option is usually used in the early stage of lung cancers. Some patients may also require adjuvant therapies after a resection surgery to reduce the risk of lung cancer re-emergence. These treatments include chemotherapy, radiation and targeted, pharmacological therapy. Chemotherapy [53] is usually proposed after a resection therapy [54]. However, approximately 40% of newly diagnosed lung cancer patients are in an advanced stage [36]. For these patients, chemotherapy using platinum as the cytotoxic agent is typically prescribed [55]. Other lung cancer patients use radiotherapy, which uses high-energy beams to target DNA within cancer cells to eventually killing them [56, 57]. This treatment is offered to patients that would not benefit from surgical resection. Based on known mutations associated with NSCLC, many new therapies focus on personalized medicine which targets known oncogenes and proto-oncogenes. For example, EGFR, a tyrosine kinase receptor, is a well-known mutation leading to uncontrolled cellular division. Inhibitors targeting the mutation in exon 19, the most commonly mutated region in EGFR, have been shown to be effective in lung cancer patients with a response rate of 70% to gefitinib or erlotinib [58].

Lung cancers are a burden on our society and cigarette smoking is one of the main risk factors for its development. Carcinogens found in cigarette lead to mutagenesis and may ultimately induce uncontrolled cellular growth. Many therapies are available and additional therapies are continually being investigated to treat lung cancer, for instance immunotherapy and vaccines [36]. Other than lung cancers, smokers may also develop devastating interstitial lung diseases.

Cigarette smoking and interstitial lung diseases

Individuals who smoke may also develop diverse diffuse interstitial and bronchiolar disorders named interstitial lung diseases. In 2012, Vassallo and Ryu decided to classify various smoking-related ILDs (Figure I). They characterized the different ILDs as follows: very likely to be caused by cigarette smoking (group 1), may be precipitated by cigarette smoking (group 2) and more prevalent in smokers (group 3) [59]. Cigarette smoking is broadly known to be the primary cause of different ILDs such as bronchiolitis-associated ILD (RB-ILD), desquamative interstitial pneumonia (DIP) and pulmonary Langerhans cell histiocytosis (PLCH) found in the group 1 classification [23, 60-63]. In group 2, we can find acute eosinophilic pneumonia and pulmonary hemorrhage syndrome. Cigarette smoking is also a risk factor of idiopathic pulmonary fibrosis (IPF) and rheumatoid arthritis associated ILD, two ILDs in group 3 [64, 65]. However, ILDs are rare diseases considering the high prevalence of smoking, suggesting that cigarette smoking is not the only factor leading to the development of most diffuse lung diseases. Genetics factors, microbial infections or allergens could also be required to induce such ILDs [59].

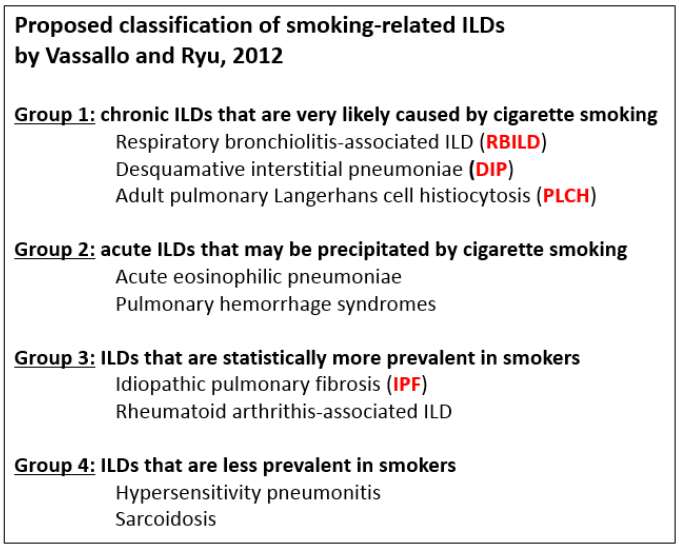

Figure I: Proposed classification of many smoking-related interstitial lung diseases by Vassallo and Ryu.

Group 1 being highly associated with cigarette smoking compared to group 4.

A key feature of most ILDs, such as RB-ILD is that this disease affects mainly young smokers, between 30 and 40 years of age. RB-ILD is slightly more predominant in men [66]. Classic symptoms of RB-ILD are chronic couch, dyspnea, while inspiratory crackles, abnormal lung sounds during inhalation, may also be present in approximately half of cases [23]. High-resolution computed tomography (CT) is used to characterize most ILD patients. Usual RB-ILD CT findings are centrilobular nodules, ground-glass opacities and a thickening of the bronchial walls [59, 66]. Lung slices of RB-ILD patients show

pigmented macrophages and inflammation near bronchioles and alveoli [66]. As RB-ILD patients usually have a good prognosis, smoking cessation remains the only management option. Corticosteroids may also be prescribed although there is little evidence of any significant beneficial effect [59, 66].

In the case of DIP, smokers are usually diagnosed in their 4th or 5th decade of life. Compared to RB-ILD, men have twice the risk of developing DIP compared to women [66]. Similar to RB-ILD, DIP symptoms include dry cough, dyspnea and respiratory crackles in half of patients [23, 59]. DIP is characterized by abnormal CT findings such as diffuse ground-glass opacity compared to RB-ILD and often the presence of cysts [67]. DIP patients have an increased number of pigmented macrophages in alveolar spaces. RB-ILD and DIP share common features but the main difference being the diffuse versus centric ground-glass opacities [66]. Smoking cessation is also the most important therapeutic strategy for DIP and corticosteroid may also be given to patients. Survival rates after 5 years and 10 years are 95.2% and 69.6% respectively [23].

PLCH patients are usually diagnosed around 20 to 40 years of age. Men and women have equal risks of developing this type of ILD [68]. Most common symptoms are dyspnea and non-productive cough. In some patients, fever, night sweats and weight loss may also occur [69]. In 10 to 15% PLCH patients will develop a pneumothorax [59, 66]. CT analysis reveals the presence of nodules and cysts in upper or mid airways [66]. PLCH is characterized by an increase in CD1a-positive dendritic cells [59]. Physicians also use aggressive tobacco cessation strategies in order to stop the progression of this ILD [59]. Corticosteroids can be given to patients. Chemotherapeutic agents may be used if multiple organs are involved [68].

Briefly, IPF is also an ILD related to cigarette smoking. Compared to the previous IDLs, this disease is usually diagnosed in patients aged 60 to 70 [66]. Progressive symptoms such as cough, dyspnea, and respiratory crackles are seen in IPF patients [70]. The key radiological features of IPF is the presence of honeycombing due to progressive fibrosis. Histologic findings can be summarized as follows: the presence of fibroblast clusters and immature connective tissue in the lung [71]. The most important clinical feature of IPF is the deterioration of the patient's condition associated with an average survival

of only 2-3 years post-diagnosis [72] and treatments are mostly to manage symptoms. This is due to the poor responsiveness to corticosteroids and the lack of effective pharmacological therapies available [66].

The mechanisms linking cigarette smoking and the development of ILDs are still unknown. However, excessive inflammation seems to be a hallmark of the ILDs related to cigarette smoking [73, 74]. As stated before, some ILDs are marked by an accumulation of pigmented macrophages in the lung [75, 76]. The cause of this increase in macrophage number is still unknown, though potentially due to intense recruitment and increased secretion of differentiating factors by epithelial cells, improved survivability of macrophages or reduced apoptosis [77]. In addition, a key feature of ILD patients is that their epithelial cells produce disproportionate levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine involved in the proliferation and activation of macrophages and dendritic cells [78, 79].

Although these diseases are rare, they clearly have a significant impact on the quality of life and they contribute to the burden of smoking on the health system. It has been estimated that ILDs will become more frequent worldwide with the rise of cigarette smoking prevalence in most developing countries.

Cigarette smoking and chronic obstructive lung disease

Besides lung cancer and interstitial lung diseases, smokers may also develop a COPD. COPD is a preventable and treatable disease marked by persistent respiratory symptoms and irreversible obstruction of airflow usually caused by exposure to chronic noxious particles or gases [24]. Classic symptoms of COPD are coughing, dyspnea and sputum production. COPD patients also often face periods of acute symptoms worsening called exacerbations known to be associated with increased hospitalization rate [24]. Most exacerbations are due to microbial infections, either bacterial or viral [80, 81], and lead to alteration of the pulmonary microbiome [82, 83]. An important negative impact of exacerbations is their effect on the patient's quality of life [84].

COPD is currently the 4th leading cause of death worldwide. In 2012, more than 3 million deaths per year, representing approximately 6% of worldwide deaths, were attributed to COPD [24]. According to the Burden of Obstructive Chronic Diseases, a program built to assess the prevalence of COPD, yearly worldwide deaths associated with COPD will reach approximately 4.5 million by 2030 [24]. It was also estimated in 2010 that the worldwide prevalence of COPD was 11.7% [85]. However, the prevalence of COPD is probably underestimated as this disease is underdiagnosed in the general population [86]. Morbidity from COPD is also affected by other chronic diseases such as cardiovascular disease [87], musculoskeletal impairments [88, 89] and diabetes mellitus [24]. The economic burden associated with COPD is worrisome. In the European Union, 56% of total healthcare budget is due to COPD (38.6 billion euros), while in the US approximately 30 billion and 20 billion dollars are disbursed due to the direct and indirect medical costs of COPD, respectively [90, 91].

Cigarette smoking is the primary causal agent of COPD in North America [92]. However, in the Eastern world, exposure to air pollution and biomass fuels are the predominant cause [93]. Other factors including genetic, sex, respiratory infections such as tuberculosis, airway hyper-responsiveness, aging and poor lung development during childhood are also associated with the development and progression of COPD [24, 94-100]. Nonetheless, the exact factors leading to the development of COPD are still unknown since only up to 50% of smokers will be diagnosed with this disease [101-105].

Suspected COPD patients that exhibit shortness of breath, cough and mucus hypersecretion are diagnosed using spirometry, the gold standard diagnostic tool [24]. Spirometry is a non-invasive method used to measure the airflow limitation/obstruction. Post-bronchodilator spirometry allows for the measurement of two clinical parameters: the volume of air exhaled during the first second (forced expiratory volume in one second, FEV_1) and the volume of air exhaled by force at maximal inspiration (forced vital capacity, FVC). The ratio FEV_1/FVC, also called the Tiffeneau index, is considered normal when the value is higher than 0.7, while a ratio under 0.7 indicates airflow obstructions [24, 106]. COPD patients are also asked to answer questionnaires on breathlessness and symptoms for instance the Modified British medical research council (mMRC) questionnaire and the COPD assessment test, respectively (CAT; [24]. COPD is further classified by severity: mild, moderate, severe and very severe following the Global initiative for chronic Obstructive Lung Disease (GOLD) guidelines. This classification is based on the patient's FEV_1 compared to that of the general population, expressed as

a percentage of their predicted FEV_1. Patients are categorized as follows: GOLD 1 $FEV_1 \geq 80\%$, GOLD 2 $50\% \leq FEV_1 < 80\%$, GOLD 3 $30\% \leq FEV_1 < 50\%$ and GOLD 4 $FEV_1 < 30\%$ [24] (Figure II).

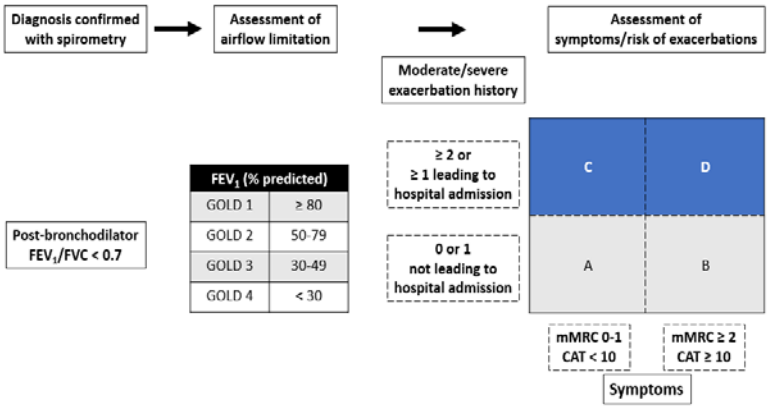

Figure II: ABCD assessment tool for COPD patients.

COPD patients are classified based on lung functions, exacerbations and symptoms. This guide, however, does not integrate genetic factors.

Genetic analysis is a great tool which has led to the discovery of genes associated with COPD and may ultimately lead to new therapeutic possibilities [107]. It is now known that a rare autosomal mutation to alpha-1 antitrypsin is found in 1-2% of COPD patients. Deficiency in alpha-1 antitrypsin (AAT1), a key circulating protease inhibitors, was the first mutation associated with COPD [108, 109]. During cigarette smoke exposure, AAT1 inhibits serine proteases from different immune cells, such as neutrophils. Other single genes, for instance matrix metalloproteinase 12 (MMP12), have been associated with a decline in lung function [110]. Similarly, genetic loci near the alpha-nicotinic acetylcholine receptor and the hedgehog interacting protein may also be associated with the development of COPD [111, 112]. Siblings of severe COPD patients have a higher risk of developing airflow limitations, suggesting that a combination of genetic and environmental factors may influence the risk of developing COPD [113].

Chronic airflow limitation in COPD patients is usually due to two different airway diseases: chronic bronchitis and destruction of lung parenchyma (emphysema). Interestingly, these clinical presentations vary among COPD patients; while some patients only exhibit signs of emphysema, other patients have pronounced obstructive bronchiolitis [24]. Chronic bronchitis is characterized by an exaggerated production of mucus in the respiratory tract mainly caused by the remodeling of the bronchial epithelium, the replacement of ciliated cells with mucus cells and the hypertrophy of secretory mucus glands [114]. Emphysema is characterized by the destruction and the loss of elasticity of alveoli, which

is the main area for gas exchange [115, 116]. This phenomenon leads to non-ventilated pulmonary zones. Patients with emphysema must breathe more often in order to meet the necessary levels of circulating oxygen required for metabolic processes but also to expel toxic carbon dioxide [117].

Another important characteristic of COPD is the presence of chronic inflammation and its persistence even after smoking cessation. The mechanisms responsible for the intensified response to cigarette smoke in COPD patients remain poorly understood [24]. However, the oxidative stress and the surplus of proteinase activity may influence lung inflammation [118-120]. Cigarette smoke and activated immune cells such as macrophages and neutrophils are the main pulmonary sources of reactive oxygen species (ROS) in COPD patients. It was shown that oxidative stress markers are increased in the systemic circulation, exhaled breath condensate and sputum of COPD patients. Exacerbations also lead to increased levels of oxidative stress [121]. In addition, excessive protease secretion from immune cells and epithelial cells have been observed in COPD patients. Their role in the destruction of elastin, a vital connective tissue in lung parenchyma, is thought to be a key mechanism leading to the development of emphysema [108, 109, 122].

Another hallmark of COPD is the presence of an increased number of inflammatory cells, most importantly macrophages, neutrophils and activated lymphocytes [123]. These cells, combined with epithelial and structural cells, release a variety of inflammatory mediators including chemotactic factors, proinflammatory cytokines and growth factors [118, 124]. Inflammation might also precede the progression towards fibrosis. COPD patients may show signs of peribronchiolar fibrosis and interstitial opacities [125] and exhibit increased levels of growth factors. The repeated injury/repair of lung parenchyma induced by cigarette smoking could underlie the excessive proliferation of muscle and fibrous tissue [126].

Other than smoking cessation, most available COPD treatments aim to reduce symptoms (coughing, sputum, and dyspnea) and exacerbations [24]. Pharmacological therapies rely mostly on bronchodilators and corticosteroids [24]. Bronchodilators such as $beta_2$-agonists are used to improve the FEV_1 of COPD patients by relaxing the airway smooth muscle and thus blocking bronchoconstriction [127, 128]. Antimuscarinic drugs may also be prescribed as they also block

bronchoconstriction [129]. Furthermore, COPD patients have access to inhaled corticosteroids which are thought to reduce the frequency and severity of exacerbations. The combination of bronchodilators and corticosteroids is more effective than either components alone at improving the health status, ameliorating lung function and reducing the number of exacerbations [130-132]. Phosphodiesterase-4 inhibitors may also be given to COPD patients as they reduce inflammation by inhibiting the breakdown of cyclic adenosine monophosphate (cAMP) [133]. These inhibitors have been shown to reduce exacerbations and improve lung function when combined with corticosteroids and bronchodilators, respectively [134, 135]. It is recommended that COPD patients receive the influenza vaccine as it was shown to significantly reduce the number of exacerbations and deaths caused by the flu [136, 137]. Additionally, pneumococcal vaccines are suggested to COPD patients, especially the 13-valent and 23-valent pneumococcal vaccines [24, 138].

COPD is a major societal burden and we will likely see a rise in the incidence of this disease in developing countries due to the increased prevalence of cigarette smoking. There are still a lot of unanswered questions regarding COPD such as susceptibility factors, initiating mechanisms, etc. Currently, a broad range of treatments are in use or under investigation. However, most of the therapies available to patients today merely attempt to reduce symptoms.

As you can see, cigarette smoking is a global burden leading to unprecedented societal, economic and health problems. The exposure to cigarette smoke leads to the development of major pulmonary diseases such as lung cancer, interstitial lung diseases and chronic obstructive pulmonary disease, as well as several other systemic diseases (Figure III, adapted from https://www.cdc.gov/tobacco/infographics/health-effects/index.htm#smoking-risks). Many of these diseases are progressive, life-threatening or even fatal. This thesis will focus on macrophages, one of the main inflammatory cells involved in the response to cigarette smoke exposure that have been shown to be associated with most of the aforementioned diseases.

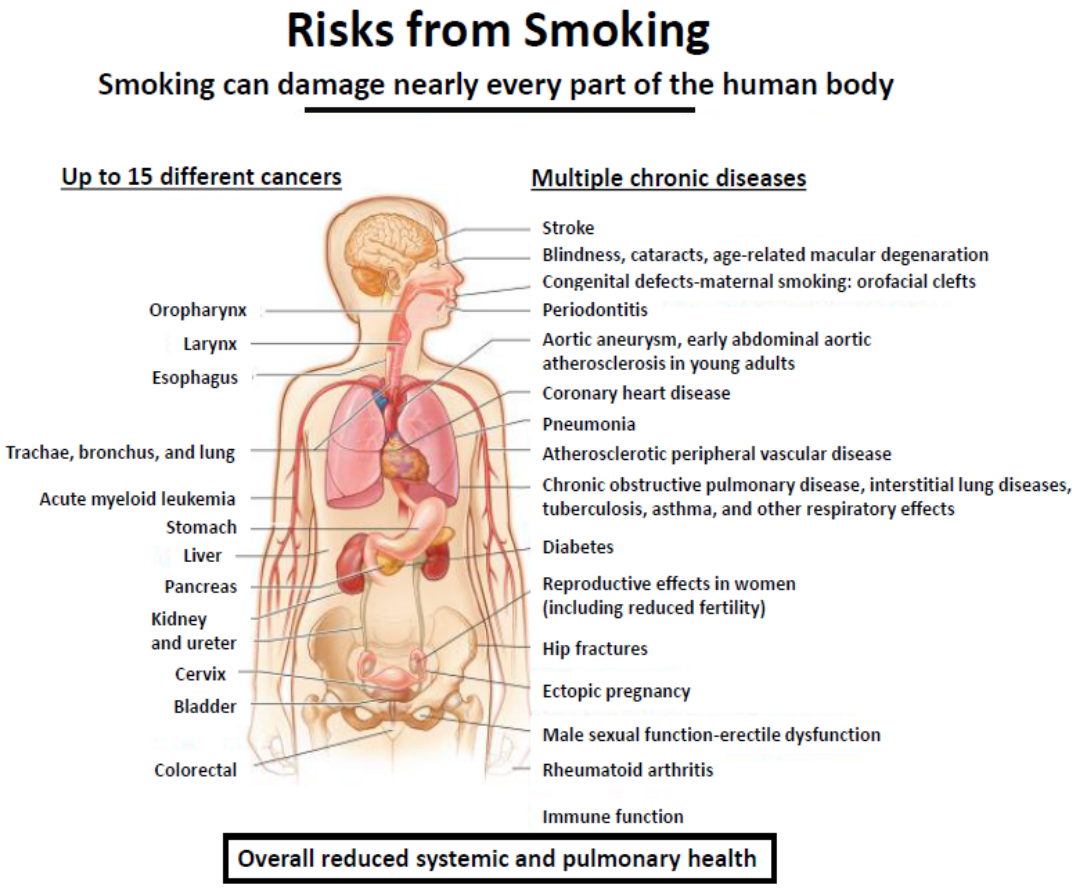

Figure III: Cigarette smoking is a major risk factor for numerous systemic and pulmonary diseases.

Generally, this habit leads to unprecedented health problems. To date cigarette smoking is associated with up to 15 different cancers and multiple chronic diseases.

Inflammation and pulmonary macrophage responses to cigarette smoke

Inflammation is a process found in all of the pulmonary diseases described above. One of the main characteristics of lung inflammation induced by cigarette smoke exposure is the increased number of pulmonary macrophages [139]. This phagocyte has many immune functions (for instance efferocytosis [140], phagocytosis [141], microbicidal activities [142], recognition functions [143], etc.) which are all negatively affected by cigarette smoking. In this section, general information related to inflammation will be presented. The acute and chronic pulmonary effects of cigarette smoke exposure will be

described, followed by its impact specifically on pulmonary macrophages. To conclude, the development of a “foamy” pulmonary macrophage phenotype induced by cigarette smoke exposure will be introduced.

What is inflammation?

Inflammation is a vital process that has been recognized since ancient times. Our ancestors already knew that inflammation involved four different mechanisms: *rubor* (redness), *tumor* (swelling), *calor* (heat), and *dolor* (pain) [144]. By the 1850s, Virchow introduced *Functio laesa*, or the dysfunction of organs, as the fifth feature [144]. Inflammation can be found throughout the body, for instance in the lung when facing diverse insults [145].

Following a variety of pulmonary insults, e.g. bacteria, viruses, air pollution, allergens, tissue injury or cigarette smoke, etc., there will be the activation of the immune system which is crucial for host protection and for the initiation of repair mechanisms [146]. Depending on the nature of the insult, the lung will mount an appropriate immune response against microorganisms or against pollution particles, allergens, cigarette smoke, etc. Responses to microorganisms are orchestrated via the detection of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs; [147], which will rapidly activate the innate immune response and later, the adaptive immune response.

Briefly, the innate immune response is a rapid response coordinated by resident pulmonary cells like pulmonary macrophages, epithelial cells, neutrophils and antigen-presenting cells (APCs) like dendritic cells [148] [149]. PRRs are found on macrophages, for instance toll-like receptors (TLRs), and are crucial in pathogen recognition [150]. Diverse PAMPs such as lipopolysaccharide (LPS) and modified low-density lipoproteins (LDLs) can bind TLRs [151]. Hallmarks of the innate immune response are the recruitment of pulmonary macrophages and neutrophils and the production of inflammatory cytokines and chemokines, for instance interleukin-1 (IL-1) and tumour necrosis factor alpha (TNFa) [147]. The main goal of the innate immune response is to mount an efficient response in order to eliminate diverse microbial threats and, if required, ultimately activate the adaptive immune response [152]. Adaptive immune response is mainly activated via APCs and the presentation of diverse antigens to naive T

cells [153]. Contrary to the innate immune response, the adaptive immune response is a slow and well-organized response relying on the production of specific antibodies against the antigen and the ability to build an immunological memory [144, 148]. The latter feature is crucial during reinfection with the same microorganism.

Interestingly, these PRRs can also be activated by non-microbial signals called danger-associated molecular patterns (DAMPs). DAMPs are the molecular signatures of sterile inflammation [147]. Cell death and tissue injury led to the release of DAMPs in the environment triggering a sterile inflammation response [147]. Binding of DAMPs to PRRs will activate downstream signaling pathways for instance the nuclear factor-κb (NFkB) and mitogen-activated protein kinase (MAPK), which will result in the upregulation of pro-inflammatory cytokines and chemokines [147]. Cigarette smoke exposure is a sterile stimulus that causes the release of DAMPs [154-156]. DAMPs can also be located extracellularly [147]. For instance, extracellular matrix degradation generates DAMPs, such as hyalorunan, biglycan, etc. These molecules are the products of proteolysis by enzymes released during cell death or via proteases secreted by pulmonary macrophages and neutrophils [157]. The latter mechanism becomes important under cigarette smoke conditions [158]. Similar to the immune response against microorganisms, responses to sterile stimuli lead to the recruitment of neutrophils and macrophages as well as the release of pro-inflammatory cytokines such as IL-1α [159, 160]. IL-1α, a pro-inflammatory cytokine, is also released in the pulmonary environment during necrosis, further promoting inflammation [161]. IL-1α is a key player involved in the initiation of sterile inflammation [162] and cigarette smoke-induced inflammation [163]. Overall, cigarette smoke triggers a sterile lung inflammation response with multiple consequences. The next section will focus on the pulmonary response to cigarette smoke and on pulmonary macrophages, a major player involved in maintaining lung homeostasis during inflammation.

The pulmonary response to cigarette smoke

Scientific and medical knowledge regarding the effects of cigarette smoke has greatly grown since the first Surgeon General in 1964 [164]. It is now known that nicotine is a lipophilic, addictive molecule found in cigarette smoke that is detected in the respiratory tract during cigarette smoke inhalation. Furthermore, nicotine binds within 10 second to the nicotinic acetylcholine receptor (nAChR) in the

brain, passing via the blood-brain barrier, to activate dopamine release [1, 165]. Dopamine leads to appetite loss, lower stress, anxiety, pain and mild euphoria [1, 165]. Other than the effects of nicotine and cigarette smoke on body composition [166], birth weight [167, 168], fetal development [169], central nervous system [170-173], metabolic and fertility problems [169], this section will focus on the cigarette smoke exposure systems available for animal research and the pulmonary outcomes of acute and chronic cigarette smoke exposure in these models.

Cigarette smoke exposure models

As acute cigarette smoke impact studies in humans are few due to ethic issues, one must turn to animal models. Animal models are therefore used to test new hypothesis, drug toxicity, side effects, and efficiency, but also to establish solid proofs of concepts. Cigarette smoke impacts are mostly studied in mice [174-177] but also in rats and guinea pigs [178]. However, molecular engineering is far more advanced in mice conferring an easier access to genetically modified mice strains [179].

Many cigarette smoke exposure systems are available. They differ in the type of exposure, which can be either nose only or whole-body exposure. Furthermore, mice can be exposed to mainstream primary smoke or secondary smoke. Most studies use acute, short-term exposure times or chronic protocols, with up to six months of cigarette smoke exposure. The former is used to characterize the pulmonary inflammatory response to cigarette smoke and to understand the initiating mechanisms leading to inflammation [180, 181]. On the contrary, subchronic and chronic models are primarily used to investigate the impact of cigarette smoke on the pulmonary structure and the long-term immune response [182, 183]. While acute cigarette smoke exposure models are known to generate an observable inflammatory response [180, 181], long-term exposure to cigarette smoke will result in a milder pulmonary inflammatory response. Though many variables can interact with the pulmonary outcomes of cigarette smoke exposure, several studies, utilizing various cigarette smoke exposure protocols, have established that cigarette smoke exposure has a negative impact on the lung.

Acute effects of cigarette smoke on the lung

Acute cigarette smoke exposure has multiple consequences on pulmonary immune functions: for instance pathogen clearance, inflammatory mediator secretion, host defense, inflammatory cell recruitment and oxidative stress, etc. The latter is known to be increased during cigarette smoke exposure (reviewed: [184]). In breath condensate and exhaled air from smokers, 8-isoprostane, a lipid peroxidation product, was increased after a short 15-minute cigarette smoke exposure [185], while hydrogen peroxide levels were increased after a 30-minute cigarette smoke exposure [186]; both of these molecules are oxidative stress markers. Furthermore, levels of glutathione, a key antioxidant enzyme, were reduced in the lung tissue and in the BALF of rats immediately and after a short cigarette smoke exposure [187, 188]. Other oxidative stress markers, such as 4-hydroxynonenal (4-HNE) a lipid peroxidation byproduct, and iNOS or eNOS mRNA levels were increased in mice and rats, respectively during acute cigarette smoke exposure [189, 190]. Overall, acute cigarette smoke exposure increases levels of oxidative stress in smokers and diverse animal models.

Acute cigarette smoke exposure also has important effects on inflammatory cells [184]. In mice, pulmonary epithelial cells and resident pulmonary macrophages, a key immune cell which will be further described in the upcoming section, secrete many chemotactic molecules following acute cigarette smoke exposure for instance C-C motif chemokine ligand 2 (CCL2, also named MCP-1) [191-193], CCL7, macrophage inflammatory proteins (MIP-1α, 1β, 1γ, 2, 3β) [180] and C-X-C chemokine motif 5 (CXCL5) [194]. These molecules activate many pathways that lead to the recruitment of immune cells to the lungs of smokers [184] and cigarette smoke-exposed mice [180, 181, 183, 184, 195].

Neutrophils are the most abundant immune cells recruited to the lung after acute or chronic cigarette smoke exposure in smokers [184, 196-199] and in mice [180, 200]. Neutrophils are phagocytes known to be a crucial for pathogen clearance via degranulation or formation of extracellular traps (NETs, [201]. Myeloperoxidase (MPO) and lactoferrin, two proteins found in neutrophil granules, and MMP-9 are used by neutrophils to eliminate pathogens and/or to degrade the extracellular matrix [201]. Another key enzyme involved in host defense and tissue destruction is neutrophil elastase [202], which was shown to be more active in smokers [203] and extremely important in the pathogenesis of emphysema [158]. While the neutrophil seems to have an important role in the response to cigarette smoke

exposure, its precise role is unclear. Further characterization of neutrophils during cigarette smoking is necessary to decipher its role.

In the context of cigarette smoke, dendritic cells (DCs) are activated and their numbers are also increased [204]. However, DC maturation and function are impaired after cigarette smoke exposure [205]. These immature DCs may exhibit altered antigen presentation to immature T cells. Natural killer cells (NKs) have an exacerbated immune response when exposed to cigarette smoke [206]. NK cell activity was also shown to be reduced in heavy smokers compared to non-smokers [207]. One of the main outcomes of this immune response is the massive increase in the secretion of multiple cytokines and inflammatory mediators such as IL-1α, IL-5, IL-6, IL-18, granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, MMP9, TIMP-1, MIP, TNF, among others [180, 208, 209]. As this immune response is initiated by a sterile stimulus, the mechanism leading to this response is still not fully understood.

Pulmonary alveolar permeability, measured as the rate of clearance of diethylenetriamine pentaacetic acid (99mTcDTPA), is also increased in smokers [210, 211] and in acutely exposed rabbits [212] and guinea pigs [213]. Furthermore, acute cigarette smoke exposure leads to an altered pulmonary transcriptome [214]. Maunders *et al*. showed that cigarette smoke disrupted the pulmonary epithelial transcriptome of smokers by altering xenobiotic metabolism, oxidants/antioxidants balance and DNA damage and repair genes [214]. Long-term cigarette smoke also negatively impacts the lung.

Chronic effects of cigarette smoke on the lung

Multiple mouse studies have shown that chronic cigarette smoke exposure leads to the development of lung tissue damage, a phenotype observed in patients with COPD [183, 215-217]. Chronic inflammation, oxidative stress, and proteases/anti-proteases balance are the most common mechanisms proposed to be involved in the destruction of the lung parenchyma [218-221]. The modification of the lung parenchyma leads to altered lung function in smokers and in cigarette smoke-exposed animal models [182, 222, 223]. The lung of cigarette smoke-exposed mice is more compliant accompanied by a reduction in tissue elastance [222]. Their inspiratory capacity is also increased

compared to unexposed mice [224]. Smokers and cigarette smoke-exposed mice have an increased mean linear intercept value, a measure used to characterize tissue destruction/emphysema [225, 226], compared to unexposed groups [215-217, 227].

The immune response is also altered by cigarette smoking [181, 196, 217, 228]. In mice, DC numbers are reduced after subchronic cigarette smoke exposure [228] and, as stated during acute exposure, they also have an impaired maturation in the lymph nodes [229]. Cigarette smoking reduced the expression of major histocompatibility class 2 and costimulatory molecules such as CD80 and CD86, ultimately leading to altered T cell responses [229]. The number of B and T cells is increased [196, 217]. Furthermore, chronic cigarette smoke exposure leads to the generation of pathogenic T lymphocytes [230]. B and T cells are extremely important to the development of tertiary lymphoid tissues (TLTs), a key autonomous and localized immunological structure that persists in the lung during cigarette smoke exposure [183, 196, 216, 231, 232]. COPD patients and mice exposed to cigarette smoke for six months develop TLTs [183, 216]. At the 2018 American thoracic society (ATS) conference, it was shown that mice genetically modified to have blunted B cell production do not develop TLTs [233]. Interestingly, these TLT structures have the ability to produce autoantibodies [183, 216, 234], further promoting the autoimmunity hypothesis in smokers [235-238]. Furthermore, Thayaparan *et al.* demonstrated that cigarette smoke leads to the generation of antibodies against oxidized lipids in mice [235]. To conclude, cigarette smoke exposure alters the lung immune response.

Cigarette smoke exposure is also known to increase the expression of diverse genes of proteins involved in detoxification, for instance the aryl hydrocarbon receptor repressor [239, 240], and P450 cytochrome family (cytochrome P450 family 1 subfamily A member 1 and cytochrome P450 family 1 subfamily B member 1, etc.) [241]. The expression of these genes is altered due to the presence of polyaromatic hydrocarbons in cigarette smoke [239, 242]. Another key gene that is overexpressed during cigarette smoking is the MMP12 [243-245], which has been proposed to be central to the development of emphysema [221, 246]. Another study investigated the pulmonary gene expression similarities between humans and mice exposed to cigarette smoke [247]. Morissette *et al*. found 16 genes that were affected in both humans and mice. These genes were mainly associated with detoxification, immune response and lipid metabolism [247]. To conclude, cigarette smoke has major acute and chronic effects on multiple pulmonary cells, on the pulmonary structure, and on the oxidative

stress response. (Figure IV). The next sections will focus on one of the most important cell types involved in the response to cigarette smoke exposure: the pulmonary macrophage.

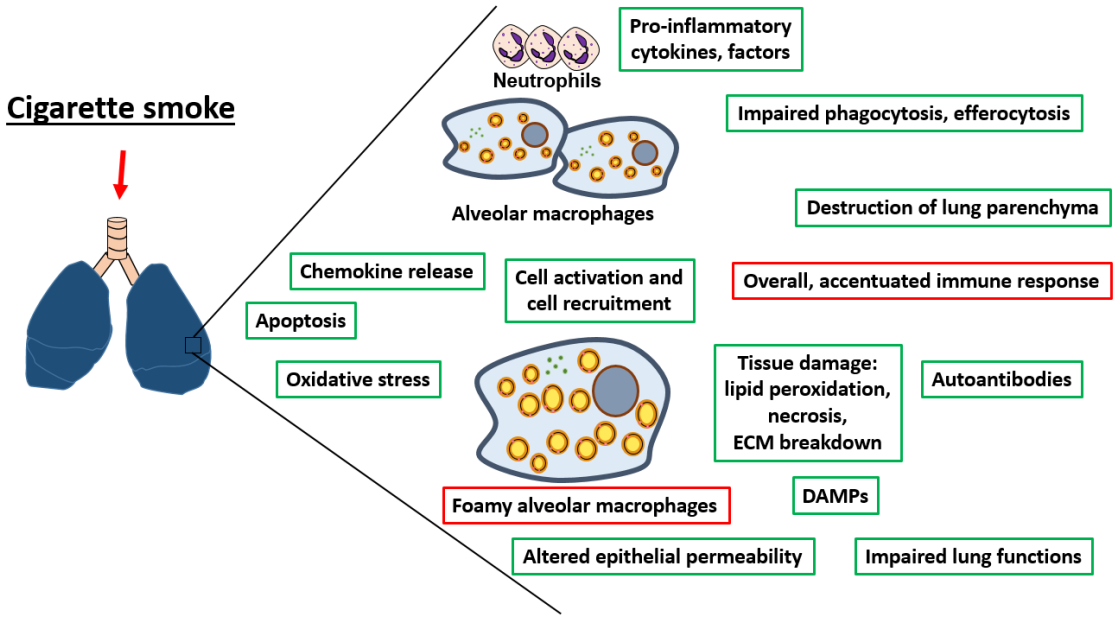

Figure IV: Cigarette smoke-induced lung alterations.

Acute and chronic cigarette smoke exposure leads to tremendous pulmonary outcomes from an exacerbated immune response due to an intensive cell recruitment to long-term parenchyma damage caused by oxidative stress and sustained inflammation. One persistent phenotype is the presence of foamy alveolar macrophages under cigarette smoke exposure conditions. A phenomenon described 50 years ago and forgotten.

Pulmonary macrophages

Under homeostatic conditions, pulmonary macrophages are one of the first lines of defense against pathogens and are the most abundant cells in the bronchoalveolar lavage [248]. This cell type has a slow turnover rate, approximately 40% per year [249, 250]. As previously stated, these phagocytes have multiple surface receptors involved in pathogen detection, making them ideal sentinel cells. They are also involved in the clearance of inhaled toxic particles, cellular debris, apoptotic cells and allergen particles [251]. They also have receptors involved in lipid capture, called scavenger receptors [252].

The initial colonization of the lung with pulmonary macrophages starts a couple of days after birth through the differentiation of fetal monocytes [253]. Interestingly, these macrophages have a great capacity for self-renewal and population replenishment [254, 255]. Bone marrow hematopoietic stem cells are also involved in the generation of circulating monocytes; which, when recruited to the lung, can further differentiate into pulmonary macrophages [256]. Overall, the lung has three different macrophage populations: resident macrophages, macrophages derived from circulating monocytes and another population of macrophages that persists post-inflammation [256]. The alveolar environment is in constant flux due to pressure or microbial changes; therefore, macrophages must adapt their response appropriately [256]. In the presence of inflammation, pulmonary macrophages release a wide array of cytokines and chemokines [251]. Cigarette smoke exposure is known to activate pulmonary macrophages and perpetuate lung inflammation, ultimately disrupting lung homeostasis.

Pulmonary macrophage responses to cigarette smoke

Cigarette smoke exposure has many negative effects, as previously described. Cigarette smoking also negatively affects pulmonary macrophages. Though their numbers are increased in smokers [257, 258] and in cigarette smoke-exposed mice [180, 181, 259], their functions are impaired. Murray *et al*. proposed that the increased in pulmonary macrophage numbers could be due to an accentuated recruitment of monocytes and differentiation into pulmonary macrophages, a higher local proliferation of resident macrophages, or a higher survival rate of pulmonary macrophages during cigarette smoking [260]. However, Aoshiba *et al.* demonstrated *in vitro* that isolated alveolar macrophages from humans, mice and rats exhibit increased apoptosis in the presence of cigarette smoke extract [261]. This suggests that cigarette smoke can lead to macrophage apoptosis, discounting the hypothesis of increased survival rate during cigarette smoking. As mentioned, the ability of macrophages to clear bacteria, phagocytosis of pathogens such as *Pseudomonas* and/or inorganic particles is impaired during cigarette smoking [141, 262-265]. *In vitro* experiments also demonstrated decreased alveolar macrophage protein synthesis after cigarette smoke extract exposure in rabbits [266, 267]. The latter being a potential mechanism explaining the impaired macrophage phagocytic abilities.

Foamy pulmonary macrophages following cigarette smoke exposure

Exposure to cigarette smoke leads to an altered pulmonary macrophage phenotype. They become larger compared to unexposed pulmonary macrophages [181]. This phenotypic change was observed in pulmonary macrophages isolated from both smokers and cigarette smoke-exposed mice [181, 268-271]. In mice, this phenotype can be detected after an acute or chronic cigarette smoke exposure [181]. Interestingly, this phenotype is also found in other pulmonary and extra-pulmonary diseases, for instance pulmonary fibrosis [272], PAP [273], asthma [274], ILDs [275], and atherosclerosis [276].

As mentioned above, this macrophage phenotype is also found in atheromatous plaques; furthermore, it was demonstrated that these larger pulmonary macrophages are laden with lipids, named foamy macrophages [181]. Similar mechanisms are initiated in atherosclerosis and cigarette smoke exposure. This foamy pulmonary macrophage phenomenon can be observed acutely, after four days of cigarette smoke exposure, but also chronically, after six months of exposure [181]. Interestingly, the foamy phenotype persists following prolonged cigarette smoke cessation [181]. Moreover, these lipid-laden macrophages are also found in pulmonary fibrosis, asthma and ILDs [272, 274, 275]. Using oil red O, BODIPY or electron microscopy, these studies demonstrated the presence of intracellular lipids in pulmonary macrophages. The mechanism behind this phenotype is not fully understood. Although it was discovered in the 1970s, few research groups have attempted to decipher and characterize this phenomenon. To conclude, cigarette smoke exposure leads to the development of pulmonary foam cells.

Morissette *et al.* further demonstrated that mice lacking IL-1α expression exhibited cloudy bronchoalveolar lavage (BAL), marked by high levels of phosphatidylcholine, an important pulmonary lipid [181]. Similar results were also shown when GM-CSF was blocked using a neutralizing antibody [181]. GM-CSF is a key cytokine involved in the differentiation of fetal monocytes into alveolar macrophages [253]. Furthermore, GM-CSF knockout mice have immature alveolar macrophages that display an accumulation of surfactant proteins and altered surfactant catabolysis [277]. Therefore, these two cytokines seem to be involved in the activation of mechanisms related to lipid recycling, uptake and/or elimination. One can hypothesize that intracellular lipids found in pulmonary

macrophages may come from the pulmonary surfactant, a lipid-rich structure with a high risk of oxidation.

Pulmonary surfactant

The lung is a complex structure that is required for the supply of oxygen (O_2), as O_2 is essential to maintain life and metabolism. While it may seem irrelevant, the human body cannot store O_2. Therefore, it is vital that O_2 be continuously supplied to mitochondria. In humans, O_2 is captured through the air and binds hemoglobin in the circulating erythrocytes, which is ultimately transported to cells throughout the body [278]. Since the average human inhales 10,000 liters of gas per day, the lungs represent a point of entry for many airborne environmental insults such as pathogens, organic and inorganic particles, cigarette smoke, allergens, pollutants, etc. [279, 280]. However, evolution has granted the human species with an efficient barrier against intruders without disturbing lung structure and gas exchange [281]. That structure is called the pulmonary surfactant.

The pulmonary surfactant is a vital structure found at the air-water interface of pulmonary alveoli. It is in close proximity to various pulmonary cells such as types 1 and 2 pneumocytes and alveolar macrophages. The main role of the pulmonary surfactant is to reduce surface tension during respiratory cycles and prevent atelectasis (alveolar collapse; [282, 283]). The pulmonary surfactant is mainly composed of lipids (90%) and proteins (10%) [284]. The major lipids found in this structure are phospholipids (approximately 95%) and neutral lipids such as cholesterol. Phospholipids can be further subdivided as follows: phosphatidylcholine (PC, representing more than 65% of surfactant phospholipids), phosphatidylglycerol, phosphatidylinositol (PI), phosphatidylethanolamine (PE), and phosphatidylserine. PC can also be categorized into disaturated PC, such as dipalmitoyl phosphatidylcholine (DPPC), and unsaturated PC [285]. DPPC is the most important phospholipid involved in reducing alveolar surface tension [283].

The liquid phase in the lung tissue and alveolar space is composed of many strong cohesive forces. The pulmonary surfactant, as stated before, reduces the net contractile force via a mon-molecular layer of lipids [286]. DPPC is the most important lipid involved in this purpose [285]. DPPC is composed of

a choline head, which is polar and hydrophilic, and palmitic acid tails, which are nonpolar and hydrophobic. Since palmitic acids are saturated, DPPC forms a straight chain making it easier to compact when the surface tension is lowered [286]. This property of DPPC improves the stability of pulmonary surfactant. Other than lipids, surfactant proteins also help in the solidity of pulmonary surfactant.

Surfactant proteins

The surfactant proteins include surfactant proteins A, B, C and D (SP-A -B -C, and –D) [283]. The main difference between surfactant proteins is that SP-B and SP-C are small and highly hydrophobic compared to hydrophilic SP-A and SP-D. SP-B and SP-C are associated with the structure and stability of pulmonary surfactant, while SP-A and SP-D are involved in its host defense functions as part of the collectin family [287, 288]. SP-A is a 32 kDa glycoprotein and is the most abundant surfactant protein [289]. SP-A has a long extracellular domain linked to a globular region, which contains a calcium-dependent carbohydrate recognition domain (CRD). This domain gives SP-A the ability to bind lipids, LPS and some microorganisms [283]. Fully operational SP-A is represented by 18 monomers which resemble a bouquet of flowers and it is expressed in type 2 pneumocytes, bronchial club cells and airway submucosal gland cells [283]. It was shown that SP-A accelerates the incorporation of phospholipids in the surfactant, stimulates host defense by targeting opzonized particles and seems to be involved in the inhibition of surfactant secretion and uptake by type 2 pneumocytes [290-292].

SP-D is a 43 kDa glycoprotein directly linked to the CRD domain. This protein has a cross shape and is synthesized by type 2 pneumocytes and bronchial club cells [283]. Just like SP-A, SP-D can bind LPS and different microorganisms [280]. Compared to other surfactant proteins who are bound to lipids, most of SP-D is dissolved in surfactant fluids [283]. SP-A and SP-D have many immune functions such as pathogen aggregation, phagocyte induction, and inflammatory mediator regulation [280].

SP-B and SP-C are both hydrophobic proteins with a molecular weight of 8 kilodaltons (kDa) and 4 kDa respectively [293]. SP-C being a very hydrophobic protein mostly due to the presence of a valine-rich region [293, 294]. SP-B is expressed by type 2 pneumocytes and bronchial club cells while SP-C

is exclusively found in type 2 pneumocytes [283, 294]. Both of these proteins play a critical role in the maintenance of surfactant lipid biophysical activity [283]. SP-B mostly accelerates the synthesis of surfactant by increasing the adsorption rate of lipids. SP-B is of importance as its deletion or mutation is deleterious in mice and human infants. Lack of SP-B expression disrupts lung homeostasis leading to respiratory failure in newborn SP-B knockout mice [295, 296] and fatal neonatal respiratory disease in humans [297]. SP-C knockout mice are viable and have been used to determine its role in surfactant function. Compared to SP-B, SP-C seems to be involved primarily in the stabilization of the pulmonary surfactant at low lung volumes [294].

As stated before, structural surfactant protein depletion leads to respiratory distress and may be fatal [298], such as in newborn respiratory distress syndrome (NRDS) and acute respiratory distress syndrome. These deficiencies are typically caused by mutation in *sftpb*, *sftpc* or *abca3* genes, key genes associated with lung diseases such as acute respiratory distress syndrome [293, 299, 300]. One of the main SP-B mutations is the 120ins2 mutation, a substitution leading to a 2-base pair insertion [293, 299]. The latter mutation was responsible for two thirds of NRDS cases identified in 2004 [293, 299]. SP-B deficiencies are hereditary compared to SP-C deficiencies [293, 299] and mainly impact newborns while SP-C deficiencies can also be found in adults. Many therapies involve the use of synthetic surfactant in order to replace the dysfunctional pulmonary surfactant in patients with surfactant protein mutations [301].

Surfactant synthesis and turnover

The pulmonary surfactant, including PC, is produced and secreted by type 2 pneumocytes [302]. PCs can merge with the actual pulmonary surfactant or be excreted by type 2 pneumocytes into the circulation [303]. Surfactant packaging starts in endoplasmic reticulum and matures in Golgi apparatus to form lamellar bodies, mediated by a member of the adenosine triphosphate (ATP)-Binding Cassette (ABC), ABCA3 [287]. ABCA3 is a transmembrane protein involved in the transportation of PCs and is exclusively expressed in type 2 pneumocytes [304, 305]. Briefly, *abca3* mutations are also associated with NRDS but also lung disease in adults, like chronic ILDs [306]. Once lamellar bodies are formed,

they can be excreted into the air-liquid interface of pulmonary alveoli to integrate the lipid monolayer of the surfactant.

The pulmonary surfactant is one of the first barriers against airborne irritants and pathogen, making it susceptible to oxidation. Due to different inhaled particles, pulmonary cells can produce ROS to defend the lung, such as superoxide and hydroxyl radicals [307], in an attempt to kill the pathogen. Unfortunately, ROS also damage surfactant by oxidizing phospholipids. Disaturated PCs are the most common of the surfactant phospholipids and are thus at high risk for ROS damage. They can be oxidized by different mechanisms such as peroxidation, while unsaturated PC are modified via electrophilic attacks. These unsaturated PCs are prone to oxidation due to the instability of double-bonds found on acyl chains. The peroxidation of disaturated PC leads to different phospholipids depending on the phospholipid backbone [307]. Disturbance in the surfactant composition due to oxidation leads to altered surfactant function. Therefore, the modified phospholipids must be cleared and replaced in order to overcome these effects (Figure V).

Pulmonary cells such as type 2 pneumocytes and alveolar macrophages are involved in the recycling and degradation of damaged surfactant [308]. Recycled lipids are trafficked and reincorporated into lamellar bodies [309]. Compared to epithelial cells, alveolar macrophages mostly degrade surfactant rather than recycling it [310]. It was also shown that neutrophils can interact and participate in the clearance of surfactant-associated phospholipids and proteins [311, 312].

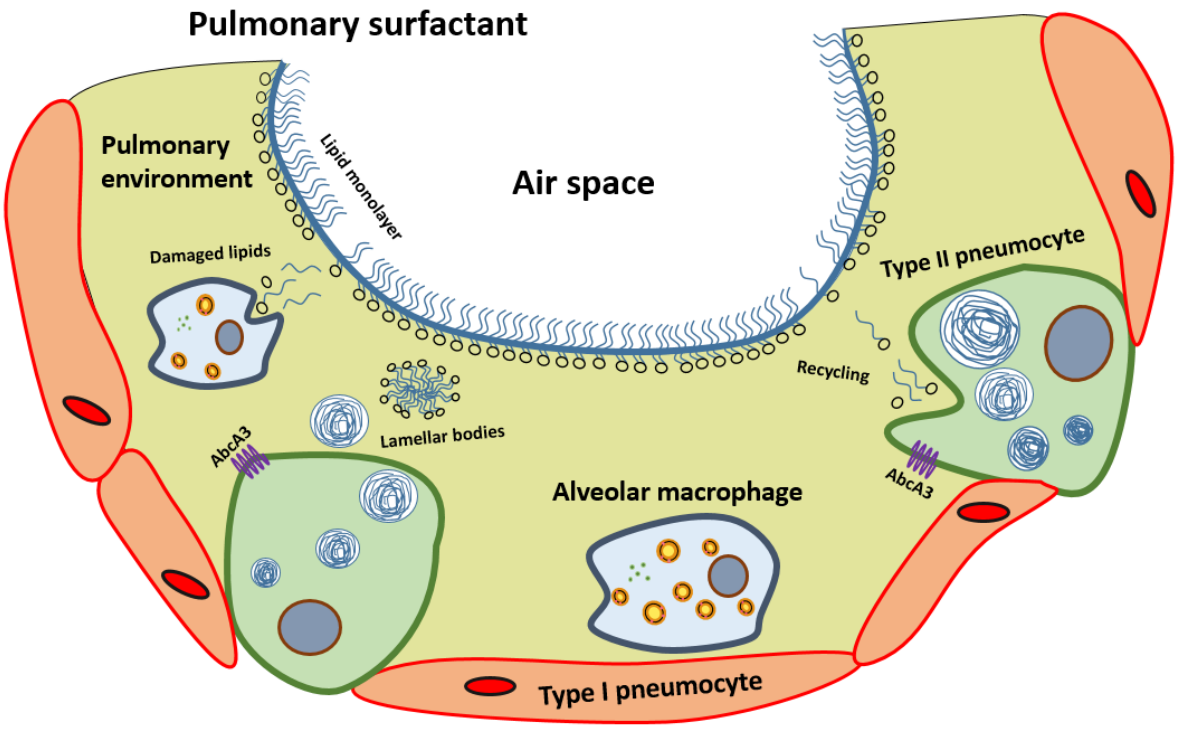

Figure V: The pulmonary surfactant.

Surfactant lipids are synthesized in the endoplasmic reticulum and are further processed in the Golgi apparatus of type 2 pneumocytes. Mature phospholipids are reorganized and excreted in the pulmonary environment in the form of lamellar bodies. Lamellar bodies can then integrate the monolayer of lipids called the pulmonary surfactant. However, due to its proximity to the airspace, the pulmonary surfactant can be oxidized by multiple sources such as normal breathing or cigarette smoke and air pollution. Therefore, type 2 pneumocytes are also involved in the recycling of these damaged phospholipids. Alveolar macrophages, on the other hand, are mostly involved in the catabolism of these oxidized lipids.

Data about the pulmonary surfactant turnover rate are scarce. Baritussio *et al*. showed that pulmonary surfactant turnover is approximately 4 to 11 hours [313]. Most methods used are based on radioactive precursors, such as palmitate, choline or phosphates and the estimated turnover rate depends on which precursor is used [286]. However, studies have established that surfactant turnover must be rapid and tightly controlled. Exercise such as swimming was shown to accelerate pulmonary surfactant

turnover in rats [314]. Furthermore, high frequency oscillation ventilation, mechanical ventilation, does not alter the pulmonary surfactant turnover [315]. The pulmonary surfactant is a complex and dynamic structure that requires much more attention in the context of pulmonary injury and disease. The next section will describe our current knowledge of how cigarette smoke interferes with pulmonary surfactant.

Cigarette smoke and pulmonary surfactant

Investigation into the direct effects of cigarette smoking on pulmonary surfactant began in the 1960s and 1970s. Finley *et al.* showed that pulmonary surfactant levels in smokers were reduced in BAL compared to non-smokers, while levels returned to normal after smoking cessation [316]. Total phospholipid levels were also decreased in the BAL of smokers [317, 318]. It was then hypothesized that a reduction in the "active and stable surfactant" may explain why smokers may develop emphysema [316, 319, 320]. Studies performed by Finley, Cook, Webb and Clement point in the same direction; cigarette smoking seems to impair the ability of surfactant to reduce pulmonary surface tension [270, 316, 321-323]. Subramaniam *et al.* also proposed that cigarette smoke could reduce the secretion of pulmonary surfactant by type 2 pneumocytes [324], a phenomenon observed in cigarette smoke-exposed rats [325]. During the ATS conference in 2017, it was described that cigarette smoke induced a unique oxidized lipid signature, mainly affecting phosphatidylcholine [326].

Cigarette smoke is known to generate an impressive number of oxidants, and to increase peroxidation [184, 189, 327]. As previously stated, oxidized lipids are replaced, recycled or catabolized by type 2 pneumocyte and/or alveolar macrophages. Cigarette smoke was shown to induce foamy alveolar macrophages that increased in size following cigarette smoke exposure [181]. This suggests that the intracellular lipid accumulation in macrophages is the result of upregulated clearance of damaged pulmonary surfactant by alveolar macrophages following cigarette smoke exposure.

The pulmonary surfactant is much more than a mechanical fluid lining as it is critical to host defense and seems to have a major role during cigarette smoke exposure. The high concentration of PC in the pulmonary surfactant and the elevated oxidant levels during cigarette smoke exposure increases its

susceptibility to oxidation and damage. Therefore, alveolar macrophages but also type 2 pneumocytes must increase the clearance, elimination and/or replenishment of these lipids in order to maintain surfactant homeostasis. Upon detection of these oxidized lipids, alveolar macrophages upregulate pathways involved in the uptake, trafficking and export of surfactant lipids.

Lipid uptake to lipid export by macrophages

Reverse cholesterol transport (RCT) is described as the transport of intracellular lipids from different cells, such as macrophages, to the liver, the most important organ in lipid regulation [328]. This process begins directly in the macrophages with the uptake of lipids by different scavenger receptors. In this section, key pulmonary macrophages scavenger receptors will be introduced. Furthermore, their pulmonary roles in health and in response to cigarette smoke will be described followed by a brief description of the processes behind intracellular lipid droplet formation. Key nuclear receptors involved in the transcription of major lipids export protein will also be introduced. To conclude this section, active and passive lipid export mechanisms will be presented, accompanied by an outline of the impact of cigarette smoking on these nuclear receptors and export mechanisms.

Macrophages and scavenger receptors

The discovery of scavenger receptors in the 1970s by Brown and Goldstein completely changed the perception of macrophages by the scientific community [329-331]. It is now well known that macrophages have multiple scavenger receptors at their cell surface, which gives them the ability to detect PAMPs (associated with pathogens), DAMPs (molecular danger signals), proteins and endo- and exogenous lipids, etc. [252]. Collagenous or noncollagenous scavenger receptors, integrins, immunoglobulin superfamily, and TLRs are some examples of the diversity of receptors found on the surface of macrophages [332]. Within the scavenger receptor family, cluster of differentiation 36 (CD36), macrophage scavenger receptor 1 (MSR1), scavenger receptor class B type 1 (SR-B1) and macrophage receptor with collagenous structure (MARCO) will be further described in this section as these receptors are involved in lipid uptake.

CD36

CD36, or SR-B2, is an 88 kDa membrane glycoprotein [333] whose expression is mostly under the control of the peroxisome proliferator-activated receptor gamma (PPARγ), a well-known nuclear receptor implicated in glucose and lipid metabolism. This scavenger receptor is found on many cell types such as monocytes, different macrophage subtypes, platelets, micro-vascularized endothelial cells, adipocytes, hepatocytes, kidney epithelial cells and cardiac myocytes [333]. CD36 has many functions and is involved in the development of several diseases such as atherosclerosis [333], characterized by progressive lipid accumulation within aortic endothelial walls, dysregulated triglyceride metabolism [334], angiogenesis, and cognitive behavior development [335]. Multiple ligands are associated with CD36: thrombospondin-1, amyloid beta (Aβ) peptides, LDLs and oxidized phospholipids [333]. Of interest, CD36 has been shown to be involved in the development of foamy macrophages found in atheromatous plaques. Furthermore, mice lacking CD36 seem to be protected against atherosclerosis when fed a high-fat diet [333]. Briefly, it was also demonstrated that hepatic overexpression of CD36 in mice is associated with an increase in liver triglyceride content [336].

In the lung, the presence of CD36 was associated with a better phagocytic response during viral infection [337]. However it was shown that during an influenza infection, the expression of CD36 was reduced [338]. In the presence of pulmonary surfactant and *Mycobacterium tuberculosis*, it was demonstrated that mice lacking CD36 had a greater bacterial burden compared to wild-type counterparts [339]. Interestingly, the administration of a synthetic CD36 peptide was able to overcome some of the adverse effects in a mouse model of bleomycin-induced pulmonary fibrosis. CD36-treated mice had diminished levels of transforming growth factor beta (TGF-β), lung inflammation and fibrosis-associated gene expressions (collagen type 1 alpha 1, collagen type 3 alpha 1, and fibrillin 1). These mice also regained weight faster compared to untreated bleomycin mice [340]. This study suggests that the activation of CD36 would be beneficial in the context of pulmonary fibrosis. It was also shown *in vitro* that treating isolated alveolar macrophages from patients with a PPARγ agonist was associated with an increase in CD36 expression and a better phagocytic response in the presence of apoptotic neutrophils compared to untreated macrophages [341]. Isolated alveolar macrophages from patients

with idiopathic interstitial diseases, especially DIP patients, have increased CD36 expression compared to control patients [342].

Few studies have evaluated the importance of CD36 during cigarette smoking. It was shown that CD36 expression in isolated alveolar macrophages from healthy smokers and COPD patients were unchanged compared to non-smokers. However, the CD36 expression was higher on circulating monocytes from healthy smokers and COPD patients compared to non-smokers [343]. This observation was also seen in two other COPD patient studies [344, 345]. CD36 has many important roles; however, further research is required in order to decipher its role in the response to cigarette smoking.

MSR1

MSR1, or scavenger receptor class A type 1, or CD204, is an approximately 80 kDa transmembrane glycoprotein composed of one large extracellular domain, giving it the ability to recognize several ligands and to perform diverse functions [346]. MSR1 is expressed on numerous cells such as different macrophage subtypes, endothelial cells, pulmonary epithelial cells, microglia, astrocytes, fibroblasts, and vascularized smooth muscle cells [346, 347]. Oxidized or acetylated LDLs (oxLDLs or acLDLs), apoptotic cells, and LPS are the most important ligands of MSR1 [346, 347]. MSR1 has diverse roles in immunity, affecting pathways involved in host defense, viral infection, inflammation, and apoptosis. This scavenger receptor is also implicated in foam cell formation, Alzheimer's disease, bone metabolism, and cardiovascular disease like atherosclerosis [346].

The importance of MSR1 depends on the context since, in most pulmonary models of microbial infection its deletion seems to be beneficial. However, in the context of sepsis, or in cerebral ischemia models, its deletion is deleterious [346]. Regardless, pulmonary effects of MSR1 has been widely investigated. In asthma, MSR1 expression is increased on dendritic cells after ovalbumin challenges [348]. It was further demonstrated that MSR1 had the ability to reduce the number of DC antigen delivered to lymph nodes, ultimately dampening the inflammatory response to allergens. Alveolar macrophages from IPF patients have also been shown to express increased levels of MSR1 [349].

Stahl *et al*. showed that collagen type 1 binding via MSR1 was able to shift the polarity of alveolar macrophages towards a profibrotic phenotype. Furthermore, MSR1 was shown to reduce pulmonary macrophage activation, which led to attenuated hyperoxic lung injury [350]. MSR1 is also known to capture environmental particles [351]. Mice lacking MSR1 fail to develop pulmonary fibrosis following silica exposure compared to wild-type mice [352] and are protected from lung damage in an organic dust extract exposure model [353].

MSR1 has an important role in immunity, especially host defense against various microorganisms. During H1N1 influenza infection, the expression of MSR1 is reduced [338]. Interestingly, aging is also associated with reduced MSR1 expression, neutrophil retention during viral infection, and alveolar macrophage neutrophil phagocytosis [354]. Targeting MSR1 may provide a new therapeutic strategy. MSR1 also has a role in the response to *Pneumocystis carinii*, an opportunistic fungal pathogen. Adult mice deficient in MSR1 cleared the fungal pathogen faster than wild-type animals due to increased T helper cell recruitment and heightened inflammatory response [355]. Furthermore, alveolar macrophages isolated from these knockout mice had unchanged phagocytic ability and secreted lower levels of TNF-α when challenged with LPS compared to wild-type controls. MSR1 seems to be a part of the inflammatory response to *Pneumocystic carinii*; however, the mechanism is unclear.

The impact of LPS on the expression of MSR1 is controversial. It has been shown that in the presence of LPS, MSR1 expression on pulmonary macrophages was increased, which led to more effective phagocytosis of apoptotic cells [356, 357]. However, another study showed that LPS did not induce any changes in the expression of MSR1 on alveolar macrophages compared to untreated mice [358]. Differences in doses and duration of exposure to LPS may explain these variations. The latter study demonstrated that in a combined model of LPS, elastase, and nontypeable *Haemophilus influenza* (NTHi), MSR1 expression was blunted [358], further suggesting that various microbial infections lead to reduced MSR1 expression.

MSR1 seems to interact with different components of the pulmonary surfactant. The ability of alveolar macrophages to phagocytose and clear *Streptococcus pneumoniae* and *Staphylococcus aureus* are increased in the presence of SP-A and this effect appears to be associated with MSR1 [359, 360].

Another study proposed that MSR1 may be involved in the clearance/uptake of malondialdehyde and SP-D adducts. Malondialdehyde-aldehyde (MAA) is an aldehyde chemically modified in the presence of alcohol and cigarette smoke [361]. Mice lacking MSR1 had lower BAL total and neutrophil counts after repeated intranasal instillations of MAA and SP-D. Moreover, MAA-adduct staining in the lung epithelium was inferior in knockout mice compared to wild-type animals suggesting that MSR1 is involved in the regulation of the lung inflammation in the presence of MAA adducts [362]. It was shown *in vitro* that incubation of alveolar macrophages with surfactant did not significantly affect MSR1 expression [363]. However, upon surfactant and phosphatidylcholine incubation with circulating monocytes, there was an increased in the expression of different receptors, such as MSR1 [364].

MSR1 has diverse roles in cancer pathogenesis depending on the type of cancer. MSR1 knockout mice are protected against ovarian and pancreatic cancer [365]. Conversely, MSR1 seems to be of importance in lung cancer. Studies have identified different single nucleotide polymorphisms (SNPs) linked with lung cancer [366, 367]. Zhang *et al*. identified two genetic variants: rs17484273 and rs1484751 in patients with NSCLC. These SNPs were associated with a poor survival rate in the Harvard Lung Cancer Study cohort. They further demonstrated that a single transplantation of bone marrow-derived macrophages from MSR1 deficient mice to wild-type mice induced more lung metastasis compared to transplanted from wild-type to wild-type animals [366].

Other than in lung cancer, additional MSR1 SNPs were also identified and linked to COPD [367]. A study published in 2010 by Ohar *et al*. showed that the MSR1-coding SNP P275A was associated with susceptibility to COPD but also a lower percent predicted FEV_1 [368]. Another Danish study discovered that the heterozygote MSR1-coding SNP Arg293X among men was also associated with a lower percent-predicted FEV_1 and susceptibility to COPD [369, 370]. In addition, isolated alveolar macrophages from severe COPD patients demonstrated higher levels of MSR1 compared to non-smokers [371].

Dahl *et al*. showed that after an intratrachael instillation of oxidized lipids, MSR1 knockout mice exhibited an increase inflammatory response, mostly characterized with an increased flux of neutrophils compared to wild-type mice [372]. This study adds important findings to the field of

scavenger receptors and solidifies their role in pulmonary lipid homeostasis. In conclusion, MSR1 may be central in the uptake of lipids found in foamy macrophages from smokers or cigarette smoke-exposed mice.

SR-B1

SR-B1 is a transmembrane protein that was discovered in the 1990s [373] and is expressed in several tissues and cell types such as liver, intestines, placenta, different macrophage subtypes, endothelial cells, smooth muscle cells, keratinocytes, and adipocytes [374, 375]. This scavenger receptor is well conserved; found in mice and man, but also in amphibians, birds and some fish [374]. Orthologues have also been identified in *Caenorhabditis elegans* and *Drosophila melanogaster* [374]. SR-B1 has the capacity to bind various ligands such as albumin, phospholipids, high-density lipoproteins (HDLs), very low-density lipoproteins (VLDLs), LDLs, acetylated and oxidized LDLs, apoptotic cells, vitamin E, carotenoids, and silica [374-376].

One of the most important roles of SR-B1 is the capture of cholesteryl esters from lipoproteins like HDLs [376, 377]. SR-B1 can also export lipids outside of the cell [378]. In atherosclerosis, mice lacking this scavenger receptor develop atherosclerotic plaques more rapidly compared to mice overexpressing SR-B1 [374, 375]. SR-B1 knockout mice have dysfunctional HDLs due to a reduction in the activity of paraxonase 1 (PON1), a key antioxidant enzyme found on this lipoprotein [379, 380]. SR-B1 is also involved in host defense, inflammatory response, phagocytosis of apoptotic cells, hepatitis C entry, platelets function and regulation of vitamin E [374-376].

Other than its role in the capture of silica [381], SR-B1's impact on the pulmonary response to different microbial infections is well characterized. In a model of pneumonia induced by *Klebsiella pneumoniae*, mice deficient for SR-B1 have a higher mortality rate [382]. The totality of knockout mice died after 48h compared to only 40% death of wild-type after 10 days [382]. These mice were also characterized by exacerbated pulmonary neutrophil influx, defective phagocytic ability, and aggravated systemic and pulmonary bacterial burden compared to infected wild-type mice [382]. In that same study, SR-B1-deficient mice also had problems eliminating LPS from airways compared to wild-type counterparts

[382]. *In vitro*, LPS incubation led to reduced expression of SR-B1 in a macrophage cell line [383]. Furthermore, SR-B1 knockout mice displayed worsened systemic inflammation as well as liver and kidney damage in systemic LPS model [384]. In the context of an infection with *M. tuberculosis*, SR-B1 deficient mice did not exhibit any changes in pulmonary phenotypes [385]. However, reduced secretion of TNFα, interferon gamma (IFN-γ), and IL-10 were observed. Schafer *et al*. study demonstrated that SR-B1 was in fact one of the entry points used by *M. tuberculosis* [385]. Similarly, SR-B1 was also shown to be the cellular entry point of the hepatitis C virus in the liver [386].

Another important role for SR-B1 is the absorption of vitamin E in the lung during neonatal development [387]. During pulmonary surfactant synthesis, it was shown that the levels of vitamin E increased in type 2 pneumocytes. It was suggested that this mechanism was induced in order to improve the defense against oxidation [387]. One of the transporters known to deliver vitamin E to type 2 pneumocytes are the HDLs [388] via scavenger receptor SR-B1 binding. Interestingly, SR-B1 is not found at the surface of type 2 pneumocytes before birth. It was proposed that oxidative damage to the lung could be aggravated by vitamin E deficiency in newborns [389].

SR-B1 expression is modulated in different types of cancer. SR-B1 was associated with a higher degree of cancer aggressivity and a lower survival rate in prostate, breast and lung (adenocarcinoma) cancers [390, 391]. Different mechanisms have been proposed to explain this phenomenon. It was suggested that SR-B1 could feed the tumours with cholesterol. Another protumoral hypothesis is that upon binding of different ligands, SR-B1 would activate the protein kinase B pathway ultimately leading to different proliferation mechanisms [392]. There are studies currently investigating whether SR-B1 could be a potential therapeutic target in different cancers [390, 392].

Few studies have investigated the role of SR-B1 during cigarette smoking. However, it was shown that *in vitro* exposure of bronchial epithelial cells to cigarette smoke led to the translocation of SR-B1 to the surface 12h post exposure. It was further demonstrated that SR-B1 mRNA and protein levels were reduced after 24 hours of incubation [376]. The latter phenomenon was associated with higher levels of ubiquinination, a post-transcriptional mechanism that could explain this alteration [376]. As described above, COPD is characterized by chronic inflammation, apoptosis and oxidative stress [393]. It was

extrapolated that SR-B1 could be a key player in COPD since it is also involved in most of these mechanisms [393]. However, no studies have investigated the pulmonary role of SR-B1 in smokers or in COPD patients. As mentioned previously, SR-B1 is found on keratinocytes. It was demonstrated that during cigarette smoking, the expression of SR-B1 on keratinocytes was reduced [394, 395] and that SR-B1 formed adducts with aldehydes [394]. These data could explain why some smokers have skin problems such as psioriasis, dermatitis and melanoma.

Cigarette smoking is known to damage AAT1 protein [396], a key anti-protease involved in the development of emphysema in patients lacking this protein. Interestingly, AAT1 is found on circulating HDLs [397]. Recently, a new pulmonary SR-B1 role has been discovered. It was shown that HDLs were able to deliver AAT1 proteins to pulmonary endothelial cells via the scavenger receptor SR-B1 [398]. They further demonstrated that AAT1 was able to modulate the expression of SR-B1. The mechanism behind this newly discovered role is still unknown. They concluded that SR-B1 could be responsible for the uptake of up to 30 to 50% of circulating AAT1 to the lung [398]. The latter study could lead to the investigation of new therapeutic tools targeting SR-B1 in patients with mutated AAT1 protein. The exact role of SR-B1 during cigarette smoking is poorly understood; nevertheless, this scavenger receptor is of importance in lipid homeostasis.

MARCO

MARCO, or SR-A6, is a 53 kDa transmembrane protein discovered at the end of the 1990s in the rat [399] and a few years later in humans [400]. MARCO is principally expressed in different macrophage subtypes such as microglia [401], alveolar macrophages [402], peritoneal macrophages [403], macrophages from the marginal zone of the spleen [404], but also dendritic cells derived from monocytes [401]. MARCO is renowned to bind acLDLs and oxLDLs [403], silica, LPS, and different bacteria [399, 404]. MARCO is mostly involved in host defense via phagocytosis [405] and in atherosclerosis [403].

One of the main pulmonary differences between MSR1 and MARCO is the ability of MARCO to bind non-opsonized particles [402]. Many studies have explored the role of MARCO in the binding of silica,

inorganic and environmental particles [351, 406, 407]. Murthy *et al*. characterized patients with asbestosis, showing that the alveolar macrophage expression of MARCO was higher in patients compared to healthy controls [408]. Mice deficient for MARCO also displayed an exacerbated pulmonary cellularity and an aggravated secretion of pro-inflammatory cytokines in a silica exposure model compared to wild-type counterparts [407].

The literature on MARCO and its role on host defense is extensive. It was demonstrated that alveolar macrophages had the capacity to bind LPS via MARCO [409]. Mice deficient for MARCO had a higher death rate and a harder time eliminating *Streptococcus pneumoniae* during this bacterial infection [410]. They further showed that these mice had an aggravated pulmonary inflammatory response and an accentuated secretion of pro-inflammatory cytokines compared to wild-type controls [410]. The same research group proved that compared to other scavenger receptors, MARCO was one of the most important human alveolar macrophage receptors involved in the capture of microorganisms and particles [411]. Another study showed that MARCO was required for an optimal TLR2 response in the nasopharynx of mice ultimately leading to the clearance of *S. pneumoniae* in this infectious model [412]. In *Legionella pneumophila* [413] and LPS/elastase supplemented with NTHi [358] infection models, the expression of MARCO was reduced. Bacteria may therefore reduce the expression of MARCO in order to increase their survivability.

Three MARCO SNPs were associated with a greater susceptibility to tuberculosis; rs4491733, rs12998782, and rs13389814, while one SNP was associated with a higher resistance to tuberculosis; rs7559955 [414]. Martinez *et al*. showed that during M. tuberculosis infection, the expression of MARCO is reduced on alveolar macrophages, leading to a defective sentinel function [415]. Another interesting study revealed that isolated sputum macrophages from cystic fibrosis patients had lower MARCO and mannose receptor mRNA and protein levels compared to healthy controls [416]. This reduction in MARCO could lead to a defective clearance capacity of macrophages and induce additional inflammation to the cystic fibrosis lung. All of these studies clearly point to the critical role of MARCO in host defense.

Compared to bacterial infections, MARCO seems to be deleterious during viral infections. The presence of MARCO during adenoviral infection exacerbated the pulmonary response [417]; a similar phenomenon was observed during influenza infection [418, 419]. Stichling *et al*. demonstrated that MARCO could be the entry point of the adenovirus [420]. One possible explanation for the negative effect of MARCO during viral infections is that IFNγ reduces alveolar macrophage expression of MARCO [421]. Since viral infections affect MARCO's expression, it was proposed that secondary infections to bacteria could be due to a lack of MARCO's ability to scavenge bacteria [422, 423]. Few data are available on the interactions between MARCO and fungal infections; however, MARCO seems to be involved [424, 425].

Other than host defense, MARCO is also involved in diverse pulmonary diseases. It was demonstrated that tumour-associated macrophages (TAMs) display elevated MARCO expression in NSCLC patients [426]. This study concluded that TAMs and MARCO could be a new therapeutic option to overcome deleterious aspects of lung cancer. This scavenger receptor is also implicated in the maturation of dendritic cells in the context of cancer [401, 427], and in asthma [348].

It was shown in alveolar macrophages isolated from COPD patients and cigarette smoke-exposed mice that MARCO is under the direct control of the nuclear erythroid-related factor 2 (NrF2). Upon transcription, MARCO would then facilitate pathogen phagocytosis [428]. Baqir *et al*. proposed that pulmonary macrophages had a reduced capacity to phagocyte pathogens due to a reduction in the expression of MARCO induced by an exposition to cigarette smoke extract [429]. Compared to MSR1, nine MARCO genetic variations were discovered in the Danish population study. However, none of these genetic variations were associated with lung function, susceptibility to COPD or susceptibility to infection [370, 430]. As stated before, one of the most important studies on MSR1 was conducted by Dahl *et al.* showing an increased pulmonary response after intratrachael instillations of oxidized lipids in SR-A1 knockout mice. Similarly, mice deficient for MARCO exhibited an exacerbated inflammatory response to inhaled oxidized lipids [372]. MARCO is clearly a key scavenger receptor and could potentially be involved in the uptake of lipids during cigarette smoke exposure.

To conclude, this section introduced four key alveolar macrophage scavenger receptors. All of these receptors are involved in many pathways, such as lipid uptake and host defense. The pulmonary roles of these scavenger receptors were also presented. Additionally, the effects of cigarette smoke on these receptors were described. Upon oxidized lipid uptake, macrophages must organize these lipids into packages in order to export them out of the cell. The last step before lipid export is the transformation of internalized lipids into lipid droplets.

Macrophage and lipid droplet formation

Lipids internalized by scavenger receptors are reorganized, transformed into lipid droplets and are either stocked [431], catabolized or exported [432]. Lipid droplets were first thought to be lipid reservoirs providing lipids for numerous processes such as membrane synthesis and cell signaling [433-435]. Briefly, lipid droplets are composed of a phospholipid monolayer, associated proteins, triacylglycerol and a neutral lipid core, mostly composed of cholesteryl esters [436-438]. A lipidomic study identified more than 160 phospholipids in the composition of lipid droplets and PC was the most abundant phospholipids of all, followed by PE and PI [438]. In non-adipocytes, intracellular lipid reorganization in lipid droplets is used to reduce their intracellular toxicity [439, 440]. First, most lipids such as cholesteryl esters are transformed into free cholesterol via acid lipase enzymes. Then, free cholesterol is esterified by the acyl-CoA:cholesterol acyltransferase 1 (ACAT1) into cholesteryl esters in the endoplasmic reticulum, which gives them the ability to merge into lipid droplets [433]. Excess free cholesterol or free fatty acids must be esterified or neutrally stored in order to improve its cellular stability. It was also shown that toxicity due to intracellular lipid accumulation is associated with higher endoplasmic reticulum stress (ER stress; [441]. Once this is accomplished, lipids can be transformed into lipid droplets [442].

Several proteins are implicated in the formation of lipid droplets [443-445]. The perilipin protein family is one of the most important [446]. Lipid accumulation leads to an increase in the expression of perilipin 2 (Plin2) [433]. This protein is ubiquitously expressed, it is bound to lipid droplets and is further protected from proteoasomal degradation [447, 448]. One of Plin2's roles is to protect lipid droplets

from degradation by the adipose triglyceride lipase (ATGL). Therefore, Plin2 reduces the affinity of ATGL for lipid droplets [433].

Two pathways have been proposed to export cholesteryl esters via lipid export mechanisms. Neutral cholesterol ester hydrolases are enzymes associated with lipid droplets. They can hydrolyze lipid droplet cholesteryl esters into free cholesterol and assist in the efflux of these lipids [433]. The second mechanism is a bit more complex as it relies on the autophagocytosis of lipid droplets, followed by a fusion with lysosomes and ultimately hydrolysis of cholesteryl esters via lysosomal acid lipase. As in the first mechanism, lipids can then be exported outside of the cell [449, 450].

Nuclear receptors and their implication in lipid transport

In parallel to lipid droplet formation, intracellular entry of oxidized lipids, such as oxidized fatty acids or oxidized cholesterol, also activates two classes of nuclear receptors: PPARγ and the liver X receptor alpha or beta (LXRα,β) [451]. The binding of oxidized lipids to these nuclear receptors results in the transcription of several genes involved in lipid homeostasis and lipid export [452]. Their pulmonary roles will also be described in this section.

PPARs and PPARγ

Nuclear receptors are a superfamily of proteins that are considered ligand-dependent transcriptional regulators [453]. The human genome contains 48 nuclear receptors [454]. One family of nuclear receptors are called orphan as their natural ligands are still unknown; PPARs are a group of nuclear receptors that falls into this category [453]. They consist of three isoforms named PPARα, PPARβ/δ and PPARγ [455]. These nuclear receptors are involved in many pathways such as lipid and glucose metabolism, lipoprotein formation, lipid catabolism and adipogenesis [455, 456].

PPARα is mostly expressed in tissues involved in lipid and cholesterol metabolism such as the liver, kidneys and macrophages, while PPARβ/δ is ubiquitously expressed [457, 458]. Their exact pulmonary

roles remain unknown. PPARα has several ligands such as prostaglandins, leukotrienes, eicosanoids and fibrate drugs [459]. However, it was shown that PPARα-knockout mice developed a more severe inflammatory response and a higher level of fibrosis compared to wild-type mice in a bleomycin model of lung fibrosis [460]. They further demonstrated that a PPARα agonist, WY-14643, was able to limit lung fibrosis in this model. Even though the mechanism is unclear, PPARα seems to be involved in molecular pathways associated with pulmonary fibrosis.

Saturated and polyunsaturated fatty acids and eicosanoids are natural ligands of PPARβ/δ [453]. One of the most important roles of PPARβ/δ is during wound healing. It was demonstrated that PPARβ/δ-knockout mice have clearly defective *in vivo* wound healing mechanisms [461, 462]. Furthermore, they showed *in vitro* that knockout keratinocytes displayed migration and adhesion defects. It was also proposed that PPARβ/δ may be involved in regulating the transition between the inflammatory response and the beginning of wound healing [463]. Perhaps this suggested mechanism may also be linked to pulmonary fibrosis repair mechanisms [464]. However, additional studies are required.

One of the most important isoforms in lung lipid homeostasis is PPARγ. The deletion of this nuclear receptor leads to pulmonary lipid dysregulation [465, 466]. PPARγ is expressed on a variety of cells such as monocytes, several macrophage subtypes, neutrophils, natural killers, dendritic cells, mastocytes, eosinophils, lymphocytes and platelet cells [455, 467]. This nuclear receptor has different ligands: endogenous fatty acid and pharmacological agents, such as the thiazolidinedione drug family. Rosiglitazone and pioglitazone are the two most studied activators of PPARγ [456]. PPARγ forms a heterodimer with the retinoid X receptor (RXR), another nuclear receptor. This complex is usually associated with corepressors [467]. Upon ligand binding to PPARγ, the corepressors are removed from PPARγ and co-activators bind to the complex. This newly formed complex can bind to DNA sequences called peroxisome proliferators response elements found near promoters which ultimately leads to the transcription of many genes [456, 467]. CD36 is a well-known scavenger receptor that is directly expressed upon PPARγ activation [333].

The pulmonary roles of PPARγ are diverse. It was shown that upon chronic hypoxia in mice, TGF-β leads to suppression of PPARγ expression in the lung [468, 469]. In a mouse model of cystic fibrosis,

rosiglitazone treatment led to a reduction in disease severity and a diminished mucus retention in the lung [470]. It was also shown that PPARγ and NrF2 are related. The latter being an important transcription factor involved in the antioxidant response upon acute lung injuries such as hyperoxia [471, 472]. PPARγ activation was also shown to modify the p53 response to cytotoxic cancer therapy [473].

Activation of this nuclear receptor is of importance during cigarette smoke exposure. Emphysema is a key component of cigarette smoke-induced lung disease. PPARγ activation was shown to be protective in different emphysema-like mouse models of cigarette smoke exposure [474-476]. Upon cigarette smoke exposure and bacterial infection with *H. influenzae*, it was shown that a PPARγ agonist reduces lung neutrophil counts and did not exacerbate the pulmonary bacterial burden [195]. Specific depletion of PPARγ in pulmonary macrophages is known to disrupt pulmonary lipid homeostasis leading to the formation of foam cells [466]. These pulmonary macrophages mostly accumulate free cholesterol and phospholipids. PPARγ is a key pulmonary regulator of inflammation, antioxidant response and lipid homeostasis. LXR is another major nuclear receptor involved in pulmonary lipid homeostasis and inflammation.

LXRα,β

LXR is also part of the nuclear receptor family; however, they are no longer called orphan receptors as their natural ligands were discovered. It is composed of two isoforms named alpha and beta. LXRα is mostly expressed in active biological tissues such as liver, adipose tissue, intestines, and several macrophage subtypes. On the other hand, LXRβ is ubiquitously expressed [477]. LXR has many ligands, such as endogenous oxysterols like 27-hydroxycholesterol and 25-hydroxycholesterol, and can be activated by pharmacological compounds like the GW3965 or T0901317 [477]. Like PPARγ, LXR also forms a heterodimer with RXR in the nucleus. Corepressors, such as silencing mediator of retinoic acid and thyroid hormone receptor [478], are also attached to the LXR/RXR complex and result in repression of transcription. However, upon ligand activation of LXR, the complex undergoes conformational change and the corepressors are replaced with coactivators. This newly formed complex can now bind to AGGTCA separated by four nucleotides on the DNA [477, 479]. This

sequence is named the LXR responsive element and is found near different promoters [480]. This leads to the transcription of several genes involved in lipid homeostasis.

One of the first genes associated with the activated LXR was cytochrome P450 family 1 subfamily A member 7. This cytochrome is part of the P450 families and is involved in the transformation of cholesterol to bile acid. It was shown that mice lacking LXRα accumulated cholesteryl esters in their livers when fed a high-fat diet [481, 482]. Interestingly, LXRβ deficient mice fed a high-fat diet did not display this phenotype [483]. This discovery led to the identification of LXR as a key cholesterol sensor. Since LXR was found to be a master regulator of cholesterol homeostasis, it was afterward discovered that many LXR targets were involved in cholesterol efflux pathways. Many genes found in the ABC superfamily of membrane transporters such as *abca1*, *abcg1*, *abcg5*, *abcg8* and the apolipoprotein (Apo) E (ApoE) gene, are under the control of LXR [477, 479, 484]. Some of these genes, *abca1*, *abcg1* and *apoe*, are of importance in the lung.

Cholesterol levels are mostly controlled by two different transcription factors: LXR and sterol regulatory element binding proteins (SREBPs) [477]. Agonists, such as statins, can modulate the SREBP pathway. As stated above, LXR can be activated by pharmacological agonists such as GW3965 and T0901317. These compounds have an affinity for both LXRα and LXRβ [477].

It was proved *in vitro* and *in vivo* that the use of these agonists could modulate the expression of key genes involved in atherosclerosis. The inducible degrader of the LDL receptor (IDOL), an important protein involved in cholesterol regulation, is another gene controlled by LXR. It was shown that upon LXR activation, IDOL triggers the ubiquitination of the LDL receptor (LDLR), the main hepatic entry molecule for cholesterol, thereby making it a target for degradation [485]. This mechanism further demonstrates the role of LXR in cholesterol homeostasis by either increasing its efflux or by suppressing the uptake of cholesterol by the liver [485]. It was further suggested that LXR could be a new therapeutic target in cardiovascular diseases, such as atherosclerosis, to facilitate the removal of excess lipids found in atheromatous plaques [477, 479].

These previous studies demonstrate the role of LXR mostly in the liver; however, it also plays a role in lung homeostasis. This nuclear receptor is known to be involved in multiple pulmonary models, such as acute lung injury, asthma, fibrosis and cancer [486-491]. In acute lung injury, LXR activation seemed to be protective as it reduced pulmonary neutrophil infiltration and facilitated the clearance of bacteria [492-497]. It was shown that in asthma models, activation of LXR led to reduced inflammatory response to different allergens. However, the impact of LXR on airway remodeling is still debatable [498-502]. Furthermore, upon bleomycin delivery, T0901317, a pharmacological agent able to activate LXR, was able to dampen the pulmonary inflammatory response and fibrosis severity in mice [503]. LXR activation was also shown to reduce tumour growth in different cancer models. The mechanisms are still unclear; however, LXR activation either led to the production of IFNγ, a key mediator in tumour growth control, or suppressed the NF-κB/MMP-9 signaling pathways [486-491, 504].

Dai *et al*. published one of the most important studies describing the role of LXR in the lung. They showed that mice lacking both LXRα and LXRβ spontaneously developed lung cancer compared to wild-type mice, especially in squamous cells [505]. They further demonstrated that after one year of age, double knockout mice exhibited accumulated lipids in alveolar walls, type 2 pneumocytes and alveolar macrophages, a phenotype not observed in single knockout mice. It was shown that this phenomenon was progressive: from the age of 3 to 14 months the lipid accumulation expanded from the periphery towards the center of the lung. They called this phenotype the golden coat. These mice also exhibited increased inflammatory cell infiltration in the lung compared to wild-type counterparts. This study indicates that LXR contributes significantly to pulmonary lipid homeostasis.

Few studies have investigated the role of LXR in the response to cigarette smoke. Higham *et al*. are the only ones who studied the role of LXR in COPD patients [506]. They showed that protein levels and gene expression of LXR were increased in the resected lung tissues of smokers and COPD patients compared to non-smokers. However, isolated alveolar macrophages did not display any changes in mRNA or protein levels of LXR. Furthermore, they showed *in vitro* that the use of GW3965 on isolated alveolar macrophages from non-smokers, smokers, and COPD patients was able to increase the expression of *abca1* and *abcg1* [506].

To conclude this section on nuclear receptors, PPARγ and LXRα,β are three extremely important transcription factors involved in lipid homeostasis and several pulmonary diseases. As their deletion leads to dramatic lipid accumulation in the lungs, PPARγ and LXRα,β are critical to maintain lung lipid homeostasis. Compared to PPARγ, LXR seems to be of importance in the context of cigarette smoking due to the presence of foamy macrophages. This nuclear receptor is responsible for the transcription of *abca1* and *abcg1*, two main proteins involved in pulmonary lipid export.

Once lipids are captured by different scavenger receptors (i.e. MSR1, MARCO, CD36 or SR-B1), they are processed into lipid droplets via perillipin. Furthermore, nuclear receptors (i.e. PPARγ and LXRα,β) activate the transcription of key lipid export genes and macrophages are now ready to efflux lipids outside of the cell.

Lipid export by macrophages

Lipid export is defined as the efflux of intracellular lipids to the extracellular environment. In order to accomplish this task, cells can use diverse active or passive mechanisms. One of the most important active mechanisms relies on the use of membrane transporters from the ABC family [507, 508]. ABC transporters are found in several species such as bacteria, plants, yeast, fish, bugs and mammals [509]. So far, up to 48 ABC membrane transporters have been identified in the human genome [328]. Among them, ABCA1 and ABCG1 are the most important and involved in the active transport of lipids by macrophages [507, 508]. In addition, macrophages can use SR-B1 or passive mechanisms to export lipids.

ABCA1

ABCA1 is a 2261 amino acid protein with two ABC domains and two transmembrane domains. This protein is part of the ABC superfamily [510]. This family of proteins uses ATP to export lipids (mostly cholesterol and phospholipids) or metabolites [511]. It is important to note that ABCA1 and ABCG1 can

only export, whereas SR-B1 also has the ability to capture/import lipids. ABCA1 is located at the surface of the plasma membrane [511] and has also been detected intracellularly [510].

The human *abca1* gene was sequenced at the beginning of the 21st century [512], although it was recognized as an extremely important protein involved in lipid transport at the end of the 1990s. This gene is associated with Tangier disease, an autosomal recessive and extremely rare disease (less than 1 case per 1,000,000 people) [513]. Patients afflicted with Tangier disease have a mutation in the *abca1* gene [511, 514, 515], which leads to major lipid transport problems characterized by extremely low levels of HDL and significant accumulation of esterified cholesterol in tissues [514]. Patients are also more likely to develop atherosclerosis [516, 517], as it was shown that low levels of HDL is associated with a higher risk of cardiovascular disease [511].

The discovery of Tangier disease led to enormous work in the field of lipid transport via ABCA1. It was shown that ABCA1 levels correlated with levels of HDL [518]. As mentioned above, ABCA1 export mechanism is unidirectional. It mostly exports cholesterol and phospholipids such as phosphatidylcholine into apolipoprotein A-1 (ApoA-1). ABCA1 is the first step involved in reverse cholesterol transport [519]. Two models have been proposed in the export of lipids via ABCA1. The first model suggests that intracellular lipids form lipid vesicles in the Golgi apparatus and are transported to the cellular membrane to be exported to apolipoproteins via ABCA1. This model is called exocytosis [519]. The second model proposes that ABCA1 and apolipoproteins are internalized in vesicles and ultimately HDL is generated in the endosomal compartment. The newly formed HDL is then secreted and this process is named retro-endocytosis [510, 520]. However, the validity of the second model is debated [521].

ABCA1 is expressed in the lung, placenta, liver, brain, intestines, and kidneys and by different macrophage subtypes [519]. Other than pulmonary macrophages in the lungs, ABCA1 is also expressed by type 1 pneumocytes [522], smooth muscle cells of the respiratory tract [499], and type 2 pneumocytes [523]. This lipid transporter is known to be regulated by multiple mechanisms. The activation of different nuclear receptors such as LXRα,β, RXR or PPARγ and the supplementation of

cAMP leads to the upregulation of *abca1* transcription [511]. *Abca1* expression is also regulated by different post-transcriptional mechanisms, such as the proteolysis of fatty acids [524].

Data on the role of ABCA1 in the lungs are limited [525]. It is known that ABCA1 has an important role in the function of type 2 pneumocytes, since this transporter is essential to the export of extra phosphatidylcholine into the circulation [303, 526]. Mice deficient for ABCA1 show key pulmonary characteristics such as foamy alveolar macrophage cells, abnormal type 2 pneumocytes and accumulation of esterified cholesterol in the lungs [527]. Mice genetically modified to express the human *abca1* gene have a lower number of neutrophils in their BAL, lower levels of lung inflammation, reduction in the production of immunoglobulin E and lower thickening of the airways in an asthma model compared to wild-type mice [528]. ABCA1 is considered a crucial systemic and pulmonary transmembrane lipid transporter. Within the ABC family, another lipid transporter is well characterized to have a major role in lipid transport: ABCG1.

ABCG1

ABCG1 is composed of 678 amino acids and is also a member of the ABC superfamily. Compared to other ABC transporters, ABCG1 is considered a half transporter since it only contains one ABC domain and one transmembrane domain [328]. ABCG1 is expressed in many tissues such as the lung, spleen, brain, heart, adrenal glands and macrophages [529]. The localization of this receptor is not unanimous. In contrast to ABCA1, ABCG1 is not found at the surface of the cell as previously described [328], but instead it is associated with intracellular endosomes [509, 530]. Many mechanisms are known to modulate the expression of ABCG1; for instance, the presence of acLDL and oxLDL, the activation of LXR and RXR or the presence of oxysterols are known to increase the transcription of ABCG1 [530].

ABCG1 mainly export lipids to HDLs, LDLs, and phospholipid vesicles compared to ABCA1, which only transports lipids to low-density apolipoproteins such as ApoA-1, ApoE and ApoC2 [531]. One model proposed by Tarling *et al*. in 2011 suggested that ABCG1, found in endosomes, was able to transfer lipids to the inner phospholipid layer of the plasma membrane before they merge with the membrane

of intracellular vesicles. These lipids could subsequently be exported by desorbing non-specifically to lipid acceptors such as HDLs, LDLs and phospholipid vesicles [509].

Mice deficient in ABCG1 show an abundant accumulation of lipids in many tissues [531]. In contrast to the deletion of ABCA1 which leads to Tangier disease and to an important reduction in the levels of HDL (~ 95%), ABCG1 deficiency does not affect the levels of systemic lipoproteins [328]. Nevertheless, murine ABCG1-deficiency also leads to an accumulation of lipids in the lungs. The levels of total cholesterol, esterified cholesterol and phospholipids found in the lung tissue are higher compared to wild-type mice [532, 533]. This pulmonary phenomenon only develops after six months of age [532, 533], suggesting that early compensatory mechanisms may be capable of overcoming this lipid accumulation in the lungs. This phenotype is also seen when the ABCG1-deficiency is specific to different cell types, such as macrophages [534], alveolar macrophages and type 2 pneumocytes [535].

The presence of foam cells and cholesterol clefts in the pulmonary environment clearly indicates pulmonary lipid dysregulation [532, 536, 537]. Furthermore, type 2 pneumocytes have enlarged and very dense lamellar bodies and exhibit alteration in lipid secretion compared to wild-type mice [532, 537]. Surprisingly, there are no known diseases associated with the direct mutation of *abcg1*. However, in pulmonary alveolar proteinosis (PAP), ABCG1 is almost undetectable [538].

PAP is a rare disease that was first described in 1958, characterized by an accumulation of lipids and protein material associated with the pulmonary surfactant in the lungs [539]. In up to 90% of cases, this disease is associated with the presence of immunoglobulin G antibodies against GM-CSF [540]. A well-designed study showed that alveolar macrophages from PAP patients and mice deficient in GM-CSF were also PPARγ-deficient, an important nuclear receptor involved in lipid and glucose homeostasis [538]. To compensate the loss of PPARγ, these alveolar macrophages overexpress LXR, another key nuclear receptor involved in lipid homeostasis and the transcription of *abca1* and *abcg1*. However, ABCG1 levels remain extremely low [538].

Other than in PAP and pulmonary lipid homeostasis, ABCG1 is also implicated in diverse biological lung functions [541]. LPS-treated, ABCG1-deficient mice exhibit increased pulmonary neutrophil counts

compared to wild-type counterparts [542]; a phenomenon also observed in an ovalbumin asthma model [543]. In a bleomycin-induced fibrosis model, *abcg1*$^{-/-}$ mice displayed increased pulmonary fibrosis compared to wild-type mice [272]. In addition, studies have found an important role for ABCG1 in B lymphocyte homeostasis [544] and in the differentiation of T lymphocytes in atherosclerosis [545]. Furthermore, various *abcg1* genetic variations have been associated with NSCLC [546] and one study described *abcg1* as a potential oncogene [547]. Overall, ABCG1 is a key transmembrane protein involved in several crucial lungs processes, such as lipid transport.

This section introduced two important transmembrane transporters involved in active lipid export. These two ABC family members are critical to the maintenance of pulmonary lipid homeostasis. Cells like alveolar macrophages can also use other lipid export mechanisms that do not require the use of ATP.

SR-B1 and passive lipid transport

As mentioned in previous sections, SR-B1 is an important scavenger receptor involved in lipid capture and can also facilitate lipid efflux [548, 549]. Compared to ABCA1 and ABCG1 that only export lipids, SR-B1 facilitates bidirectional cholesterol flux. It was further demonstrated that SR-B1 preferentially exports free cholesterol to HDLs with higher phospholipid content [550].

Lipids can also be exported without using mechanisms that require energy, such as passive cholesterol transport [551]. Passive diffusion is proportional to the lipid gradient between the cellular membrane and the extracellular environment [552], meaning that a high concentration of cholesterol inside macrophages will result in automatic passive diffusion when the extracellular environment contains low levels of cholesterol. In contrast, cholesterol release by this process is negligible when compared to active transport mechanisms via ABCA1 and ABCG1.

To conclude this section, alveolar macrophages have at their surface four scavenger receptors involved in diverse processes such as the antimicrobial response and the capture of oxidized lipids. Upon lipid capture, intracellular mechanisms must be well organized in order to reduce potential toxicity induced

by intracellular lipid accumulation. Once lipid droplets are formed, three nuclear receptors (PPARγ and LXRα,β) upregulate the expression of two transmembrane transporters, ABCA1 and ABCG1, to activate lipid export mechanisms (Figure F). Most of the exported lipids will merge with circulating lipoproteins, such as LDLs, VLDLs and HDLs.

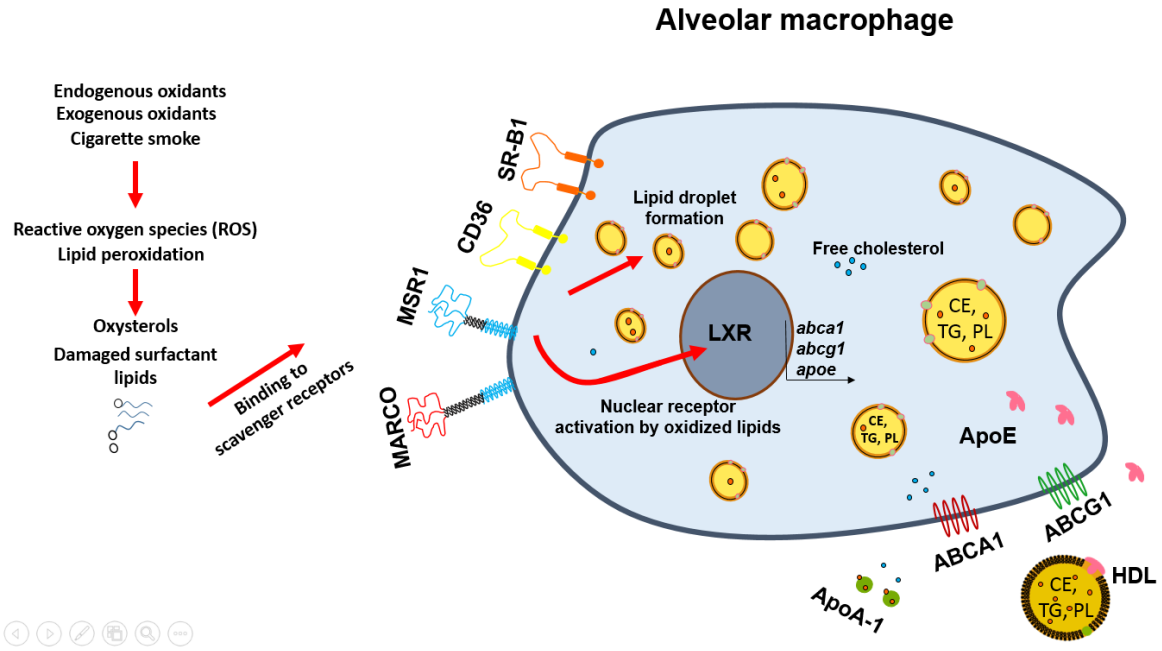

Figure VI: From lipid capture to lipid export by alveolar macrophages.

This illustration identifies key steps involved in the processing of lipids by alveolar macrophages. Generation of oxidized surfactant phospholipids and oxysterols induced by oxidants from cigarette smoking leads to the catabolism of these lipids by alveolar macrophages. Oxidized lipids bind to key scavenger receptors for instance MARCO, MSR1, CD36 and SR-B1. Within alveolar macrophages, these lipids are reorganized into lipid droplets in order to reduce intracellular toxicity. Meanwhile, lipids can also bind to nuclear receptors such as LXR, thus activating the transcription of lipid export genes (i.e. AbcA1, AbcG1 and ApoE). Lipid droplets can then be exported to circulating apolipoproteins and lipoproteins, such as ApoA-1 and HDLs.

Systemic lipid transport

In the circulation, lipids must be transported as part of complexes due to their hydrophobicity and insolubility. Therefore, cholesterol and triglycerides bind circulating proteins such as lipoproteins in order to be transported systemically. Lipoproteins are diverse in size and composition and each has different functions. These lipoproteins include chylomicrons, VLDLs, IDLs, LDLs and HDLs [553].

Briefly, chylomicrons are very large particles formed in the intestines and are involved in the transport of dietary cholesterol and triglycerides to the liver and peripheral tissues. Upon triglycerides delivery, chylomicrons undergo modification and form chylomicron remnants: smaller particles enriched in cholesterol. VLDLs are synthesized in the liver and are triglyceride-rich particles. They are smaller than chylomicrons. Upon exchange or delivery of triglycerides to muscle or adipose tissue, VLDL particles evolve into intermediate density lipoproteins (IDLs). This newly formed lipoprotein is now enriched in cholesterol esters and smaller than VLDLs [553]. IDLs and VLDLs are the precursors of LDLs. In humans, LDL carries most of the circulating cholesterol and are highly enriched in cholesterol esters. LDLs are smaller than IDLs, VLDLs and chylomicrons. Circulating LDL levels are associated with cardiovascular disease. LDL is typically defined as “bad cholesterol” and is considered the major pro-atherogenic lipoprotein [554, 555]. HDLs will be further described in the upcoming section.

Lipoprotein transport and transformation is a dynamic process. This is mostly attributable to diverse enzymes bound on their surfaces or in the circulation. Lipoprotein lipase (LPL) is the enzyme involved in the hydrolization of triglycerides from chylomicrons and VLDLs, which results in the production of fatty acids/chylomicron remnants and IDLs, respectively [556]. These fatty acids can then be captured by different cells. Hepatic lipase is a liver enzyme that hydrolyzes triglycerides and phospholipids from IDLs, LDLs and HDLs, further modifying their structure [557]. Cholesterol ester transfer protein (CETP) is a key enzyme synthesized by the liver that is involved in the transfer of cholesterol esters from HDLs to VLDLs, chylomicrons and LDLs but also in the transfer of triglycerides from VLDLs and chylomicrons to HDLs [558]. To conclude, systemic lipid transport is similarly an extremely dynamic process.

High-density lipoproteins

Thorough characterization of HDL started in the 1970s with the use of ultracentrifugation and density gradients, which led to HDL isolation [559]. Studies on HDLs rapidly progressed when HDL cholesterol (HDLc) was found to be inversely associated with cardiovascular events [560]. This led to the belief that HDLs are to be considered “good cholesterol”. However, the Framingham Heart Study demonstrated that some of the patients who had cardiovascular events also had normal HDLc [380], suggesting that HDLc is not the ultimate marker of cardiovascular risk. Nevertheless, HDLs do have many beneficial biological functions, such as enabling reverse cholesterol transport, antioxidant activity and anti-inflammatory properties. RCT is the most important of HDL’s functions and was discovered in the 1960s [380, 560-564]. RCT has become an interesting therapeutic avenue for research, especially in the field of cardiology. Mimetic peptides, recombinant HDL proteins, and other strategies have been developed to enhance systemic lipid transport. In the upcoming sections, HDLs and their components will be introduced. Furthermore, different approaches that increase systemic lipid transport will be described.

HDL composition and structure

HDLs are highly heterogeneous, mainly due to apolipopotein A-I versatility. HDLs can be classified in terms of its size (small, medium or large), density, electrophoretic mobility (charge), composition and capacity to bind and transport diverse molecules. Their size and density vary between 8 and 10 nm and 1.063-1.21 g/mL, respectively [562]. HDLs can be divided into subpopulations based on their density: HDL_2 (1.063-1.125 g/mL) and HDL_3 (1.125-1.21 g/mL). They are mostly composed of lipids and apolipoproteins. Lipids represent 40 to 60% of HDLs, such as cholesterol, esterified cholesterol, triglycerides, and phospholipids (especially phosphatidylcholine and sphingomyelin) [380, 562]. Using mass spectrometry, proteomics analysis revealed more than 50 proteins associated to HDL. Among them, there are apolipoproteins (ApoA-I, ApoA-II, ApoA-IV, ApoA-V, ApoC-I to -IV, ApoD, ApoE, ApoF, ApoJ, ApoL-I, and ApoM), enzymes (lecithin–cholesterol acyltransferase (LCAT), PON1, platelet-activating factor acetylhydrolase, and glutathione peroxidase 3) and proteins involved in the transfer of lipids such as phospholipids transfer protein (PLTP) and CETP. Acute-phase proteins like serum amyloid A-1, complement 3, AAT1 [561, 562], carotenoids, hormones, vitamins and RNA are found in

low quantity on HDLs [561]. In the upcoming section, two important apolipoproteins will be described: ApoA-1 and ApoE.

Apolipoprotein A-1

ApoA-1 is the most important protein found on HDL. It was first described in 1992 that mice lacking *apoa1* were viable and demonstrated lower levels of circulating total cholesterol and HDLc compared to wild-type counterparts [565]. Heterozygous mice (*apoa-1*$^{+/-}$) showed an intermediate cholesterol phenotype. While cholesterol levels are modified, HDL size remains normal. However, their composition is changed [566, 567]: shift towards an increase in triglycerides and free fatty acid with a decrease in cholesteryl esters. This lipid difference may be explained by a reduction in LCAT activity. This enzyme transforms free cholesterol into cholesteryl esters, a more hydrophobic lipid, which constitutes the lipid core of HDLs. However, LCAT cannot be activated without ApoA-1 [566, 567]. Interestingly, *apoa-1*$^{-/-}$ mice only have a 4% increase in HDLc when fed a high-fat diet compared to 56% increase in wild-type controls [566, 567], suggesting a key role for ApoA-1 in lipid transport. Furthermore, their cholesterol efflux capacity is reduced by 50% compared to wild-type mice [568].

Studies investigating the *apoa1* deletion in the lung are rare. However, it was shown that knockout mice had higher levels of HDL oxidation [569]. This is mostly attributed to an alteration in the activity of PON1, an antioxidant enzyme found on HDLs. Wang *et al.* also demonstrated that *apoa1*-knockout mice exhibited an exacerbated response to methacholine, a higher level of pulmonary inflammatory cells and increased collagen deposition compared to wild-type animals [569]. In a mouse model of LPS challenge, *apoa1*-deficient mice showed increased lung recruitment of neutrophils compared to wild-type controls [570]. While few studies have evaluated the deletion of *apoa1* in the lung, its antioxidant and anti-inflammatory properties seem extremely important.

ApoA-1 and ApoA-2 constitute approximately 70% and 20% of HDL proteins, respectively. These two proteins are transferable between HDLs, increasing their complexity and their dynamics. Their flexibility is of importance as both of them are required for HDL biosynthesis [380, 560]. This is in contrast to

ApoB, a static apolipoprotein associated to LDLs [380, 561]. As stated before, HDLs are not static proteins and are continuously evolving.

Apolipoprotein E

ApoE is a 34 kDa apolipoprotein that has two structural domains. These domains confer ApoE the ability to bind to the LDLR and lipids [571]. By binding to LDLR, ApoE is involved in the elimination of circulating chylomicron and VLDL remnants [572]. Just like ApoA-1, ApoE can interact with ABCA1 to produce nascent HDL particles [573]. This protein is expressed in many tissues and organs such as the brain, liver, lung, adipose tissue, and several macrophage subtypes. ApoE expression is upregulated in the presence of ApoA-1, oxysterols, HDLs, and cholesterol, but it is repressed by statins and many cytokines, such as GM-CSF and IL-1 [574]. ApoE is also involved in RCT, inhibition of LDL oxidation, platelet aggregation, T cell inhibition, smooth muscle cells and endothelial cell proliferation, among other functions [574]. ApoE may be involved in the uptake of HDL by the liver, as it can bind to LDLR [575]. Many of ApoE's functions and properties were discovered in the context of Alzheimer's disease, a neurodegenerative disease. It was also well studied in the context of cardiovascular disease since ApoE-knockout mice develop atherosclerosis when fed a high-fat diet [576].

Nervous system and ApoE

ApoE is expressed by astrocytes and microglia, a subtype of macrophages found in the brain. Its focus is to regulate lipid homeostasis by transporting lipids to neurons [576], but ApoE is also involved in neuron growth, synaptic plasticity and cellular membrane repair [577]. More than 20 ApoE genetic variations have been identified [574]. However, three isoforms are predominantly found and studied in Alzheimer's disease: ApoE ε2, ε3, and ε4. The only difference being the presence of a cysteine or arginine at position 112 or 158 (ε2 Cys112; Cys158, ε3 Cys112; Arg158 and ε4 Arg112; Arg158) [578]. The isoform ε3 is considered wild-type and the most frequently observed isoform in the population. However, ε4 was defined as the most important genetic factor associated with a higher risk of developing Alzheimer [576, 579], while the ε2 isoform has beneficial properties [579].

Alzheimer's disease is a progressive disease characterized by a loss of memory and cognitive function [580]. Hyperphosphorylation of Tau protein and an altered amyloid β (Aβ) metabolism are features of this disease. $A\beta_{42}$ is a highly neurotoxic peptide due to its ability to form large aggregates or amyloid plaques in the brain, a hallmark of this disease. It is therefore the most abundant peptide isolated from Alzheimer's disease patients [581].

As mentioned above, ApoE is needed for lipid homeostasis. Its lipidation in the brain, just like ApoA-1, requires an interaction with ABCA1, which will generate a lipoprotein-like particle [576]. Other than lipids, ApoE also has the capacity to bind Aβ and facilitate its uptake by LDLR and LDL receptor-related protein 1 [576]. It was shown that the ε4 isoform had altered Aβ clearance compared to ApoE-ε2 and ApoE-ε3.

Cardiovascular system and ApoE

Atherosclerosis is a progressive disease triggered by damage to the vascular endothelium. This newly formed wound will increase the expression of cellular adhesive molecules, such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) [582, 583], thus activating the recruitment of monocytes, NK cells and T lymphocytes. Monocytes will differentiate into macrophages and exacerbate the local inflammatory response. These macrophages will phagocyte cell debris and lipids [582, 583]. A hallmark of atherosclerosis is the impressive number of lipids, mostly lipoproteins, such as LDLs that are trapped in the arterial wall. The mechanisms behind this phenotype are still poorly understood and unfortunately, LDLs in the vessel wall are oxidized, further exacerbating the inflammatory response [584]. As lipids accumulate and the disease progresses, macrophages will evolve into lipid-laden macrophages, also known as foam cells. In addition, increased collagen deposition and smooth muscle cell proliferation lead to the formation of atheromatous plaques. These plaques can be extremely fragile and burst, initiating clotting mechanisms in the artery that can lead to a vessel occlusion, which can be fatal [585].

Human deficiency in ApoE increases the incidence of atherosclerosis [586, 587]. This discovery led to the development of the *apoe*-knockout mouse model in 1992 [588, 589]. This is one of the most studied

models of atherosclerosis [590]. ApoE-knockout mice display systemic hypercholesterolemia (500 mg/dL) compared to wild-type controls (60-90 mg/dL) [591]. Without ApoE, LDLs cannot be catabolized by the liver, which leads to massive cholesterol-enriched LDL particles [588, 589]. ApoE-knockout mice spontaneously develop atherosclerosis, even when fed a low-cholesterol, low-fat diet. However, when they are fed a high-fat diet, they rapidly develop atherosclerosis lesions [574]. These lesions can be treated or reversed when *apoe*$^{-/-}$ mice are injected with wild-type ApoE. This can also be achieved with an adenoviral vector [592], with a transgenic model overexpressing human ApoE [593], and with injections of synthetic ApoE [594].

Respiratory system and ApoE

In comparison to ApoA-1, the pulmonary roles of ApoE are well described. Knockout mice exhibit a unique pulmonary phenotype. Male knockout mice display alveolarization problems, altered pulmonary function and rapid loss of mechanical lung function compared to wild-type mice [595, 596]. However, female mice do not exhibit any abnormal pulmonary phenotypes compared to controls [595, 596]. This sexual dimorphism seems to be independent of ApoE and is more likely due to different developmental processes [571, 595, 596]. Deficient young mice (1-month old) have altered septation compared to wild-type controls [595]. This impairment is still present in 3-month-old deficient mice [595]. At 8 months of age, *apoe*$^{-/-}$ mice display increased pulmonary volume and compliance compared to wild-type animals; however, these phenotypes are not present at 3 months of age [595]. ApoE-knockout mice also display elevated systemic levels of VLDLs and triglycerides, which seems to be associated with an increase in pulmonary phosphatidylcholine levels [597].

ApoE has been studied in different pulmonary disease models, such as in house dust mite (HDM) asthma models [598], mycobacterial infection model [599], *Chlamydia* model *[600]*, LPS model [601], and septicemia [602]. It was also characterized in acute pulmonary damage caused by nanoparticles [603], hyperoxia [604], and acid aspiration [605]. It was further explored in pulmonary cancer [606, 607] and emphysema [608, 609]. Briefly, ApoE-deficient mice display increased bronchial hyperreactivity and goblet cell metaplasia during HDM challenges in a mouse model of asthma [598]. In acute lung injury models, *apoe*$^{-/-}$ mice display exacerbated neutrophilia and increased oxidative stress [603-605].

Arunachalam *et al.* demonstrated that unexposed knockout mice displayed higher lung compliance and greater alveolar spacing compared to wild-type controls [608]. They also showed that exposing ApoE-deficient mice to cigarette smoke exacerbated their compliance and alveolar spacing compared to unexposed counterparts [608]. Cigarette smoke-exposed knockout mice had increased pulmonary neutrophil infiltration, peroxidation byproduct levels (4-HNE and MDA) and MMP-9 and MMP-12 levels in their BAL and lung tissue [608]. In these models, ApoE seems protective in the lung. However, in the context of lung cancer, the presence of this apolipoprotein seems to be associated with increased tumour growth [606, 607], potentially by facilitating cholesterol delivery to tumour cells [610].

Two important apolipoproteins were described in the previous sections. While ApoE is more widely studies in diverse disease contexts compared to ApoA-1, the latter is definitely the most important in HDL biosynthesis. The following sections will focus on HDL biosynthesis and function as well as the impact of cigarette smoke on these processes.

HDL biosynthesis

ApoA-1 is synthesized in the liver and intestines [380] (Figure G, step 1). When secreted, ApoA-1 is an immature protein, which has the capacity to incorporate phospholipids and cholesterol. Upon lipid fusion, the nascent lipoprotein is autonomous and stable [562]. It will eventually evolve into a pre-β-1 HDL and interact with ABCA1 to capture additional lipids (Figure G, step 2). Pre-β-1 HDL is crucial for lipid export [611]. The discoidal form of pre-β-1 HDL evolves into a spherical HDL form upon incorporation of phospholipids and cholesterol [380]. The LCAT enzyme also helps in the maturation of HDLs, via the creation of a lipid core rich in cholesteryl esters. Mature HDLs interact with ABCG1 to absorb lipids (Figure G, step 3). This lipid-rich lipoprotein now returns to the circulation and binds to SR-B1 in the liver, in order to deliver lipids (Figure G, step 4). These lipids can be recycled, stocked or excreted via the bile [380]; Figure G, step 5). This process is extremely dynamic, therefore the remaining HDLs, carrying fewer lipids, can return in the circulation or the ApoA-1 can dissociate in order to restart the cycle (Figure G). HDL catabolism is principally done in the liver [612] and kidneys, where ApoA-1 is degraded by cubulin [613].

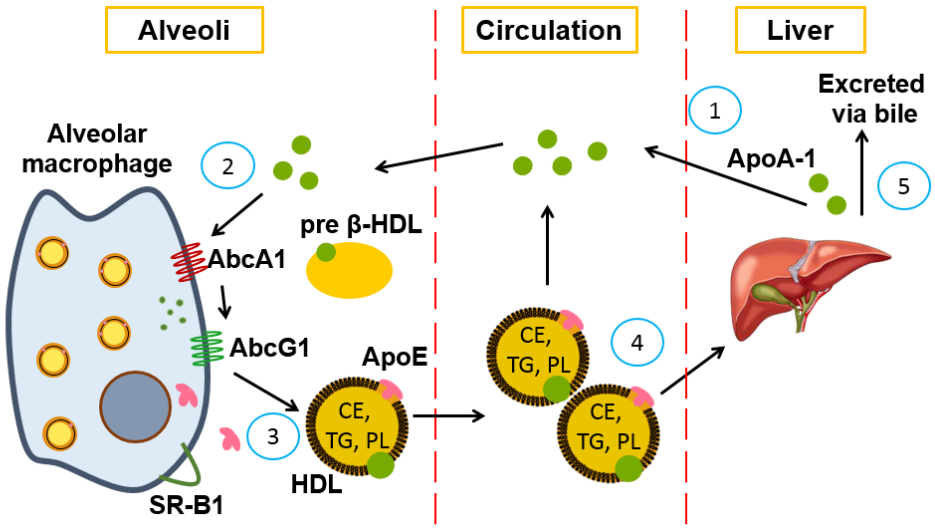

Figure VII: Schematic representation of reverse cholesterol transport (RCT).

This illustration represents a general overview of lipid transport and reverse cholesterol transport by ApoA-1, HDLs, macrophages, and the liver. The first step of lipid transport is the secretion of ApoA-1 by the liver (1). ApoA-1 enters circulation, reaches the pulmonary system and interacts with ABCA1 to capture lipids (2). The latter step induces ApoA-1 conformational change and forms a pre β-HDL. This immature lipoprotein complex further capture lipids via ApoE binding to ABCG1 and SR-B1 to form a mature HDL (3). This mature lipoprotein re-enters circulation (4). ApoA-1 can then dissociate to restart the cycle or HDLs can return to the liver to either store lipids or excrete them via bile (5).

HDL functions

Other than their main function in RCT, HDLs are considered anti-inflammatory [380], as they can sequester LPS [614] and protect against apoptosis [615, 616]. Furthermore, they have the ability to decrease the expression of ICAM-1 and VCAM-1. These adhesion molecules are found on endothelial cells and are involved in the migration of immune cells [617]. HDLs are also involved in the regulation of nitric oxide and CCL2 production as well as nicotinamide adenine dinucleotide phosphate oxidase and TNF activity [380].

As previously stated, HDLs are equipped with an important antioxidant enzyme, PON1. It has been shown that PON1 has the capacity to prevent lipid oxidation [618]. Moreover, this enzyme protects

against LDL oxidation, the main lipoprotein found in atheromatous plaques. ApoA-1 on its own can also intervene to protect from LDL oxidation [619, 620]. These antioxidant properties are important as they limit the formation of inflammatory HDLs. However, upon sustained inflammation/oxidation, lipids and proteins found at the surface of HDLs can be oxidized or enzymatically modified [619, 620], ultimately impairing their anti-inflammatory abilities. The chlorination of ApoA-1 via myeloperoxidase enzymes is one example of oxidation/denaturation that can occur. Besides inflammatory protection, the cholesterol efflux capacity via ABCA1 is also altered upon oxidation of ApoA-1 [621-625]. Interestingly, cigarette smoke exposure is known to lead to increased secretion of myeloperoxidase by neutrophils [626].

Impact of cigarette smoke on HDLs

It is well known that cigarette smoking reduces HDLc levels. In the Framingham cohort, Garrisson *et al.* showed that HDLc levels were 10% lower in male and female smokers compared to non-smokers [627]. These results were also observed in a 312 cohort of men and women. Furthermore, their ApoA-1 levels were diminished compared to non-smokers [628]. Another study following 105 subjects by Batic-Mujanovic *et al*. showed similar conclusions [629]. Recently, it was described that ApoA-1 levels were reduced in the lungs of COPD patients and cigarette smoke-exposed mice [630] as well as in the sputum of COPD patients [631]. These studies suggest that HDL levels are affected by cigarette smoke exposure.

Additionally, cigarette smoke alters the HDL subpopulation proportions. It was characterized by Moffatt *et al*. that cigarette smoking reduced HDL_2 levels in smokers compared to non-smokers, especially in women [632]. Secondhand smoke exposure also lead to a reduction in HDLc levels in children [633]. As mentioned above, LCAT is a key enzyme involved in HDL synthesis and maturation and cigarette smoking was associated with reduced LCAT levels and activity [634]. Furthermore, hepatic HDL uptake or its catabolism was found to be impaired following cigarette smoke exposure in pigeons [612]. Nevertheless, smoking cessation rapidly restored HDLc levels, which remained stable over time [635, 636]. Overall, cigarette smoking alters HDLc and ApoA-1 levels by promoting an inflammatory environment prone to HDL oxidation.

HDLs are one of the most important lipid transporters in our body. They face many challenges under cigarette smoking conditions, such as oxidative stress. They are crucial to many biological processes in the cardiovascular, nervous and pulmonary systems. Given their extremely diverse and complex roles, there is clearly a pharmacological therapeutic potential. Most of the molecules that have been designed (i.e. recombinant proteins, mimetic peptides, etc.), which will be described in the upcoming section, were created for use in cardiovascular and pulmonary disease settings.

Therapeutic approaches to increasing lipid transport

Many therapeutic strategies have been developed or are currently used to increase lipid transport. These strategies may use wild-type isolated HDL from humans or animals. Furthermore, developed complexes, for instance recombinant HDLs, are either combined with wild-type or mutated. ApoA-1 and ApoE mimetic peptides are also used. Other less common strategies, such as LXR pharmacological agonists, HDL delipidation or HDL phospholipidation will also be introduced.

First ApoA-1 therapies and recombinant HDLs

The first ApoA-1 supplementation slowed atheromatous plaque progression reduced plaque volume and changed plaque composition in rabbits fed a high-cholesterol diet [637, 638]. Furthermore, a 4-hour ApoA-1 infusion in humans induced an increase in pre-β-1 HDLs [639]. Eriksson *et al.* found that ApoA-1 administration increased RCT, leading to higher levels of cholesterol in the feces [640]. These pioneer works led to the development of recombinant proteins by pharmacological companies [641-643]. Many recombinant proteins were synthesized; however, CSL-111 and MDCO-216 are the most important ones.

CSL-111 (Commonwealth Serum Laboratories) is a recombinant HDL designed to improve cardiovascular and metabolic outcomes. It is composed of wild-type ApoA-1 purified and isolated from human plasma and reconstituted with soybean phosphatidylcholine (ratio 1:150; [641]. The benefit compared to the use of recombinant HDLs with mutated ApoA-1 is the relatively low-cost of purification

and isolation of wild-type ApoA-1. The first clinical trial of CSL-111 using 4 infusions of either 40 mg/kg or 80 mg/kg found a 3.4% reduction in the atheromatous plaque size in the low-dose group [644]. However, this difference was not statistically significant compared to the placebo group (1.6% reduction). Furthermore, patients who received the higher dose (80 mg/kg) dropped out of the study due to hepatic problems, exhibiting a 2-log increase in transaminase levels compared to placebo-treated controls. Nevertheless, coronary artery score and plaque characterization index were significantly improved despite a lack of change in plaque size [644].

CSL-111 was also investigated in the context of diabetes mellitus. Diabetes can lead to cardiovascular outcomes and these patients also display hyperreactive platelets, increasing their resistance to antithrombotic agents [645]. CSL-111 led to a 50% reduction in platelet aggregation *ex vivo* after 4-hour infusions. Furthermore, it reduced platelet response and thrombus formation by 50% *in vitro,* using cells isolated from diabetes patients [645]. Another study, with diabetic patients showed that CSL-111 had atheroprotective properties, marked by reduced inflammation and increased cholesterol efflux [646].

Wild-type HDL and pre-β-1 HDL levels and cholesterol efflux capacity via ABCA1 were increased in mice after CSL-111 administrations [647]. A more recent study by Gebhard *et al.* demonstrated a cardiovascular repair role for CSL-111. They showed that four CSL-111 infusions for one week led to an increased number of CD34 positive cells, key bone marrow-derived progenitor cells, and increased levels of stromal cell-derived factor 1, a chemokine involved in progenitor cell recruitment [648]. Therefore, CSL-111 showed promising results in a cardiovascular context.

ApoA-1 Milano was the first apolipoprotein A-1 mutation discovered and it is characterized by the replacement of the arginine 173 with a cysteine [649]. This confers the protein the possibility to form a homodimer or a heterodimer with other proteins like ApoA-2 [650]. ApoA-1 Milano carriers have lower HDLc levels, a reduced carotid thickness compared to non-mutated subjects, and they display higher longevity [651-653]. These findings led to the development of ETC-216, by Esperion Therapeutics, a recombinant ApoA-1 Milano protein overexpressed by *Escherichia coli* and fused with phospholipids [654]. The first clinical trial in 47 subjects revealed that ETC-216 administration (15 mg/kg or 45 mg/kg;

weekly 5-hour infusions over five weeks) led to reduced atheromous plaque size (4.2% decrease) compared to the placebo group (0.14% increase) [655]. Other studies have proposed that a higher dose (150 mg/kg) could be even more beneficial since rabbit data suggested that higher doses were better than low doses at accelerating atheromatous plaque regression [656].

Now under the name of MDCO-216 (The Medicines Company), this compound has also been shown to increase cholesterol efflux capacity in monkeys [657]. As with HDLs, MDCO-216 is equally dynamic and its native form is undetectable after 10 minutes of incubation with human serum. It was proposed that MDCO-216 binds to wild-type HDLs and this process leads to the removal of wild-type ApoA-1, ultimately increasing pre-β-1 HDL levels [658]. These results were also confirmed in a clinical study with stable angina pectoris patients who received MDCO-216 infusions for 2 hours [659].

A single infusion of MDCO-216 was shown to rapidly modify HDL quantity and composition in patients with coronary arterial disease and healthy controls, some of these differences persisted 7 days post infusion [660]. Kallend *et al.* further demonstrated that a single 2-hour infusion led to an increase in cholesterol efflux capacity via ABCA1 in coronary arterial disease patients compared to the placebo group [661]. MDCO-216 infusions did not lead to any significant side effects. Furthermore, MDCO-216 did not have any impact on body temperature, circulating leucocytes, C-reactive protein levels, nor inflammatory cytokine levels (IL-6, TNFα, etc.) [662]. Compared to CSL-111, MDCO-216 seems to be very beneficial in the context of cardiovascular diseases.

Other recombinant HDLs exist such as the CER-101 (Cerenis Therapeutics, France), a negatively charged lipoprotein complex associated with two different phospholipids: sphingomyelin and disphosphatidylglycerol. This molecule was designed to mimic pre-β-1 HDL. However, it did not have any beneficial effects on atheromatous plaque size [663, 664]. The ApoA-1 trimeric, developed by Roche, has three ApoA-1 proteins per complex and led to reduced arterial lesion severity but had no effect on the number of lesions [665].

Pulmonary impacts of recombinant HDL administrations with CSL-111 or with MDCO-216 have never been investigated. However, many mouse studies have evaluated the impact of wild-type human ApoA-

1 on the lung [666]. It has been shown to have therapeutic potential in bleomycin- [667] and silica-induced pulmonary fibrosis [668] due to its anti-inflammatory and antifibrotic properties. It was also beneficial in allergic asthma by enhancing the epithelial cell junction repair [669] and in cancer by reducing tumour size [670]. Kim *et al.* also showed that mice overexpressing ApoA-1 in the lung were protected from cigarette smoke-induced emphysema [630]. Wild-type ApoA-1 has positive impacts in different pulmonary disease models. Exploiting recombinant HDLs could therefore be a new option to treat pulmonary diseases such as cigarette smoke-induced emphysema.

ApoA-1 mimetic peptides

Recombinant HDLs can be disadvantageous due to their production cost, route of administration and long duration of infusion time, making them less than ideal for long-term therapy [641]. Therefore, research groups have focused on the use of amphipathic ApoA-1 synthetic peptides [671]. These peptides are usually composed of 20 amino acids that are able to mimic the complete ApoA-1 protein. Most of mouse studies have investigated APP018, also known as D-4F, peptide synthesized by Novartis [672]. This peptide is known to increase macrophage cholesterol efflux [673]. It is an amphipathic, alpha-helical peptide composed of 18 amino acids with four phenylalanines in D conformation, conferring intestinal proteolysis resistance [641]. A phase I clinical trial showed no toxicity with D-4F *per os* in humans, however, only 1% of the peptide was absorbed [674].

5F is another peptide that reduced atherosclerosis in a mouse model [675]. Additional peptides have also been tested *in vivo*: 5A, a peptide that stimulates cholesterol and phospholipid efflux through ABCA1 [676]; ETC-642 peptides, which inhibit atheromatous plaque formation [560]; and ATI-5261 peptides that also increased cholesterol efflux capacity [380].

Compared to recombinant HDLs, ApoA-1 mimetic peptides have been studied in diverse pulmonary diseases [571, 666]. The L-4F peptide, a copy of D-4F peptide under L conformation, led to reduced pulmonary hypertension and oxidized lipid levels in a pulmonary hypertension model [677] and decreased BAL neutrophil levels post-LPS instillation [570]. 5A ApoA-1 mimetic peptides are amphipathic molecules with two helices: one with five alanines, which reduces its hydrophobicity [666,

676]. This peptide was shown to reduce neutrophilic inflammation in an ovalbumin asthma model by reducing G-CSF levels [678]. Furthermore, 5A reduced bronchial hyperresponsiness, pulmonary inflammatory response and collagen expression in an HDM model of asthma [679, 680]. Another mimetic peptide, D-4F, lowered systemic inflammation by reducing IL-6 levels and pulmonary viral titers post-influenza infection [681]. D-4F, just like 5A, reduced oxidative stress, bronchial hyperreactivity, eosinophil levels and collagen deposition in an ovalbumin mouse model [682]. To conclude, ApoA-1 mimetic peptides display therapeutic potential suggesting that recombinant HDLs could also be beneficial under diverse pulmonary disease.

ApoE mimetic peptides

Pulmonary therapeutic strategies involving ApoE are rare compared to ApoA-1 protein. However, ApoE peptides or the complete protein have been involved in some pulmonary diseases [666]. Treatment with a 19 amino acids ApoE peptide attenuated pulmonary inflammation and bronchial hyperresponsiveness in a mouse model of asthma [598]. Three studies investigating 11 amino acids (COG1410) and 16 amino acid peptides showed lower pulmonary inflammation in a LPS model [570] and lower systemic inflammation in sepsis models [683, 684]. In addition, administration of the complete ApoE protein reduced pulmonary artery smooth muscle cell proliferation in a pulmonary hypertension model [685]. Overall, recombinant HDLs, ApoA-1 and ApoE mimetic peptides have potential in the treatment of diverse pulmonary pathologies and require additional investigation. As stated before, these molecules have never been investigated during cigarette smoke exposure. Other than recombinant HDLs, ApoA-1 and ApoE mimetic peptides, other strategies focusing on alternative pathways to increase lipid transport are available.

HDL delipidation or phospholipidation

HDLs delipidation is a new strategy to increase RCT. This *ex vivo* approach, proposed in 2004, relies on eliminating mature HDLs from the circulation of patients. To do so, the blood is drawn, depleted and reinfused into the patients. Without mature HDLs, the system will now biosynthesize new ApoA-1, pre-β-HDLs and eventually mature HDLs [686]. The efficacy of this hypothetical strategy was demonstrated

in 2009 by Sacks *et al*. The study revealed that specific HDL delipidation led to increased *in vitro* and *in vivo* RCT in African monkeys [687]. Furthermore, delipidated and reinfused plasma tended to reduce atherosclerosis induced by a high-fat diet in subjects with cardiovascular disease [687]. Nevertheless, additional studies are required to fully decipher its therapeutic potential.

On the other hand, HDL phospholipidation involves the enrichment of HDLs with phospholipids, which will increase the phospholipid/cholesterol ratio. This method was shown, *in vitro*, to increase RCT via SR-B1 [688, 689]. However, it has not been investigated in any *in vivo* animal models or in humans.

Pharmacological LXR agonists

As described in a previous section, liver X receptor or LXR is a key nuclear receptor involved in the transcription of diverse genes controlling lipid transport mechanisms. ABCA1, ABCG1 and ApoE are all proteins that can be expressed when LXR is activated. Therefore, many agonists were designed to activate LXR and to ultimately promote lipid transport and RCT. T0901317 and GW3965 were the first agonists to be created, although others exist such as LXR-623, GW6340, AZ876, and ATI-111 [690]. LXR activation leads to an increase in ABCA1 and ABCG1 protein and mRNA expression [691], and promotes RCT [692]). T0901317 was used *in vivo* in a model of atherosclerosis where mice were fed a high-cholesterol diet. These agonists were shown to inhibit atherosclerosis progression [693-695] and to facilitate plaque regression [696].

As observed in models of LPS challenge and pulmonary infection, LXR activation during atherosclerosis seems to be anti-inflammatory. LXR activation induces recruitment of negative co-regulatory proteins, which were proposed to interfere with NF-κB at diverse immune gene promoters by protein-protein interactions [697]. However, repetitive LXR activation with agonists leads to undesirable hepatic side effects [560, 690, 698]. It is now known that LXR activation stimulates the SREBP1c, a key protein involved in fatty acid synthesis. Unfortunately, this leads to a rapid hepatic lipid accumulation called hepatic steatosis [699]. Nonetheless, LXR activation stimulates RCT, a pathway that is significantly affected during cigarette smoke exposure. Development of alternative LXR agonists which fail to induce the unwanted hepatic effects would be an interesting therapeutic avenue.

In this section, diverse strategies surrounding HDLs or RCT were shown to promote lipid transport. New therapeutical approaches were also described. These methods, for instance recombinant HDLs were mostly studied in the context of cardiovascular disease, while mimetic peptides have also been studied in diverse pulmonary diseases. However, these therapies have not been investigated during cigarette smoke exposure. As cigarette smoke-exposed macrophages also display a foamy phenotype, promoting RCT with pharmacological agents may be beneficial to smokers.

Nutrition and respiratory health

RCT could not function without lipoproteins, apolipoproteins, nuclear receptors, transmembrane export proteins and lipid capture receptors. ApoA-1 and ApoE are crucial to RCT and macrophage lipid transport, respectively. Mutation in any RCT step will lead to lipid transport alterations and may cause dysregulated pulmonary lipid homeostasis, as seen in ApoA-1, ABCA1 and LXR deficiencies. These altered RCT functions are due to genetic variation causing a defective or absent protein. However, other factors, such as unhealthy nutrition, can also lead to the modification of lipid transport mechanisms. Studies investigating the impact of malnutrition on the lung are extremely scarce. This last section will focus on nutrition and its relevance to respiratory health.

Nutrition is a well-known factor that contributes to the development and progression of cardiovascular and metabolic diseases [700, 701]. Often described as a Western diet, the nutritional habits of Western Europe and North America leave much to be desired. Unfortunately, globalization has meant that the Eastern world is increasingly influenced by Western food culture. The Western diet, especially fast food, is high in fat and sugar, leading to exacerbation of cardiometabolic health problems [702]. It is associated with an increased risk of developing type 2 diabetes and higher coronary heart disease mortality rates [702]. Nutrition affects risk of cardiovascular disease development and mortality mainly through its significant impact on lipid transport. It is known that a high-fat diet increases circulating lipoprotein levels [703]; while a low-fat diet reduces them [704] to adjust for lipid transport. However, studies investigating the importance of nutrition on pulmonary health, especially during cigarette smoking, are uncommon. The next section will introduce diverse studies that have investigated the

impact of different nutrients (i.e. vitamins) on pulmonary health and whether diet modification can protect against cigarette smoke exposure outcomes.

Lung health and benefits from nutrition?

In 1995, Sridhar published an editorial entitled “Should people at risk of COPD eat more fruit and vegetables?” suggesting that nutrition might be important in respiratory health [705]. However, few studies have actually attempted to answer this question. One studied proposed that polyunsaturated fatty acid consumption might help smokers [706]. Strachan *et al.*, presented data suggesting that a lower fruit and vegetable intake during winter decreased FEV_1 by approximately 80 mL, after multifactorial adjustments, compared to the high-intake group [707] The latter study and others have associated this positive effect to vitamin C. Britton *et al.* have shown a positive effect of vitamin C intake on FEV_1 and this observation was accentuated in the older age group [708]. Several respiratory symptoms like wheezing and bronchitis were also negatively associated with serum vitamin C, independent of smoking status [709]. Sridhar *et al*. postulated that the antioxidant properties of this vitamin could therefore be important to lung health, especially during cigarette smoke exposure [705]. Furthermore, similar conclusions were observed concerning antioxidant vitamins in the MORGEN study [710]. However, as stated in his editorial, association clearly does not mean causation. Although vitamin C levels were shown to be reduced in smokers compared to non-smokers [711], one cannot discount the possibility that the increased oxidative environment caused by cigarette smoke exposure accelerates its consumption thus leading to reduced circulating vitamin C levels [712]. After adjustment for age, height and sex, data presented by Kelly *et al.* showed a positive relationship between fruit and vegetable consumption and FEV_1 [713]. In this study, vitamin C, vitamin E, A, and β-carotene levels were measured. Fruit and vitamin E were associated with reduced production of sputum over three months [713].

On the contrary, it was shown that fast food can exacerbate the pulmonary inflammatory response [714]. Asthmatic patients fed with a high-fat diet had higher neutrophil numbers, TLR-4 mRNA expression and pulmonary dysfunction compared to the low-fat intake group [714]. The Western diet, based on refined sugars, red meats, and high-fat products, was associated with higher risk of

exacerbation in asthma patients [715]. Varraso further demonstrated that the Western diet was also associated with an increased risk of developing COPD in men and women [716-718]. On the contrary, Mediterranean diet seems to be beneficial for COPD [719]. Favorable outcomes of this diet mostly rely on a high intake of antioxidant and anti-inflammatory nutrients and vitamins [720, 721]. Curcumin, isolated from turmeric, has been shown to have diverse antioxidant properties [722]. Curcumin intake, at least once per month, was positively associated with a greater FEV_1 and FEV_1/FVC ratio in smokers compared to the non-smoking group in a Chinese population-based study [722]. Additional nutrients have been studied: for instance, vitamins A, D, E, flavonoids, fats, N-acetylcysteine, etc. [720, 721]. Although, the evidence regarding the impact of diet on pulmonary healthy is increasing, there are still no recommendations on diet or supplementation to help prevent cigarette smoke-associated pulmonary diseases.

Most of the above studies and review papers either focus on antioxidant properties of food or the impact of supplementation in the context of pulmonary disease [720, 721]. However, we barely know what effect nutrient deprivation has on respiratory health. Key dietary components are required in order to maintain lung homeostasis. For instance, the lung surfactant is a vital structure composed of diverse lipids and proteins. As previously mentioned, phosphatidylcholine (PC) is the most abundant lipid found in pulmonary surfactant. Therefore, adequate choline intake, a precursor to PC synthesis, is crucial for maintenance of pulmonary PC levels. To my knowledge, only one study has evaluated the impact of PC depletion on lung homeostasis. Yost *et al.* investigated the effect of the methionine and choline deficient (MCD) diet on the pulmonary PC levels in rats [723]. They concluded that PC levels are not modified in rats fed the MCD diet compared to the control group. They suggested that PE methylation could overcome the circulating choline deficiency and activate local compensatory choline synthesis [724]. However, total lung extracts were used to measure PC levels [723] and contamination with cell membranes and lipoproteins could affect PC measurement; therefore, the amount of PC actually found in the pulmonary surfactant cannot be inferred.

Choline

Choline was recognized as an essential vitamin (B4) by the US Institute of Medicine in the late 1990s [725]. This vitamin can be acquired via diet or *de novo* via PE methylation. However, *de novo* synthesis is unable to meet daily human requirements. Eggs, meat, poultry, liver, fish and peanuts are great sources of choline [721]. Daily intake recommendations for men and women are 550 mg and 425 mg, respectively [725]. Choline is involved in many molecular pathways: for instance, acetylcholine synthesis, a crucial neurotransmitter [726]; PC and sphingomyelin production, key lipids involved in cellular membrane synthesis [727]; VLDL biosynthesis [728]; methyl group donor cycle [729]; and pulmonary surfactant synthesis.

Diverse genetic variations have been identified in choline metabolism [730]. One of the most important is a SNP located in phosphatidylethanolamine methyltransferase, the enzyme involved in *de novo* choline synthesis [730]. Da Costa *et al.* demonstrated that over half of the general population had at least one allele with this polymorphism [731]. Interestingly, women with this genetic mutation were 7 times more likely to develop choline deficiency problems, especially when dietary intake is low [731]. Interestingly, choline deficiency was shown to increase peripheral lymphocyte apoptosis and DNA damage [732]. Surprisingly, aging is associated with an increase in circulating choline levels [733-735]. Inadequate food consumption can also lead to low choline levels and one of the most studied models to investigate this deficiency is the MCD diet.

Methionine choline deficient diet

MCD diet model is mostly used because rodents rapidly develop hepatic steatosis. Therefore, this model can help understand mechanisms leading to non-alcoholic fatty liver disease (NAFLD), an important human hepatic disease [736]. Hepatic steatosis is characterized by hepatic lipid accumulation caused by failed lipid export by the liver in the absence choline and methionine [736]. Hepatic steatosis is marked by altered VLDL biosynthesis mechanisms leading to low VLDL levels, a key lipoprotein involved in fatty acid transport [737]. As mentioned in a previous section, HDL biosynthesis occurs mainly in the liver. Stankovic *et al.* demonstrated that HDL levels are also reduced

when animals are fed the MCD diet [737]. Furthermore, MCD diet leads to hepatic fibrosis, increased oxidative stress, inflammation and hepatocyte ballooning, a pathway that activates apoptosis [738, 739]. In contrast to human NAFLD, MCD-fed mice lose weight and are not insulin resistant [739]. Overall, this model allows us to study the impact of choline and methionine deficiencies and investigate impaired lipoprotein homeostasis in diverse disease contexts.

Methionine

Methionine is an essential amino acid [740]. It can be synthesized *de novo* via homocysteine and vitamin B6 or by using the folic acid pathway [741]. However, it is not sufficient to meet daily requirements (approximately 13 mg/kg/day for adults; [741]. Therefore, dietary intake must be adequate. Methionine can be acquired from nuts, eggs, red meats, soya, fish and dairy products [742]. This amino acid is involved in multiple biological processes, such as protein synthesis and methyl donation. The latter mechanism is implicated in over a hundred reactions and is activated with the help of diverse enzymes like methionine adenosyltransaminase, involved in generating S-adenosylmethionine from methionine [740, 743]. Furthermore, aminopropylation and transulfuration of methionine leads to the generation of polyamines and glutathione synthesis [740]. Glutathione is a key protector against oxidative stress that has the ability to interact with ROS and nitrogen species in order to neutralize them using its thiol group [744, 745].

The methionine deficient diet has been well characterized in mice. It leads to drastic weight lost mostly due to increased energy consumption (37% increase) compared to the control group [746, 747]. Furthermore, this deficiency is responsible for hepatic fibrosis, inflammation, and oversecretion of stress enzymes in the circulation (e.g. instance aspartate transaminase and alanine aminotransferase), a sign of hepatic damage [747].

Choline deficiency has also been characterized in humans and mice. It was demonstrated that patients developed hepatic steatosis after years of parenteral nutrition, feeding via intravenous administration of nutrients [748]. At that time, choline was not present in the diet, which led to the development of hepatic lipid accumulation. A simple choline supplementation was enough to overcome hepatic

problems in most of the patients [748]. Furthermore, fasting induced a choline deficiency [749]. A simple choline supplementation was also sufficient to restore normal choline levels [750]. In the MCD diet, Caballero *et al.* showed that choline deficiency alone was responsible for hepatic steatosis [747]. As mentioned above, choline is required for VLDL synthesis, the principal hepatic triglyceride vehicle; therefore, without this lipoprotein, triglycerides accumulate in the liver [728].

To conclude, nutrition and its pulmonary health effects have been scarcely studied. Studies investigating the impact of different diet deprivations on pulmonary homeostasis, on cigarette smoke exposure, or on pulmonary lipid transport mechanisms are either rare or nonexistent. Interestingly, the MCD diet reduces systemic lipid transport, a process known to be important during cigarette smoking. Therefore, the effects of choline and/or methionine deprivation can be investigated in the MCD model and may reveal an important role for these nutrients in the pulmonary response to cigarette smoke exposure and the maintenance of pulmonary homeostasis.

Rationale, hypothesis, and objectives

Cigarette smoking leads to significant pulmonary damage and inflammation and is associated with increased risk of developing lung disease. One of the most intriguing phenomena, first characterized in the 1970s and further studied by Morissette *et al*. is the presence of lipid-laden pulmonary macrophages following cigarette smoke exposure [181]. This foamy pulmonary macrophage phenotype has been described in smokers and in cigarette smoke-exposed mice and was shown to persist following smoking cessation [181]. Therefore, mechanisms involved in pulmonary lipid transport seem to be impaired by cigarette smoking.

Mounting evidence suggests that these intracellular lipids may come from the pulmonary surfactant, a vital lipid-rich lung structure. Cigarette smoking is known to intensify the oxidative stress response, which could lead to increased levels of oxidized lipids, from the surfactant, in the pulmonary environment. One can hypothesize that increased lipid capture response by macrophage scavenger receptors in the presence of oxidized lipids underlines the foamy phenotype observed. Furthermore, cigarette smoke exposure may also impair the ability of pulmonary macrophages to export lipids via lipid transporters (e.g. ABCA1, ABCG1, etc.). In addition, nuclear proteins, such as LXRs and PPARs, may also be affected during cigarette smoke exposure, thus promoting the inability of macrophages to clear lipids. However, it has been described that cigarette smoking leads to reduced levels of ApoA-1 and HDL, responsible for systemic lipid transport. The reason why cigarette smoking results in intracellular accumulation of lipids in macrophages remains unclear and requires additional investigation.

As mentioned in the introduction, smoking may lead to the development of many lung diseases. The discovery of factors influencing the susceptibility of smokers to develop these lung diseases remains a topic of research. Many environmental and genetic factors have been found and some suggest that pulmonary lipid homeostasis may be involved in the development of cigarette smoke associated lung disease. Consequently, therapies focusing on improving the pulmonary lipid transport may be a new approach available for ex-smokers. The main objective of this thesis is to characterize the impact of cigarette smoke exposure on pulmonary lipid transport. More precisely, this thesis will focus on

answering this general question: Why is the pulmonary lipid transport altered during cigarette smoking and can we improve it?

Aim 1: Many proteins involved in lipid capture and export were described in the introduction. The impact of cigarette smoking on the expression of these genes has not been fully described in humans and in mice. Another interesting question is how fast can cigarette smoke exposure modulate the expression of these pulmonary lipid transport genes? The impressive size of pulmonary macrophages following cigarette smoke exposure clearly suggests that pulmonary lipid transport is altered. Therefore, investigating the importance of pulmonary lipid transport using knockout mouse models during cigarette smoking, for instance *apoa*-1$^{-/-}$, could lead to new insights into how lipid transport is central to lung homeostasis. As mentioned above, HDLs and ApoA-1 levels are reduced in smokers and cigarette-smoke-exposed mice. These two molecules are crucial to lipid transport, thus emphasizing the need to investigate the impact of an HDL raising therapy during cigarette smoking. These questions and hypotheses will be explored in Objective 1.

Objective 1: To investigate the impact of cigarette smoke exposure on the pulmonary lipid transport

The sub-aims described in Chapter 1 are:

1.1: To characterize the impact of cigarette smoking on the gene expression profile of diverse pulmonary lipid export proteins in humans and in mice.

1.2: To investigate how an acute exposure to cigarette smoke impacts the lipid efflux capacity of the BALF.

1.3: To underline the role of ApoA-1 on 1) the pulmonary response to cigarette smoke and 2) the size of pulmonary macrophages following cigarette smoke exposure using a knockout mouse model.

1.4: To explore the therapeutic potential of raising HDL levels, using a recombinant HDL, on 1) the pulmonary response to cigarette smoke, 2) the size of pulmonary macrophages, 3) lung function, and 4) the body composition following cigarette smoke exposure.

Aim 2: Nuclear receptors such as PPARγ and LXRα,β are key players promoting the transcription of lipid transport-associated genes and their deletion leads to obvious pulmonary lipid disorders. However, LXR is directly involved in the regulation of lipid export gene expression, for instance ABCA1, ABCG1 and ApoE. The use of pharmacological agonists to activate LXR has been shown to increase the expression of *abca1* and *abcg1* in diverse models. Therefore, the hypothesis here is that activation of LXR via the use of T0901317 to promote lipid export mechanisms, will ultimately facilitate the export of intracellular lipids found in pulmonary macrophages. While Aim 1 is based on an exogenous supplementation therapy, Aim 2 focuses on activating endogenous lipid export mechanisms.

Objective 2: <u>To investigate the therapeutic potential of a systemic liver X receptor activation during cigarette smoking</u>

The sub-aims described in Chapter 2 are as follows:

2.1: To characterize the impact of LXR activation with T0901317 on the gene expression profile of the lung tissue and alveolar macrophages in room air- or cigarette smoke-exposed mice.

2.2: To investigate the effects of LXR activation on the responses of lung and alveolar macrophage to cigarette smoke exposure.

2.3: To explore the consequences of LXR activation on pulmonary surfactant homeostasis in room air- and cigarette smoke-exposed mice.

Aim 3: Though nutrition is a key factor altering the cardiometabolic health, its impact on pulmonary health and on the response to cigarette smoke is poorly understood. Methionine and choline are two nutrients involved in the biosynthesis of phosphatidylcholine, a crucial lipid found in the pulmonary surfactant. While some studies have investigated the effects of diverse nutrient supplementation, few have explored the impact of dietary deficiencies on pulmonary health. The methionine choline deficient diet is a well-known model that rapidly induces hepatic steatosis and decreases levels of circulating lipoproteins; however, the pulmonary outcomes of this diet are unknown. The goal of this last chapter is to explore how nutrition can affect lung homeostasis with or without cigarette smoke.

Objective 3: To investigate the impact of methionine and/or choline dietary deficiency on pulmonary health and cigarette smoke exposure.

The sub-aims described in Chapter 3 are:

3.1: To characterize the impact of the methionine and choline deficient diet on lung function, lung tissue expression, and the pulmonary inflammatory response to cigarette smoke.

3.2: To investigate the effects of methionine/choline dietary deficiencies on pulmonary surfactant homeostasis.

3.3: To explore whether the pulmonary effects of these dietary deficiencies, if any exist, are reversible when mice are fed a control diet.

3.4: To characterize which nutrient, choline and/or methionine, impacts pulmonary health and the pulmonary response to cigarette smoke.

Chapter 1: Interplay between cigarette smoking and pulmonary reverse lipid transport

1.1 Foreword

The scientific article presented in Chapter 1 “Interplay between cigarette smoking and pulmonary reverse lipid transport” was published in the European Respiratory Journal in 2017 (PMID: 28889112). This study describes the impact of cigarette smoking on the pulmonary lipid transport. Furthermore, it shows that cigarette smoke exposure affects the expression of key lipid transport genes, that pulmonary lipid transport is important under cigarette smoking conditions, and that it can be improved via recombinant HDL supplementation.

1.1.1 Author contributions

I am the principal author of this article. I conceived and performed all techniques associated to the animal protocols: MCDO-216 administration, cholesterol efflux, qPCRs and ELISA assay, and Dexa-Scan Piximus analyses. Maude Talbot, Mélanie Hamel-Auger and Marie-Josée Beaulieu helped during mouse euthanasia. Jean-Christophe Bérubé performed and analyzed the data concerning the human lung expression. Michaël Maranda-Robitaille helped with the murine qPCRs. Sophie Aubin exposed the mice to cigarette smoke and Marie-Ève Paré was involved in diverse laboratory analyses. David G. Kallend provided the MDCO-216. Benoît Arsenault helped with the study design. Yohan Bossé helped with the interpretation of the human pulmonary expression data set. Mathieu C. Morissette also conceived the study design and participated to the manuscript redaction.

1.2 Résumé

Le transport inverse des lipides est crucial au maintien de l'homéostasie. Le tabagisme induit une accumulation rapide de lipides au niveau des macrophages pulmonaires suggérant que les mécanismes impliqués dans le transport inverse des lipides sont altérés. Dans cette étude, nous avons investigué l'interaction entre le tabagisme et le transport inverse des lipides et les conséquences de celui-ci sur les dommages induits au poumon et en périphérie.

Un jeu de données clinique d'expression pulmonaire et du tissu pulmonaire de souris exposées à la fumée de cigarette ont été utilisés pour investiguer la relation entre le tabagisme et le transport inverse des lipides. Nous avons aussi caractérisé un modèle de souris avec un transport lipidique réduit, les souris ApoA-1$^{-/-}$, et une thérapie d'amélioration du transport lipidique en utilisant un HDL recombinant le complexe d'ApoA-1 Milano et de phospholipides le MDCO-216. Des analyses fonctionnelles et inflammatoires ont été faites sur le poumon et l'impact sur la composition corporelle a aussi été étudié.

La fumée de cigarette modifie l'expression pulmonaire de gènes impliqués dans le transport inverse des lipides, dont *abca1*, *abcg1*, *apoe* et *scarb1* chez l'homme et la souris. La capacité d'efflux de cholestérol par les macrophages est augmentée en présence de lavage bronchoalvéolaire et de sérum de souris exposées à une seule exposition de 2 heures. Les souris ApoA-1$^{-/-}$ déficientes ont une plus grande neutrophilie, de plus gros macrophages pulmonaires et une plus grande perte de masse maigre en contexte tabagique, comparativement aux souris traitées avec le MDCO-216 qui démontrent une réduction dans la taille de leur macrophage et une plus grande quantité de masse maigre lors de l'exposition à la fumée de cigarette.

Pour conclure, cette étude démontre une interaction fonctionnelle entre la fumée de cigarette et le transport inverse des lipides et ouvre de nouvelles pistes pour mieux comprendre ce qui relie les maladies métaboliques et pulmonaires associées au tabagisme.

1.3 Abstract

Reverse lipid transport is critical to maintain homeostasis. Smoking causes lipid accumulation in macrophages, therefore suggesting sub-optimal reverse lipid transport mechanisms. In this study, we investigated the interplay between smoking and reverse lipid transport and the consequences on smoking-induced lung and peripheral alterations.

To investigate the relationship between smoking and reverse lipid transport, we used a clinical lung gene expression data set and a mouse model of cigarette smoke exposure. We also used ApoA-$1^{-/-}$ mice, with reduced reverse lipid transport capacity, and a recombinant ApoA-1 Milano/Phospholipid complex (MDCO-216) to boost reverse lipid transport. Cellular and functional analyses were performed on the lungs and impact on body composition was also assessed.

Smoking affects pulmonary expression of *abca1*, *abcg1*, *apoe* and *scarb1* in both mice and humans, key genes involved in reverse lipid transport. In mice, the capacity of bronchoalveolar lavage fluid and serum to stimulate cholesterol efflux in macrophages was increased after a single exposure to cigarette smoke. ApoA-$1^{-/-}$ mice showed increased lung neutrophilia, larger macrophages and greater loss in lean mass in response to smoking, whereas treatment with MDCO-216 reduced the size of macrophages and increased the lean mass of mice exposed to cigarette smoke.

Altogether, this study shows a functional interaction between smoking and reverse lipid transport, and opens new avenues for better understanding the link between metabolic and pulmonary diseases related to smoking.

INTERPLAY BETWEEN CIGARETTE SMOKING AND PULMONARY REVERSE LIPID TRANSPORT

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Take home message: Smoking affects reverse lipid export mechanisms, represent a new pathological mechanism and therapeutic target

Running title: Smoking and pulmonary reverse lipid transport

1.4 Introduction

Reverse lipid transport first starts with the export of intracellular phospholipids and cholesterols to acceptors such as high-density lipoproteins (HDLs) present in the cellular environment (1). HDLs then access the peripheral circulation, reaching the liver where they are capture and processed, and the lipid content repackaged or excreted. The archetype lipid transporters ABCA1 and ABCG1 are especially important in initiating this process by the exporting cell as they recognize and bind apolipoprotein A-1 (ApoA-1), the most abundant apolipoprotein in HDLs (2). ABCA1 has special affinity for lipid-poor HDLs, such as pre-HDL, while ABCG1 interacts with more mature HDL molecules (2). Reverse lipid transport therefore integrates in the great scheme of systemic lipid transport, preventing problematic lipid accumulation in peripheral tissues.

Reverse lipid transport is extremely important to pulmonary cells, especially alveolar macrophages and type II pneumocytes, as they are processing a significant load of lipids on a daily basis. At the alveolar level, most of these lipids originate from the pulmonary surfactant, a high-turnover vital structure composed of phospholipids (~85%), cholesterols (~10%) and proteins (~5%) that lowers alveolar surface tension (3). Type II pneumocytes are responsible for pulmonary surfactant synthesis and recycling while alveolar macrophages take care of its degradation when damaged (4). In the lungs, deficiencies in reverse lipid transporters ATP-binding cassette A1 (ABCA1) and ABCG1 rapidly lead to lipid accumulation in alveolar macrophages and type II pneumocytes, as well as intraalveolar pulmonary surfactant build-up, ultimately causing lung dysfunction (5–7). Therefore, reverse lipid transport is a key function to the lung, pulmonary surfactant and alveolar macrophage homeostasis.

Given the importance of reverse lipid transport in the lungs and the pathological consequences of its dysfunction, environmental exposures that damage lipid structures like the pulmonary surfactant may require increased surfactant degradation by macrophages and, as a consequence, acceleration of reverse lipid transport. Cigarette smoking has been well documented for causing pulmonary lipid damage (8–10). In fact, upon exposure to cigarette smoke, alveolar macrophages become chronically enlarged, showing extensive intracellular lipid accumulation coupled with the release of numerous inflammatory mediators (11), suggesting sub-optimal capabilities in reverse lipid transport. A recent

pre-clinical study proposed that this foamy macrophage phenotype and early inflammatory response to cigarette smoke exposure is due, at least in part, to direct damage made to pulmonary lipids (11). Similarly to pulmonary surfactant and cellular lipids, lipoproteins are exposed to biochemical damage by cigarette smoke. Lipoproteins damaged by cigarette smoke have compromised functionality, reducing their capacity to promote and support reverse lipid transport (12). This could be relevant to chronic smoking-related lung disease, such as chronic obstructive pulmonary disease (COPD), where lung diffusion is reduced, small airways are dysfunctional often leading to hyperinflation, and increased alveolar surface tension caused by compromised surfactant integrity could lead to excessive alveolar stretching and damage. Thus, we postulate that the progressive and constant pressure applied by cigarette smoke inhalation on pulmonary reverse lipid transport mechanisms may represent an underappreciated contributor to smoking-related lung pathologies, preventing macrophages from properly disposing of intracellular lipids and further promoting local inflammation. Moreover, improving the maintenance of pulmonary lipid homeostasis could have beneficial effects on lung functions but also on the peripheral manifestations of smoking, such as lean mass loss.

In this study, we first investigated the impact of cigarette smoke exposure on the levels of key genes involved in reverse lipid transport using clinical and pre-clinical samples and found that cigarette smoke exposure modulates the expression of the lipid transporters *abcg1*, *abca1*, and *scarb1* as well as the apolipoprotein E (*apoe)* in the lungs of both humans and mice. Using our pre-clinical model of cigarette smoke exposure, we found that cigarette smoke exposure elicits a rapid increase in the ability of bronchoalveolar (BAL) fluid and serum to promote reverse lipid transport in macrophages. To investigate the impact of a dysfunctional reverse lipid transport capacity, mice lacking ApoA-1, the main apolipoprotein of HDLs, showed an exacerbated pulmonary and systemic response when exposed to cigarette smoke. Finally, administration of MDCO-216, a recombinant ApoA-1 Milano/Phospholipid complex, was able to dampen the pulmonary and systemic effects of cigarette smoke exposure.

1.5 Methods

1.5.1 Human lung gene expression cohort and analyses

Messenger RNA levels of key genes involved in reverse lipid transport, namely *abcg1*, *abca1, scarb1,* and *apoe*, were investigated in a human lung gene expression dataset previously described (13). Briefly, microarray data from 465 non-tumor lung specimens were obtained from patients that underwent lung cancer resection. Gene expression levels were adjusted for sex and age, and compared among never, former and current smokers. The elapsed time between smoking cessation and tissue collection was also considered among former smokers. Differences in gene expression levels were tested using a one-way ANOVA followed by a Tukey's multiple comparison tests.

1.5.2 Mice, cigarette smoke exposure and treatments

Female six to eight weeks old BALB/c (Charles River, St-Constant, QC, Canada), C57BL/6 (Charles River), C57BL/6J (Jackson Laboratories, Bar Harbor, ME, USA) and ApoA1-deficient (Jackson Laboratories) mice were exposed to the mainstream smoke of 3R4F research cigarettes (University of Kentucky, Lexington, KY, USA) for two consecutive hours/day, five days/week, for two or eight weeks using the Promech SIU24 whole-body exposure system (Promech Lab AB, Vintrie, Sweden). MDCO-216 (20 or 100 mg/kg in 200 µl; kindly provided by The Medicines Company, Parsippany, NJ, USA), a recombinant ApoA-1 Milano/Phospholipid complex, and saline were delivered intraperitonealy two hours after every cigarette smoke exposure (2-week exposure protocol) or every Monday, Wednesday, and Friday during the last four weeks of exposure (8-week exposure protocol). Mice were housed according to the Canadian Council for Animal Care (CCAC) guidelines and Université Laval's Animal Research Ethics Board approved all procedures (Animal utilization protocol #2014121-2).

1.5.3 Lung harvesting and processing

When lung function assessment was not required, mice were anesthetised with isoflurane. The lungs were removed from the chest cavity, the trachea was canulated, the right multi-lobed lung was attached with a suture string and the left lobe subjected to bronchoalveolar lavage (BAL), performing a first lavage with 250µl and a second with 200µl of cold PBS. The right lung was snap frozen in liquid nitrogen and kept at -80°C for qPCR analyses. After BAL, the left lobe was inflated with 10% formalin

for paraffin embedding and tissue histology. BAL total cell concentration was determined using a hemacytometer. BAL was then centrifuged at 800g for 10 min to pellet the cells and perform cytospins for cell differentials (Diff-Quik; Fisher Scientific, Ottawa, ON, Canada). At least 300 cells per cytospin were manually counted using the Image J software (v1.44o). The size of pulmonary macrophages was determined as previously described (11). Thirty-five macrophages per cytospin per mouse were randomly picked and the surface area measured using the Image J Software. Lymphocytes (bright blue nucleus and small cytoplasm) were excluded from the analysis. The average size of the pulmonary macrophages from room air (RA)-exposed mice was set as 100%. BAL fluid was collected in a separate tube and kept at -80°C.

1.5.4 Human ApoA-1 ELISA

Human ApoA-1 was assessed as a surrogate for MDCO-216 levels, a complex composed of recombinant human ApoA-1 Milano and phospholipids. Human ApoA-1 concentrations were assessed in mouse BAL fluid and serum samples using the human ApoA-1 ELISA kit (Abcam, Cambridge, UK) according to manufacturer's instructions.

1.5.5 Quantitative PCR (qPCR)

Lung lobes were homogenized in 1 ml of Trizol (Fisher Scientific) using PowerGen 125 polytron (Fisher Scientific). RNA was extracted according to manufacturer's instructions. RNA concentration and purity was determined using Take3 Trio Micro-Volume plate and Synergy H1 Hybrid Reader (BioTek, Winooski, VT, USA). RNA integrity was verified using agarose gel electrophoresis. Reverse transcription was performed on 1 μg of RNA using the iScript™ Advanced cDNA Synthesis Kit for quantitative PCR (qPCR) (Bio-Rad, Mississauga, ON, Canada) according to manufacturer instructions. *Abca1*, *abcg1, scarb1* and *apoe* lung mRNA expression was assessed by qPCR and normalized to *hprt* and *rplp0* reference genes using the ΔΔCq method. All reactions were performed in duplicate or triplicate using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) and primers at 10 μM. The primers and qPCR conditions used for amplifications were optimised for each gene (*abca1* [NM_013454]: Fwr CCA TAC CGA AAC TCG TTC ACC - Rev CCG CAG ACA TCC TTC AGA ATC - annealing T° 56°C; *abcg1* [NM_009593]: Fwr ACT GTT CTG ATC CCC GTA CT - Rev TCT CCA ATC

TCG TGC CGT A - annealing T° 59°C; *apoe* [NM_009696]: Fwr CAC TCG AGC TGA TCT GTC AC - Rev TGG AGG CTA AGG ACT TGT TTC - annealing T° 60°C; *scarb1* [NM_016741]: Fwr CTT GCT GAG TCC GTT CCA TT - Rev GTA CCT CCC AGA CAT GCT TC - annealing T° 59°C; *hprt* [NM_013556]: Fwr AGC AGG TCA GCA AAG AAC T - Rev CCT CAT GGA CTG ATT ATG GAC A - annealing T° 57°C; *rplp0* [NM_007475.5]: Fwr ATC ACA GAG CAG GCC CTG CA - Rev CAC CGA GGC AAC AGT TGG GT - annealing T° 57°C). Quantitative PCR were performed using a Rotor-Gene 6000 series (QIAGEN, Toronto, ON, Canada) and data acquired/analysed with the on-board Rotor-Gene series Software version 1.7. All qPCR efficiencies were between 90-110%, with R^2 values ranging between 0.97-1.00. The thermo-protocol was as follows: 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds and 57-60°C for 30 seconds and followed by a melting curve.

1.5.6 Lung function assessment

Lung functions were assessed using the FlexiVent® (SCIREQ, Montreal, PQ, Canada). Mice were anaesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine; tracheotomised with an 18-gauge blunted needle, mechanically ventilated at a respiratory rate of 150 breaths/minutes and a tidal volume of 10 ml/kg, with a pressure limit of 30 cmH_2O. Muscle paralysis was achieved using pancuronium (2 mg/kg, Sandoz, Boucherville, PQ, Canada) to prevent respiratory efforts during the measurement. The following sequence of measures was repeated three times: Deep inflation, Snapshot-150, Quick Prime-3 and Pressure/Volume-loop to obtain lung inspiratory capacity, resistance, hysteresis, compliance and elastance.

1.5.7 Body composition analyses

Mice were anaesthetized with isoflurane. Body composition was assessed using the Lunar PIXImus dual-energy x-ray absorptiometry (DEXA) system.

1.5.8 In vitro cholesterol efflux assay

J774A.1 macrophages (ATCC TIB-67) were grown to 75% confluence (DMEM, 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine) in 48-well plates. They were then loaded with ^{3}H-cholesterol overnight (0.5 µCi/well; PerkinElmer, Waltham, MA, USA) in DMEM with 1% FBS. On the next day,

cells were thoroughly washed with warm PBS and exposed for 4h to polyethylenglycol-treated human or mouse serum (2.8% v/v final) or 10-fold concentrated mouse bronchoalveolar lavage (20% v/v final). Supernatant was collected and macrophages were lysed. Total ^{3}H -cholesterol was assessed in both supernatant and macrophage lysate. Percentage of cholesterol efflux was calculated as follow: supernatant/(supernatant + lysate)*100. In the context of this study, this assay gives information on how the capacity of BAL fluid or serum lipoprotein acceptors to accept cholesterol is affected by cigarette smoke exposure. Please refer to Low *et al.* (14) for a complete description of the technique and data interpretation.

1.5.9 Statistical analysis

Two-group comparisons were performed using unpaired student T-tests. Multiple group comparisons were performed using a One-way ANOVA followed, if applicable ($p<0.05$), by a *post hoc* Tukey's multiple comparisons test. Tests were performed using Prism 7 from GraphPad Software, Inc.

1.6 Results

1.6.1 Impact of cigarette exposure on key genes involved in the regulation of reverse lipid transport

To investigate whether cigarette smoke exposure impacts the expression of important genes involved in regulating reverse lipid transport, we compared the expression levels of *abca1*, *abcg1*, *scarb1*, and *apoe* in clinical lung samples from individuals with different smoking status and history (13). We found that *abcg1, scarb1*, and *apoe* expressions were higher in active smokers compared to never-smokers, whereas *abca1* expression was significantly lower (Figure 1A). Smoking-induced alterations in *abcg1*, *abca1*, *scarb1* and *apoe* lung expression persisted in ex-smokers and progressively reached the levels of never-smokers with increased duration of smoking cessation (Figure 1A). With regard to the mouse model of cigarette smoke exposure, *abcg1* expression was rapidly increased in BALB/c mice following 2 weeks of exposure whereas *abca1* and *apoe* expression was only increased following 24 weeks of exposure (Figure 1B). *Apoe* and *abcg1* levels remained elevated following cessation while *abca1* levels returned to baseline (Figure 1B). Levels of *scarb1* were reduced from 8 weeks of exposure to cigarette smoke and remained lower despite cessation (Figure 1B). Altogether, these results show that *abcg1*, *abca1*, scarb1, and *apoe*, central genes involves in reverse lipid transport, are affected by cigarette smoke exposure in both humans and mice.

1.6.2 Impact of cigarette smoke exposure on pulmonary and circulating lipid export capacity

Since cigarette smoke exposure affects the expression of key genes involved in reverse lipid transport, we investigated the ability of the pulmonary extracellular milieu and peripheral circulation to promote reverse lipid transport. Using an *in vitro* cholesterol efflux assay, we found that BAL fluid and serum samples collected from BALB/c mice 2h after a single 2h-exposure to cigarette smoke had increased ability to promote reverse lipid transport compared to room air-exposed controls (Timepoint A; Figure 2A-C). This phenomenon was also observed in BAL and serum samples collected 2h after the last exposure of a 4-day protocol (Timepoint B; Figure 2A-C). Interestingly, only BAL fluid samples collected 21h after the last cigarette smoke exposure of a 4-day protocol showed higher reverse lipid transport stimulation capacity, whereas no differences were observed between serum samples from room air and cigarette smoke-exposed mice (Timepoint C; Figure 2A-C). These data show that, in

response to cigarette smoke exposure, there is a rapid biological drive to increase local and peripheral reverse lipid export capacity.

1.6.3 ApoA1 deficiency exacerbates pulmonary and systemic responses to cigarette smoke exposure

ApoA1-deficient mice show a marked reduction in reverse lipid transport capacity as measured using the cholesterol efflux assay (Figure 3A). To investigate the impact of dysfunctional reverse lipid transport on the pulmonary and systemic response to cigarette smoke exposure, wild-type C57BL/6J and Apoa1-deficient mice were exposed to mainstream cigarette smoke for 8 weeks. We found that Apoa1 deficiency led to an increase in cigarette smoke-induced BAL total cell and neutrophil numbers compared to wild-type animals (Figure 3B). Despite similar numbers in mononuclear cells between Apoa1-deficient and wild-type mice exposed to cigarette smoke, a greater proportion of pulmonary macrophages from ApoA1-deficient mice were considered enlarged (Figure 3C). ApoA1-deficient mice also had lower pulmonary levels of *abca1*, but smoking-induced changes in *abca1*, *abcg1*, *scarb1* and *apoe* were not different between C57BL/6J and ApoA1-deficient mice (Figure S1A). With regard to extra-pulmonary aspects, Apoa1-deficient mice remain significantly smaller than wild-type mice and cigarette smoke exposure inhibited weight gain similarly in both strains (Figure 3D). In wild-type mice, cigarette smoke exposure prevents lean mass gain, while adiposity remains similar (Figure 3E-F). However, Apoa1-deficient mice gain significantly less lean and fat mass (Figure 3E-F). Altogether, these data show that a reduced capacity to support reverse lipid export can exacerbate the pulmonary and some systemic effects of cigarette smoke exposure.

1.6.4 Impact of MDCO-216 therapy on the pulmonary response to cigarette smoke

As ApoA-1-deficient mice appear to be more susceptible to cigarette smoke exposure, we investigated whether boosting lipid export could dampen the pulmonary and systemic effects of cigarette smoke exposure. To do so, we used a recombinant ApoA-I Milano/Phospholipid complex (MDCO-216), which has the ability to promote reverse lipid transport (15), in conjunction to our cigarette smoke exposure models. We found that MDCO-216 injected intraperitonealy rapidly reaches the lungs (Figure 4A) and that cigarette smoke exposure does not interfere with its translocation to the pulmonary environment (Figure 4B). In the 2-week protocol, cigarette smoke exposure lead to a significant increase in BAL

total cellularity and mononuclear cells, which was not observed in MDCO-216-treated animals (20 and 100 mg/kg) (Figure 4C). However, cigarette smoke-induced neutrophilia was not impacted by MDCO-216 treatment (Figure 4C). In the 2-week protocol, MDCO-216 significantly reduced BAL pulmonary macrophage size compared to vehicle-treated animals (Figure 4D). With regard to the 8-week cigarette smoke exposure protocol, the therapeutic treatment with MDCO-216 had no significant impact on BAL total cell, mononuclear cell, and neutrophil numbers compared to the vehicle treatment, and BAL pulmonary macrophage size remained similar. MDCO-216 therapeutic delivery increased *abca1* and reduced *scarb1* pulmonary mRNA levels, but smoking-induced changes in *abca1*, *abcg1*, *scarb1* and *apoe* were not different between vehicle and MDCO-216-treated mice (Figure S1B). These finding are mitigated by the fact that both room air and cigarette smoke-exposed mice that received MDCO-216 started producing antibodies against the compound (human ApoA-I), likely interfering with its biological activity (Figure S2). Altogether, these results show that systemic administration of a recombinant ApoA-I Milano/Phospholipid complex (MDCO-216) can reach the lungs and has the potential to dampen mononuclear cells/macrophages recruitment in the lungs as well as limit their size.

1.6.5 Impact of MDCO-216 therapy on lung functional alterations caused by cigarette smoke

To investigate the impact of MDCO-216 administration on lung functional alterations caused by cigarette smoke exposure, we performed FlexiVent analyses in mice exposed to cigarette smoke or room air for 8 weeks and treated with either vehicle or MDCO-216 for the last 4 weeks. As expected, cigarette smoke exposure led to significant alterations in the general pressure-volume loop dynamics (Figure 5A) and increased lung inspiratory capacity, hysteresis and compliance while reducing lung elastance (Figure 5B-E). MDCO-216 treatment only significantly reduced the impact of cigarette smoke exposure on lung hysteresis. These results show that MDCO-216 can have partial effects on lung functional alterations caused by cigarette smoke exposure.

1.6.6 Impact of MDCO-216 therapy on body composition alterations caused by cigarette smoke

To investigate the impact of MDCO-216 treatment on body composition alterations caused by cigarette smoke exposure, we performed whole-body DEXA densitometry analyses before the initiation of the

treatments (week 5) and the day before sacrifice (week 8). Vehicle and MDCO-treated mice exposed to cigarette smoke were lighter after 8 weeks of exposure than their room air-exposed counter parts (Figure 6A). During the treatment phase, vehicle-treated mice exposed to room air significantly gained more weight than mice exposed to cigarette smoke (Figure 6B), largely explained by a gain of fat mass (Figure 6C) rather than lean mass (Figure 6D). MDCO-216-treated room air-exposed mice show a profile very similar to vehicle-treated room air-exposed mice, MDCO-216 alone having no significant independent effect on body composition. Interestingly, treatment with MDCO-216 induced a marked gain in lean mass in cigarette smoke-exposed mice (Figure 6D). These data show that MDCO-216 treatment can have positive effects on body composition of mice exposed to cigarette smoke exposure, notably by increasing lean mass.

1.7 Discussion

Results from several studies suggest that cigarette smoke exposure affects pulmonary lipid homeostasis, notably by directly damaging pulmonary lipids, reducing pulmonary surfactant levels, increasing levels of anti-oxidized lipids antibodies, and causing lipid accumulation in pulmonary macrophages (8, 9, 11, 16–18). Reverse lipid transport is also a crucial function in lung homeostasis, allowing pulmonary macrophages and structural cells to properly export intracellular lipid. Similar to cigarette smoke exposure, deficiencies in ABCA1 and ABCG1, two instrumental lipid transporters involved in reverse lipid transport, cause lipid accumulation in pulmonary macrophages and chronic lung inflammation (5–7). Therefore, we investigated the impact of cigarette smoke exposure on aspects of reverse lipid transport in human and mouse lungs as well as the impact of poor reverse lipid transport capacity on the pulmonary and systemic response to cigarette smoke exposure in an *in vivo* mouse model. We also investigated the potential benefits of promoting reverse lipid transport using a recombinant ApoA-I Milano/Phospholipid complex (MDCO-216).

Smoking modulates pulmonary mRNA levels of *abcg1, abca1, scarb1* and *apoe* in both humans and mice. From the human lung data, we can see that the effects on *apoe* levels persist for over a decade following cessation, while *abcg1, scarb1* and *abca1* levels take between 5 and 10 years to return to never smokers levels. Mouse data show that *abcg1* is rapidly induced upon cigarette smoke exposure, while the effects on *abca1*, *scarb1*, and *apoe* are more progressive in nature. This transcriptional profile associated with smoking in both humans and mice is highly consistent with the notion of increased reverse lipid transport, or at least a biological will to do so. Functionally, ABCA1 exports lipids to lipid-poor HDLs (pre β HDLs) trough ApoA-1 binding while ABCG1 and SR-B1 (protein of the gene *scarb1*) export lipids to more mature HDLs. Literature is extremely scarce on the use of HDL lipoproteins subsets by the lungs but our data suggest that, upon cigarette smoke exposure, the lungs activate mechanisms to increase its capacity to export lipids to more mature HDLs trough ABCG1 and SR-B1 for instance, pre HDLs likely being less accessible for ABCA1-mediated processes. While the regulating mechanisms behind *abcg1*, *abca1*, *scarb1*, and *apoe* expression in the smoker's lungs remain to be clarified, the expression profile of these four genes crucial to reverse lipid transport supports a strong interaction between smoking and pulmonary reverse lipid transport.

Cigarette smoke exposure rapidly affects reverse lipid transport-stimulating capacity in the mouse lungs and circulation. In fact, an increase in BAL fluid and serum cholesterol efflux-stimulating capacity can be observed as early as two hours after a single exposure to cigarette smoke. In humans, reduced reverse lipid transport is observed in individuals with coronary artery disease (CAD), of which smoking is an important risk factor. A very limited number of studies, however, looked at the specific effects of smoking in 'healthy' young/middle-aged smokers (19). Smoking is notoriously known to reduce circulating HDL and ApoA-1 levels, strongly supporting a negative impact on reverse lipid transport. Discrepancies between the effects of smoking on reverse lipid transport may be attributable to differences between species, the HDL/LDL ratio being much lower in humans (~0.5) than in mice (~2.5). Nevertheless, our data suggest that cigarette smoke exposure triggers a local and systemic drive to promote reverse lipid transport capacity in mice. However, this increase in reverse lipid transport-stimulating capability does not appear to be sufficient to prevent pulmonary macrophages from accumulating intracellular lipids.

Mice deficient in ApoA-1 have a reverse lipid transport capacity reduced to about 50% of wild-type mice and show an exacerbated immune response to cigarette smoke exposure and increased pulmonary macrophage size. On the other end, treatment with MDCO-216 showed potential for reducing the inflammatory response to cigarette smoke, especially mononuclear cell accumulation and pulmonary macrophage size, and aspects of smoke-induced lung function alterations. This displays the significant impact reverse lipid export capacity can have on the magnitude of the pulmonary response to cigarette smoke exposure. Our data is consistent with the study from Kim et al. (20), showing that mice overexpressing human ApoA-1 in the alveolar epithelium are protected from lung emphysema. It also aligns with findings in experimental atherosclerosis, where ApoA-1 deficiency exacerbates the pathology (21, 22), while introduction of a human ApoA-1 transgene confers protection (23, 24). In both pathologies, the proposed and most likely mechanism of action is promotion/reduction of reverse lipid export. However, HDL molecules and ApoA-1 have been shown to have numerous other functions, including anti-inflammatory and anti-oxidant properties (25). While each of these mechanisms could have a significant impact on smoking-induced lung inflammation, macrophage lipid accumulation, and lung function alterations, we currently do not have the data to support mechanistic explanations of our findings.

Aside from the pulmonary phenotype, ApoA-1 deficiency and MDCO-216 treatment significantly affected the variations in mouse total weight and body composition attributable to cigarette smoke exposure. ApoA-1 deficient mice are smaller than wild-type mice at baseline, a difference largely attributable to a reduced lean mass, while MDCO-216 did not significantly affect total weight or body composition. The main differences between ApoA-1 deficiency and MDCO-216 treatment in mice exposed to cigarette smoke were that ApoA-1 deficiency caused a significant loss in both lean and fat mass, while MDCO-216 treatment markedly increased lean mass, leaving fat mass unaffected. ApoA-1 is known to affect numerous metabolic functions, including the stimulation of glucose uptake by skeletal muscle (26, 27). It has also been shown that ApoA-1 can increase mitochondrial biogenesis in vitro in myotubes (28). While these findings are consistent with an increase in lean mass, we currently cannot explain why this phenotype is only observed in mice exposed to cigarette smoke. It is likely that MDCO-216 favours a regain of the lost lean mass caused by cigarette smoke exposure; potential mechanisms being improved glucose uptake and myogenesis. Additional research is needed to understand the cellular and molecular mechanisms. Clinically, reduced lean/muscle mass has significant adverse effects on quality of life and mortality rate in patients with COPD. Moreover, only a proportion of individuals with COPD show significant limb muscles functional alterations. Reverse lipid transport capacity may represent a novel avenue of investigation to explain the variability in limb muscle phenotypes in smokers with and without COPD and restore limb muscle functionality.

A limitation of this study, inherent to the use of human protein in mice, is the apparition of antibodies against MDCO-216. This very likely reduced the efficacy of the MDCO-216 treatment in the last days or weeks of the protocol, likely having more pronounced impacts in the 8-week protocol and potentially explaining the reduced impact of MDCO-216 in this setting.

Overall, this study shows that smoking affects mechanisms of pulmonary reverse lipid transport and also how the biological capacity to promote reverse lipid transport impacts the intensity of the pulmonary response to cigarette smoke. This tight interaction between smoking and pulmonary reverse lipid transport aligns with the pathological mechanisms of coronary artery disease (CAD) and dyslipidemia, of which smoking is a major risk factor. Research using a more comprehensive approach is required to investigate smoking-related/associated pathologies as a whole as they tend to coexist in many individuals.

1.8 Acknowledgements

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1.9 References

1. Favari E, Chroni A, Tietge UJ, Zanotti I, Escolà-Gil JC, Bernini F. Cholesterol efflux and reverse cholesterol transport. Handb Exp Pharmacol. 2015;224:181-206.
2. Phillips MC. Molecular mechanisms of cellular cholesterol efflux. J Biol Chem. 2014;289:24020-24029.
3. Carey B, Trapnell BC. The molecular basis of pulmonary alveolar proteinosis. Clin Immunol. 2010;135:223-235.
4. Goss V, Hunt AN, Postle AD. Regulation of lung surfactant phospholipid synthesis and metabolism. Biochim Biophys Acta. 2013;1831:448-458.
5. Baldán A, Gomes AV, Ping P, Edwards PA. Loss of ABCG1 results in chronic pulmonary inflammation. J Immunol. 2008;180:3560-3568.
6. Wojcik AJ, Skaflen MD, Srinivasan S, Hedrick CC. A critical role for ABCG1 in macrophage inflammation and lung homeostasis. J Immunol. 2008;180:4273-4282.
7. Bates SR, Tao JQ, Collins HL, Francone OL, Rothblat GH. Pulmonary abnormalities due to ABCA1 deficiency in mice. Am J Physiol Lung Cell Mol Physiol. 2005;289:L980-9.
8. Rahman I, van Schadewijk AA, Crowther AJ, Hiemstra PS, Stolk J, MacNee W, De Boer WI. 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2002;166:490-495.
9. Aoshiba K, Koinuma M, Yokohori N, Nagai A. Immunohistochemical evaluation of oxidative stress in murine lungs after cigarette smoke exposure. Inhal Toxicol. 2003;15:1029-1038.
10. Malhotra D, Thimmulappa R, Navas-Acien A, Sandford A, Elliott M, Singh A, Chen L, Zhuang X, Hogg J, Pare P, Tuder RM, Biswal S. Decline in NRF2 Regulated Antioxidants in COPD Lungs due to Loss of its Positive Regulator DJ-1. Am J Respir Crit Care Med. 2008
11. Morissette MC, Shen P, Thayaparan D, Stämpfli MR. Disruption of pulmonary lipid homeostasis drives cigarette smoke-induced lung inflammation in mice. Eur Respir J. 2015;46:1451-1460.
12. Ueyama K, Yokode M, Arai H, Nagano Y, Li ZX, Cho M, Kita T. Cholesterol efflux effect of high-density lipoprotein is impaired by whole cigarette smoke extracts through lipid peroxidation. Free Radic Biol Med. 1998;24:182-190.
13. Bosse Y, Postma DS, Sin DD, Lamontagne M, Couture C, Gaudreault N, Joubert P, Wong V, Elliott M, van den Berge M, Brandsma CA, Tribouley C, Malkov V, Tsou JA, Opiteck GJ, Hogg JC, Sandford AJ, Timens W, Pare PD, Laviolette M. Molecular signature of smoking in human lung tissues. Cancer Res. 2012;72:3753-3763.
14. Low H, Hoang A, Sviridov D. Cholesterol efflux assay. J Vis Exp. 2012e3810.
15. Kallend DG, Reijers JA, Bellibas SE, Bobillier A, Kempen H, Burggraaf J, Moerland M, Wijngaard PL. A single infusion of MDCO-216 (ApoA-1 Milano/POPC) increases ABCA1-mediated cholesterol efflux and pre-beta 1 HDL in healthy volunteers and patients with stable coronary artery disease. Eur Heart J Cardiovasc Pharmacother. 2016;2:23-29.
16. Pratt SA, Finley TN, Smith MH, Ladman AJ. A comparison of alveolar macrophages and pulmonary surfactant(?) obtained from the lungs of human smokers and nonsmokers by endobronchial lavage. Anat Rec. 1969;163:497-507.
17. Finley TN, Ladman AJ. Low yield of pulmonary surfactant in cigarette smokers. N Engl J Med. 1972;286:223-227.
18. Thayaparan D, Shen P, Stämpfli MR, Morissette MC. Induction of pulmonary antibodies against oxidized lipids in mice exposed to cigarette smoke. Respir Res. 2016;17:97.

19. Kralova Lesna I, Poledne R, Pagacova L, Stavek P, Pitha J. HDL and apolipoprotein A1 concentrations as markers of cholesterol efflux in middle-aged women: interaction with smoking. Neuro Endocrinol Lett. 2012;33 Suppl 2:38-42.
20. Kim C, Lee JM, Park SW, Kim KS, Lee MW, Paik S, Jang AS, Kim DJ, Uh S, Kim Y, Park CS. Attenuation of Cigarette Smoke-Induced Emphysema in Mice by Apolipoprotein A-1 Overexpression. Am J Respir Cell Mol Biol. 2016;54:91-102.
21. Moore RE, Kawashiri MA, Kitajima K, Secreto A, Millar JS, Pratico D, Rader DJ. Apolipoprotein A-I deficiency results in markedly increased atherosclerosis in mice lacking the LDL receptor. Arterioscler Thromb Vasc Biol. 2003;23:1914-1920.
22. Moore RE, Navab M, Millar JS, Zimetti F, Hama S, Rothblat GH, Rader DJ. Increased atherosclerosis in mice lacking apolipoprotein A-I attributable to both impaired reverse cholesterol transport and increased inflammation. Circ Res. 2005;97:763-771.
23. Plump AS, Scott CJ, Breslow JL. Human apolipoprotein A-I gene expression increases high density lipoprotein and suppresses atherosclerosis in the apolipoprotein E-deficient mouse. Proc Natl Acad Sci U S A. 1994;91:9607-9611.
24. Pászty C, Maeda N, Verstuyft J, Rubin EM. Apolipoprotein AI transgene corrects apolipoprotein E deficiency-induced atherosclerosis in mice. J Clin Invest. 1994;94:899-903.
25. Rosenson RS, Brewer HB, Ansell BJ, Barter P, Chapman MJ, Heinecke JW, Kontush A, Tall AR, Webb NR. Dysfunctional HDL and atherosclerotic cardiovascular disease. Nat Rev Cardiol. 2016;13:48-60.
26. Domingo-Espín J, Lindahl M, Nilsson-Wolanin O, Cushman SW, Stenkula KG, Lagerstedt JO. Dual Actions of Apolipoprotein A-I on Glucose-Stimulated Insulin Secretion and Insulin-Independent Peripheral Tissue Glucose Uptake Lead to Increased Heart and Skeletal Muscle Glucose Disposal. Diabetes. 2016;65:1838-1848.
27. Cochran BJ, Ryder WJ, Parmar A, Tang S, Reilhac A, Arthur A, Charil A, Hamze H, Barter PJ, Kritharides L, Meikle SR, Gregoire MC, Rye KA. In vivo PET imaging with [(18)F]FDG to explain improved glucose uptake in an apolipoprotein A-I treated mouse model of diabetes. Diabetologia. 2016;59:1977-1984.
28. Song P, Kwon Y, Yea K, Moon HY, Yoon JH, Ghim J, Hyun H, Kim D, Koh A, Berggren PO, Suh PG, Ryu SH. Apolipoprotein a1 increases mitochondrial biogenesis through AMP-activated protein kinase. Cell Signal. 2015;27:1873-1881.

1.10 Figure legends

FIGURE 1. Cigarette smoking affects pulmonary expression levels of key genes involved in reverse lipid transport in both humans and mice. A) Pulmonary mRNA expression (microarray) of *ABCG1*, *ABCA1*, *SCARB1*, and *APOE* in human never smokers (NevSm), active smokers (ActSm), former smokers (FormSM) who stopped smoking for 5 years or less (≤5y), 6 to 10 years ago (6-10y) or over 10 years ago (>10y). B) Pulmonary mRNA expression (qPCR) of *abcg1*, *abca1*, *scarb1* and *apoe* in control room air-exposed BALB/c mice and BALB/c mice exposed to mainstream cigarette smoke for 2 weeks (2wks), 8 weeks (8wks), 24 weeks (24 wks), or 24 weeks followed by a 60 day cessation period (24 wks + 60 cess). *p<0.05; **0<0.01; ***p<0.001; ****p<0.0001.

FIGURE 2. Impact of cigarette smoke exposure on pulmonary and systemic reverse lipid export capacity. A) Female 6-8 weeks old BALB/c mice (n=5/group) were exposed to room air (RA) or mainstream cigarette smoke (CS) and sacrificed at three different timepoints for bronchoalveolar lavage (BAL) and serum collection. Cholesterol efflux assay was performed using B) BAL fluid and C) serum samples collected at timepoint A, B, and C. NS = not significant; *p<0.05; **0<0.01.

FIGURE 3. Deficiency in ApoA1 exacerbates the response to cigarette smoke. C57BL/6J and ApoA1-deficient (C57BL/6J background) mice (n=4-5/group) were exposed to room air or mainstream cigarette smoke for 8 weeks prior to sacrifice the day after the last exposure. A) Cholesterol efflux assay was performed on serum collected at sacrifice. B) BAL total cell counts, cell differentials (mononuclear cells and neutrophils), and C) macrophage size measurements were performed (the 166% value represents the 95th-percentile and 332% the 95th-percentile times 2). D) Mice were weighted on a weekly basis (Monday; weeks 0 to 7) and the day prior to sacrifice (week 8) along with E-F) body composition assessment by DEXA densitometry. NS = not significant; *p<0.05; **0<0.01.

FIGURE 4. Impacts of prophylactic and therapeutic MDCO-216 treatment on the pulmonary response to cigarette smoke. A) Human ApoA1 levels (surrogate for MDCO-216) were measured in the bronchoalveolar lavage (BAL) and serum of C57BL/6 mice (n=5/group) sacrificed 1, 6, and 24 hours after a single intraperitoneal injection of 20 and 100 mg/kg of MDCO-216. BALB/c mice (n=4-5/group)

were exposed to room air or mainstream cigarette smoke for 2 weeks, injected with vehicle, 20 or 100 mg/kg of MDCO-216 on weekdays and sacrificed 18h after the last exposure. B) Human ApoA1 levels were measured in the BAL fluid and serum, and C) BAL total cell numbers, differentials and D) macrophages size measurements were performed. BALB/c mice (n=9-10/group) were exposed to room air or mainstream cigarette smoke for 8 weeks, injected with vehicle or 100 mg/kg of MDCO-216 every second-day of weeks 5 to 8, and sacrificed 18h after the last exposure. E) BAL total cell numbers, differentials and F) macrophages size measurements were performed. NS = not significant; *p<0.05; **0<0.01; ****p<0.0001.

FIGURE 5. Therapeutic MDCO-216 treatment reduces aspects of cigarette smoke-induced lung function alterations. BALB/c mice (n=9-10/group) were exposed to room air or mainstream cigarette smoke for 8 weeks, injected with vehicle or 100 mg/kg of MDCO-216 every second-day of weeks 5 to 8, and sacrificed 18h after the last exposure. Lung mechanical properties were then assessed using FlexiVent. A) Average pressure-volume loops for each group. B) Inspiratory capacity. C) Hysteresis. D) Lung elastance. E) Lung compliance. NS = not significant; *p<0.05; **0<0.01; ***p<0.001;****p<0.0001.

FIGURE 6. Administration of MDCO-216 improves aspects of cigarette smoke-induced changes in body composition. BALB/c mice (n=9-10/group) were exposed to room air or mainstream cigarette smoke for 8 weeks, injected with vehicle or 100 mg/kg of MDCO-216 every second-day of weeks 5 to 8. Body composition was assessed by DEXA densitometry the day before the initiation of treatments and the day before sacrifice. A) Weekly weight measures. Variations between the end of the protocol and the day the treatments were initiated for mouse B) total weight, C) fat mass, and D) lean mass. NS = not significant; *p<0.05; **0<0.01; ****p<0.0001.

1.11 Figures

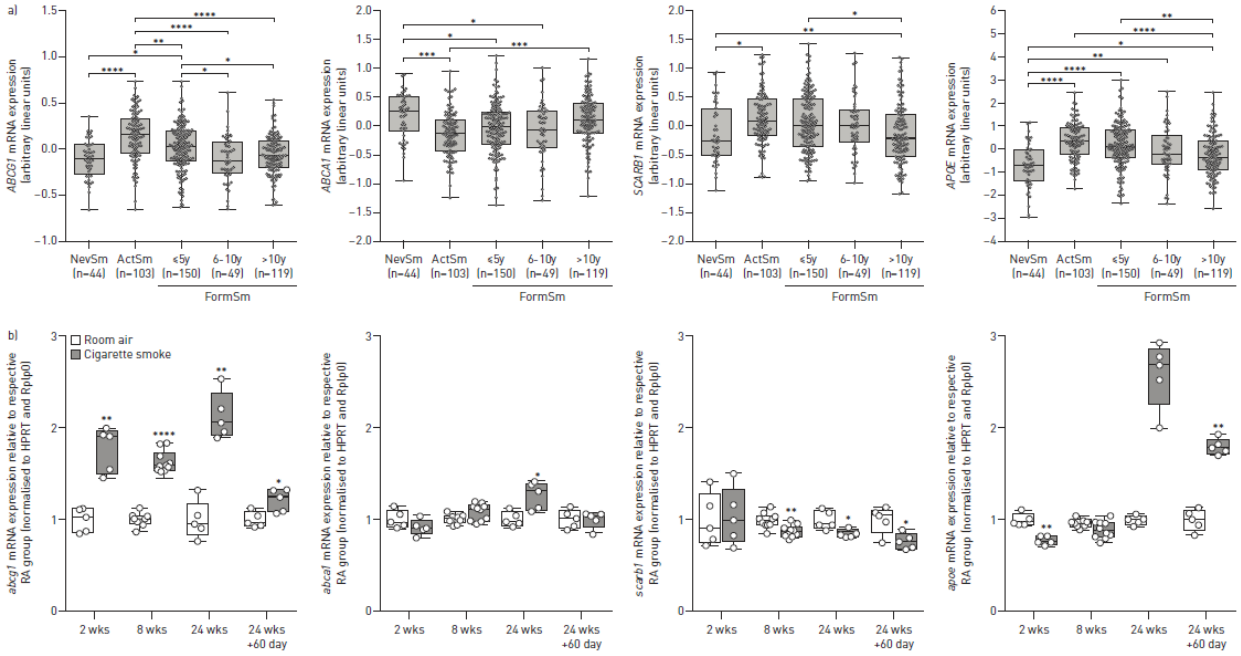

Figure 1. 1: Cigarette smoking affects pulmonary expression levels of key genes involved in reverse lipid transport in both humans and mice.

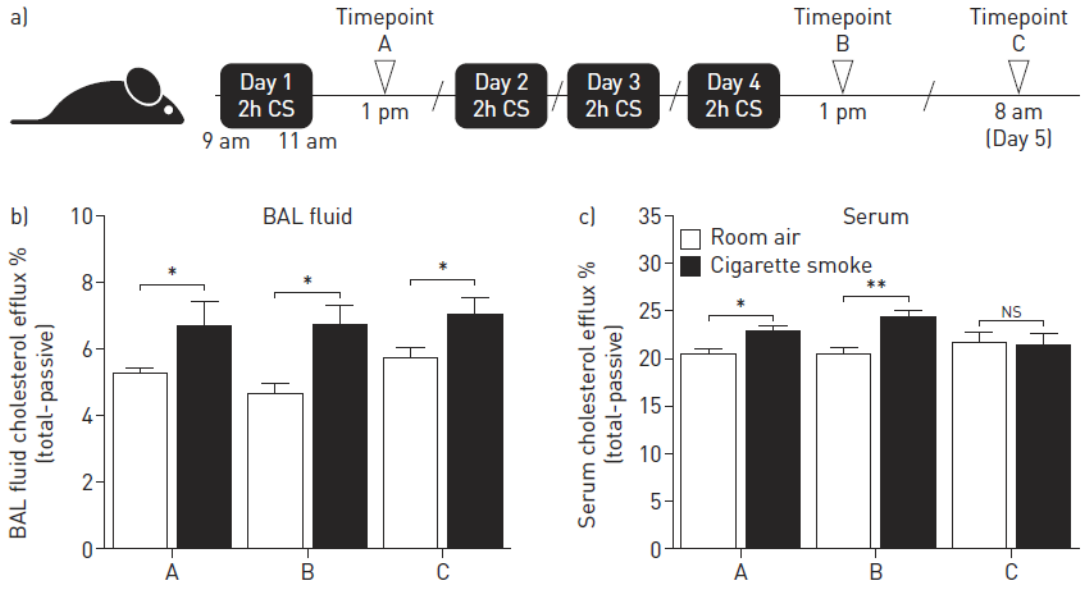

Figure 1. 2: Impact of cigarette smoke exposure on pulmonary and systemic reverse lipid export capacity.

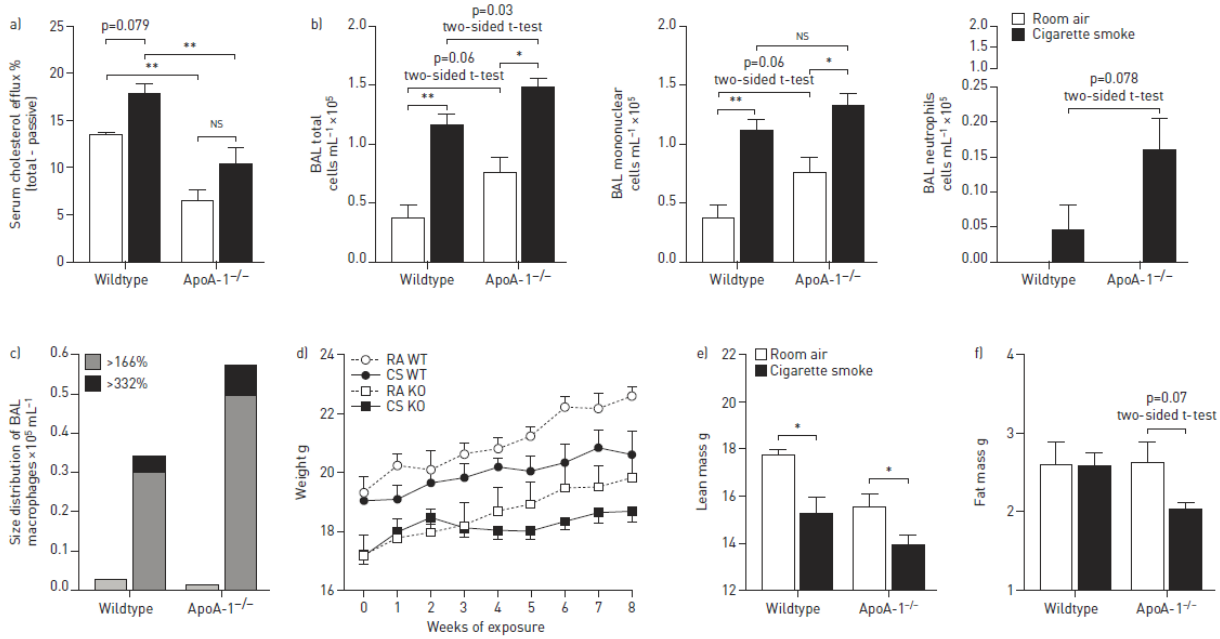

Figure 1. 3: Deficiency in ApoA1 exacerbates the response to cigarette smoke.

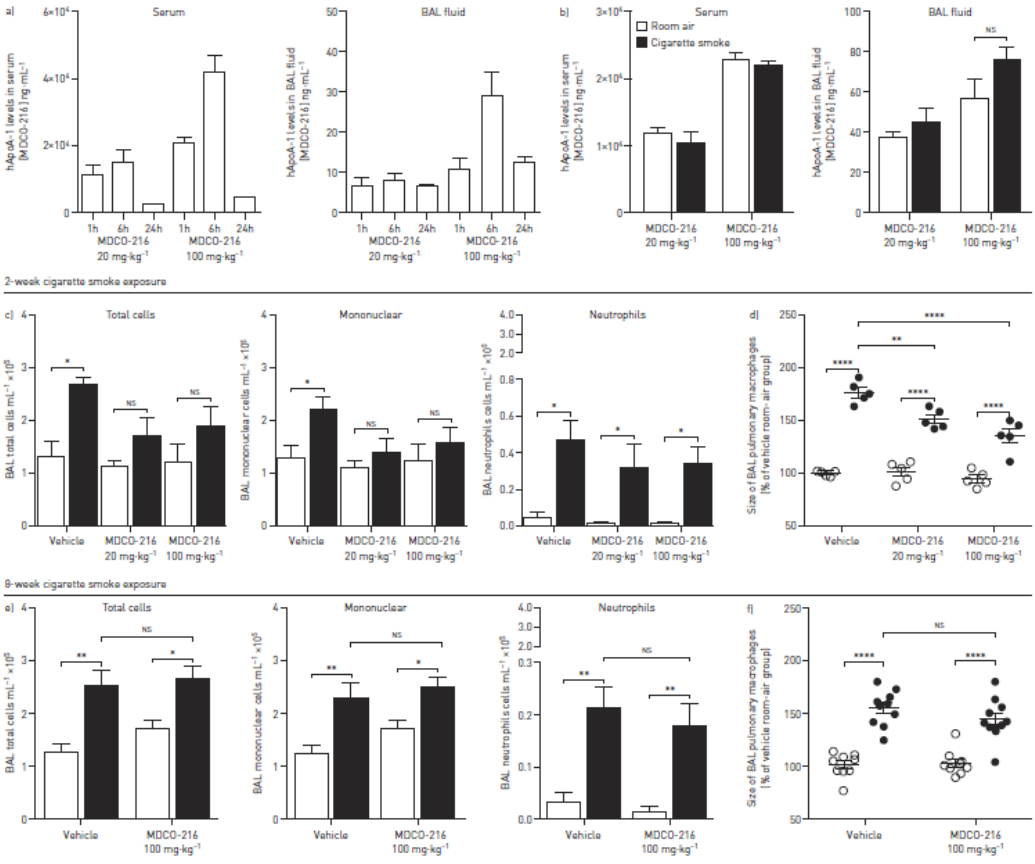

Figure 1. 4: Impacts of prophylactic and therapeutic MDCO-216 treatment on the pulmonary response to cigarette smoke.

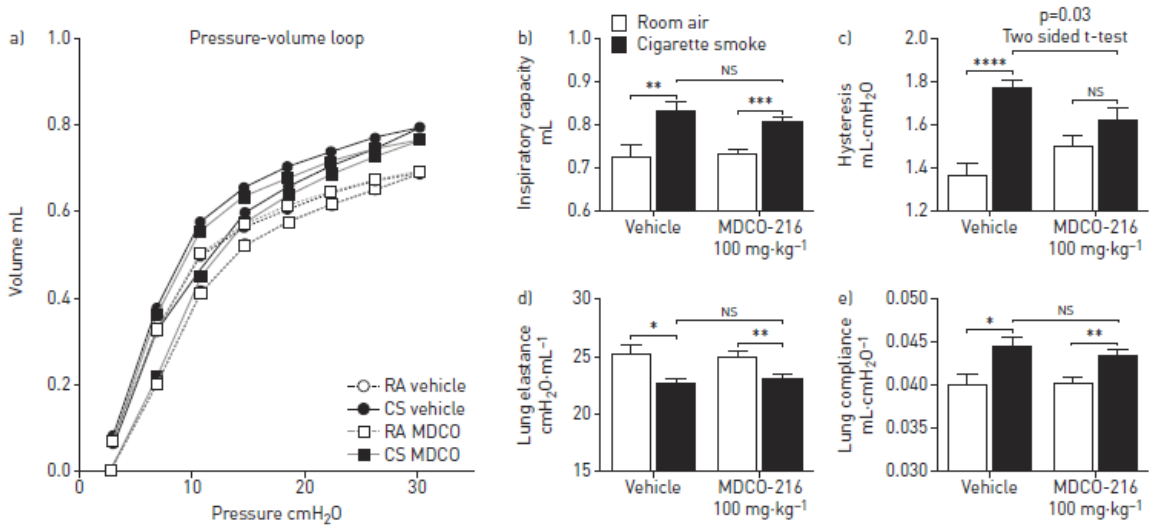

Figure 1. 5: Therapeutic MDCO-216 treatment reduces aspects of cigarette smoke-induced lung function alterations.

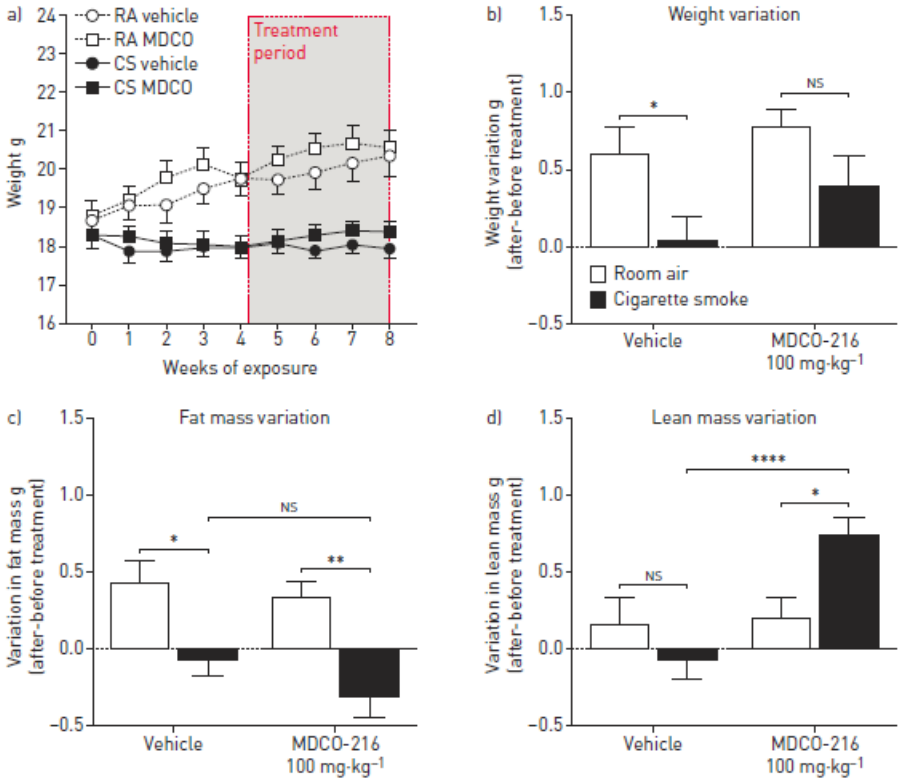

Figure 1. 6: Administration of MDCO-216 improves aspects of cigarette smoke-induced changes in body composition.

1.12 Supplementary data

1.12.1 Supplementary Methods

1.12.1.1 Measurement of antibodies against MDCO-216

Wells of a 96-well plate were coated overnight with 1 µg/100 µl of MDCO-216 in PBS. Wells were blocked with 200 µl of PBS-0.05% Tween + 1% BSA (Sigma-Aldrich, Oakville, ON, Canada) for 1 hour. Wells were incubated at room temperature with diluted serum (1:2000 in PBS-0.05% Tween + 1% BSA) for 2 hours. Wells were then washed 5 times with PBS-0.05% Tween. For detection, wells were incubated with the goat anti-mouse IgG+IgM+IgA H&L coupled to biotin (0.25 µg/ml in PBS-0.05% Tween + 1% BSA; AbCam, Toronto, ON, Canada) for 1 hour, washed, incubated with Streptavidin-HRP (1:40; R&D systems, Minneapolis, MN, USA) for 30 minutes, washed and incubated with TMB substrate reagent BD OptEIA™ (BD Biosciences, San Jose, CA, USA) for 20 minutes. The reaction was stopped after 30 minutes with 2N H_2SO_4 and the absorbance at 450 nm was read using Synergy H1 Hybrid Reader (BioTek, Winooski, VT, USA).

1.12.2 Supplementary Figures Legends

FIGURE S1. Impact of ApoA-1 deficiency and MDCO-216 administration on the pulmonary expression levels of key genes involved in reverse lipid transport in response to cigarette smoke exposure. A) Pulmonary mRNA levels (qPCR) of *abcg1*, *abca1*, *scarb1* and *apoe* were assessed in C57BL/6J and ApoA1-deficient (C57BL/6J background) mice (n=4-5/group) that were exposed to room air or mainstream cigarette smoke for 8 weeks prior to sacrifice the day after the last exposure. B) Pulmonary mRNA levels (qPCR) of *abcg1*, *abca1*, *scarb1* and *apoe* were assessed in BALB/c mice (n=9-10/group) that were exposed to room air or mainstream cigarette smoke for 8 weeks, injected with vehicle or 100 mg/kg of MDCO-216 every second-day of weeks 5 to 8, and sacrificed 18h after the last exposure. *p<0.05; **0<0.01; ***p<0.001.

FIGURE S2. Presence of antibodies against MDCO-216 in mice chronically injected with the compound. Total immunoglobulins against MDCO-216 were measured in the serum (diluted 1:2000)

of A) BALB/c mice (n=4-5/group) exposed to room air or mainstream cigarette smoke for 2 weeks, injected with vehicle, 20 or 100 mg/kg of MDCO-216 on weekdays and sacrificed 18h after the last exposure and B) BALB/c mice (n=9-10/group) exposed to room air or mainstream cigarette smoke for 8 weeks, injected with vehicle or 100 mg/kg of MDCO-216 every second-day of weeks 5 to 8, and sacrificed 18h after the last exposure.

1.12.3 Supplementary Figures

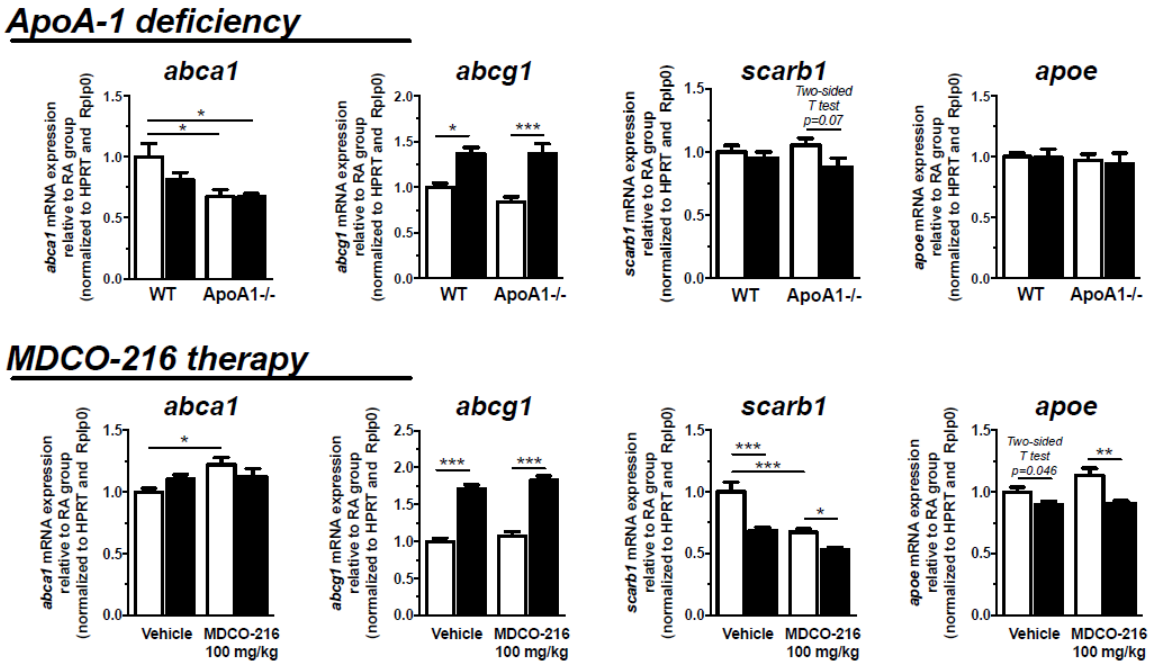

Figure 1. 7 Supplementary 1: Impact of ApoA-1 deficiency and MDCO-216 administration on the pulmonary expression levels of key genes involved in reverse lipid transport in response to cigarette smoke exposure.

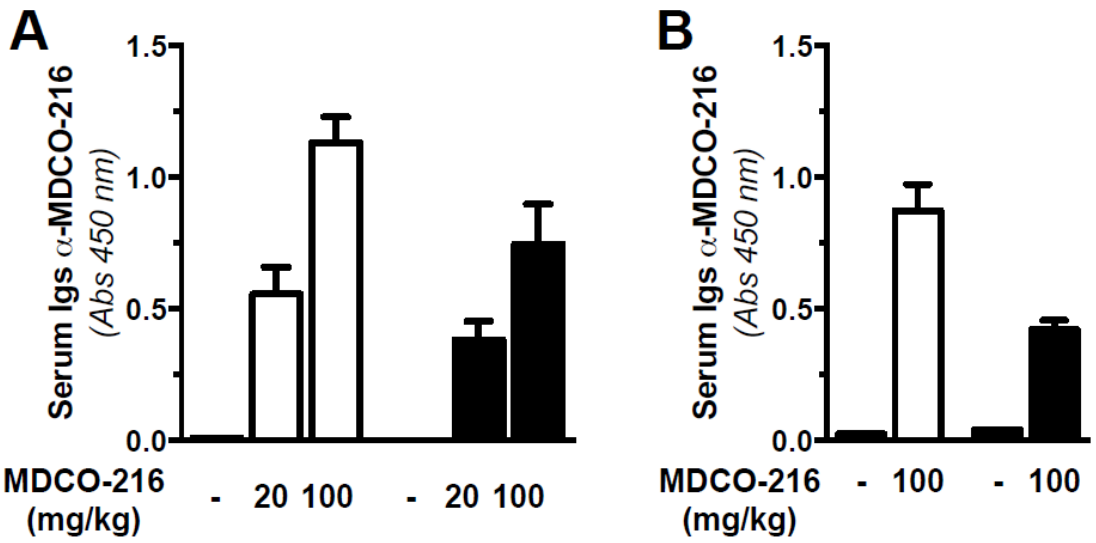

Figure 1. 8 Supplementary 2: Presence of antibodies against MDCO-216 in mice chronically injected with compound.

Chapter 2: Pharmacological activation of Liver X Receptor during cigarette smoke exposure adversely affects alveolar macrophages and pulmonary surfactant homeostasis.

2.1 Foreword

The scientific article presented in Chapter 2 “Pharmacological activation of Liver X Receptor during cigarette smoke exposure adversely affects alveolar macrophages and pulmonary surfactant homeostasis” was published in the American Journal of Physiology-Lung Cellular and Molecular Physiology in 2019 (PMID: 30702343). This study describes the impact of LXR hyperactivation during cigarette smoking. It shows that LXR activation leads to an increase in the lung and alveolar macrophage expression of key lipid transport genes. However, LXR activation during cigarette smoking exacerbates pulmonary inflammatory response, a phenomenon associated with a pro-inflammatory alveolar macrophage response to cigarette smoke. Furthermore, hyperactivation of LXR leads to pulmonary surfactant depletion under cigarette smoke exposure conditions.

2.1.1 Author contributions

I am the principal author of this article. I conceived and performed all experiments associated with the animal protocols, T0901317 administration, cholesterol efflux, qPCR, phosphatidylcholine measurements, ELISA assays, alveolar macrophage cell culture and Western Blot analyses. Joanie Routhier and Marie Pineault helped with alveolar macrophage cell culture, ELISA assays and with some animal work. Michaël Maranda-Robitaille helped with the first T0901317 protocols and qPCR analyses. Nadia Milad helped with phosphatidylcholine assays and revised the manuscript. Maude Talbot and Marie-Josée Beaulieu contributed to animal protocols. Sophie Aubin exposed the mice to cigarette smoke and Marie-Ève Paré was involved in Western Blot analyses. Mathieu Laplante helped with the interpretation of results concerning endoplasmic reticulum stress. Mathieu C Morissette also helped design the study and prepare the manuscript.

2.2 Résumé

Le tabagisme altère le transport inverse des lipides pulmonaires et mène à une accumulation intracellulaire de lipides dans les macrophages alvéolaires. Nous avons investigué si la stimulation du transport inverse des lipides avec un agoniste du récepteur nucléaire *liver X receptor* (LXR) pouvait réduire l'accumulation de lipides dans les macrophages alvéolaires et réduire la réponse inflammatoire pulmonaire à la fumée de cigarette.

Les souris ont été exposées à la fumée de cigarette et traitées avec des injections intraperitonéales d'un agoniste de LXR le T0901317. L'expression de gènes impliqués dans la capture et l'export lipidique a été mesurée dans le tissu pulmonaire et dans les macrophages alvéolaires. L'inflammation pulmonaire a été caractérisée dans le lavage bronchoalvéolaire (LBA). Finalement, la capacité d'efflux de cholestérol et les niveaux de surfactant pulmonaire ont été mesurés.

Chez les souris non exposées, le T0901317 a augmenté l'expression des gènes impliqués dans l'export lipidique au niveau du poumon et des macrophages alvéolaires. De plus, le T09013317 a augmenté la capacité d'efflux de cholestérol sans induire d'inflammation pulmonaire ni d'altérer la synthèse du surfactant pulmonaire. Cependant, les souris exposées à la fumée de cigarette et traitées au T0901317 ont une forte augmentation de neutrophiles, d'IL-1α, de CCL2 et de G-CSF dans leur LBA. Le traitement au T0901317 n'a pas augmenté la capacité d'efflux de cholestérol des macrophages alvéolaires, en plus d'induire une exacerbation de la sécrétion d'IL-1α chez les macrophages alvéolaires de souris exposées à la fumée de cigarette. Pour finir, le traitement au T0901317 a mené à une réduction de la quantité de surfactant pulmonaire chez les souris exposées à la fumée de cigarette.

Cette étude démontre qu'une hyperactivation de LXR et des mécanismes de capture et d'export lipidique n'a pas d'effet majeur lorsqu'il n'y a pas d'exposition à la fumée de cigarette. Cependant, en contexte tabagique et d'oxydation accrue du surfactant pulmonaire, l'hyperactivation de LXR induit des effets significatifs démontrant encore une fois le rôle central de l'homéostasie des lipides pulmonaires lors de la réponse pulmonaire à la fumée de cigarette.

2.3 Abstract

Smoking alters pulmonary reverse lipid transport and leads to intracellular lipid accumulation in alveolar macrophages. We investigated whether stimulating reverse lipid transport with an agonist of the Liver X receptor (LXR) would help alveolar macrophages limit lipid accumulation and dampen lung inflammation in response to cigarette smoke.

Mice were exposed to cigarette smoke and treated intraperitonealy with the LXR agonist T0901317. Expression of lipid capture and lipid export genes was assessed in lung tissue and alveolar macrophages. Pulmonary inflammation was assessed in the bronchoalveolar lavage (BAL). Finally, cholesterol efflux capacity and pulmonary surfactant levels were determined.

In room air-exposed mice, T0901317 increased the expression of lipid export genes in macrophages and the whole lung, and increased cholesterol efflux capacity without inducing inflammation or affecting the pulmonary surfactant. However, cigarette smoke-exposed mice treated with T0901317 showed a marked increase in BAL neutrophils, IL-1α, CCL2 and G-CSF levels. T0901317 treatment in cigarette smoke-exposed mice failed to increase the ability of alveolar macrophages to export cholesterol, and markedly exacerbated IL-1α release. Finally, T0901317 led to pulmonary surfactant depletion only in cigarette smoke-exposed mice.

This study shows that hyperactivation of LXR and the associated lipid capture/export mechanisms only have minor pulmonary effects on the normal lung. However, in the context of cigarette smoke exposure, where the pulmonary surfactant is constantly oxidized, hyperactivation of LXR has dramatic adverse effects; once again showing the central role of lipid homeostasis in the pulmonary response to cigarette smoke exposure.

Pharmacological activation of Liver X Receptor during cigarette smoke exposure adversely affects alveolar macrophages and pulmonary surfactant homeostasis

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Running head: LXR activation exacerbates the pulmonary response to smoking

Take home message: During acute cigarette smoke exposure in mice, pharmacological activation of LXR leads to pulmonary adverse effects, including increased neutrophilia, alveolar macrophage activation and pulmonary surfactant depletion.

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2.4 Introduction

The pulmonary immune response induced by exposure to cigarette smoke has been extensively characterized in humans and pre-clinical models; the inflammatory pathways involved in this response being deciphered with increasing precision (selected reviews(8, 22, 27)). Increased neutrophil infiltration and the presence of enlarged pigmented macrophages in sputum or bronchoalveolar lavage are early immune hallmarks of cigarette smoke exposure which develop well before any serious symptoms or sign of disease(2, 19, 20, 23, 35).

Lipid transport to and from the lungs is important for supporting the lipid species diversity required to maintain healthy lung homeostasis. Cells within the lungs express numerous genes involved in lipid transport, including, but not restricted to, lipid-scavenging genes *msr1*, *cd36* and *marco*, lipid uptake genes *ldlr* and *scarb1*, as well as lipid export genes *abca1*, *abcg1* and *apoe* (The Human Protein Atlas; www.proteinatlas.org). They are highly expressed by alveolar macrophages and/or alveolar type II cells, both of which are highly active in lipid transport to maintain pulmonary surfactant homeostasis(6, 18, 21, 30).

Lipid transport is regulated by transcription factors that include the liver X receptor or LXR(32). LXR is a nuclear receptor closely related to PPARs, FXR, and RXR, and both isoforms, LXRα and LXRβ, are expressed in human and mouse lungs(4, 9). Natural ligands for LXR are oxysterols, making it an important sensor and regulator of cholesterol levels by controlling the expression of lipid export genes *abca1*, *abcg1* and *apoe*, among other functions(32). Deletion of both LXRα and LXRβ in mice leads to massive lipid accumulation in alveolar macrophages(9), symptomatic of defective lipid export capabilities. Activation of LXR in alveolar macrophages is therefore critical for intracellular lipid clearance and maintenance of pulmonary lipid homeostasis.

Several publications by our group have recently highlighted the relationship between pulmonary lipid homeostasis processes and the response to cigarette smoke (13, 15–17, 31). In this study, we hypothesized that stimulating reverse lipid transport would help alveolar macrophages limit intracellular lipid accumulation and ultimately reduce lung inflammation in response to cigarette smoke

exposure. To test our hypothesis, we investigated the impact of promoting reverse lipid transport systemically with an LXR agonist on the pulmonary response to cigarette smoke, hoping it would improve maintenance of lipid homeostasis, reduce the intracellular lipid burden on macrophages, and dampen the early pulmonary immune response. We used a well-established acute mouse model of cigarette smoke exposure as well as the LXR agonist T0901317, an activator of both LXRα and β isoforms. In 'room air' conditions, we found that the LXR agonist T0901317 had the ability to stimulate transcription of lipid export genes *abca1*, *abcg1* and *apoe* in alveolar macrophages and in the whole lung with no obvious adverse effects. LXR activation had similar effects in mice exposed to cigarette smoke, especially in alveolar macrophages. However, LXR agonist was associated with a marked exacerbation of cigarette smoke-induced neutrophilia, largely attributable to an aggravated alveolar macrophage inflammatory phenotype. Finally, activation of LXR during cigarette smoke also led to pulmonary surfactant depletion.

2.5 Methods

2.5.1 Animals

Female 6- to 8-week-old C57BL/6 mice were purchased from Charles River Laboratories (Montréal, Canada). Mice were housed following recommendations from the Guide for the Care and Use of Laboratory Animals of the Canadian Council on Animal Care (CCAC). Animal protocols were approved by the Committee on the Ethics of Animal Experiments of Université Laval (#2017-101-1).

2.5.2 Cigarette smoke exposure and T0901317 administration

Mice were exposed every morning for 2 h and 4 consecutive days to room air or mainstream smoke from 3R4F reference cigarettes (University of Kentucky, Lexington, KY, USA) without filters, using a whole-body exposure system (SIU24; Promech Lab Holding AB, Vintrie, Sweden) as previously described (13). Stock solution of T0901317 (Cayman, Ann Arbour, MI, USA) was made in dimethyl sulfoxide (DMSO; Fischer Scientific, Fair Lawn, NJ, USA) and diluted in corn oil before being administered intraperitoneally (Sigma, USA) (20 $mg \cdot kg^{-1}$/day in 150µL). Sham mice received vehicle only: corn oil containing DMSO. All intraperitoneally injections were performed daily 2 h after cigarette smoke exposure.

2.5.3 Bronchoalveolar lavage and lung processing

Mice were anesthetised with isoflurane and euthanized by exsanguination. Lungs were removed from the thoracic cavity, the trachea was cannulated and the right lobes were tied off. Lungs were lavaged once with 250 µL of ice-cold PBS and a second time with 200 µL. Tied-off lobes were collected and immediately frozen in liquid nitrogen and kept at −80°C until RNA extraction. Total cell counts were performed on the bronchoalveolar lavage (BAL) using a hemocytometer. BAL was then centrifuged (800 g, 10 min, 4°C) and the BAL fluid kept at -80°C for phosphatidylcholine measurements, cholesterol efflux assay and ELISA assays. Cells were then re-suspended in PBS to perform cytospins. Cytospins were prepared and stained using the Hema 3 protocol (Fisher, USA) for differential cell counts. A total of 300 cells were counted per cytospin using the Image J software (v1.6; ImageJ, http://imagej.nih.gov/ij).

2.5.4 Bronchoalveolar lavage and alveolar macrophages culture

To recover and culture alveolar macrophages, lungs were lavaged twice with 500 µL of ice cold PBS and then three more times with 1 mL PBS to recover as many cells as possible. BAL was then centrifuged (800 g, 10 min, 4°C), re-suspended in RPMI, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S) (Wisent Bioproducts, St-Bruno, QC, Canada) and alveolar macrophages were isolated by adherence to a culture-treated 96-well plate (5×10^4 alveolar macrophages per well; 1 h at 37°C, 5% CO_2). Alveolar macrophages were then washed twice with PBS. Cells were incubated for 24 h at 37°C, 5% CO_2 in RPMI, 10% FBS, 1% P/S for secretion assays. Supernatants were then recovered and stored at -80°C for ELISA analyses. A portion of alveolar macrophages was also lysed directly after adherence isolation with 300 µL of RLT buffer (RNeasy Mini Kit; QIAGEN, Toronto, ON, Canada) containing 1% β-mercaptoethanol (Sigma, USA). Lysates were kept at −80°C until RNA extraction.

2.5.5 Alveolar macrophage size

Alveolar macrophage size was measured using the ImageJ software (v1.6; ImageJ, http://imagej.nih.gov/ij) as previously described (13). Briefly, 30 macrophages per cytospin per mouse were randomly selected using the grid plugin and the surface area was measured. Macrophage size was normalized to mean area of room air-exposed mice.

2.5.6 Serum sampling and processing

Prior to euthanasia under isoflurane, orbital blood was recovered. Blood samples were then centrifuged (12000 g, 4°C) and serum was stored at −80°C until ELISA or cholesterol efflux analysis.

2.5.7 Mouse IL-1α, CCL2 and G-CSF ELISA

Mouse IL-1α, CCL2 and G-CSF levels were assessed in mouse BAL fluid and/or alveolar macrophage culture supernatants using the mouse CCL2 and IL-1α DuoSet® ELISA kits (R&D systems, Minneapolis, MN, USA) and the mouse G-CSF ELISA kit (Abcam, Cambridge, MA, USA) according to manufacturer's instructions.

2.5.8 Lungs and alveolar macrophages RNA extractions and cDNA synthesis

Lung RNA was extracted from 1 frozen lobe using Trizol (Fisher, USA) and from the alveolar macrophages using the QIAGEN RNeasy Mini Kit (QIAGEN, Canada). Lung RNA was re-suspended in 50 µL of Tris-EDTA (Fisher, USA) and quantified using the BioTek Synergy H1 (BioTek, Winooski, VT, USA). Lung RNA integrity and 28S/16S ratios were analyzed on agarose gels before cDNA synthesis. Optional centrifugation was performed with the RNeasy Kit and RNA was eluted twice with 40 µL of RNase-free water (provided with the kit).

Lung cDNA was prepared with 1 µg of total RNA and alveolar macrophage cDNA was prepared with 10-15 µL of RNA using the iScript cDNA synthesis kit following manufacturer's instructions (Bio-Rad, Mississauga, ON, Canada).

2.5.9 Quantitative PCR (qPCR) gene expression of lung tissue and alveolar macrophages

Lung and alveolar macrophage gene expression was evaluated with the SsoAdvanced universal SYBR green Supermix (Bio-Rad, Canada). Gene-specific primers were used to amplify *abca1*, *abcg1*, *apoe*, *scarb1*, *msr1*, *marco*, *cd36*, *il-1α*, *cxcl5*, *mmp12*, *grp78*, *atf6*, *ddit3* and two reference genes; *rplp0* and *hprt* (IDT, Skokie, IL, USA). The primers and qPCR conditions used for amplification are described in Table 1. Each qPCR reaction volume was composed of: 10 µL of SsoAdvanced universal SYBR green Supermix 2X, 10 µM forward primer (0.6 µL), 10 µM reverse primer (0.6 µL), 5 µL of cDNA template, and RNAse-free water (GE Healthcare, South Logan, UT, USA) up to the final volume of 20 µL. The thermo-protocol was as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 56-60°C for 30 s. All samples were analyzed in duplicate and RNase-free water (GE Healthscare, USA) was used as a no template control. Reactions were manually loaded into 0.1 mL PCR tube strips (Ultident, St. Laurent, QC, Canada). Amplifications were performed using a Rotor-Gene 6000 series (QIAGEN, Canada) and data acquired/analyzed with the on-board Rotor-Gene series Software version 1.7. Before qPCR assays, thermal gradients and efficiencies were performed. All qPCR efficiencies were between 90-110%, with R^2 values ranging between 0.96-0.99. Melt curves and qPCR products on agarose gels were analyzed for specificity and no template controls were detected passed 35 Cq. Threshold from the efficiency assays were used to determine the Cq and the ΔΔCq method was used to normalize our qPCR results.

2.5.10 Measurement of phosphatidylcholine in the bronchoalveolar lavage fluid and serum

Serum and bronchoalveolar lavage fluid phosphatidylcholine levels were measured using the Phosphatidylcholine Colorimetric Assay Kit according to manufacturer's instructions (Cayman, USA).

2.5.11 Bronchoalveolar lavage fluid SP-B Western blot analysis

20 µL (15 µL of BAL fluid + 5µL Laemmli 4X Sample buffer (Bio-Rad, Canada)) was subjected to 15% SDS-Page and transferred to a nitrocellulose membrane. Western blotting was performed with the anti-pro + mature surfactant protein B antibody (ab408076; Abcam, Cambridge, MA, USA) diluted 1/200. Goat anti-rabbit secondary antibody was diluted 1/2000 (IRDYE 800CW 926-32211, LI-COR, Lincoln, NE, USA). Imaging analysis was performed using the Odyssey (LI-COR, USA) with the Image Studio V5.2 (LI-COR, USA).

2.5.12 *In vitro* and *ex vivo* cholesterol efflux assay

Cholesterol efflux assay was performed as previously described (13). Briefly, J774A.1 macrophages (ATCC TIB-67) were grown to 70-75% confluence in 48-well plates. Cells were then loaded with 3H-cholesterol for 18h (0.5 µCi per well; PerkinElmer, Waltham, MA, USA) in DMEM containing 1% FBS. On the next day, cells were washed with warm PBS and exposed to polyethylene glycol-treated mouse serum (2.8% v/v final) or BALF for 4 h. Percentage of cholesterol efflux was then calculated, ratio of: supernatant/(supernatant+lysate).

Cholesterol efflux assay was also performed with alveolar macrophages isolated from C57BL/6 mice. Cells were isolated by adherence in RPMI (2×10^5 cells/well; 12-well plate). Cells were then loaded for 5 h with 0.5 µCu ^{3}H-cholesterol in RPMI containing 1% FBS. The assay was then performed as previously described in RPMI.

2.5.13 Statistical analysis

Statistical analyses were performed using GraphPad Prism Software V. 6 (La Jolla, CA, USA). Two-group comparisons were made using an unpaired t-test ($p \leq 0.05$). Experimental protocols with more than two groups were compared using a one-way ANOVA ($p \leq 0.05$) followed by a Bonferroni multiple comparisons post-hoc test. Two-group unpaired t-tests were also performed in protocols with more than two groups when indicated.

2.6 Results

2.6.1 Pharmacological activation of LXR modulates the expression of key lipid transport genes in alveolar macrophages and lung tissue

To investigate the impact of LXR activation on key lipid transport gene expression, C57BL/6 mice were treated with T0901317 (20 mg/kg) for four consecutive days. Alveolar macrophages from *in vivo*-treated mice had a different expression profile compared to *ex vivo*-treated alveolar macrophages, showing a marked increase in *apoe* levels as well as *abca1*, *abcg1*, *scarb1*, *msr1* and *il-1α* levels (Figure 1A). Finally, whole lung tissue showed similarities with alveolar macrophages, except for reduced *marco* expression (Figure 1B). While markedly affecting expression of lipid transport gene and increasing the ability of alveolar macrophages to export cholesterol *ex vivo* (Figure 1C), T0901317 did not induce any pulmonary immune response, as no increase in total cell number, mononuclear cells or neutrophils was observed (Figure 1D). BALF IL-1α, CCL2 and G-CSF levels were also not affected (Figure 1E).

2.6.2 Pharmacological activation of LXR promotes expression of lipid transport genes during acute cigarette smoke exposure

We previously showed that lung tissue expression of pulmonary lipid transport genes was rapidly and, for some genes, negatively affected by acute cigarette smoke exposure (13). To determine whether LXR activation could overcome the effects of cigarette smoke exposure on the expression levels of lipid transport genes, we treated cigarette smoke-exposed mice with T0901317 and assessed lung tissue and alveolar macrophage expression of lipid export genes *abca1*, *abcg1*, *apoe*, *scarb1* and lipid capture genes *msr1*, *marco* and *cd36*. Acute cigarette smoke exposure led to decreased lung tissue mRNA levels of *abca1*, *apoe* and *cd36* while increasing *abcg1*, *msr1* and *marco* expression (Figure 2A). We show a similar expression pattern in alveolar macrophages from cigarette smoke-exposed mice, except for *abcg1* and *scarb1* while *cd36* expression is unchanged during acute cigarette smoke exposure (Figure 2B). LXR activation with T0901317 increased the expression of *abca1*, *abcg1*, *apoe*, *scarb1*, and *msr1* in the lung and in alveolar macrophages, while *marco* and *cd36* expression were either decreased or unchanged (Figure 2A-B). Therefore, cigarette smoke exposure does not prevent LXR activation by T0901317.

2.6.3 LXR activation exacerbates the pulmonary immune response to acute cigarette smoke exposure

To investigate the impact of LXR activation on lung inflammation in cigarette smoke-exposed mice, we analyzed BAL total and differential cell counts. We also quantified BAL fluid protein levels of IL-1α, a cytokine required for smoke-induced neutrophilia (5, 17), CCL2, a chemokine rapidly induced by smoking (16), and G-CSF, an important myeloid/granulocyte growth factor(10). Finally, we assessed lung mRNA levels of *mmp12*, a key metalloproteinase in smoking-induced lung pathologies (7), and *cxcl5*, an important neutrophil chemoattractant (1). Although we showed that T0901317 causes no lung inflammation on its own (Figure 1), it markedly affected the inflammatory response to cigarette smoke. While numbers of mononuclear cells were reduced (Figure 3A), macrophage-associated mediators such as IL-1α, CCL2, G-CSF and *mmp12* were markedly induced by T0901317 in BAL fluid and lung tissue (Figure 3B-C). Cigarette smoke-induced lung neutrophilia was exacerbated in T0901317-treated mice (Figure 3A), possibly explained by the marked increase in IL-1α levels and *cxcl5* expression (Figure 3B-C). Therefore, the effects of T0901317 exhibited an environment-specific phenotype, only exacerbating pulmonary inflammation in a cigarette smoke exposure context.

2.6.4 LXR activation affects alveolar macrophages during cigarette smoke exposure

As shown in Figure 3, reduced numbers of mononuclear cells, increased BAL IL-1α and CCL2 levels and elevated lung tissue *mmp12* expression suggest a significant involvement of alveolar macrophages in cigarette smoke-exposed, T0901317-treated mice. To specifically investigate the impact of LXR activation on alveolar macrophages during cigarette smoke exposure, we measured BAL alveolar macrophages size, *il-1α* mRNA levels and *ex vivo* secretion levels, *mmp12* expression as well as expression of the endoplasmic reticulum (ER) stress-associated genes *grp78*, *atf6,* and *ddit3*. We found that mean alveolar macrophage size was significantly increased in cigarette smoke-exposed mice, but not room air-exposed mice treated with T0901317. While increasing cholesterol efflux in alveolar macrophages from room air-exposed mice *ex vivo*, T0901317 failed to have the same effect in alveolar macrophages from cigarette smoke-exposed (Figure 4A). Interestingly, a further increase in alveolar macrophage size was observed in cigarette smoke-exposed, T0901317-treated mice (Figure 4B), suggesting additional intracellular lipid accumulation. T0901317 treatment caused a marked increase in *il-1α* mRNA levels (Figure 4C) and *ex vivo* IL-1α secretion (Figure 4D) in alveolar

macrophages from cigarette smoke-exposed mice. T0901317 also caused a drastic increase in *mmp12* expression in alveolar macrophages from cigarette smoke-exposed mice (Figure 4E) compared to alveolar macrophages from cigarette smoke-exposed vehicle-treated mice. Regarding ER stress, T0901317 on its own had no effect on *grp78*, *atf6* and *ddit3* mRNA expression (Figure 4F). However, it exacerbated the effects of cigarette smoke exposure, further expressing the expression of ER stress-associated genes in alveolar macrophages (Figure 4F) and suggesting LXR activation leads to ER stress during cigarette smoke exposure, but not during normal conditions. Altogether, T0901317 administration in cigarette smoke-exposed mice led to increased levels of inflammatory and disease-associated mediators as well as elevated ER-stress markers in alveolar macrophages without improving their lipid export capacity *ex vivo*.

2.6.5 LXR activation induces pulmonary surfactant depletion during cigarette smoke exposure

Systemic activation of LXR is known to promote reverse lipid transport from the periphery to the liver. We wanted to investigate in room air- and cigarette smoke-exposed mice the impact of T0901317 on pulmonary and systemic cholesterol efflux capacity as well as phosphatidylcholine levels, a critical constituent of lipoproteins and pulmonary surfactant. As expected, T0901317 treatment led to increased serum cholesterol efflux capacity (Figure 5A) and also increased circulating levels of phosphatidylcholine (Figure 5B) in both room air and cigarette smoke-exposed mice. In the lungs, cholesterol efflux capacity of BAL fluid was only increased in T0901317-treated, cigarette smoke-exposed mice, again showing a cigarette smoke-specific effect of T0901317 (Figure 5C). Interestingly, BAL fluid phosphatidylcholine levels were drastically reduced in T0901317-treated, cigarette smoke-exposed mice (Figure 5D). In BAL fluid, phosphatidylcholine levels are largely representative of the amount of pulmonary surfactant. T0901317 alone slightly but significantly affected the expression of surfactant protein genes *sftpa*, *sftpb* and *sftpc* as well as the lipid transporter *abca3* (Figure 5E), which is important for pulmonary surfactant packaging by alveolar type II cells(3). In cigarette smoke-exposed mice, T0901317 caused a significant decrease in *sftpb, sftpc* and *abca3* expression levels (Figure 5F). Reduction in BAL fluid surfactant B protein levels was further confirmed by Western blot in T0901317-treated, cigarette smoke-exposed mice (Figure 5G). Altogether, these data suggest that T0901317 administration leads to a depletion in pulmonary surfactant only in cigarette smoke-exposed mice.

2.7 Discussion

LXR is an important regulator of pulmonary lipid homeostasis, where mice deficient in LXRα and LXRβ exhibit significant cholesterol accumulation in the lungs(9). In several macrophage subsets, LXR is known to stimulate lipid export, notably by increasing *abca1*, *abcg1* and *apoe* expression (32). Cigarette smoke exposure leads to intracellular lipid accumulation in alveolar macrophages, initiating an inflammatory cascade that can become chronic and ultimately damaging to the lungs. Therefore, we investigated the impact of systemic pharmacological LXR activation on the pulmonary response to acute cigarette smoke exposure, expecting that it would improve pulmonary lipid homeostasis and reduce the inflammatory response.

As anticipated, systemic pharmacological LXR activation led to increased expression of lipid export genes in alveolar macrophages and lung tissue of both room air-exposed and cigarette smoke-exposed mice. Increased *abca1* mRNA levels in bone marrow-derived macrophages treated with T0901317 has been previously reported (26). However, T0901317 failed to alleviate the alveolar macrophage phenotype typically induced by cigarette smoke exposure, and in fact exacerbated the pulmonary inflammatory response to cigarette smoke. This is contradictory to the effects of LXR agonists on inflammatory stimuli such as lipopolysaccharides (LPS) on the lungs and alveolar macrophages (12, 25), suggesting the inflammation caused by smoking and microbial components are affected in opposite direction by LXR activation. The exacerbated inflammation observed during cigarette smoke exposure is potentially caused by the increased intracellular lipid burden induced by LXR activation. While the impact of T0901317 on *abca1*, *abcg1* and *apoe* mRNA levels is similar in alveolar macrophages isolated from room air-exposed and cigarette smoke-exposed mice, levels of lipid capture genes such as *msr1* and *marco* were higher in alveolar macrophages from cigarette smoke-exposed mice. This imbalance between import and export capacity could in part explain why alveolar macrophages from T0901317-treated, cigarette smoke-exposed mice are larger. Alveolar macrophages are very important in eliminating damaged pulmonary surfactant, which mainly contains phospholipids and cholesterol. Exposure to room air does not induce significant damage to the pulmonary surfactant, which would require elimination by alveolar macrophages, keeping alveolar macrophages within a homeostatic range without the need for an inflammatory response. However, cigarette smoke exposure is well known to cause lipid oxidation. Therefore, in the context of cigarette

smoke exposure, the amount of damaged lipids available for capture by alveolar macrophages is markedly increased. Hyperactivation of LXR appears to cause alveolar macrophages to capture an excessive amount of damaged lipids, significantly exacerbating the imbalance between lipid import and export.

We observed that LXR activation led to a reduced number of mononuclear cells in the BAL of cigarette smoke-exposed mice. This has also been documented by Sonett *et al.*(26). However, we found that alveolar macrophages were more activated, larger and released more IL-1α and CCL2. We also found that LXR activation during cigarette smoke exposure increased mRNA levels of *grp78* and especially *ddit3* in alveolar macrophages, two important markers of endoplasmic reticulum (ER) stress. CHOP (*ddit3)* is known to induce apoptosis during ER stress and intracellular accumulation of lipids is well established to be an ER stressor (24, 29, 33). Moreover, IL-1α is released by macrophages under ER stress(14). Therefore, it is possible that the reduced number of mononuclear cells caused by the pharmacological activation of LXR during cigarette smoke exposure would in part cause ER stress-mediated apoptosis in alveolar macrophages, exacerbating the release of IL-1α and the related inflammatory cascade. ER stress has been investigated in alveolar macrophages *in vitro* using cigarette smoke extract(11). However, this approach completely removes the pulmonary surfactant dimension from alveolar macrophage biology. Our data strongly suggest that the increased ER stress caused by excessive intracellular lipid accumulation in alveolar macrophages can exacerbate the inflammatory response to cigarette smoke. Further investigations are required to fully dissect the underlying pathways.

Systemic pharmacological activation of LXR led to increased cholesterol efflux capacity as well as elevated phosphatidylcholine levels in the circulation. This is in accordance with previous publications on LXR agonists(28, 34). We also observed a significant increase in BAL fluid cholesterol efflux capacity, although the reason for this requires further study. Interestingly, LXR activation caused a marked reduction in BAL fluid phosphatidylcholine levels, a phenomenon not observed in room air-exposed mice. In the BAL fluid, phosphatidylcholine levels are representative of the pulmonary surfactant. Looking at the surfactant proteins at the mRNA and protein levels, we also noticed that LXR activation reduced surfactant protein levels, most notably SP-B, suggesting local pulmonary surfactant depletion. This is a previously unreported phenomenon. The effects of LXR activation on alveolar

macrophages and alveolar type II cells could in part explain this phenomenon. LXR activation appears to prime alveolar macrophages for additional lipid uptake in cigarette smoke-exposed mice, leading to increased *msr1* levels increased size. Therefore, the reduction in BAL fluid surfactant levels may be due to an increase in surfactant internalization by alveolar macrophages. Moreover, pharmacological LXR activation during cigarette smoke exposure led to increased *abcg1* mRNA levels in the lung tissue, not in alveolar macrophages, and decreased *abca3* mRNA levels in the lung tissue. ABCG1 is an important lipid transporter involved in cellular lipid efflux and ABCA3 is crucial to alveolar type II cells formation of intracellular lamellar bodies secreted as pulmonary surfactant(3, 6). It is therefore possible that pharmacological LXR activation in alveolar type II cells promotes a reduction in pulmonary surfactant release. Altogether, the impact of LXR hyperactivation on alveolar macrophages and alveolar type II cells could have a detrimental impact on pulmonary surfactant homeostasis.

This study adds to the increasing body of literature supporting a strong involvement of pulmonary lipid homeostasis in the response to cigarette smoke. While our data suggest that pharmacological activation of LXR could be detrimental in active smokers, it is possible that former smokers may behave differently. Further investigation is required to fully decipher the reason behind LXR's adverse effects during acute cigarette smoke exposure.

2.8 Table

TABLE 1. Primers for quantitative PCR analysis

Genes	Sequence	Gene Symbol	Accession number	Amplicon Length (bp)	Annealing temperature (°C)
rplp0	ATC ACA GAG CAG GCC CTG CA	RPLP0	NM_0074475.5	96	56
	CAC CGA GGC AAC AGT TGG GT				
hprt	AGC AGG TCA GCA AAG AAC T	HPRT1	NM_013556	125	57
	CCT CAT GGA CTG ATT ATG GAC A				
abca1	CCA TAC CGA AAC TCG TTC ACC	ABCA1	NM_013454	121	56
	CCG CAG ACA TCC TTC AGA ATC				
abcg1	ACT GTT CTG ATC CCC GTA CT	ABCG1	NM_009593	113	59
	TCT CCA ATC TCG TGC CGT A				
apoe	GTCATAGTGTCCTCCATCAGTG	APOE	NM_009696	144	57
	AACCGCTTCTGGGATTACC				
scarb1	CTT GCT GAG TCC GTT CCA TT	SCARB1	NM_3iso[1]	148	59
	GTA CCT CCC AGA CAT GCT TC				
msr1	GCA CGT TCA ATG ACA GCATC	MSR1	NM_001113326	108	60
	CAC AAG GAG GTA GAG AGC AAT G				
marco	ATT GTC CAG CCA GAT GTT CC	MARCO	NM_010766.2	95	57
	ATG ATG CGA CTG TCT TCT GTC				
cd36	ACT GTA CAT CTT ATG GTG TGC T	CD36	NM_001159555	105	57
	GTT CTG AAA CAT CTG GAC TTG C				
mmp12	GCT CCT GCC TCA CAT CAT AC	MMP12	NM_008610	112	59
	GGC TTC TCT GCA TCT GTG AA				
il-1α	ACA AAC TTC TGC CTG ACG AG	IL1A	NM_010554.4	113	60
	CTG CAG TCC ATA ACC CAT GA				
cxcl5	TTC TGT TGC TGT TCA CGC T	CXCL5	NM_009141	139	59
	ATC ACC TCC AAA TTA GCG ATC A				
hspa5	ACTTCAATGATGCCCAGCGA	HSPA5	NM_001163434.1	362	60
	AGCCTTTTCTACCTCACGCC				
atf6	GAACTTCGAGGCTGGGTTCA	ATF6	NM_001081304.1	204	60
	TCCAGGGGAGGCGTAATACA				
ddit3	TGCAGATCCTCATACCAGGC	DDIT3	NM_007837.4; NM_001290183.1	176	60
	CCAGAATAACAGCCGGAACCT				
sftpa	AGACTTTATCCCCCACTGACA	SFTPA1	NM_023134	102	56
	AGGAGCTTCAGACTGCACT				
sftpb	GTGCTGTTCCACAAACTGTTC	SFTPB	NM_147779	125	56
	TCTGCAAGTCTGTGATCAACC				
sftpc	ACCACAACCACGATGAGAAG	SFTPC	NM_011359	123	56
	GGACATGAGTAGCAAAGAGGT				
sftpd	GCTCTTCATTTCTGCTCCCA	SFTPD	NM_009160	146	56
	CATAAATAGCCGAGCCTGACA				
abca3	GTACGTGGAGGCTGTCTTC	ABCA3	NM_001039581	107	56
	CCTTCCCTACGACAGTTCTTG				

[1]NM_001205082, NM_001205083, NM_016741

2.9 References

1. Balamayooran G., Batra S., Cai S., Mei J., Worthen G. S., Penn A. L., Jeyaseelan S. Role of CXCL5 in Leukocyte Recruitment to the Lungs During Secondhand Smoke Exposure. *Am J Respir Cell Mol Biol* 2012.
2. Balter M. S., Toews G. B., Peters-Golden M. Multiple defects in arachidonate metabolism in alveolar macrophages from young asymptomatic smokers. *J Lab Clin Med* 114: 662-673, 1989.
3. Beers M. F., Mulugeta S. The biology of the ABCA3 lipid transporter in lung health and disease. *Cell Tissue Res* 367: 481-493, 2017.
4. Birrell M. A., Catley M. C., Hardaker E., Wong S., Willson T. M., Mccluskie K., Leonard T., Farrow S. N., Collins J. L., Haj-Yahia S., Belvisi M. G. Novel role for the liver X nuclear receptor in the suppression of lung inflammatory responses. *J Biol Chem* 282: 31882-31890, 2007.
5. Botelho F. M., Bauer C. M., Finch D., Nikota J. K., Zavitz C. C., Kelly A., Lambert K. N., Piper S., Foster M. L., Goldring J. J., Wedzicha J. A., Bassett J., Bramson J., Iwakura Y., Sleeman M., Kolbeck R., Coyle A. J., Humbles A. A., Stampfli M. R. IL-1alpha/IL-1R1 expression in chronic obstructive pulmonary disease and mechanistic relevance to smoke-induced neutrophilia in mice. *PLoS One* 6: e28457, 2011.
6. Chai A. B., Ammit A. J., Gelissen I. C. Examining the role of ABC lipid transporters in pulmonary lipid homeostasis and inflammation. *Respir Res* 18: 41, 2017.
7. Churg A., Wang R. D., Tai H., Wang X., Xie C., Dai J., Shapiro S. D., Wright J. L. Macrophage metalloelastase mediates acute cigarette smoke-induced inflammation via tumor necrosis factor-alpha release. *Am J Respir Crit Care Med* 167: 1083-1089, 2003.
8. Crotty Alexander L. E., Shin S., Hwang J. H. Inflammatory Diseases of the Lung Induced by Conventional Cigarette Smoke: A Review. *Chest* 148: 1307-1322, 2015.
9. Dai Y. B., Miao Y. F., Wu W. F., Li Y., D'errico F., Su W., Burns A. R., Huang B., Maneix L., Warner M., Gustafsson J. Å. Ablation of Liver X receptors α and β leads to spontaneous peripheral squamous cell lung cancer in mice. *Proc Natl Acad Sci U S A* 113: 7614-7619, 2016.
10. Furze R. C., Rankin S. M. Neutrophil mobilization and clearance in the bone marrow. *Immunology* 125: 281-288, 2008.
11. Geraghty P., Wallace A., D'armiento J. M. Induction of the unfolded protein response by cigarette smoke is primarily an activating transcription factor 4-C/EBP homologous protein mediated process. *Int J Chron Obstruct Pulmon Dis* 6: 309-319, 2011.
12. Higham A., Lea S., Plumb J., Maschera B., Simpson K., Ray D., Singh D. The role of the liver X receptor in chronic obstructive pulmonary disease. *Respir Res* 14: 106, 2013.
13. Jubinville É., Talbot M., Bérubé J. C., Hamel-Auger M., Maranda-Robitaille M., Beaulieu M. J., Aubin S., Paré M. È., Kallend D. G., Arsenault B., Bossé Y., Morissette M. C. Interplay between cigarette smoking and pulmonary reverse lipid transport. *Eur Respir J* 50: 2017.
14. Kandel-Kfir M., Almog T., Shaish A., Shlomai G., Anafi L., Avivi C., Barshack I., Grosskopf I., Harats D., Kamari Y. Interleukin-1α deficiency attenuates endoplasmic reticulum stress-induced liver damage and CHOP expression in mice. *J Hepatol* 63: 926-933, 2015.
15. Morissette M. C., Lamontagne M., Berube J. C., Gaschler G., Williams A., Yauk C., Couture C., Laviolette M., Hogg J. C., Timens W., Halappanavar S., Stampfli M. R., Bosse Y. Impact of cigarette smoke on the human and mouse lungs: a gene-expression comparison study. *PLoS One* 9: e92498, 2014.
16. Morissette M. C., Shen P., Thayaparan D., Stampfli M. R. Impacts of peroxisome proliferator-activated

receptor-gamma activation on cigarette smoke-induced exacerbated response to bacteria. *Eur Respir J* 45: 191-200, 2015.
17. Morissette M. C., Shen P., Thayaparan D., Stämpfli M. R. Disruption of pulmonary lipid homeostasis drives cigarette smoke-induced lung inflammation in mice. *Eur Respir J* 46: 1451-1460, 2015.
18. Nakayama M. Macrophage Recognition of Crystals and Nanoparticles. *Front Immunol* 9: 103, 2018.
19. Nicola M. L., Carvalho H. B., Yoshida C. T., Anjos F. M. D., Nakao M., Santos U. P., Cardozo K. H. M., Carvalho V. M., Pinto E., Farsky S. H. P., Saldiva P. H. N., Rubin B. K., Nakagawa N. K. Young "healthy" smokers have functional and inflammatory changes in the nasal and the lower airways. *Chest* 145: 998-1005, 2014.
20. Niewoehner D. E., Kleinerman J., Rice D. B. Pathologic changes in the peripheral airways of young cigarette smokers. *N Engl J Med* 291: 755-758, 1974.
21. Palecanda A., Kobzik L. Receptors for unopsonized particles: the role of alveolar macrophage scavenger receptors. *Curr Mol Med* 1: 589-595, 2001.
22. Polverino F., Seys L. J., Bracke K. R., Owen C. A. B cells in chronic obstructive pulmonary disease: moving to center stage. *Am J Physiol Lung Cell Mol Physiol* 311: L687-L695, 2016.
23. Roth M. D., Arora A., Barsky S. H., Kleerup E. C., Simmons M., Tashkin D. P. Airway inflammation in young marijuana and tobacco smokers. *Am J Respir Crit Care Med* 157: 928-937, 1998.
24. Sanda G. M., Deleanu M., Toma L., Stancu C. S., Simionescu M., Sima A. V. Oxidized LDL-Exposed Human Macrophages Display Increased MMP-9 Expression and Secretion Mediated by Endoplasmic Reticulum Stress. *J Cell Biochem* 118: 661-669, 2017.
25. Smoak K., Madenspacher J., Jeyaseelan S., Williams B., Dixon D., Poch K. R., Nick J. A., Worthen G. S., Fessler M. B. Effects of liver X receptor agonist treatment on pulmonary inflammation and host defense. *J Immunol* 180: 3305-3312, 2008.
26. Sonett J., Goldklang M., Sklepkiewicz P., Gerber A., Trischler J., Zelonina T., Westerterp M., Lemaître V., Okada Y., D'armiento J. A critical role for ABC transporters in persistent lung inflammation in the development of emphysema after smoke exposure. *FASEB J* fj201701381, 2018.
27. Stämpfli M. R., Anderson G. P. How cigarette smoke skews immune responses to promote infection, lung disease and cancer. *Nat Rev Immunol* 9: 377-384, 2009.
28. Stein Y., Stein O., Dabach Y., Halperin G., Ben-Naim M. LXR activation and cholesterol efflux from a lipoprotein depot in vivo. *Biochim Biophys Acta* 1686: 24-29, 2004.
29. Sun Y., Zhang D., Liu X., Li X., Liu F., Yu Y., Jia S., Zhou Y., Zhao Y. Endoplasmic Reticulum Stress Affects Lipid Metabolism in Atherosclerosis Via CHOP Activation and Over-Expression of miR-33. *Cell Physiol Biochem* 48: 1995-2010, 2018.
30. Thakur S. A., Hamilton R. F., Holian A. Role of scavenger receptor a family in lung inflammation from exposure to environmental particles. *J Immunotoxicol* 5: 151-157, 2008.
31. Thayaparan D., Shen P., Stämpfli M. R., Morissette M. C. Induction of pulmonary antibodies against oxidized lipids in mice exposed to cigarette smoke. *Respir Res* 17: 97, 2016.
32. Wang B., Tontonoz P. Liver X receptors in lipid signaling and membrane homeostasis. *Nat Rev Endocrinol* 2018.
33. Yao S., Tian H., Miao C., Zhang D. W., Zhao L., Li Y., Yang N., Jiao P., Sang H., Guo S., Wang Y., Qin S. D4F alleviates macrophage-derived foam cell apoptosis by inhibiting CD36 expression and ER stress-CHOP pathway. *J Lipid Res* 56: 836-847, 2015.
34. Zanotti I., Potì F., Pedrelli M., Favari E., Moleri E., Franceschini G., Calabresi L., Bernini F. The LXR agonist T0901317 promotes the reverse cholesterol transport from macrophages by increasing plasma

efflux potential. *J Lipid Res* 49: 954-960, 2008.
35. Zijlstra F. J., Vincent J. E., Mol W. M., Hoogsteden H. C., Van Hal P. T., Jongejan R. C. Eicosanoid levels in bronchoalveolar lavage fluid of young female smokers and non-smokers. *Eur J Clin Invest* 22: 301-306, 1992.

2.10 Figure legends

FIGURE 1. T0901317 treatment impacts key lipid transport genes in lung tissue and alveolar macrophages without causing pulmonary inflammation. A) Alveolar macrophages from C57BL/6 mice were treated *ex vivo* with vehicle (DMSO) or T0901317 for 6 h at 50 nM and mRNA levels of *abca1*, *abcg1*, *apoe*, *scarb1*, *msr1*, *marco*, *cd36*, *il-1α* and *mmp12* were assessed by quantitative PCR. C57BL/6 mice were treated for 4 days with vehicle (corn oil/DMSO) or T09013017 (20 mg/kg; i.p.); B) alveolar macrophages were isolated *ex vivo* from bronchoalveolar lavage (BAL) and C) non-lavaged lung tissue was collected. D) Cholesterol efflux assay was performed *ex vivo* on isolated alveolar macrophages from vehicle- or T0901317-treated mice. E) BAL total, mononuclear and neutrophil cell counts and F) BAL fluid IL-1α, CCL2 and G-CSF levels were assessed. *abca1*: ATP-Binding Cassette A-1; *abcg1*: ATP-Binding Cassette G-1; *apoe*: apolipoprotein E; *scarb1*: Scavenger receptor class B member 1; *msr1*: macrophage scavenger receptor 1; *marco*: macrophage receptor with collagenous structure; *cd36*: CD36 molecule; *il-1α*/IL-1α: Interleukin 1 alpha; *mmp12*: Matrix metallopeptidase 12; *cxcl5*: C-X-C motif chemokine 5; CCL2: C-C Motif Chemokine Ligand 2; G-CSF: Granulocyte-colony stimulating factor. Data are presented as mean±SEM. n=5 per group. *p<0.05; **0<0.01; ***p<0.001.

FIGURE 2. T0901317 treatment restores the expression key pulmonary lipid transport genes altered by cigarette smoke exposure. C57BL/6 mice were exposed to room air (RA) or cigarette smoke (CS) for 4 days and treated daily with vehicle (corn oil/DMSO) or T0901317 (20 mg/kg; i.p.). mRNA levels of *abca1*, *abcg1*, *apoe*, *scarb1* and *msr1*, *marco*, *cd36* were assessed by quantitative PCR in A) non-lavaged lung tissue and B) alveolar macrophages isolated from bronchoalveolar lavage *ex vivo*. *For Figure 2A and 2B, data from room air-exposed T0901317-treated mice from matching protocols are presented in Figure 1B and 1A, respectively. abca1*: ATP-Binding Cassette A-1; *abcg1*: ATP-Binding Cassette G-1; *apoe*: apolipoprotein E; *scarb1*: Scavenger receptor class B member 1; *msr1*: Macrophage scavenger receptor 1; *marco*: Macrophage receptor with collagenous structure; *cd36*: CD36 molecule. Data are presented as mean±SEM. n=5 per group. *p<0.05; **0<0.01; ***p<0.001.

FIGURE 3. **Pharmacological activation of LXR during acute cigarette smoke exposure exacerbates the neutrophilic inflammatory response.** C57BL/6 mice were exposed to room air (RA) or cigarette smoke (CS) for 4 days and treated daily with vehicle (corn oil/DMSO) or T0901317 (20 mg/kg; i.p.). A) Bronchoalveolar lavage (BAL) total, mononuclear and neutrophil cell counts and B) BAL fluid IL-1α, CCL2 and G-CSF levels were assessed. C) mRNA levels of *mmp12* and *cxcl5* were measured in the lung tissue by quantitative PCR. *For Figure 3A, 3B and 3C, data from room air-exposed T0901317-treated mice from matching protocols are presented in Figure 1D, 1E and 1B, respectively.* IL-1α: Interleukin 1 alpha; CCL2: C-C Motif Chemokine Ligand 2; G-CSF: Granulocyte-colony stimulating factor; *mmp12*: Matrix metallopeptidase 12; *cxcl5*: C-X-C motif chemokine 5. Data are presented as mean±SEM. n=5 mice per group. *p<0.05; **0<0.01; ***p<0.001.

FIGURE 4. **Alveolar macrophages from T0901317-treated mice exhibit an exacerbated inflammatory phenotype as well as signs of endoplasmic reticulum stress.** C57BL/6 mice were exposed to room air (RA) or cigarette smoke (CS) for 4 days and treated daily with vehicle (corn oil/DMSO) or T0901317 (20 mg/kg; i.p.). A) Alveolar macrophages were isolated from bronchoalveolar lavage (BAL) and used to assessed their cholesterol efflux capacity. B) Size of alveolar macrophages was assessed from cytospins of BAL cells. Alveolar macrophages were isolated from BAL and used to assess C) secretion of IL-1*α* over 24h, D) mRNA levels of *il-1α* and E) mRNA levels of *mmp12*. F) Impact of T0901317 treatment on the expression of *grp78*, *atf6* and *ddit3* was assessed by quantitative PCR on alveolar macrophages isolated from room air-exposed and cigarette smoke-exposed mice. *For Figure 4A, 4D and 4E, data from room air-exposed T0901317-treated mice from matching protocols are presented in Figure 1C, 1A and 1A, respectively. il-1α*: Interleukin 1 alpha; *mmp12*: Matrix metallopeptidase 12; *grp78*: glucose related protein 78kDa; *atf6*: activating transcription factor 6; *ddit3*: DNA Damage Inducible Transcript 3. Data are presented as mean±SEM. n=5 mice per group. *p<0.05; **0<0.01; ***p<0.001.

FIGURE 5. **T0901317 treatment causes pulmonary surfactant depletion.** C57BL/6 mice were exposed to room air (RA) or cigarette smoke (CS) for 4 days and treated daily with vehicle (corn oil/DMSO) or T0901317 (20 mg/kg; i.p.). A) Serum cholesterol efflux capacity and B) phosphatidylcholine levels were measured. C) Cholesterol efflux capacity of alveolar macrophages isolated from bronchoalveolar lavage (BAL) and D) BAL fluid phosphatidylcholine levels were also

measured. Key pulmonary surfactant genes were measured by quantitative PCR in E) RA- and F) CS-exposed mice were measured by qPCR. Finally, G) mature SP-B protein levels were measured by Western blot of BAL fluid. Data are presented as mean±SEM. n=5 mice per group. *p<0.05; **0<0.01; ***p<0.001.

FIGURE 6. Schematic representation of the distinctive impact of pharmacological LXR activation under normal homeostatic conditions and during exposure to cigarette smoke.

A) In normal homeostatic conditions, [1] pharmacological activation of LXR increases transcription of lipid transporters *abca1*, *abcg1* and *scarb1*, the apolipoprotein *apoe* and the scavenger receptor *msr1* by alveolar macrophages. [2] However, since the pulmonary surfactant is not damaged or oxidized, this does not increase surfactant uptake by macrophages, pulmonary surfactant pool remains intact and no inflammatory response is induced. B) During exposure to cigarette smoke, [1] pharmacological activation of LXR increases transcription of *abca1*, *abcg1*, and *apoe*, compensating for the effects of cigarette smoke on these genes. [2] However, the concomitant impact of cigarette smoke exposure and LXR activation further increases *msr1* and *marco* levels, likely increasing surfactant uptake and macrophage size, and reducing the overall pulmonary surfactant pool. [3] This increase in pulmonary surfactant uptake causes additional endoplasmic-reticulum stress. [4] With an increased metabolic pressure on macrophages, they release additional IL-1, which in turn increases *cxcl5* transcription and leads to an exacerbated recruitment of neutrophils.

2.11 Figures

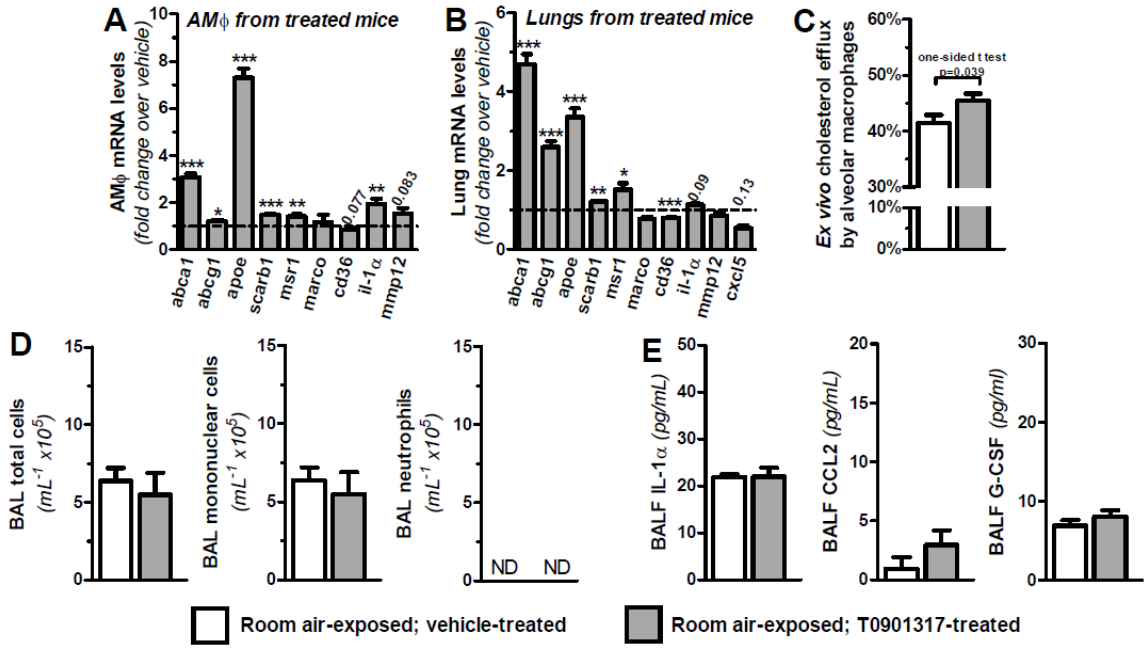

Figure 2. 1: T0901317 treatment impacts key lipid transport genes in lung tissue and alveolar macrophages without causing pulmonary inflammation.

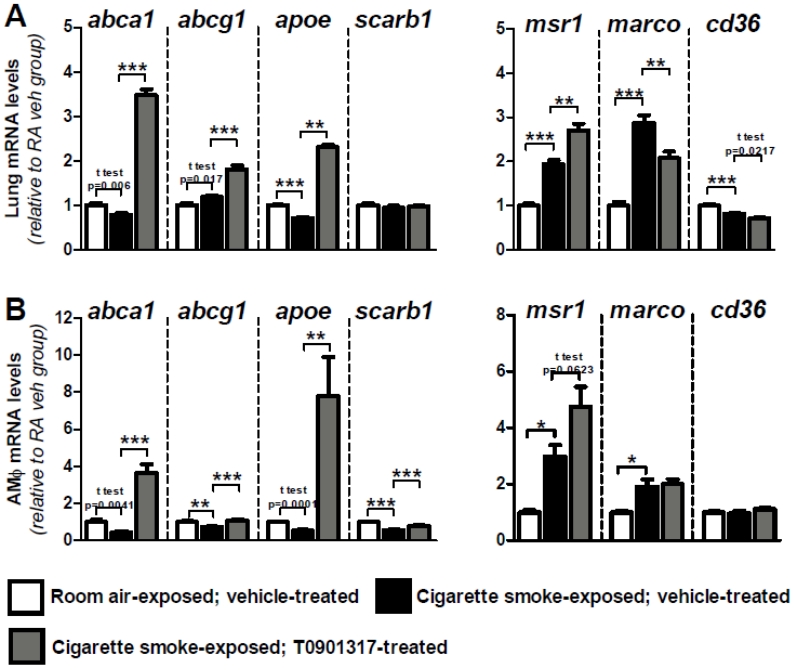

Figure 2. 2: T0901317 treatment restores the expression of key pulmonary lipid transport genes altered by cigarette smoke exposures.

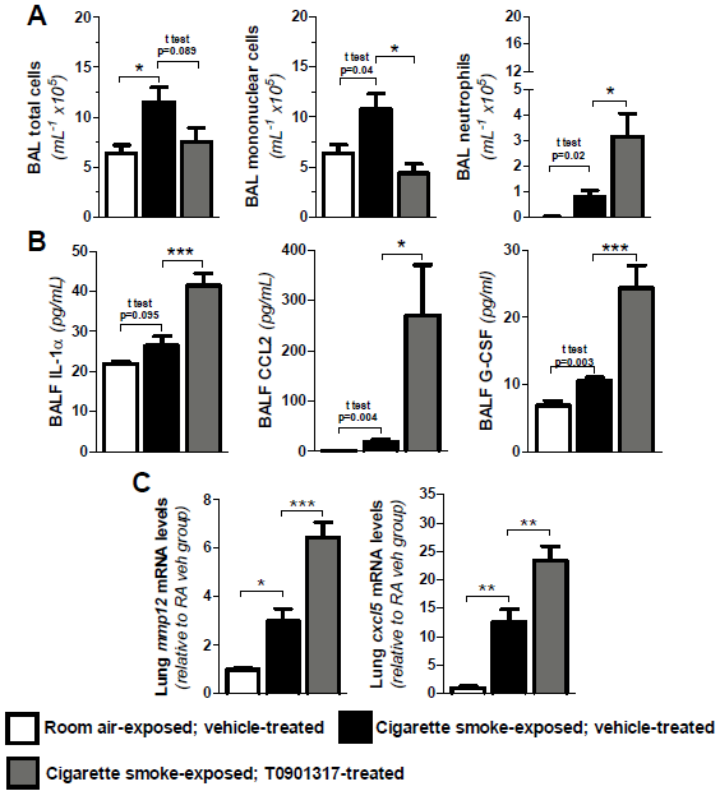

Figure 2. 3: Pharmacological activation of LXR during acute cigarette smoke exposure exacerbates the neutrophilic inflammatory response.

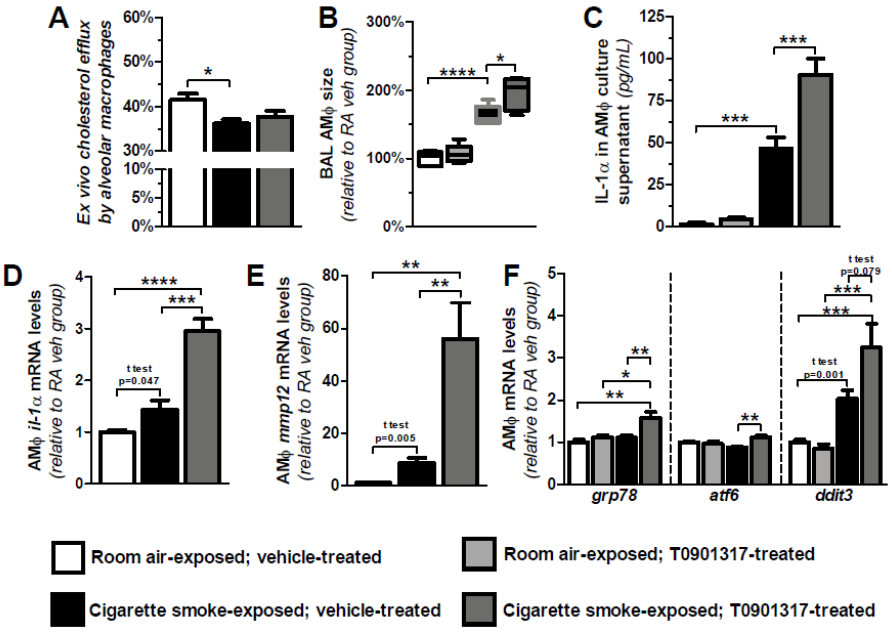

Figure 2. 4: Alveolar macrophages from T0901317-treated mice exhibit an exacerbated inflammatory phenotypes as well as signs of endoplasmic reticulum stress.

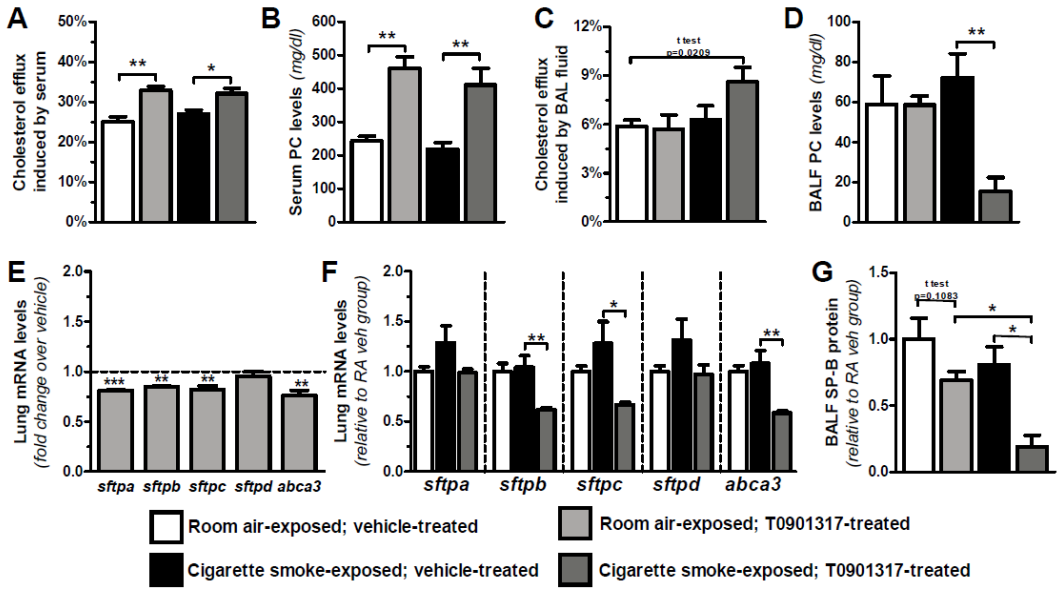

Figure 2. 5: T0901317 treatment causes pulmonary surfactant depletion.

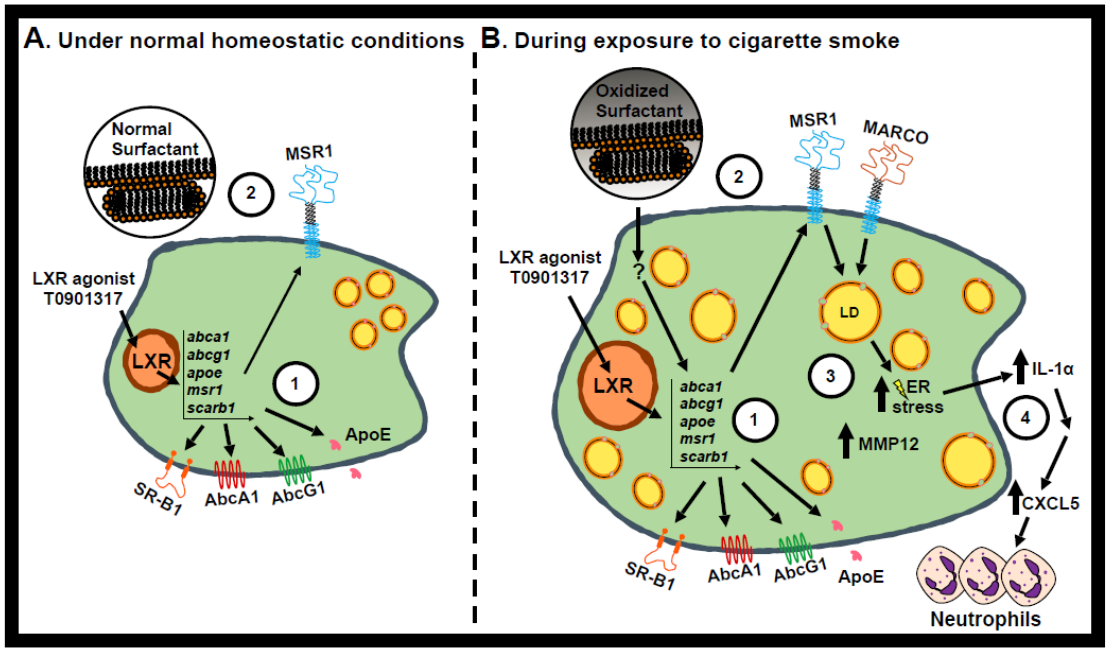

Figure 2. 6: Schematic representation of the distinctive impact of pharmacological LXR activation under normal homeostatic conditions and during exposure to cigarette smoke.

Chapter 3: Critical importance of dietary methionine and choline in maintenance of lung homeostasis during normal and cigarette smoke exposure conditions

3.1 Foreword

The scientific article presented in Chapter 3 “Critical importance of dietary methionine and choline in maintenance of lung homeostasis during normal and cigarette smoke exposure conditions in mice” was submitted to the European Respiratory Journal in February 2019. This study describes the impact of methionine and choline dietary deficiency on lung health and on the response to cigarette smoke exposure. The methionine choline deficient diet led to dramatic alterations in pulmonary function and inflammation, extracellular matrix dysregulation, and pulmonary surfactant depletion. These lung phenotypes were shown to be quickly reversible when mice are subsequently fed the control diet. Interestingly, the methionine deficient diet induced a restrictive lung profile compared to the emphysematous-like profile induced by the choline deficient diet. This study found a novel link between nutrition and pulmonary homeostasis, especially during cigarette smoke exposure, and opens new avenues in the field of “nutri-respiratory health”.

3.1.1 Author contributions

I am the principal author of this article. I conceived and performed most of the techniques associated with animal protocols, qPCRs, phosphatidylcholine measurements and ELISA assays. Marc-Alexandre Lafrance helped with most of the protocols and generated the data regarding the impact of surfactant pre- and post-bronchoalveolar lavage. Joanie Routhier and Marie Pineault helped with ELISA assays and with some animal work. Nadia Milad helped with phosphatidylcholine assays and reviewed the manuscript. Jennifer Lamothe performed the bioinformatic analysis. Marie-Josée Beaulieu contributed to animal protocols, especially with FlexiVent measurements. Sophie Aubin exposed the mice to cigarette smoke and helped with body weight measurements and Marie-Ève Paré was involved in many laboratory analyses. Mathieu Laplante helped with the interpretation of results concerning the methionine choline deficient diet. Mathieu C Morissette also helped design the study and prepare the manuscript.

3.2 Résumé

Les prédispositions génétiques et les expositions environnementales sont bien connues pour être des facteurs de risque importants dans les maladies respiratoires. Les effets néfastes d'une mauvaise alimentation ont été très bien caractérisés dans les maladies cardiovasculaires, l'obésité et le diabète de type 2, toutefois ils sont très peu documentés dans les maladies chroniques respiratoires. Les déficiences en choline et en méthionine sont fréquentes dans la population, toutefois leur impact sur l'homéostasie pulmonaire est inconnu.

Des souris ont été nourries avec la diète déficiente en choline et/ou méthionine, en plus d'être exposées ou non à la fumée de cigarette jusqu'à 4 semaines. Les fonctions pulmonaires ont été analysées avec le FlexiVent. Des analyses transcriptionnelles sur le poumon ont été effectuées avec des biopuces microarray et la PCR quantitative. La quantité de cellules immunitaires, de cytokines et de phosphatidylcholine a été quantifiée dans le lavage bronchoalvéolaire.

Dans cette étude, nous avons démontré qu'après quelques semaines, la diète déficiente en méthionine et/ou en choline induisait des changements réversibles au niveau des fonctions pulmonaires des souris. Il y avait aussi des effets significatifs sur l'expression des gènes associés à la matrice extracellulaire et sur les niveaux de phosphatidylcholine associé au surfactant pulmonaire. Ces diètes ont aussi altéré la réponse pulmonaire à la fumée de cigarette en plus de modifier les fonctions pulmonaires et l'inflammation pulmonaire.

Ces résultats démontrent qu'une carence alimentaire en élément nutritif essentiel comme la choline et la méthionine peut mener à des effets pathophysiologiques pulmonaires et modifier les altérations pulmonaires induites par la fumée de cigarette. L'amélioration de nos connaissances dans le domaine du « nutri-respiratoire » permettrait de découvrir le rôle potentiel des nutriments essentiels dans la santé et les maladies respiratoires et ainsi devenir potentiellement un nouveau facteur de prédisposition comme la génétique et les expositions environnementales.

3.3 Abstract

Genetic predispositions and environmental exposures are regarded as the main predictors of respiratory disease development. However, the impact of dietary essential nutrients deficiencies as a pathogenic factor has been largely studied in cardiovascular diseases, obesity and type II diabetes but remains poorly explored in chronic respiratory diseases. Dietary choline and methionine deficiencies are common in the population and their impact on pulmonary homeostasis is currently unknown.

Mice were fed choline and/or methionine deficient diets while being exposed to room-air or cigarette smoke for up-to 4 weeks. Lung functions were assessed using the FlexiVent. Pulmonary transcriptional activity was assessed using gene expression microarrays and quantitative PCR. Immune cells, cytokines and phosphatidylcholine were quantified in the bronchoalveolar lavage.

In this study, we found that short-term dietary choline and/or methionine deficiencies significantly affect lung function in mice in a reversible manner. It also reduced transcriptional levels of collagens and elastin as well as pulmonary surfactant phosphatidylcholine levels. We also found that dietary choline and/or methionine deficiency markedly interfered with the pulmonary response to cigarette smoke exposure, modulating lung functions and dampening inflammation.

These findings clearly show that dietary choline and/or methionine deficiencies can have dramatic pathophysiological effects on the lungs and can also affect the pathobiology of cigarette smoke-induced pulmonary alterations. Expanding our knowledge in the field of “nutri-respiratory research” may reveal a crucial role for essential nutrients in pulmonary health and diseases, which may be as relevant as genetic predispositions and environmental exposures.

Critical importance of dietary methionine and choline in maintenance of lung homeostasis during normal and cigarette smoke exposure conditions

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Take home message: This study shows that dietary choline and/or methionine deficiencies can have dramatic pathophysiological effects on the lungs and can also affect the pathobiology of cigarette smoke-induced pulmonary alterations.

3.4 Introduction

Diet is critical to cardiovascular and metabolic health (1–3), a fact that is not as well established for respiratory health. The lung is a complex structure directly exposed to the environment and thus is subjected to continuous insult and injury from agents such as microbes, air pollution or tobacco smoke. Therefore, the maintenance of lung homeostasis during health and disease is likely affected by changes in the supply of essential nutrients acquired through diet.

Our group has been investigating the interaction between smoking and pulmonary lipid homeostasis (4–6), progressively unraveling the critical role of the pulmonary surfactant in the response to cigarette smoke exposure. Phosphatidylcholine is a crucial pillar of the pulmonary surfactant, representing over 70% of its lipid content (7–9). During surfactant biogenesis, the required building blocks are captured by type II alveolar cells from the circulation allowing for the synthesis of phosphatidylcholine species essential to a fully functional pulmonary surfactant (7, 10). Phosphatidylcholine is in high demand throughout the body. Therefore, a reduced capacity to produce phosphatidylcholine, in addition to the overall high demand, may jeopardise pulmonary surfactant homeostasis, and consequently, lung function.

Choline and methionine are two essential nutrients, which have to be acquired through diet to meet our daily requirements (11, 12). Choline is important for the synthesis of the neurotransmitter acetylcholine and the phospholipid phosphatidylcholine, among other crucial biological functions (11). Methionine is critical to protein synthesis, as it initiates translation of every mammalian protein, and can also be used to synthetize phosphatidylcholine from phosphatidylethanolamine (12). Therefore, these two essential nutrients contribute to phosphatidylcholine biosynthesis and can compensate for one another for some of their shared metabolic functions.

Simultaneous dietary choline and methionine deficiency is known to have drastic effects. In mice, a diet deficient in methionine and choline rapidly leads to non-alcoholic hepatic steatosis followed by hepatic damage due to increased inflammation and fibrosis (13–15). It also markedly reduces very-low-density lipoprotein (VLDLs) biogenesis by the liver and decreases systemic lipid transporters such

as high-density lipoproteins (HDLs) (16, 17). Dietary choline insufficiency also leads to lipid accumulation in the liver in humans (18). It is estimated that the majority of individuals do not acquire enough choline through the diet to meet daily requirements (19). Because circulating lipoprotein homeostasis and phosphatidylcholine availability are important factors which influence lung function, we hypothesized that dietary deficiency in choline and methionine would impact pulmonary homeostasis.

In this study, we used a mouse model to investigate the impact of a dietary methionine and choline deficiency on lung homeostasis during normal and cigarette smoke exposure conditions. We observed that dietary methionine and choline deficiency progressively and reversibly affected lung function in both room air and cigarette smoke-exposed mice, leading to a restrictive pulmonary phenotype. The inflammatory response to cigarette smoke was also altered by this dietary deficiency, exhibiting a markedly reduced neutrophil infiltration. Finally, dietary deficiency in methionine and choline drastically reduced pulmonary surfactant levels, which partially explains the observed susceptibility to atelectasis conferred by the dietary deficiency.

3.5 Methods

3.5.1 Animals, cigarette smoke exposure and dietary protocols

3.5.1.1 Mice - Female 6- to 8-week-old C57BL/6 mice were purchased from Charles River Laboratories (Montréal, PQ, Canada). Mice were housed following recommendations from the Guide for the Care and Use of Laboratory Animals of the Canadian Council on Animal Care (CCAC). Mouse protocols were approved by the Committee on the Ethics of Animal Experiments of Université Laval (#2017-101-1).

3.5.1.2 Cigarette smoke exposure - Mice were exposed every morning for 2h, 5 days/week, for 2, 3 or 4 weeks to either room air or the mainstream smoke of 3R4F reference cigarettes (University of Kentucky, Lexington, KY, USA) without filters, using an established whole-body exposure system (SIU24; Promech Lab, Vintrie, Sweden) (4, 20).

3.5.1.3 Diets - Mice were fed *ad libidum* a control diet (21% fat; 66% Carbohydrates; 17% proteins; 2 g/kg choline and 3 g/kg methionine; Research Diets inc. A02082003BY), a methionine-deficient diet (Research Diets inc. A17091101), a choline-deficient diet (Research Diets inc. A17091102) or a methionine/choline-deficient diet (Research Diets inc. A02082002B) for 2, 3 or 4 weeks.

3.5.2 Blood collection and processing

Prior to lung function assessment, orbital blood was collected under ketamine/xylazine anaesthesia. Blood samples were then centrifuged (12000g, 4°C) and the serum stored at -80°C for future measurements.

3.5.3 Lung function assessment

3.5.3.1 Standard assessment - Lung function parameters were measured using the FlexiVent® (SCIREQ, Montreal, PQ, Canada). Mice were anaesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine; tracheotomised with an 18-gauge blunted needle, mechanically ventilated at a respiratory rate of 150 breaths/minutes and a tidal volume of 10 ml/kg, with a pressure limit of 30 cmH_2O. Muscle paralysis was achieved using pancuronium (2 mg/kg, Sandoz, Boucherville, PQ, Canada) to prevent

respiratory efforts during manoeuvres. The following sequence of measurements was repeated three times: Deep inflation, Snapshot-150, Quick Prime-3 and Pressure/Volume-loop (PV-loop) to obtain lung function parameters.

3.5.3.2 Pre and post bronchoalveolar lavage (BAL) assessments - These experiments were performed on a separate cohort of mice and could not be used for any other assessment. Under the same anaesthetic conditions of the 'standard assessment' described above, the following sequence of measurements was repeated three times: Deep inflation, Snapshot-150, Quick Prime-3 and Pressure/Volume-loop (PV-loops) to obtain 'Pre-BAL' lung function parameters. The cannula was then removed, lungs lavaged *in situ* once with 1 ml of warm PBS with an approximate 900µl recovery to remove pulmonary surfactant. Mice were then reconnected to the FlexiVent® and ventilated for 3 minutes. To assess 'Post-BAL' lung function parameters, the following sequence of measurements was repeated three times: Deep inflation, Snapshot-150, Quick Prime-3 and PV-loop.

3.5.4 Bronchoalveolar lavage and lung tissue collection

After lung function assessment, mice were euthanized by exsanguination. Lungs were removed from the thoracic cavity, the trachea was cannulated and right lobes were tied off. Bronchoalveolar lavage (BAL) was performed with a first injection of 250 µL of cold PBS and a second injection of 200 µL. The right lobes were then collected, immediately frozen in liquid nitrogen and kept at -80°C. For histological assessment, the left lung was inflated with 10% formalin and fixed for 3 days prior transfer to 70% ethanol and paraffin-embedding. Lung total cell count was performed on the fresh BAL using a hemacytometer. BAL cells were then centrifuged (800g, 4°C), the supernatant (BAL fluid) collected and kept at -80°C, and the cells were re-suspended in PBS for cytospins. Cytospins were prepared and stained using the Hema 3 protocol (Fisher, USA) for differential cell counts. A total of 300 cells were counted per cytospin.

3.5.5 RNA extraction, quantitative PCR and gene expression microarray analysis

3.5.5.1 RNA extraction - Lung total RNA was extracted from one frozen lobe using Trizol (Fisher, USA) according to manufacturer's instructions. Lung RNA was re-suspended in 50 µL of Tris-EDTA

(Fisher, USA) and quantitated using the BioTek Synergy H1 (BioTek, Winooski, VT, USA). Lung RNA integrity and 28S/16S ratios were analyzed on agarose gels before cDNA synthesis.

3.5.5.2 Quantitative PCR (qPCR) - Lung cDNA was prepared with 1 µg of total RNA using the iScript cDNA synthesis kit following manufacturer's instructions (Bio-Rad, Mississauga, ON, Canada). Lung gene mRNA levels was assessed using the SsoAdvanced universal SYBR green Supermix (Bio-Rad, Canada). Two reference genes; *Rplp0* and *Hprt* and gene-specific primers were used to amplify *Col1a1*, *Col3a1*, *Eln*, *Lum*, *Cxcl5* and *Mmp-12* (IDT, Skokie, IL, USA). All qPCR reactions were composed of: 10 µL of SsoAdvanced universal SYBR green Supermix 2X, 10 µM forward primer (0.6 µL), 10 µM reverse primer (0.6 µL), 5 µL of cDNA template, and were completed with RNAse-free water to the final volume of 20 µL (GE Healthcare, South Logan, UT, USA). Thermo-protocols were as follow: 95°C for 3 min, followed by 40 cycles of 95°C for 10s and 56-60°C for 30s. All samples were run in duplicate and RNase-free water (GE Healthscare, USA) was used as a no template control. Reactions were manually loaded into 0.1 mL PCR tube strips (Corning, Corning, NY, USA). Amplifications were performed using a Rotor-Gene 6000 series (QIAGEN, Canada) and data acquired/analyzed with the on-board Rotor-Gene series Software version 1.7. Thermal gradients and efficiencies were all performed prior to qPCR assays. qPCR efficiencies were between 90-110%, with R^2 values ranging between 0.96-0.99. Melt curves and qPCR products on agarose gels were analyzed for specificity and no template controls were detected passed 35 Cq. Threshold from the efficiency assays were used to determine the Cq and the ΔΔCq method was used to normalize all qPCR results.

3.5.5.3 Gene expression microarray analysis - Lung total RNA was sent to GenomeQuebec (Montreal, PQ, Canada) to perform Affimetrix mouse Clariom D microarrays. The differential analysis was performed using the R software and packages *affy*, *oligo* and *limma*.

3.5.6 BAL fluid and serum biomarkers

3.5.6.1 IL-1α and CCL2 in BAL fluid - IL-1α and CCL2 levels were assessed in mouse bronchoalveolar lavage fluid using the mouse IL-1α and CCL2 DuoSet® ELISA kits (R&D systems, Minneapolis, MN, USA) following manufacturer's instructions.

3.5.6.2 Phosphatidylcholine levels in serum and BAL fluid - Serum and BAL fluid levels of phosphatidylcholine were measured using the Phosphatidylcholine Assay Kit following manufacturer's instructions (STA-600, Cell Biolabs, San Diego, CA, USA). Dilutions of 1:4 and 1:100 were used, respectively, for BAL fluid and serum.

3.5.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism Software V.6 (La Jolla, CA, USA). Two group comparisons were made using an unpaired t-test ($p \leq 0.05$). Experimental protocols with more than two groups were compared using a one-way ANOVA ($p \leq 0.05$) followed by a Bonferroni multiple comparisons post-test.

3.6 Results

3.6.1 Dietary deficiency in choline and methionine reversibly affects lung function

To investigate the impact of dietary deficiency in choline and methionine on the lungs, we fed C57BL/6 mice with a control or a methionine/choline deficient (MCD) diet for 3 weeks. To assess reversibility, we switched half of the mice on MCD diet to control diet while the other half remained on MCD diet for one additional week. We assessed pulmonary functions at 3 (before diet switch) and 4 weeks (one week after diet switch). We found that the MCD diet progressively affected pulmonary functions causing a restrictive phenotype, as shown by the lowering of the PV-loop as well as the reduced inspiratory capacity and compliance (Figure 1A). Unchanged Newtonian resistance and increased tissue damping suggest lung tissue rather than conductive airways is affected (Figure 1A). As seen with the MCD diet-induced changes in bodyweight (Figure 1B), reintroduction of control diet completely reverses the lung function phenotype within one week (Figure 1A - red bars). Since a lung restrictive phenotype can result from pulmonary fibrosis, we investigated the presence of pulmonary fibrosis via tissue histology. No signs of established fibrotic processes were detected in the lung tissue of mice fed the MCD diet (Figure 1C), thus excluding lung fibrosis as the cause of the MCD diet-induced lung phenotype. This is also in accordance with the relatively rapid renormalisation of lung functions upon reintroduction of the control diet, since lung fibrosis generally requires a longer period to resolve.

3.6.2 Dietary choline and methionine deficiency strongly reduces pulmonary expression of extracellular matrix-related genes

To better understand the underlying mechanisms behind the impact of dietary choline and methionine deficiency on the lungs, we performed gene expression microarrays on lung tissue from mice fed control or MCD diet for 4 weeks. We found that levels of genes encoding extracellular matrix proteins were markedly reduced by the MCD diet (Figure 2A). We confirmed 4 important pulmonary extracellular matrix genes by quantitative PCR (Figure 2B): *col1a1*, *col3a1*, *elastin* and *lum*, respectively encoding collagen type I alpha 1 chain, collagen type III alpha 1 chain, elastin and lumican. As observed in the case of lung function alterations (Figure 1A), mRNA levels of *col1a1*, *col3a1*, *elastin* and *lum* fully normalized after one week of control diet reintroduction. Since cigarette smoke exposure progressively leads to a reduction in lung extracellular matrix, we investigated lung expression of *col1a1*, *col3a1*, *elastin* and *lum* in mice exposed to room air or cigarette smoke for 2 and 4 weeks while

on control or MCD diets during this period. Cigarette smoke exposure markedly reduced elastin expression, with no or only a marginal effect on the other genes (Figures 2C and 2D). Interestingly, mRNA levels of all four genes were higher in the MCD-fed cigarette smoke-exposed group compared to the MCD-fed room air-exposed group. Altogether, this suggest that MCD diet markedly affects mRNA levels of key genes encoding for extracellular matrix proteins and that MCD diet and cigarette smoke exposure affect each others respective effects.

3.6.3 Dietary choline and methionine deficiency markedly affects cigarette smoke-induced changes in lung function and inflammation

Since dietary deficiency in choline and methionine markedly affected lung functions as well as the expression of genes associated to extracellular matrix, we investigated how this nutritional deficiency could impact the pulmonary response to cigarette smoke. A 2-week exposure to cigarette smoke did not affect lung function, while the MCD diet-fed animals showed signs of early lung function changes (Figure 3A). However, the MCD diet markedly blunted the neutrophilia induced by cigarette smoke exposure (Figure 3B). BAL fluid IL-1α and CCL2 as well as tissue *cxcl5* and *mmp12* mRNA levels were all increased by cigarette smoke exposure, while only *cxcl5* mRNA levels were affected by the MCD diet. Regarding the 4-week exposure to cigarette smoke, lung functions were altered as previously observed: increased inspiratory capacity but yet no effect on compliance or tissue damping (Figure 3E). The MCD diet had a significant effect on cigarette smoke exposure, leading to a discrepancy between cigarette smoke-exposed mice fed the control diet and those fed the MCD diet (Figure 3E). The MCD diet maintained its blunting effect on cigarette smoke-induced neutrophilia, with an additional reduction in mononuclear cells and, consequently, total cell counts (Figure 3F). BAL fluid IL-1α and lung tissue *mmp12* mRNA levels remained unaffected by the MCD diet, while BAL fluid CCL2 levels were increased and lung tissue *cxcl5* mRNA levels were reduced (Figure 3G and 3H). Therefore, the MCD diet progressively impacted lung function with a dominant effect over cigarette smoke exposure but also rapidly blunted cigarette smoke-induced inflammation, which preceded pulmonary function alterations.

3.6.4 Dietary choline and methionine deficiency predisposes mice to small airway atelectasis due to reduced pulmonary surfactant levels and structural alterations independent of pulmonary surfactant

To investigate the role of pulmonary surfactant in the alterations in lung function caused by the MCD diet, we assessed phosphatidylcholine levels in the BAL fluid, which reflect the amount of pulmonary surfactant. We found that, similar to circulating phosphatidylcholine levels, phosphatidylcholine levels in the BAL fluid were lower in mice fed the MCD diet; a phenomenon that persisted during cigarette smoke exposure and remains fully reversible within a week (Figure 4A and 4B). In order to determine lung dynamics in the absence of pulmonary surfactant, we performed lung function tests before and after pulmonary surfactant removal via lavage. We found that the impact of pulmonary surfactant removal was worse in the lungs MCD-fed mice compared to those fed control diet, as reflected by the lower PV-loop after the lavage (Figure 4C) and the more pronounced increase in tissue damping and tissue elastance (Figure 4C). Of note, removal of pulmonary surfactant through lavage did not affect Newtonian resistance, supporting the notion that conducting airways were not affected by the procedure (Figure 4C). Altogether, these data indicate that the MCD diet reduced pulmonary surfactant levels but that structural alterations are also present, making the lungs more resistant to alveolar recruitment.

3.6.5 Choline or methionine dietary deficiencies have different effects on lung function and lung inflammation

So far, we have documented the impact of concomitant methionine and choline deficiency. Here, we wanted to investigate the respective impact of methionine or choline deficiencies on lung function and the response to cigarette smoke exposure. Mice were fed either a control, methionine-deficient (MD) or choline-deficient (CD) diet for 4 weeks and exposed to room air or cigarette smoke during that period. Methionine deficiency alone caused a pulmonary phenotype similar to MCD diet, only milder, exhibiting a restrictive lung function pattern (Figure 5A). To the contrary, choline deficiency led to the opposite phenotype: increased inspiratory capacity and compliance, and a reduced tissue damping (Figure 5A). With regard to gene expression, MD diet reduced *col1a1*, *col3a1*, *elastin* and *lum* levels, while CD diet led to reduced the expression of *col1a1*, *col3a1* and *elastin* but not *lum* (Figure 5B). Both MD and CD diets led to a similar reduction in circulating phosphatidylcholine levels but only MD-fed animals had significantly reduced BAL fluid phosphatidylcholine levels (Figure 5C). Regarding the dietary impact on

the pulmonary response to cigarette smoke exposure, CD diet had no significant effect on lung function during cigarette smoke exposure compared to control diet-fed animals, while MD diet reproduced the effects observed in MCD-fed mice (Figure 6A). Both MD and CD diets markedly reduced BAL total, mononuclear and neutrophil cell counts (Figure 6B). CD diet significantly reduces BAL fluid IL-1α and CCL2 levels (Figure 6C) as well as lung *cxcl5* and *mmp12* mRNA levels (Figure 6D). In cigarette smoke-exposed groups, CD diet had no significant impact on *col1a1*, *col3a1*, *elastin* and *lum* lung mRNA levels while MD diet reduced *col1a1*, *col3a1* and *lum* expression (Figure 6E). Finally, only CD diet reduced circulating phosphatidylcholine levels and neither affected BAL fluid phosphatidylcholine levels in the context of cigarette smoke exposure (Figure 6F). Altogether, MD and CD have different effects on lung homeostasis and both interfere with the normal pulmonary response to cigarette smoke exposure.

3.7 Discussion

This study reveals that dietary deficiencies in methionine and/or choline can affect lung function in normal homeostatic conditions and alter the pulmonary response to cigarette smoke exposure, in terms of lung function and inflammation. We also document that these effects are both progressive in nature and rapidly reversible. Finally, mechanistic insights suggest that a reduced rate of lung extracellular matrix turnover and pulmonary surfactant biosynthesis are likely involved in the pathogenesis.

Dietary methionine and choline deficiency progressively affects lung functions. These effects are also rapidly and completely reversible when the nutrients are reintroduced into the diet. This is also observed in the liver of mice fed the MCD diet, where steatosis, inflammation and fibrosis progressively and sequentially occur over 2 to 6 weeks with a high degree of reversibility (13, 17, 21–24). While the adverse effects of dietary methionine and choline deficiency on the liver stem from reduced lipid transport from the liver to the circulation due to impaired VLDL and HDL synthesis, such mechanisms are unlikely to be involved in the pulmonary effects observed in this study. There are many potential mechanisms whereby this 'essential nutrient starvation state' caused by dietary methionine and choline deficiencies can affect lung function and the response to cigarette smoke exposure.

3.7.1 The pulmonary response to an 'essential nutrient starvation state'. The MCD diet rapidly affected pulmonary transcription of genes associated with the extracellular matrix. This effect can also be observed in mice fed CD and MD diets, supporting the concept that both choline and methionine are important to maintain normal transcription of genes such as *col3a1*, *col1a1* and *elastin*. However, methionine deficiency seemed to have a more pronounced impact. With regard to the mechanism, methionine is well known for its role in the initiation of protein synthesis. It is therefore possible that reduced methionine intake leads to reduced protein synthesis capability in the lungs. When looking at the histological sections of the lungs, we could not observed any reduction in cellularity. This suggests that, in the context of methionine and choline deficiency, lung extracellular matrix synthesis may have been sacrificed to prioritize synthesis of crucial pulmonary proteins and thus maintain the integrity of structural cells. While this remains an untested hypothesis, it would explain why the mechanical properties of the lung are rapidly affected by methionine and choline restriction,

while the tissue itself remains relatively normal. The same concept can be applied to the pulmonary surfactant, where reducing extracellular matrix and pulmonary surfactant elements to a minimum to maintain the cellular integrity of the lung would also allow for rapid recovery upon reintroduction of methionine and choline in the diet. Reducing extracellular matrix and pulmonary surfactant elements to a minimum to maintain the cellular integrity of the lung would allow it to recover rapidly upon reintroduction of methionine and choline. In fact, we indeed observed a very rapid return to homeostatic conditions after one week of recovery, indicating that all the required cellular machinery was still functionally intact. While this hypothesis remains to be fully tested, it suggests an important adaptive pathway for the maintenance of lung integrity during times of essential nutrient restriction.

3.7.2 Interaction between cigarette smoke exposure and dietary methionine/choline deficiency. Dietary methionine and/or choline deficiency also has very interesting impacts on the pulmonary effects of cigarette smoke exposure. Firstly, the MCD diet, more precisely methionine deficiency, impacts lung function. These diets led to a completely different pulmonary phenotype compared to control diet mice exposed to cigarette smoke, marked by reduced tissue damping and inspiratory capacity. Choline deficiency did not have this effect. These findings suggest that human smokers under methionine restriction could be more susceptible to atelectasis and air-trapping, likely exacerbating existing symptoms. A second noticeable impact of dietary methionine and choline deficiency during cigarette smoke exposure was the blunted inflammatory response and reduced neutrophil infiltration, in particular. Deficiencies in methionine or choline alone were both associated with reduced pulmonary inflammation in response to cigarette smoke exposure. While the mechanism behind this phenomenon remains to be elucidated, MCD diet markedly dampened the neutrophilia and did so before the appearance of lung function changes. Also, we did not observe any change in circulating neutrophils (data not shown). It is therefore unlikely that the lungs of methionine- and/or choline-deficient mice would not be able to mount a proper immune response. This question, therefore, remains open. A third interesting interaction between cigarette smoke exposure and dietary methionine and choline deficiency is the effect on extracellular matrix gene mRNA levels. Lungs from mice exposed to cigarette smoke and fed the MCD diet exhibited higher mRNA levels of *col3a1*, *col1a1, elastin* and *lum* compared to room air-exposed mice fed the MCD diet. This can suggest that, to maintain lung integrity at an acceptable level, transcription of extracellular matrix genes needed to be higher in the

context of cigarette smoke exposure, thus interfering with the normal adaptation process to dietary methionine and choline restriction observed in room air-exposed, MCD-fed mice.

3.7.3 Implications for respiratory health in non-smokers and smokers. Since methionine and choline dietary insufficiency can impact the healthy lung, all lung diseases are likely impacted by such dietary deficiencies. Daily intake of choline and methionine should therefore be considered when assessing pulmonary health. With regard to cigarette smoke exposure, dietary methionine and choline deficiency was found to affect three crucial aspects of the pulmonary response: lung function, extracellular matrix homeostasis and inflammation. While we do not know if methionine and choline deficiencies have significantly deleterious effects over time in smokers, it is likely that deficiency in these essential nutrients would affect pulmonary function and symptoms linked to inflammation such as exacerbations in patients with chronic obstructive pulmonary disease (COPD).

This study adds to the emerging field of 'nutri-respiratory' research and shows that some essential nutrients, such as choline and methionine, are crucial to pulmonary health. Further research is required to fully understand the mechanisms behind the impact of dietary choline and methionine deficiency on the lungs but also to investigate other nutrients that may be essential to the respiratory health.

3.8 References

1. Gentile CL, Weir TL. The gut microbiota at the intersection of diet and human health. Science. 2018;362:776-780.
2. Getz GS, Reardon CA. Nutrition and cardiovascular disease. Arterioscler Thromb Vasc Biol. 2007;27:2499-2506.
3. Pitsavos C, Panagiotakos D, Weinem M, Stefanadis C. Diet, exercise and the metabolic syndrome. Rev Diabet Stud. 2006;3:118-126.
4. Jubinville É, Talbot M, Bérubé JC, Hamel-Auger M, Maranda-Robitaille M, Beaulieu MJ, Aubin S, Paré MÈ, Kallend DG, Arsenault B, Bossé Y, Morissette MC. Interplay between cigarette smoking and pulmonary reverse lipid transport. Eur Respir J. 2017;50
5. Morissette MC, Shen P, Thayaparan D, Stämpfli MR. Disruption of pulmonary lipid homeostasis drives cigarette smoke-induced lung inflammation in mice. Eur Respir J. 2015;46:1451-1460.
6. Thayaparan D, Shen P, Stämpfli MR, Morissette MC. Induction of pulmonary antibodies against oxidized lipids in mice exposed to cigarette smoke. Respir Res. 2016;17:97.
7. Agassandian M, Mallampalli RK. Surfactant phospholipid metabolism. Biochim Biophys Acta. 2013;1831:612-625.
8. Chroneos ZC, Sever-Chroneos Z, Shepherd VL. Pulmonary surfactant: an immunological perspective. Cell Physiol Biochem. 2010;25:13-26.
9. Goerke J. Pulmonary surfactant: functions and molecular composition. Biochim Biophys Acta. 1998;1408:79-89.
10. Goss V, Hunt AN, Postle AD. Regulation of lung surfactant phospholipid synthesis and metabolism. Biochim Biophys Acta. 2013;1831:448-458.
11. Wiedeman AM, Barr SI, Green TJ, Xu Z, Innis SM, Kitts DD. Dietary Choline Intake: Current State of Knowledge Across the Life Cycle. Nutrients. 2018;10
12. Mastrototaro L, Sponder G, Saremi B, Aschenbach JR. Gastrointestinal methionine shuttle: Priority handling of precious goods. IUBMB Life. 2016;68:924-934.
13. Rinella ME, Elias MS, Smolak RR, Fu T, Borensztajn J, Green RM. Mechanisms of hepatic steatosis in mice fed a lipogenic methionine choline-deficient diet. J Lipid Res. 2008;49:1068-1076.
14. Larter CZ, Yeh MM. Animal models of NASH: getting both pathology and metabolic context right. J Gastroenterol Hepatol. 2008;23:1635-1648.
15. Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. [editorial]. World J Gastroenterol 2012;18(19):2300.
16. Yao ZM, Vance DE. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. J Biol Chem. 1988;263:2998-3004.
17. Stanković MN, Mladenović DR, Duričić I, Šobajić SS, Timić J, Jorgačević B, Aleksić V, Vučević DB, Ješić-Vukićević R, Radosavljević TS. Time-dependent changes and association between liver free fatty acids, serum lipid profile and histological features in mice model of nonalcoholic fatty liver disease. Arch Med Res. 2014;45:116-124.
18. Buchman AL. The addition of choline to parenteral nutrition. Gastroenterology. 2009;137:S119-28.
19. Zeisel SH, da Costa KA. Choline: an essential nutrient for public health. Nutr Rev. 2009;67:615-623.
20. Talbot M, Hamel-Auger M, Beaulieu MJ, Gazzola M, Lechasseur A, Aubin S, Paré MÈ, Marsolais D, Bossé Y, Morissette MC. Impact of immunization against OxLDL on the pulmonary response to cigarette

smoke exposure in mice. Respir Res. 2018;19:131.
21. Larter CZ, Yeh MM, Williams J, Bell-Anderson KS, Farrell GC. MCD-induced steatohepatitis is associated with hepatic adiponectin resistance and adipogenic transformation of hepatocytes. J Hepatol. 2008;49:407-416.
22. Dela Peña A, Leclercq I, Field J, George J, Jones B, Farrell G. NF-kappaB activation, rather than TNF, mediates hepatic inflammation in a murine dietary model of steatohepatitis. Gastroenterology. 2005;129:1663-1674.
23. Itagaki H, Shimizu K, Morikawa S, Ogawa K, Ezaki T. Morphological and functional characterization of non-alcoholic fatty liver disease induced by a methionine-choline-deficient diet in C57BL/6 mice. Int J Clin Exp Pathol. 2013;6:2683-2696.
24. Mu YP, Ogawa T, Kawada N. Reversibility of fibrosis, inflammation, and endoplasmic reticulum stress in the liver of rats fed a methionine-choline-deficient diet. Lab Invest. 2010;90:245-256.

3.9 Figure legends

FIGURE 1. Dietary methionine and choline deficiency progressively and reversibly affects lung functions. Female C57BL/6 mice were fed control (Ctrl) diet or methionine/choline deficient (MCD) diet for 3 weeks and switched back, or not, to control diet for an additional week to assess reversibility. (A) Lung functions were assessed using the FlexiVent at 3 and 4 weeks after initiation of the diet. (B) Body weight evolution over the 4 weeks under control and MCD. (C) Histology of whole lung cross-sections (H&E stain) after 4 weeks under control or MCD diet. Data are presented as mean±SEM. n=5 per group. *p<0.05.

FIGURE 2. Dietary methionine and choline deficiency reduces pulmonary expression of extracellular matrix genes. (A) Levels of extracellular matrix genes from gene expression arrays performed on lung tissue from female C57BL/6 mice fed control (Ctrl) diet or methionine/choline deficient (MCD) diet for 4 weeks. (B) Lung mRNA levels of *col1a1*, *col3a1*, *elastin* and *lum* assessed by quantitative PCR (qPCR) in female C57BL/6 mice fed control diet or methionine/choline deficient (MCD) diet for 3 weeks and switched back, or not, to control diet for an additional week. Lung mRNA levels of *col1a1*, *col3a1*, *elastin* and *lum* assessed by qPCR in female C57BL/6 mice exposed to room air (RA) or cigarette smoke (CS) for (C) 2 weeks and (D) 4 weeks and fed control diet or MCD diet for the same period, respectively. Data are presented as mean±SEM. n=5 mice per group. *p<0.05; **0<0.01; ***p<0.001.

FIGURE 3. Dietary methionine and choline deficiency progressively alters cigarette smoke-induced lung function changes and inflammation. Female C57BL/6 mice were exposed to room air (RA) or cigarette smoke (CS) for (A-D) 2 weeks or (E-H) 4 weeks and fed control (Ctrl) diet or methionine/choline deficient (MCD) diet for the same period, respectively. (A and E) Lung functions were assessed using the FlexiVent. (B and F) Total, mononuclear and neutrophil cell counts were performed on the bronchoalveolar lavage (BAL). (C and G) Protein levels of interleukin 1 alpha (IL-1α) and monocyte chemotactic protein 1 (CCL2) were assessed in the BAL fluid (BALF). (D and H) Lung mRNA levels of *cxcl5* and *mmp12* were assessed by qPCR. Data are presented as mean±SEM. n=5 mice per group. *p<0.05; **0<0.01; ***p<0.001.

FIGURE 4. Dietary methionine and choline deficiency reduces pulmonary surfactant levels as well as lung tissue mechanical properties. (A) Serum and bronchoalveolar lavage fluid (BALF) phosphatidylcholine (PC) levels were assessed in female C57BL/6 mice fed control (Ctrl) diet or methionine/choline deficient (MCD) diet for 3 weeks and switched back, or not, to control diet for an additional week. (B) Serum and bronchoalveolar lavage fluid (BALF) PC levels were assessed in female C57BL/6 mice exposed to room air (RA) or cigarette

smoke (CS) for 4 weeks and fed Ctrl diet or MCD diet for the same period. (C) Lung functions were assessed using the FlexiVent before and after bronchoalveolar lavage (BAL) in female C57BL/6 mice fed Ctrl diet or MCD diet for 4 weeks. Data are presented as mean±SEM. n=5 mice per group. **0<0.01; ***p<0.001.

FIGURE 5. Dietary deficiencies in choline and methionine each affect differently the lungs. Female C57BL/6 mice were fed control (Ctrl) diet, methionine-deficient (MD) diet or choline-deficient (CD) diet for 4 weeks. (A) Lung functions were assessed using the FlexiVent. (B) Lung mRNA levels of *col1a1*, *col3a1*, *elastin* and *lum* were assessed by qPCR. (C) Phosphatidylcholine (PC) levels were assessed in the serum and bronchoalveolar lavage fluid (BALF). Data are presented as mean±SEM. n=5 mice per group. *p<0.05; **0<0.01; ***p<0.001; ****p<0.0001.

FIGURE 6. Dietary deficiencies in choline and methionine each affect differently the pulmonary response to cigarette smoke exposure. Female C57BL/6 mice were exposed to room air (RA) or cigarette smoke (CS) for 4 weeks and fed control (Ctrl) diet, methionine-deficient (MD) diet or choline-deficient (CD) diet for the same period. (A) Lung functions were assessed using the FlexiVent. (B) Total, mononuclear and neutrophil cell counts were performed on the bronchoalveolar lavage (BAL). (C) Protein levels of interleukin 1 alpha (IL-1α) and monocyte chemotactic protein 1 (MCP-1) were assessed in the BAL fluid (BALF). (D) Lung mRNA levels of *cxcl5* and *mmp12* were assessed by quantitative PCR. (E) Lung mRNA levels of *col1a1*, *col3a1*, *elastin* and *lum* were assessed by qPCR. (F) Phosphatidylcholine (PC) levels were assessed in the serum and BALF. Data are presented as mean±SEM. n=5 mice per group. *p<0.05; **0<0.01; ***p<0.001; ****p<0.0001.

3.10 Figures

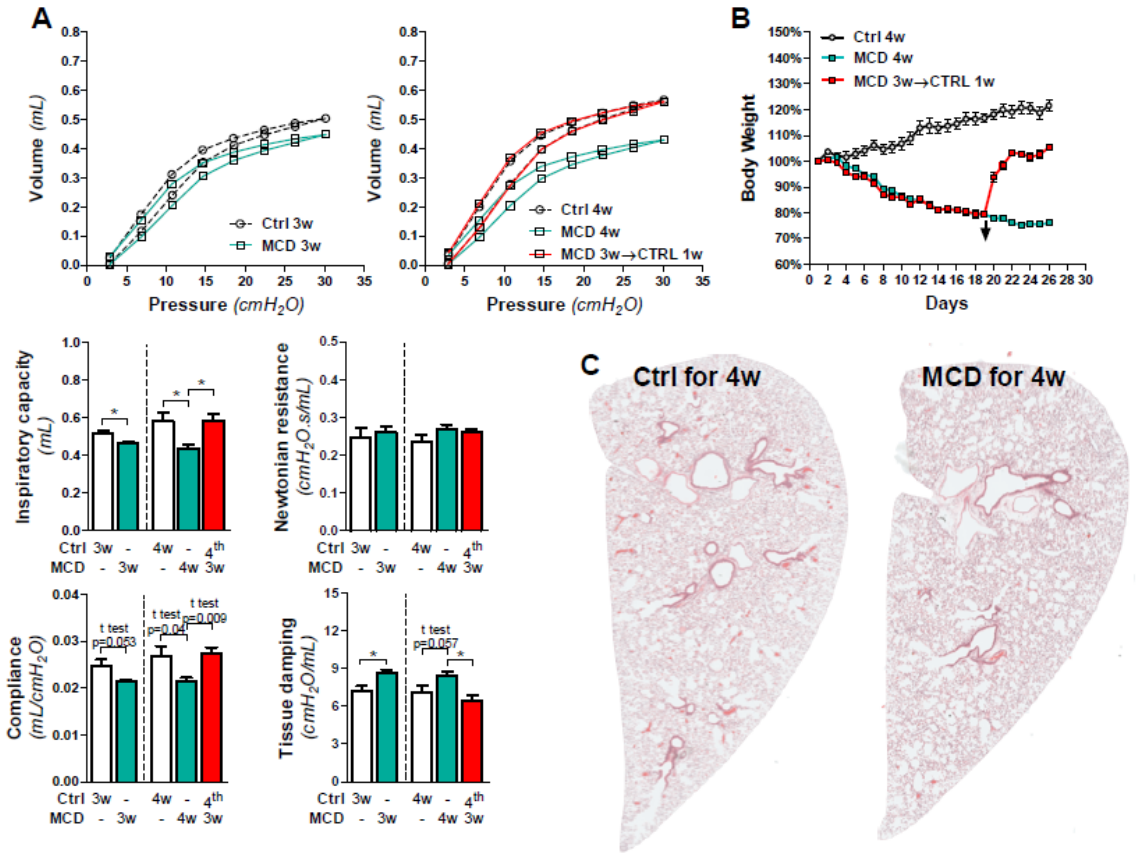

Figure 3. 1: Dietary methionine and choline deficiency progressively and reversibly affects lung functions.

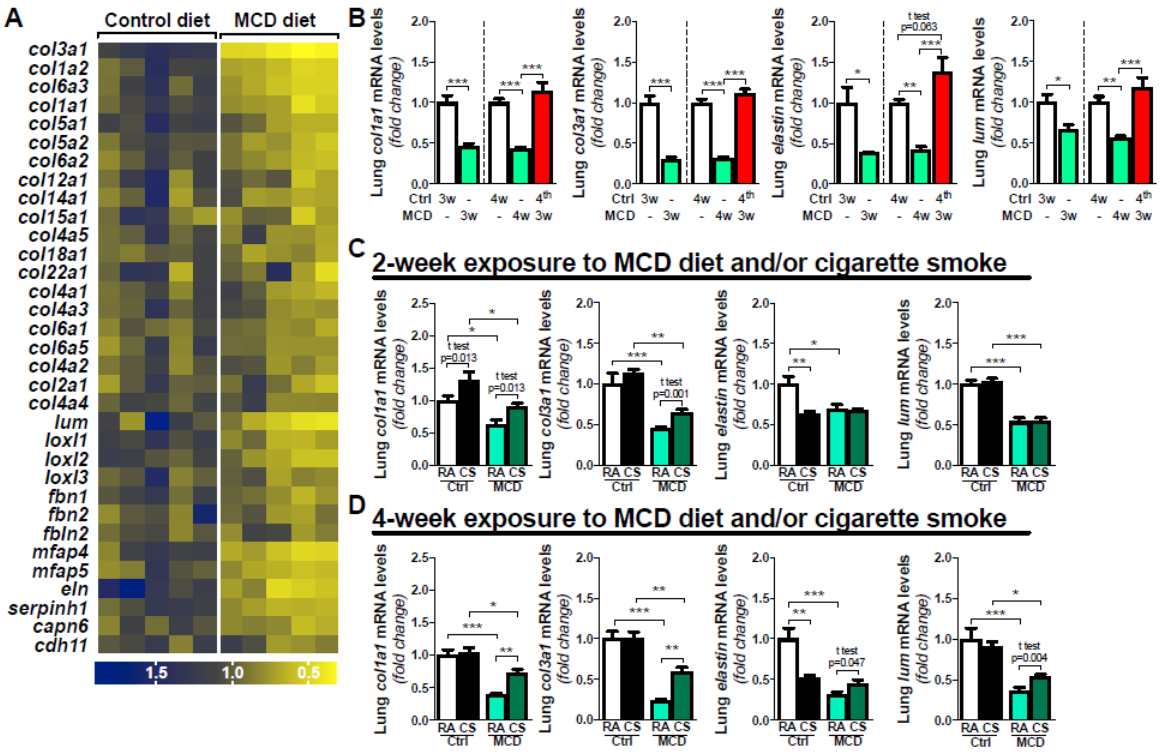

Figure 3. 2: Dietary methionine and choline deficiency reduces pulmonary expression of extracellular matrix genes.

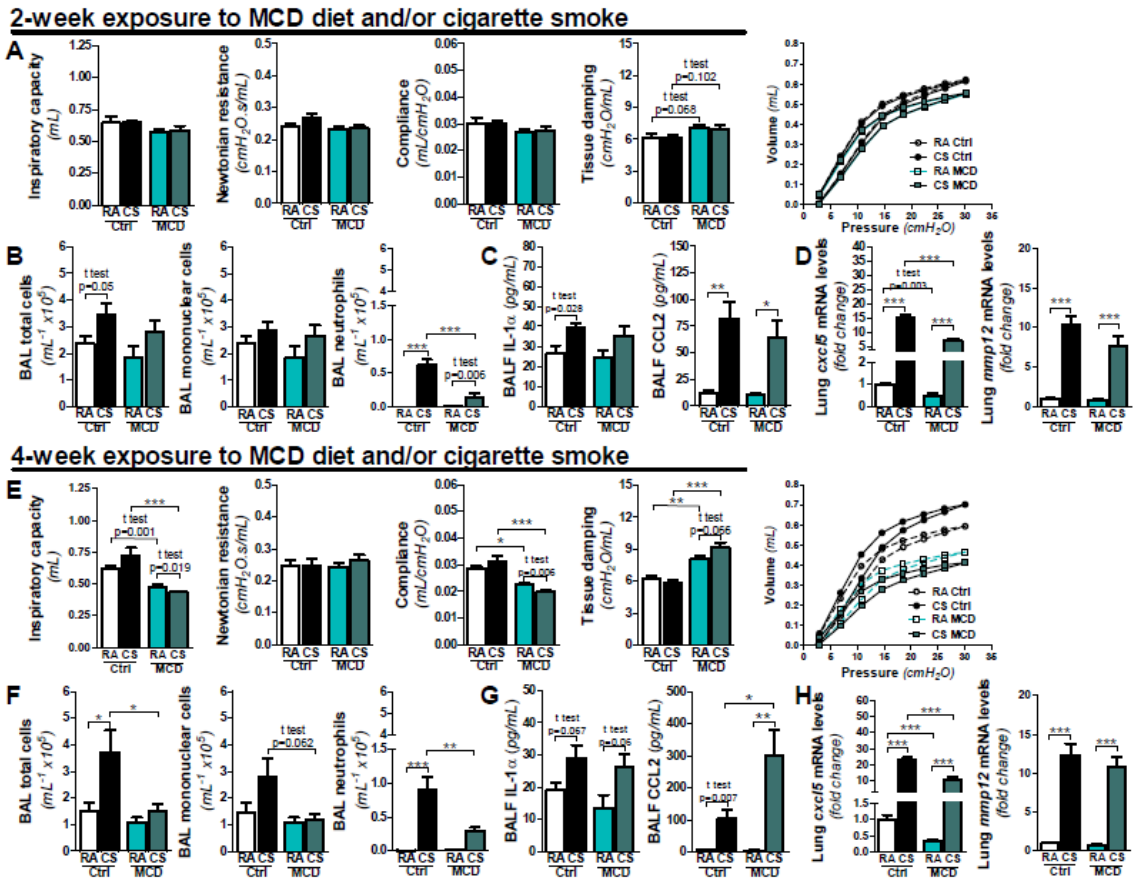

Figure 3. 3: Dietary methionine and choline deficiency progressively alters cigarette smoke-induced lung function changes and inflammation.

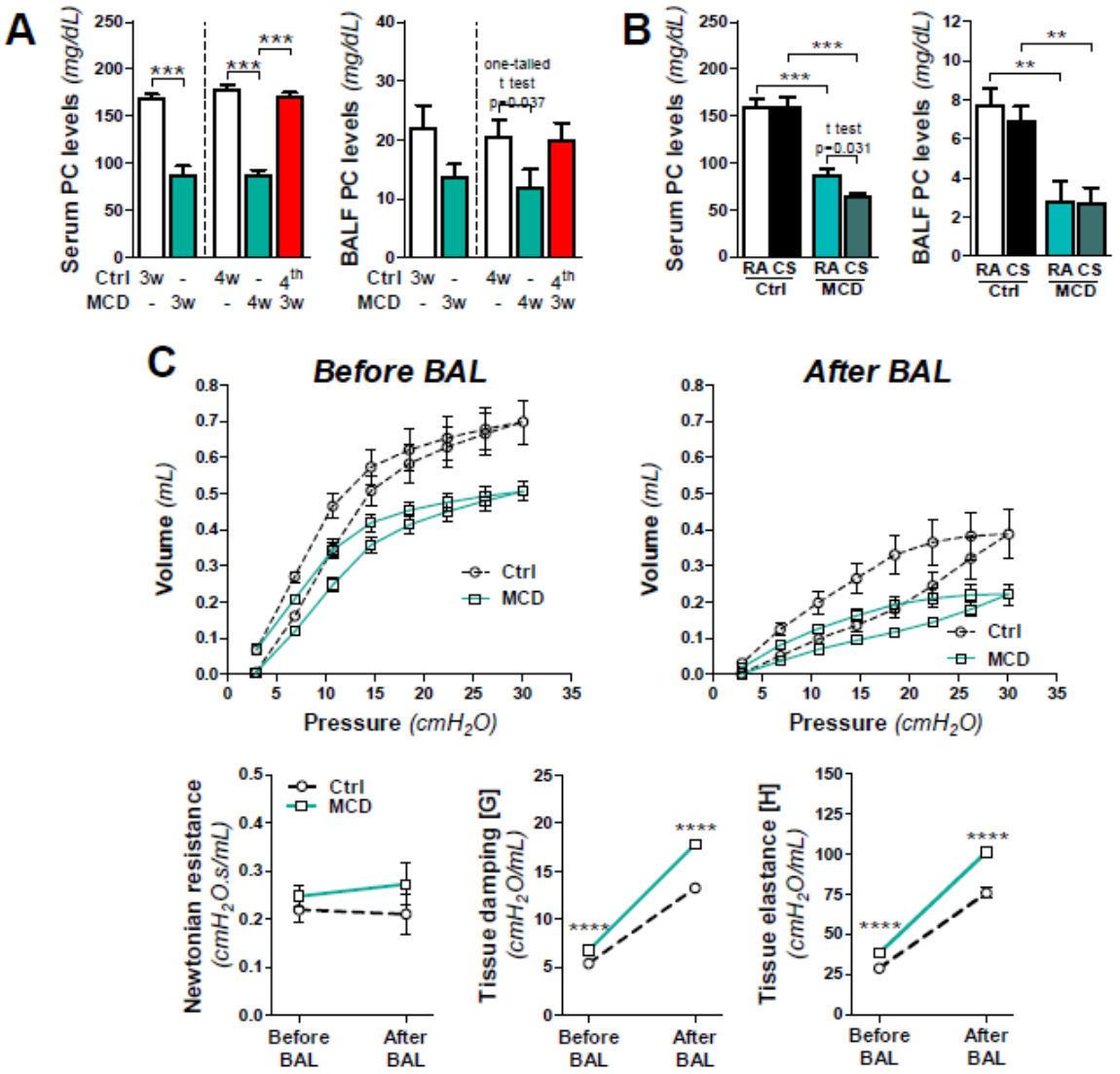

Figure 3. 4: Dietary methionine and choline deficiency reduces pulmonary surfactant levels as well as lung tissue mechanical properties.

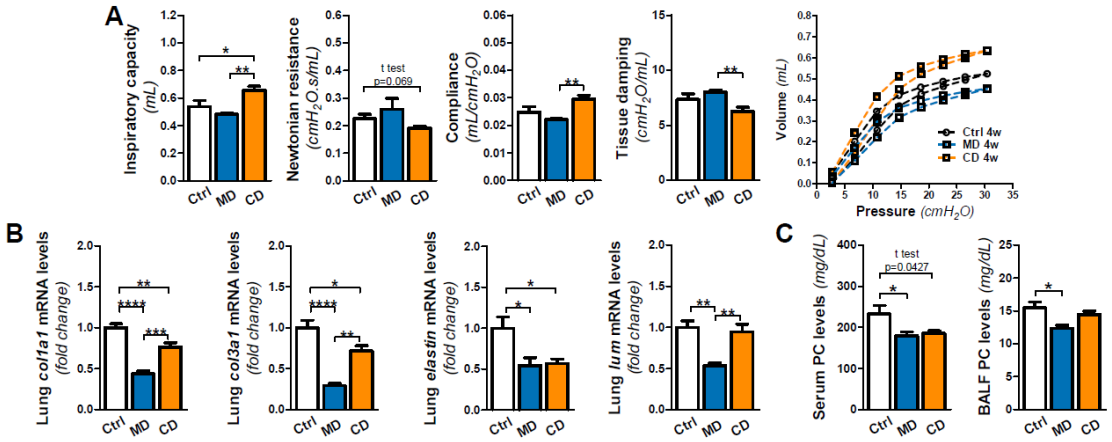

Figure 3. 5: Dietary deficiencies in choline and methionine each affect differently the lungs.

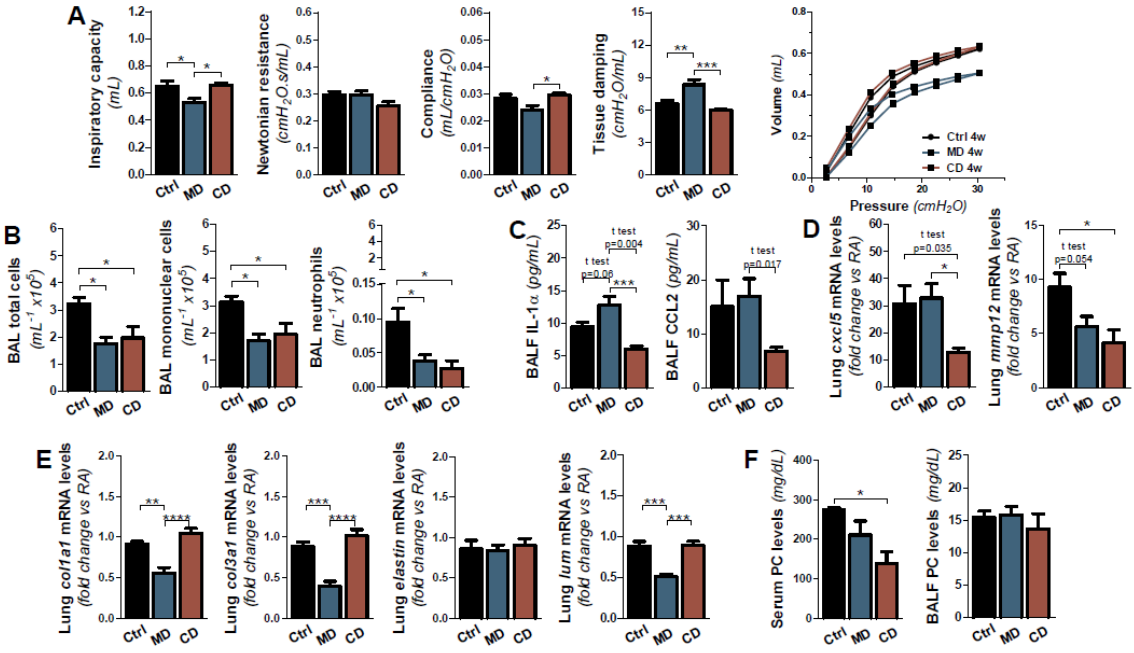

Figure 3. 6: Dietary deficiencies in choline and methionine each affect differently the pulmonary response to cigarette smoke exposure.

General discussion, limitations and perspectives

The first two chapters of this thesis had a common goal, to answer this simple question: How and why is pulmonary lipid transport altered following cigarette smoke exposure and can we improve it? It is now known that cigarette smoke exposure alters the expression of key genes involved in pulmonary lipid transport in the lung of smokers and ex-smokers, and rapidly in the lung and alveolar macrophages of cigarette smoke-exposed mice (Chapter 1 & 2). Furthermore, under acute cigarette smoke exposure conditions (two-hour exposure), we have shown that the pulmonary milieu (i.e. BALF) was adequately equipped to activate lipid efflux (Chapter 1). In Chapter 1, it was also described that removing a key apolipoprotein, ApoA-1, exacerbates the inflammatory pulmonary response to cigarette smoke. Furthermore, Chapters 1 and 2 investigated two new therapeutic strategies to overcome the negative lipid transport outcomes observed following cigarette smoke exposure. Firstly, HDL supplementation therapy exhibited great potential, as it dampened some negative aspects associated with cigarette smoking: for instance alveolar macrophage size and loss of lean mass. Secondly, LXR activation therapy was remarkable at increasing diverse pulmonary lipid transport genes; however, hyperactivation of this nuclear receptor induced acute exacerbation of the pulmonary response to cigarette smoke, especially in alveolar macrophages. For this reason, the latter therapy should not be proposed to smokers, unless a better compound with fewer inflammatory effects is developed, as it also led to decreased pulmonary surfactant levels. Additional studies are required to evaluate the therapeutic potential of these two strategies during a cessation protocol.

The final chapter on nutrition and pulmonary health suggests that alteration of pulmonary lipid homeostasis due to phosphatidylcholine deficiency via methionine and choline deprivation can drastically impact lung function, the extracellular matrix and the pulmonary inflammatory response to cigarette smoke exposure. This study sheds light on a new field of research and opens new possible explanations for diverse lung diseases involving pulmonary surfactant. In this final chapter, results from the previous chapters will be further discussed and future directions will be proposed, although additional discussion of these results can be found in the discussion of each published scientific articles.

Are alveolar macrophages or is the pulmonary environment responsible for disrupted lipid transport?

There are three possible explanations as to why alveolar macrophages become foamy under acute cigarette smoke exposure conditions. Firstly, cigarette smoke exposure may lead to an inadequate level of lipid acceptors, for instance ApoA-1 and HDLs, in the pulmonary environment, which may impair alveolar macrophages export of engulfed lipids. Secondly, if the pulmonary environment is adequately equipped with lipid acceptors, it is possible that changes in alveolar macrophage expression and function may render them unable to appropriately export lipids. This may be due to 1) an inappropriate level of proteins involved in lipid export or 2) altered nuclear receptor functions which reduce their capacity to stimulate the transcription of lipid export genes. It could also be a normal pulmonary macrophage response, perhaps macrophages want to accumulate lipids. Finally, the last option may be that cigarette smoking upregulates scavenger receptor activity and ultimately overwhelms these pulmonary cells with excessive levels of oxidized lipids, even in the presence of adequate lipid export proteins and lipid acceptors. One can also hypothesize that cigarette smoke induces a foamy alveolar macrophage phenotype by altering all of these mechanisms, leading to decreased extracellular lipid acceptors, lower lipid export proteins and increased oxidized lipid load. The upcoming section will discuss these possible hypotheses.

The pulmonary environment

Following cigarette smoke exposure, the pulmonary environment, which can be studied via bronchoalveolar lavage, contains cytokines and inflammatory cells, such as neutrophils and alveolar macrophages. In addition, the BAL fluid also contains ApoA-1 [751]. The findings from the MDCO-216 study shows that the BAL fluid of room air- and cigarette smoke-exposed mice is rapidly in contact with the recombinant HDL, typically within 6 hours of injection (Chapter 1, Figure 4A & B). In Chapter 1, it was demonstrated that the BAL fluid of cigarette smoke-exposed mice exhibited a higher lipid efflux capacity after a single two-hour cigarette smoke exposure compared to unexposed mice (Chapter 1, Figure 2B). This result suggests that there are higher levels of lipid acceptors in the BAL fluid during an acute cigarette smoke exposure. The increase in lipoproteins could be due to increased secretion from the liver [380] or from local pulmonary production [630]. Unpublished data from Chapter 3 shows

that circulating levels of ApoA-1 are higher in cigarette smoke-exposed mice compared to unexposed controls after four weeks of exposure. Despite the fact that lung ApoA-1 levels are lower in smokers with emphysema and in chronic (six months) cigarette smoke-exposed mice [630], one can hypothesize that ApoA-1 levels may rise during acute and subchronic cigarette smoke exposure to overcome the high demand for lipid export, oxidation and inflammation in the lung. Unfortunately, long-term cigarette smoke exposure adds sustained pressure on the pulmonary surfactant due to an increased oxidation and alteration of HDL integrity, thus leading to low levels of ApoA-1 and HDL [634].

Although we did not measure circulating and pulmonary ApoE levels, acute cigarette smoke exposure led to a reduction in *apoe* mRNA expression in the lung tissue (Chapter 1) and in isolated alveolar macrophages (Chapter 2) of cigarette smoke-exposed mice. Interestingly, *apoe* gene expression is increased in mice chronically exposed to cigarette smoke and in active smokers. Therefore, one can hypothesize that ApoA-1/other apolipoproteins compensate for reduced ApoE levels during acute cigarette smoke exposure and inversely, during chronic exposition, ApoE or other apolipoproteins may compensate for low ApoA-1 levels. However, additional studies are required to investigate this possibility.

The alveolar macrophage

Chapter 2 focused, in part, on the impact of cigarette smoking on the alveolar macrophage's capacity to export lipids. The expression of the four most important genes involved in pulmonary lipid export was measured in alveolar macrophages isolated from cigarette smoke-exposed mice. Transcript levels of *abca1*, *abcg1*, *apoe* and *scarb1* were reduced following acute exposure to cigarette smoke (Chapter 2, Figure 2B). Sonett *et al*. found similar results regarding mRNA expression of *abca1* in the lung and alveolar macrophages from smokers and cigarette smoke-exposed mice, respectively [752]. Despite the fact that protein levels were not assessed in our study, the direct cholesterol efflux capacity of unexposed and cigarette smoke-exposed alveolar macrophages was measured (Chapter 2, Figure 4A). This represents, to the best of our knowledge, the first time that a cholesterol efflux assay has been performed using alveolar macrophages. One striking observation is the high basal level of cholesterol efflux from alveolar macrophages, showing how important this function is in the lung. The

impaired efflux capacity of alveolar macrophages and the reduced expression of lipid export genes induced by cigarette smoke exposure strongly suggest that the main defect in lipid transport is due to impaired alveolar macrophage lipid efflux.

One can further postulate that impaired nuclear receptor activation may be the cause of this efflux dysregulation in alveolar macrophages. However, Higham *et al*. showed that LXR mRNA and protein levels were higher in whole lung tissue extract of COPD patients, a result that was not observed in isolated alveolar macrophages from the same COPD patients compared to controls [506]. Unpublished results from the human expression data set found in Chapter 1 showed that LXR mRNA levels in total lung tissue extracts did not differ between healthy controls, smokers and ex-smokers. Overall, this suggests that the nuclear receptor LXR seems to be unaltered during cigarette smoking, although additional activity assays are required to confirm this hypothesis.

Together results from Chapters 1 and 2, investigating which aspect of pulmonary lipid transport is impaired during cigarette smoke exposure, suggest that the main problem comes from alveolar macrophages.

> Impaired pulmonary lipid export mechanisms under acute cigarette smoke exposure conditions seems to be due to altered alveolar macrophage function and not inadequate lipid acceptors in the pulmonary environment.

Limitations

One limitation of studying lipid transport in mice is the difference between ApoA-1/HDL levels in humans compared to mice. The murine lipid profiles are characterized by high levels of HDLs and low levels of LDLs, while the opposite is true of human circulating lipoproteins [753]. Furthermore, it has been known that mice lack a key protein involved in lipid transport, the CETP [754]. This protein is involved in the transfer of cholesteryl esters (from HDLs to VLDLs, chylomicrons and LDLs) but also in

the transfer of triglycerides from VLDLs and chylomicrons to HDLs. Therefore, lipid dynamics differ between species, as shown in studies by Agellon *et al*. and Marotti *et al*. where HDLc levels were reduced in mice overexpressing human CETP [755, 756]. Additional studies are required in order to extrapolate our results to human smokers.

Perspectives

One path that should be further explored is the dynamic effects of cigarette smoke exposure on ApoA-1/HDL levels in the lung. Kim *et al*. measured ApoA-1 levels in the lung tissue of mice chronically exposed to cigarette smoke [757]. Measurement of ApoA-1 levels in the BAL fluid of cigarette smoke-exposed mice following acute (2 hours and 4 days) or subchronic (8 weeks) exposures could shed light on how cigarette smoking modulates this apolipoprotein. These results could also pinpoint an approximative time associated with the reduction in ApoA-1 levels following cigarette smoke exposure and could refine the pulmonary lipid transport "supplementation therapy". The next step would be to confirm this hypothesis in the BAL fluid of healthy controls, active smokers and ex-smokers.

Results from the BAL fluid (Chapter 1) and alveolar macrophages (Chapter 2) cholesterol efflux assays are of particular interest as they represent the pulmonary lipid transport capacity of cigarette smoke-exposed mice and smokers. This simple, *ex vivo*, test could become a key asset to further characterize patients who may benefit from ApoA-1/HDL and RCT targeted therapies, allowing for a more personalized approach to treatment. In mice, additional time-course (2-hour, 4 days, 8 weeks, and 24 weeks of cigarette smoke exposition) studies focused on the lipid efflux capacities of BALF and isolated alveolar macrophages from cigarette smoke-exposed mice are required. These results would shed light on how cigarette smoking affects the dynamic of pulmonary lipid transport capacity of the pulmonary environment (BALF) and the direct export capacity of alveolar macrophages.

Additional genetic analysis should also be performed since multiple SNPs have been identified in lipid transport genes: for instance in the *abca1* gene [758, 759] and the *abcg1* gene [760, 761]. Most of these SNPs identified to lipid transport genes were investigated in the context of cardiovascular diseases. It would be appropriate to further study these SNPs in the context of pulmonary health and

pulmonary lipid transport of healthy controls, smokers and COPD patients. In addition, linking these genetic variations with ApoA-1 levels or cholesterol efflux assays could be a great tool to further characterize the impact of these SNPs on pulmonary lipid transport capacity of patients.

What is the best option to promote pulmonary lipid transport in the context of cigarette smoking?

Chapters 1 and 2 also investigated the impact of HDL supplementation and LXR activation under cigarette smoke exposure conditions. Both of these therapeutic strategies had the same goal, to promote lipid efflux from alveolar macrophages and thus reduce the lipid burden. However, is there a clear benefit to reducing intracellular lipid accumulation in alveolar macrophages? It is known that lipid-laden macrophages exhibit higher levels of ER stress [441] and that this can be toxic to cellular membranes [439]. Uncontrolled ER stress leads to apoptosis [762, 763], a feature already present during cigarette smoke exposure [261]. Furthermore, alveolar macrophages are exposed to high levels of oxidative compounds due to cigarette smoking [184]. We hypothesize that overwhelmed macrophages may expend their metabolic energy on exporting lipids instead of clearing pathogens and apoptotic cells. Chapter 3 also explored the impact of nutrition on pulmonary homeostasis. The model used, the MCD diet, is known to alter lipid transport mechanisms, therefore optimizing nutrition could be an alternative strategy to modulate pulmonary lipid transport.

Can HDLs be used to prevent lung damage induced by cigarette smoke?

While RCT is the main function of HDLs [380, 560-562], they also have anti-inflammatory and antioxidative roles which could be beneficial during cigarette smoking. Many studies have investigated the impact of recombinant HDL supplementation in the context of cardiovascular disease: for instance with the CSL-111 and the MDCO-216 complexes. Unfortunately, these clinical trials using recombinant HDLs failed to demonstrate a therapeutic potential in patients with cardiovascular diseases [764-766]. In 2016, MDCO-216 production was discontinued. It was also stated by Rader *et al*. that research teams should stop focusing on HDL therapies in the cardiometabolic field [767]. However, these studies focused on the size of atheromatous plaques using IVUS [768] and plaque volume was measured in a

single and specific cardiovascular region, potentially introducing a bias. Furthermore, these studies did not explore the potential impact of recombinant HDL therapy on plaque composition.

In Chapter 1, we investigated the impact of MDCO-216, an ApoA-1 Milano and phospholipid complex, on pulmonary and systemic effects of cigarette smoking. Firstly, it was rapidly detected, between 1 and 6-hours, in the BALF of room air- and cigarette smoke-exposed mice. Secondly, prophylactic MDCO-216 injections dampened the immune inflammatory response by reducing mononuclear cells and the size of alveolar macrophages in the BAL following acute cigarette smoke exposure. Lastly, therapeutic MDCO-216 treatments had some protective effects on lung function and led to interesting changes on body composition (Chapter 1). Cigarette smoke-exposed mice treated with MDCO-216 showed an increase in lean mass compared to untreated, exposed counterparts. This muscular effect is important and requires further investigations as some COPD patients develop muscular atrophy, a loss of lean mass, which can affect their quality of life [769].

A study by Lethi *et al*. showed that HDLs were able to maintain skeletal muscular function by modulating glucose cellular respiration [770]. In their study, they showed that mice overexpressing the human ApoA-1 had a higher weight and lean mass percentage compared to wild-type controls. They further showed that HDLs increased glycolysis and rate of oxygen consumption *in vitro* [770]. In addition, they investigated whether HDLs could help prevent age-associated muscle function decline. Wild-type and transgenic mice performed an endurance test twice at two different time points: 0 and 8 weeks later. This test showed that wild-type mouse walking distance declined over time compared to mice overexpressing human ApoA-1. This result suggests that HDL/ApoA-1 supplementation could preserve skeletal muscle functions, a feature usually altered in subchronic cigarette smoke-exposed mice [769]. Therefore, HDL supplementation using the MDCO-216 could potentially be a great tool to improve the quality of life in former smokers and COPD patients with muscular atrophy. Overall, HDL supplementation with MDCO-216 showed beneficial effects on the pulmonary response to cigarette smoke with an interesting finding regarding lean mass.

Can LXR activation be beneficial during cigarette smoking?

Promoting RCT via the activation of the nuclear receptor LXR has already been investigated in the context of cardiovascular disease [477, 479]. The main idea is to increase the transcription of *abca1* and *abcg1* to stimulate the export of lipids from foamy macrophages. LXR is critical to the maintenance of pulmonary lipid homeostasis [505] and its activation is considered anti-inflammatory in diverse pulmonary diseases for instance fibrosis, asthma, acute lung injury and cancer [486-497, 503]. Therefore, studying the impact of LXR activation in an inflammatory environment and in the presence of foamy alveolar macrophages, such as during cigarette smoking, is relevant.

Treatment with T0901317, an LXR agonist, greatly increased lung and alveolar macrophage expression of *abca1* and *abcg1*. However, T0901317 failed to increase cholesterol efflux of cigarette smoke-exposed alveolar macrophages, suggesting that cigarette smoke may interfere with T0901317's ability to promote RCT *in vivo*. LXR activation also induced an exacerbated pulmonary inflammatory response to cigarette smoke and led to surfactant depletion. Please refer to Chapter 2's discussion that covers most of these aspects. An interesting hypothesis presented in Chapter 2 is the imbalance between lipid capture and lipid export. Chapter 2 showed that the expression of lipid capture genes, such as *msr1* and *marco* are increased following cigarette smoke exposure. Unpublished data from Morissette shows that *msr1*$^{-/-}$ mice have an increased pulmonary inflammatory response to cigarette smoke, mostly neutrophils, higher BALF IL-1α and MCP-1 levels, and a higher BALF turbidity, a sign of lipid accumulation compared to wild-type controls. Phenotypes that were not observed in *marco*-knockout mice exposed to cigarette smoke. Therefore, additional studies are required to fully understand the importance of MSR1 and its interaction with LXR under cigarette smoke exposure conditions.

One cannot forget that LXR activation leads to an increase in circulating and hepatic triglycerides in mice and humans [771, 772], a major side effect of LXR agonists. A lot of effort has been put towards developing new compounds that does not activate the SREBP pathway [773, 774]. *N*,*N*-dimethyl-3β-hydroxycholenamide and methylpiperidinyl-3β-hydroxycholenamide (also known as DMHCA [699] and MePipHCA [775], respectively) are new agonists that bind to LXRα,β without any adverse effects on the expression of lipogenic SREBP targets. T0901317 and GW3965 definitely helped in the

understanding of LXR's role in regulating macrophage lipid biology; however these molecules have a different structure compared to endogenous ligands [773]. Additional studies using these new agonists are required to decipher their impact on the lung during cigarette smoke exposure.

Promoting RCT via dietary modification and other pharmacological treatments

Despite the fact that we did not investigate the impacts of dietary intake and reverse cholesterol transport, we showed that nutritional deficiencies can lead to altered lung homeostasis, especially under cigarette smoke exposure conditions. We have unpublished data showing that administration of a methionine choline deficient diet leads to lower circulating ApoA-1, reduced serum cholesterol efflux capacity, and lower *abcg1* and *apoe* pulmonary mRNA levels in mice, suggesting that nutrient deprivations could alter pulmonary lipid transport. These results are in line with the reduced circulating lipoprotein levels induced by the MDC diet [737].

Nutrition and RCT is a new field of research (reviewed [776, 777]. Diverse functional foods and bioactive compounds have been tested to improve RCT, for instance cocoa [778], olive oil [779] and resveratrol [780]. Flavonoids, a group of compounds within the polyphenol family, are one class of molecules that have been shown to improve RCT [776, 777] but also to reduce inflammation [781] and to act as an antioxidant [782]. They are mostly found in vegetables and fruits [781]. Most studies investigating RCT using flavonoids were performed on cell lines and in rodent models [777] and, like many nutritional studies, results only show weak evidence. Choline and/or methionine supplementation could be a potential option to improve RCT, as deficiencies are relatively common and associated with lower VLDL and HDL levels [737]. In particular, it could be an interesting therapeutic strategy in the context of cigarette smoke since it is known that smokers have lower circulating choline levels [783]. Studies investigating methionine status in smokers do not currently exist.

One can also propose other therapeutic approaches to improve RCT: for instance the use of CEPT inhibitors [784]. The goal of this therapy is to retain cholesterol in the HDL fraction which will lead to an increase in HDL-c and HDL levels, a mechanism explained by Barter *et al*. [785]. As with recombinant HDLs and apolipoprotein mimetics, these molecules have only been tested in the context of cardiology.

Unfortunately, most of the phase 3 clinical trials with CEPT inhibitors were stopped due to lack of efficacy, such as evacetrapib [784, 786]. Nevertheless, one compound succeeded in reducing cardiovascular events: anacetrapib [787]. Although anacetrapib did not increase HDL levels but instead reduced non-HDL lipoproteins [784], it may be a new option to promote RCT under cigarette smoking exposure conditions.

Statin administrations is another strategy that could be used to improve RCT [788]. Statins inhibit the activity of hydroxymethyl glutaryl coenzyme A reductase, the rate-limiting enzyme in the biosynthesis of cholesterol [788]. Guerin *et al*. showed that atorvastatin reduced atherogenic lipoproteins (LDLs, IDLs and VLDLs) but also raised ApoA-1 levels [789]. These results are in line with studies conducted by Nissen *et al*. showing a mild increase in HDL-c levels following atorvastatin treatment [790-792]. However, Remaley *et al*. pointed out that the effects of statins on plaque volume were observed only after several years [641], which is significantly less favourable compared to the speed and ability of MDCO-216 to reduce plaque volume [655]. Therefore, MDCO-216 may be more appropriate in suppressing lipid accumulations in alveolar macrophages during cigarette smoking and also promote some cardiovascular health.

To conclude, the comparison between our different RCT promoting therapies suggest that HDL supplementation (Chapter 1) is associated with better outcomes than LXR activation (Chapter 2) under cigarette smoke exposure conditions. Although potential mechanisms were further investigated in the T0901317 study, MDCO-216 administration was beneficial to several lung outcomes compared to LXR therapy. Regarding the effects of diet modification and other pharmaceutical strategies, future studies are required to evaluate the impact of choline supplementation and CETP inhibitors on RCT in the context of cigarette smoking.

In the context of cigarette smoking, exogenous supplementation of HDL seems to be a better option to improve RCT and pulmonary outcomes in response to cigarette smoke compared to the activation of the liver X receptor

Limitations

Besides the use of a mouse model to study a human phenomenon, the main limitation in the MDCO-216 study is the presence of antibodies against MDCO-216, a human-developed complex. The generation of these antibodies likely reduced the ability of MDCO-216 to dampen the response to cigarette smoke, a topic already covered in the discussion section of Chapter 1.

As mentioned before, mice lack CETP a key enzyme involved in lipoprotein metabolism. LXRs are key cholesterol sensors involved in the transcription of lipid export genes. Furthermore, transcription of CETP is under the control of LXR [793]. LXR also activates cholesterol 7α-hydroxylase (CYP7a1) in mice but not in humans [481, 794], the first and rate-limiting enzyme in the conversion of cholesterol to bile. Groot *et al*. further demonstrated the importance of using different models when using LXR agonists [794]. They showed that in hamsters and cynomolgus monkeys, two species expressing CETP, LXR activation led to an increase in LDLc, suggesting LXR activation in these models leads to increased cardiovascular disease markers [794]. Therefore, caution must be taken when extrapolating mouse data to humans. Nevertheless, our LXR study using cigarette smoke-exposed mice revealed additional lung impacts when using T0901317.

Perspectives

One of the most relevant perspectives from the MDCO-216 study would be to investigate its potential in a clinical trial with former smokers. This ApoA-1 Milano/phospholipids complex has already been used in clinical trials [655, 766]; therefore, it would accelerate clinical trial steps involving this compound and become the first lung study using recombinant HDLs. Readouts, such as lung function, BAL and alveolar macrophage efflux capacities, and 6-minute walk test could be investigated. Furthermore, it should be tested in former smokers with or without COPD, since active smoking may alter the integrity and capacity of MDCO-216 to efflux lipids, a known effect of cigarette smoke effects HDLs.

One of the pulmonary outcomes of cigarette smoking is the increased risk of developing bacterial and viral infections [795, 796]. LPS is a key constituent of gram-negative bacteria [797] and induces an

inflammatory response by activating monocytes and endothelial cells via TLR4 [798]. Interestingly, circulating lipoproteins are able to bind and sequester LPS [799]. This binding is possible with the presence of the LPS binding protein (LBP) [614, 799]. The latter protein, with the help of PLTP, is involved in the transfer of LPS from HDLs to LDLs [614]. Furthermore, VLDLs and LDLs with LBP were also found to bind LPS and enhance the sequestration of LPS [797]. We have preliminary data showing that unexposed MDCO-216-treated mice had a better lung bacterial clearance compared to unexposed, untreated controls in a *Haemophilus influenza* model, suggesting that MDCO-216 might enhance the lung ability to eliminate gram-negative bacteria. However, additional studies are required to investigate its role under cigarette smoke exposure conditions.

As for the LXR study, most of the perspectives are mentioned above. Briefly, it is relevant to investigate the potential of other agonists that do not activate SREBP lipogenic pathways. We have not investigated these new agonists; however, we have unpublished data showing a similar but milder response to cigarette smoke when using GW3965, another LXR agonist. GW3965 also induced an exacerbated pulmonary inflammatory response to an acute cigarette smoke exposure. Miao *et al*. showed that GW3965 had a weaker LXR activity, approximately 70% compared to T091317 [800], which could explain the milder response to cigarette smoke compared to T0901317.

As already mentioned in Chapter 2's discussion, the interaction between LXR and surfactant synthesis is not well understood. Providing additional information on the interaction between cigarette smoking, pulmonary lipid transport and surfactant homeostasis would shed light on this field of research.

Regarding nutrient deprivations and pulmonary health, it would be relevant to explore the effects of other nutrients (such as lipids, carbohydrates, and other amino acids) on pulmonary functions. Finally, as mentioned above, the investigation of other pharmacological compounds, such as CETP inhibitors, may be another option to explore.

The underestimated role of pulmonary surfactant

"Pulmonary surfactant is a part of the lungs that physicians learn about as medical students and then ignore." [286]. This citation perfectly represents how surfactant is seen nowadays. As described in the introduction, it is much more than just a static component with mechanical properties. Our studies clearly show a relationship between pulmonary surfactant, nuclear receptors (Chapter 2), nutrition (Chapter 3) and cigarette smoking.

Liver X receptor and type 2 pneumocytes

As seen in Chapter 2, LXR activation led to pulmonary surfactant depletion and, as proposed in the discussion, this may be due to a decreased surfactant secretion by type 2 pneumocytes. Type 2 pneumocytes cover approximately 5% of the pulmonary epithelium compared to 95% by type 1 pneumocyte [801, 802]. Type 2 pneumocytes are responsible for surfactant secretion. Furthermore, they are involved in repairing alveolar epithelium after damage to type 1 pneumocytes [801], since they are the progenitor cells of the alveolar epithelium. Therefore, one hypothesis that could explain the potential lower secretion of pulmonary surfactant is an increased differentiation of type 2 to type 1 pneumocytes, lowering their number.

Another potential explanation concerns lipogenesis and fatty acid biosynthesis, a key step required for surfactant production and performed by type 2 pneumocytes [801]. One of the major pneumocyte differentiation factors is the keratinocyte growth factor [801]. This growth factor stimulates the expression of key transcription factors, such as SREPB-1c and CCAAT enhancer binding protein [801]. These factors will then increase mRNA levels of stearoyl CoA desaturase and fatty acid synthase, enzymes involved in fatty acid biosynthesis [801]. These fatty acids can further be used in phosphatidylcholine synthesis [803]. As mentioned in the introduction, SREBP-1 can also be activated by T0901317, an LXR agonist. Therefore, under cigarette smoke exposure conditions, the activation of LXR by T0901317 may alter diverse mechanisms involved in these pathways resulting in lower surfactant synthesis. Further, characterization of LXR activation and type 2 pneumocytes during cigarette smoking are required.

Pulmonary surfactant and nutrition

It is already known that malnutrition leads to dramatic lung problems during lung development [804]. Some nutrients were shown to be important for lung maturation. The retinoids found in vitamin A were shown to be involved in airway development and alveolization via the expression of extracellular matrix proteins [805]. Furthermore, deficiencies in vitamin D were shown to induce altered lung structure and maturation due to impaired lung alveolization and surfactant metabolism [806, 807].

As stated above, smokers have lower levels of circulating choline [783]. They also have lower surfactant/phospholipid levels in their BAL [316-318]. Furthermore, we have shown that deficiency in choline and methionine can dramatically alter lung function associated with extracellular matrix modification and surfactant depletion (Chapter 3). Therefore, one can imagine that supplementation in choline and/or methionine could be a great option for smokers; however, this has not been explored in the context of cigarette smoking.

On another note, choline supplementation in mice via inhalation, oral and intraperitoneal routes did not cause any liver and systemic toxicity during a 1-month of choline supplementation protocol [808]. The same research group showed promising results with choline supplementation in an ovalbumin mouse model of asthma [809]. Ovalbumin-challenged treated mice showed lower ROS and peroxidation levels with suppressed TNFα levels [809]. They further investigated this new therapeutic strategy in asthma patients [810] and showed that choline supplementation led to reduced inflammation and oxidative stress. Specifically, choline therapy (1500 mg) improved FEV_1, reduced IL-4, IL-5, and TNFα as well as lowered blood eosinophil counts and total IgE levels in asthma patients compared to baseline or standard pharmacotherapy after 6 months [810]. Another study showed that healthy adults who consumed more than 310 mg of choline per day had lower systemic inflammatory markers (such as CRP, IL-6 and TNFα) showing general anti-inflammatory effects of choline [811]. Choline depletion is unfortunately seen in children with cystic fibrosis which may lead to liver and muscle abnormalities [812]. Schall *et al*. investigated a lipid-choline complex supplementation in children with cystic fibrosis and found it to be a safe therapy that improved overall choline status [812]. As mentioned above, methionine supplementation studies and lung health have not been investigated.

Surfactant homeostasis was shown to be disrupted during LXR activation and dietary modification in choline and methionine, especially in the context of cigarette smoking. Surfactant studies are required to further decipher its role during cigarette smoking.

Limitations

One limitation of the MCD study is the impact of this diet on body weight, associated to the methionine deficiency [746, 747]: within 2 weeks, mice lost up to 20% of their initial weight. Due to ethical issues, protocols could last no more than 4 weeks and therefore the effects of MCD in subchronic and chronic cigarette smoke exposure conditions could not be explored. In future studies, additional factors should also be assessed, for instance food and water consumption rates, as these are greatly affected in MCD diet-fed animals.

One can also question the effect of this massive weight loss of lung function. However, as seen in Figure 3A & E, the major impact of diet on PV-loops is seen after 4 weeks compared to the 2 weeks protocol, suggesting that the effect of MCD diet on lung function is independent. In Chapter 3, we investigated the lung tissue response to diets; however, it would be relevant to explore how these methionine and choline deficient diets impact alveolar macrophages and type 2 pneumocytes, individually.

Perspectives

Overall, surfactant analysis should be routinely performed when studying lung functions, lung diseases and dietary deficiencies. Perhaps the usage of electron microscopy to characterize type 2 pneumocytes could be well suited to investigate this cell type in the context of cigarette smoking, LXR and choline deficiencies. As for the impact of human choline levels on the development of lung pathology, a study associating choline metabolism SNPs and lung function in smokers would be of interest. Furthermore,

the links between circulating choline levels, and lung gene expression could be investigated in a cohort of smokers.

A choline supplementation study will be performed by our laboratory in the upcoming year. The main objective will be to investigate the impact of choline supplementation on lung function and circulating levels in healthy subjects and smokers. To further characterize these effects, mouse models will be used to evaluate the outcomes of choline supplementation in the response to cigarette smoke. Now that these supplementation studies have already been done in asthma mouse models and patients [808, 810] and are soon to be performed in smokers, we hope that nutritional management will be considered as a new therapeutic strategy in lung disease.

Conclusion

Results from this thesis clearly show the importance of pulmonary lipid transport during cigarette smoking and add tremendous findings to the field of pulmonary lipid homeostasis during health and cigarette smoke exposure conditions. New therapeutic strategies, for instance recombinant HDLs and LXR agonist, were also explored to improve pulmonary lipid transport and revealed great potential, in the case of recombinant HDL, for ex-smokers in the future. A lot has been learned from the cardiovascular field and it would be relevant to evaluate the potential of such therapies in cigarette smoke-associated lung diseases. Of note, the alveolar macrophage was shown to be a key asset to pulmonary lipid homeostasis during cigarette smoking. The roles of pulmonary surfactant and type 2 pneumocytes are clearly underestimated in cigarette smoke-associated lung diseases and requires further attention. Furthermore, dietary intake of choline and methionine seems to be of great importance in the context of lung homeostasis and cigarette smoking. This new field of research “nutri-respiratory” opens unexplored avenues in the management of cigarette smoke-induced lung diseases but also in pulmonary health and may integrate, in the near future, the dietary recommendations to preserve lung homeostasis. Overall, this thesis revealed major impacts of cigarette smoking on pulmonary lipid homeostasis.

References

1. Services, U.S.D.o.H.a.H., *The Health Consequences of Smoking—50 Years of Progress: A Report of the Surgeon General*, U.S.D.O.H.A.H. SERVICES, Editor. 2014: Atlanta.
2. World Health Organization. *Tobacco*. 2018; Available from: https://www.who.int/en/news-room/fact-sheets/detail/tobacco.
3. Canada, S. *Tabagisme, 2016*. 2016; Available from: https://www150.statcan.gc.ca/n1/pub/82-625-x/2017001/article/54864-fra.htm.
4. Québec, I.N.d.S.P. *Le tabagisme au Québec*. 2014; Available from: https://www.inspq.qc.ca/tabagisme/le-tabagisme-au-quebec.
5. Thun, M.J., et al., *50-year trends in smoking-related mortality in the United States.* N Engl J Med, 2013. 368(4): p. 351-64.
6. Doll, R. and A.B. Hill, *The mortality of doctors in relation to their smoking habits; a preliminary report.* Br Med J, 1954. 1(4877): p. 1451-5.
7. Hammond, E.C. and D. Horn, *Smoking and death rates: report on forty-four months of follow-up of 187,783 men. 2. Death rates by cause.* J Am Med Assoc, 1958. 166(11): p. 1294-308.
8. Dorn, *The mortality of smokers and nonsmokers.* Proc Soc Stat Sect Am Stat Assn., 1958: p. 34–71.
9. World Health Organization. *Tobacco threatens us all: Health & economic impacts*. 2017; Available from: https://www.who.int/campaigns/no-tobacco-day/2017/infographic-economic-impact.png?ua=1.
10. Mackay, J.L., *The fight against tobacco in developing countries.* Tuber Lung Dis, 1994. 75(1): p. 8-24.
11. World Health Organization. *Tobacco threatens us all: poverty*. 2017; Available from: https://www.who.int/campaigns/no-tobacco-day/2017/infographic-poverty.png?ua=1.
12. World Health Organization, *Tobacco threatens us all: environment*. 2017.
13. Bonanomi, G., et al., *Cigarette butt decomposition and associated chemical changes assessed by 13C CPMAS NMR.* PLoS One, 2015. 10(1): p. e0117393.
14. Micevska, T., et al., *Variation in, and causes of, toxicity of cigarette butts to a cladoceran and microtox.* Arch Environ Contam Toxicol, 2006. 50(2): p. 205-12.
15. Dieng, H., et al., *Turning cigarette butt waste into an alternative control tool against an insecticide-resistant mosquito vector.* Acta Trop, 2013. 128(3): p. 584-90.
16. Novotny, T.E., et al., *Tobacco and cigarette butt consumption in humans and animals.* Tob Control, 2011. 20 Suppl 1: p. i17-20.
17. Hecht, S.S., *Cigarette smoking and lung cancer: chemical mechanisms and approaches to prevention.* Lancet Oncol, 2002. 3(8): p. 461-9.
18. Talhout, R., et al., *Hazardous compounds in tobacco smoke.* Int J Environ Res Public Health, 2011. 8(2): p. 613-28.
19. Barrett-Connor, E. and K.T. Khaw, *Cigarette smoking and increased central adiposity.* Ann Intern Med, 1989. 111(10): p. 783-7.
20. Shimokata, H., D.C. Muller, and R. Andres, *Studies in the distribution of body fat. III. Effects of cigarette smoking.* JAMA, 1989. 261(8): p. 1169-73.
21. Canoy, D., et al., *Cigarette smoking and fat distribution in 21,828 British men and women: a population-based study.* Obes Res, 2005. 13(8): p. 1466-75.
22. Visser, M., et al., *Past and current smoking in relation to body fat distribution in older men and women.* J Gerontol A Biol Sci Med Sci, 1999. 54(6): p. M293-8.
23. Ryu, J.H., et al., *Smoking-related interstitial lung diseases: a concise review.* Eur Respir J, 2001. 17(1): p. 122-32.
24. Disease, G.i.f.C.O.L., *Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease*. 2018.
25. Dela Cruz, C.S., L.T. Tanoue, and R.A. Matthay, *Lung cancer: epidemiology, etiology, and prevention.* Clin Chest Med, 2011. 32(4): p. 605-44.

26. Pesch, B., et al., *Cigarette smoking and lung cancer--relative risk estimates for the major histological types from a pooled analysis of case-control studies.* Int J Cancer, 2012. 131(5): p. 1210-9.
27. Ferlay, J., et al., *Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012.* Int J Cancer, 2015. 136(5): p. E359-86.
28. Devereux, T.R., J.A. Taylor, and J.C. Barrett, *Molecular mechanisms of lung cancer. Interaction of environmental and genetic factors. Giles F. Filley Lecture.* Chest, 1996. 109(3 Suppl): p. 14S-19S.
29. Field, R.W. and B.L. Withers, *Occupational and environmental causes of lung cancer.* Clin Chest Med, 2012. 33(4): p. 681-703.
30. Vineis, P. and K. Husgafvel-Pursiainen, *Air pollution and cancer: biomarker studies in human populations.* Carcinogenesis, 2005. 26(11): p. 1846-55.
31. Youlden, D.R., S.M. Cramb, and P.D. Baade, *The International Epidemiology of Lung Cancer: geographical distribution and secular trends.* J Thorac Oncol, 2008. 3(8): p. 819-31.
32. Sasco, A.J., M.B. Secretan, and K. Straif, *Tobacco smoking and cancer: a brief review of recent epidemiological evidence.* Lung Cancer, 2004. 45 Suppl 2: p. S3-9.
33. Proctor, R.N., *Tobacco and the global lung cancer epidemic.* Nat Rev Cancer, 2001. 1(1): p. 82-6.
34. Institute, N.C. *Cancer Stat Facts: Lung and Bronchus Cancer*. 2014; Available from: https://seer.cancer.gov/statfacts/html/lungb.html.
35. Sher, T., G.K. Dy, and A.A. Adjei, *Small cell lung cancer.* Mayo Clin Proc, 2008. 83(3): p. 355-67.
36. Zappa, C. and S.A. Mousa, *Non-small cell lung cancer: current treatment and future advances.* Transl Lung Cancer Res, 2016. 5(3): p. 288-300.
37. Chan, B.A. and B.G. Hughes, *Targeted therapy for non-small cell lung cancer: current standards and the promise of the future.* Transl Lung Cancer Res, 2015. 4(1): p. 36-54.
38. Travis, W.D., L.B. Travis, and S.S. Devesa, *Lung cancer.* Cancer, 1995. 75(1 Suppl): p. 191-202.
39. Kenfield, S.A., et al., *Comparison of aspects of smoking among the four histological types of lung cancer.* Tob Control, 2008. 17(3): p. 198-204.
40. Muscat, J.E., et al., *Cigarette smoking and large cell carcinoma of the lung.* Cancer Epidemiol Biomarkers Prev, 1997. 6(7): p. 477-80.
41. Auerbach, O., et al., *Changes in bronchial epithelium in relation to cigarette smoking and in relation to lung cancer.* N Engl J Med, 1961. 265: p. 253-67.
42. Kadara, H. and Wistuba, II, *Field cancerization in non-small cell lung cancer: implications in disease pathogenesis.* Proc Am Thorac Soc, 2012. 9(2): p. 38-42.
43. Hecht, S.S., *Tobacco smoke carcinogens and lung cancer.* J Natl Cancer Inst, 1999. 91(14): p. 1194-210.
44. Hecht, S.S., *DNA adduct formation from tobacco-specific N-nitrosamines.* Mutat Res, 1999. 424(1-2): p. 127-42.
45. Tang, D., et al., *Association between carcinogen-DNA adducts in white blood cells and lung cancer risk in the physicians health study.* Cancer Res, 2001. 61(18): p. 6708-12.
46. Norbury, C.J. and I.D. Hickson, *Cellular responses to DNA damage.* Annu Rev Pharmacol Toxicol, 2001. 41: p. 367-401.
47. Hanawalt, P.C., *Controlling the efficiency of excision repair.* Mutat Res, 2001. 485(1): p. 3-13.
48. Walser, T., et al., *Smoking and lung cancer: the role of inflammation.* Proc Am Thorac Soc, 2008. 5(8): p. 811-5.
49. Nakachi, K., et al., *Genetic susceptibility to squamous cell carcinoma of the lung in relation to cigarette smoking dose.* Cancer Res, 1991. 51(19): p. 5177-80.
50. Ambrosone, C.B., et al., *Lung cancer histologic types and family history of cancer. Analysis of histologic subtypes of 872 patients with primary lung cancer.* Cancer, 1993. 72(4): p. 1192-8.
51. Hwang, S.J., et al., *Lung cancer risk in germline p53 mutation carriers: association between an inherited cancer predisposition, cigarette smoking, and cancer risk.* Hum Genet, 2003. 113(3): p. 238-43.
52. Howington, J.A., et al., *Treatment of stage I and II non-small cell lung cancer: Diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines.* Chest, 2013. 143(5 Suppl): p. e278S-e313S.

53. Chan, B.A. and J.I. Coward, *Chemotherapy advances in small-cell lung cancer.* J Thorac Dis, 2013. 5 Suppl 5: p. S565-78.
54. *Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. Non-small Cell Lung Cancer Collaborative Group.* BMJ, 1995. 311(7010): p. 899-909.
55. Ramalingam, S. and C. Belani, *Systemic chemotherapy for advanced non-small cell lung cancer: recent advances and future directions.* Oncologist, 2008. 13 Suppl 1: p. 5-13.
56. Amini, A., et al., *Stereotactic body radiation therapy (SBRT) for lung cancer patients previously treated with conventional radiotherapy: a review.* Radiat Oncol, 2014. 9: p. 210.
57. Amini, A., et al., *Progress in the management of limited-stage small cell lung cancer.* Cancer, 2014. 120(6): p. 790-8.
58. Pao, W., et al., *EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib.* Proc Natl Acad Sci U S A, 2004. 101(36): p. 13306-11.
59. Vassallo, R. and J.H. Ryu, *Smoking-related interstitial lung diseases.* Clin Chest Med, 2012. 33(1): p. 165-78.
60. Fraig, M., et al., *Respiratory bronchiolitis: a clinicopathologic study in current smokers, ex-smokers, and never-smokers.* Am J Surg Pathol, 2002. 26(5): p. 647-53.
61. Friedman, P.J., A.A. Liebow, and J. Sokoloff, *Eosinophilic granuloma of lung. Clinical aspects of primary histiocytosis in the adult.* Medicine (Baltimore), 1981. 60(6): p. 385-96.
62. Ryu, J.H., et al., *Desquamative interstitial pneumonia and respiratory bronchiolitis-associated interstitial lung disease.* Chest, 2005. 127(1): p. 178-84.
63. Vassallo, R., et al., *Clinical outcomes of pulmonary Langerhans'-cell histiocytosis in adults.* N Engl J Med, 2002. 346(7): p. 484-90.
64. Miyake, Y., et al., *Occupational and environmental factors and idiopathic pulmonary fibrosis in Japan.* Ann Occup Hyg, 2005. 49(3): p. 259-65.
65. Saag, K.G., et al., *Rheumatoid arthritis lung disease. Determinants of radiographic and physiologic abnormalities.* Arthritis Rheum, 1996. 39(10): p. 1711-9.
66. Attili, A.K., et al., *Smoking-related interstitial lung disease: radiologic-clinical-pathologic correlation.* Radiographics, 2008. 28(5): p. 1383-96; discussion 1396-8.
67. Hartman, T.E., et al., *Desquamative interstitial pneumonia: thin-section CT findings in 22 patients.* Radiology, 1993. 187(3): p. 787-90.
68. Vassallo, R., et al., *Pulmonary Langerhans'-cell histiocytosis.* N Engl J Med, 2000. 342(26): p. 1969-78.
69. Caminati, A. and S. Harari, *Smoking-related interstitial pneumonias and pulmonary Langerhans cell histiocytosis.* Proc Am Thorac Soc, 2006. 3(4): p. 299-306.
70. Gross, T.J. and G.W. Hunninghake, *Idiopathic pulmonary fibrosis.* N Engl J Med, 2001. 345(7): p. 517-25.
71. Raghu, G., et al., *An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management.* Am J Respir Crit Care Med, 2011. 183(6): p. 788-824.
72. Bjoraker, J.A., et al., *Prognostic significance of histopathologic subsets in idiopathic pulmonary fibrosis.* Am J Respir Crit Care Med, 1998. 157(1): p. 199-203.
73. Myers, J.L., et al., *Respiratory bronchiolitis causing interstitial lung disease. A clinicopathologic study of six cases.* Am Rev Respir Dis, 1987. 135(4): p. 880-4.
74. Travis, W.D., et al., *Pulmonary Langerhans cell granulomatosis (histiocytosis X). A clinicopathologic study of 48 cases.* Am J Surg Pathol, 1993. 17(10): p. 971-86.
75. Remy-Jardin, M., et al., *Lung parenchymal changes secondary to cigarette smoking: pathologic-CT correlations.* Radiology, 1993. 186(3): p. 643-51.

76. Remy-Jardin, M., et al., *Morphologic effects of cigarette smoking on airways and pulmonary parenchyma in healthy adult volunteers: CT evaluation and correlation with pulmonary function tests.* Radiology, 1993. 186(1): p. 107-15.
77. Tomita, K., et al., *Increased p21(CIP1/WAF1) and B cell lymphoma leukemia-x(L) expression and reduced apoptosis in alveolar macrophages from smokers.* Am J Respir Crit Care Med, 2002. 166(5): p. 724-31.
78. Tazi, A., et al., *Role of granulocyte-macrophage colony stimulating factor (GM-CSF) in the pathogenesis of adult pulmonary histiocytosis X.* Thorax, 1996. 51(6): p. 611-4.
79. Tazi, A., et al., *Evidence that granulocyte macrophage-colony-stimulating factor regulates the distribution and differentiated state of dendritic cells/Langerhans cells in human lung and lung cancers.* J Clin Invest, 1993. 91(2): p. 566-76.
80. Sethi, S., *Bacteria in exacerbations of chronic obstructive pulmonary disease: phenomenon or epiphenomenon?* Proc Am Thorac Soc, 2004. 1(2): p. 109-14.
81. Wedzicha, J.A., *Role of viruses in exacerbations of chronic obstructive pulmonary disease.* Proc Am Thorac Soc, 2004. 1(2): p. 115-20.
82. Huang, Y.J., et al., *Airway microbiome dynamics in exacerbations of chronic obstructive pulmonary disease.* J Clin Microbiol, 2014. 52(8): p. 2813-23.
83. Jubinville, E., et al., *Exacerbation induces a microbiota shift in sputa of COPD patients.* PLoS One, 2018. 13(3): p. e0194355.
84. Miravitlles, M. and T. Grupo de trabajo de la Asociacion Latinoamericana del, *Update to the Latin American Thoracic Association (ALAT) Recommendations on Infectious Exacerbation of Chronic Obstructive Pulmonary Disease.* Arch Bronconeumol, 2004. 40(7): p. 315-25.
85. Adeloye, D., et al., *Global and regional estimates of COPD prevalence: Systematic review and meta-analysis.* J Glob Health, 2015. 5(2): p. 020415.
86. Quach, A., et al., *Prevalence and underdiagnosis of airway obstruction among middle-aged adults in northern France: The ELISABET study 2011-2013.* Respir Med, 2015. 109(12): p. 1553-61.
87. Chen, W., et al., *Risk of cardiovascular comorbidity in patients with chronic obstructive pulmonary disease: a systematic review and meta-analysis.* Lancet Respir Med, 2015. 3(8): p. 631-9.
88. Maltais, F., et al., *Skeletal muscle adaptation to endurance training in patients with chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 1996. 154(2 Pt 1): p. 442-7.
89. Maltais, F., et al., *An official American Thoracic Society/European Respiratory Society statement: update on limb muscle dysfunction in chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 2014. 189(9): p. e15-62.
90. Murphy, M.F., P. Antonini, and Z.V. Lai, *Postapproval Development Options in COPD: A Case Study in Value-Based Healthcare Systems.* Am Health Drug Benefits, 2011. 4(1): p. 19-23.
91. Guarascio, A.J., et al., *The clinical and economic burden of chronic obstructive pulmonary disease in the USA.* Clinicoecon Outcomes Res, 2013. 5: p. 235-45.
92. Decramer, M., W. Janssens, and M. Miravitlles, *Chronic obstructive pulmonary disease.* Lancet, 2012. 379(9823): p. 1341-51.
93. Torres-Duque, C., et al., *Biomass fuels and respiratory diseases: a review of the evidence.* Proc Am Thorac Soc, 2008. 5(5): p. 577-90.
94. Brandsma, C.A., et al., *Lung ageing and COPD: is there a role for ageing in abnormal tissue repair?* Eur Respir Rev, 2017. 26(146).
95. Lange, P., et al., *Lung-Function Trajectories Leading to Chronic Obstructive Pulmonary Disease.* N Engl J Med, 2015. 373(2): p. 111-22.
96. Stern, D.A., et al., *Poor airway function in early infancy and lung function by age 22 years: a non-selective longitudinal cohort study.* Lancet, 2007. 370(9589): p. 758-64.
97. Tashkin, D.P., et al., *The lung health study: airway responsiveness to inhaled methacholine in smokers with mild to moderate airflow limitation. The Lung Health Study Research Group.* Am Rev Respir Dis, 1992. 145(2 Pt 1): p. 301-10.

98. Menezes, A.M., et al., *Tuberculosis and airflow obstruction: evidence from the PLATINO study in Latin America.* Eur Respir J, 2007. 30(6): p. 1180-5.
99. Menezes, A.M. and P.C. Hallal, *Role of passive smoking on COPD risk in non-smokers.* Lancet, 2007. 370(9589): p. 716-7.
100. Jenkins, C.R., et al., *Improving the Management of COPD in Women.* Chest, 2017. 151(3): p. 686-696.
101. Afonso, A.S., et al., *COPD in the general population: prevalence, incidence and survival.* Respir Med, 2011. 105(12): p. 1872-84.
102. Labonte, L.E., et al., *Undiagnosed Chronic Obstructive Pulmonary Disease Contributes to the Burden of Health Care Use. Data from the CanCOLD Study.* Am J Respir Crit Care Med, 2016. 194(3): p. 285-98.
103. Lokke, A., et al., *Developing COPD: a 25 year follow up study of the general population.* Thorax, 2006. 61(11): p. 935-9.
104. Rennard, S.I. and J. Vestbo, *COPD: the dangerous underestimate of 15%.* Lancet, 2006. 367(9518): p. 1216-9.
105. Crighton, E.J., et al., *A spatial analysis of COPD prevalence, incidence, mortality and health service use in Ontario.* Health Rep, 2015. 26(3): p. 10-8.
106. Miller, M.R., et al., *Interpreting lung function data using 80% predicted and fixed thresholds misclassifies more than 20% of patients.* Chest, 2011. 139(1): p. 52-9.
107. Lamontagne, M., et al., *Leveraging lung tissue transcriptome to uncover candidate causal genes in COPD genetic associations.* Hum Mol Genet, 2018. 27(10): p. 1819-1829.
108. Barros-Tizon, J.C., et al., *Reduction of severe exacerbations and hospitalization-derived costs in alpha-1-antitrypsin-deficient patients treated with alpha-1-antitrypsin augmentation therapy.* Ther Adv Respir Dis, 2012. 6(2): p. 67-78.
109. Gooptu, B., U.I. Ekeowa, and D.A. Lomas, *Mechanisms of emphysema in alpha1-antitrypsin deficiency: molecular and cellular insights.* Eur Respir J, 2009. 34(2): p. 475-88.
110. Hunninghake, G.M., et al., *MMP12, lung function, and COPD in high-risk populations.* N Engl J Med, 2009. 361(27): p. 2599-608.
111. Pillai, S.G., et al., *A genome-wide association study in chronic obstructive pulmonary disease (COPD): identification of two major susceptibility loci.* PLoS Genet, 2009. 5(3): p. e1000421.
112. Cho, M.H., et al., *Risk loci for chronic obstructive pulmonary disease: a genome-wide association study and meta-analysis.* Lancet Respir Med, 2014. 2(3): p. 214-25.
113. McCloskey, S.C., et al., *Siblings of patients with severe chronic obstructive pulmonary disease have a significant risk of airflow obstruction.* Am J Respir Crit Care Med, 2001. 164(8 Pt 1): p. 1419-24.
114. Aubier, M., et al., *[COPD and inflammation: statement from a French expert group: inflammation and remodelling mechanisms].* Rev Mal Respir, 2010. 27(10): p. 1254-66.
115. Tuder, R.M., et al., *Role of lung maintenance program in the heterogeneity of lung destruction in emphysema.* Proc Am Thorac Soc, 2006. 3(8): p. 673-9.
116. Tuder, R.M., et al., *State of the art. Cellular and molecular mechanisms of alveolar destruction in emphysema: an evolutionary perspective.* Proc Am Thorac Soc, 2006. 3(6): p. 503-10.
117. Morris, D.G. and D. Sheppard, *Pulmonary emphysema: when more is less.* Physiology (Bethesda), 2006. 21: p. 396-403.
118. Barnes, P.J., *Inflammatory mechanisms in patients with chronic obstructive pulmonary disease.* J Allergy Clin Immunol, 2016. 138(1): p. 16-27.
119. Domej, W., K. Oettl, and W. Renner, *Oxidative stress and free radicals in COPD--implications and relevance for treatment.* Int J Chron Obstruct Pulmon Dis, 2014. 9: p. 1207-24.
120. Stockley, R.A., *Neutrophils and protease/antiprotease imbalance.* Am J Respir Crit Care Med, 1999. 160(5 Pt 2): p. S49-52.
121. Drost, E.M., et al., *Oxidative stress and airway inflammation in severe exacerbations of COPD.* Thorax, 2005. 60(4): p. 293-300.
122. Johnson, S.R., *Untangling the protease web in COPD: metalloproteinases in the silent zone.* Thorax, 2016. 71(2): p. 105-6.

123. Adcock, I.M., G. Caramori, and P.J. Barnes, *Chronic obstructive pulmonary disease and lung cancer: new molecular insights.* Respiration, 2011. 81(4): p. 265-84.
124. Barnes, P.J., *Cellular and molecular mechanisms of chronic obstructive pulmonary disease.* Clin Chest Med, 2014. 35(1): p. 71-86.
125. Katzenstein, A.L., S. Mukhopadhyay, and J.L. Myers, *Diagnosis of usual interstitial pneumonia and distinction from other fibrosing interstitial lung diseases.* Hum Pathol, 2008. 39(9): p. 1275-94.
126. Rennard, S.I., *Pathogenesis of chronic obstructive pulmonary disease.* Pneumonol Alergol Pol, 2011. 79(2): p. 132-8.
127. Salpeter, S.R., *Bronchodilators in COPD: impact of beta-agonists and anticholinergics on severe exacerbations and mortality.* Int J Chron Obstruct Pulmon Dis, 2007. 2(1): p. 11-8.
128. Sestini, P., et al., *Short-acting beta 2 agonists for stable chronic obstructive pulmonary disease.* Cochrane Database Syst Rev, 2002(4): p. CD001495.
129. Melani, A.S., *Long-acting muscarinic antagonists.* Expert Rev Clin Pharmacol, 2015. 8(4): p. 479-501.
130. Nannini, L.J., T.J. Lasserson, and P. Poole, *Combined corticosteroid and long-acting beta(2)-agonist in one inhaler versus long-acting beta(2)-agonists for chronic obstructive pulmonary disease.* Cochrane Database Syst Rev, 2012(9): p. CD006829.
131. Nannini, L.J., et al., *Combined corticosteroid and long-acting beta(2)-agonist in one inhaler versus placebo for chronic obstructive pulmonary disease.* Cochrane Database Syst Rev, 2013(11): p. CD003794.
132. Nannini, L.J., et al., *Combined corticosteroid and long-acting beta(2)-agonist in one inhaler versus inhaled corticosteroids alone for chronic obstructive pulmonary disease.* Cochrane Database Syst Rev, 2013(8): p. CD006826.
133. Rabe, K.F., *Update on roflumilast, a phosphodiesterase 4 inhibitor for the treatment of chronic obstructive pulmonary disease.* Br J Pharmacol, 2011. 163(1): p. 53-67.
134. Calverley, P.M., et al., *Roflumilast in symptomatic chronic obstructive pulmonary disease: two randomised clinical trials.* Lancet, 2009. 374(9691): p. 685-94.
135. Fabbri, L.M., et al., *Roflumilast in moderate-to-severe chronic obstructive pulmonary disease treated with longacting bronchodilators: two randomised clinical trials.* Lancet, 2009. 374(9691): p. 695-703.
136. Poole, P.J., et al., *Influenza vaccine for patients with chronic obstructive pulmonary disease.* Cochrane Database Syst Rev, 2006(1): p. CD002733.
137. Wongsurakiat, P., et al., *Economic evaluation of influenza vaccination in Thai chronic obstructive pulmonary disease patients.* J Med Assoc Thai, 2003. 86(6): p. 497-508.
138. Tomczyk, S., et al., *Use of 13-valent pneumococcal conjugate vaccine and 23-valent pneumococcal polysaccharide vaccine among adults aged >/=65 years: recommendations of the Advisory Committee on Immunization Practices (ACIP).* MMWR Morb Mortal Wkly Rep, 2014. 63(37): p. 822-5.
139. Holt, P.G., *Immune and inflammatory function in cigarette smokers.* Thorax, 1987. 42(4): p. 241-9.
140. Hodge, S. and P.N. Reynolds, *Low-dose azithromycin improves phagocytosis of bacteria by both alveolar and monocyte-derived macrophages in chronic obstructive pulmonary disease subjects.* Respirology, 2012. 17(5): p. 802-7.
141. Thomas, W.R., P.G. Holt, and D. Keast, *Cigarette smoke and phagocyte function: effect of chronic exposure in vivo and acute exposure in vitro.* Infect Immun, 1978. 20(2): p. 468-75.
142. Kotani, N., et al., *Exposure to cigarette smoke impairs alveolar macrophage functions during halothane and isoflurane anesthesia in rats.* Anesthesiology, 1999. 91(6): p. 1823-33.
143. Hodge, S., et al., *Smoking alters alveolar macrophage recognition and phagocytic ability: implications in chronic obstructive pulmonary disease.* Am J Respir Cell Mol Biol, 2007. 37(6): p. 748-55.
144. Libby, P., *Inflammatory mechanisms: the molecular basis of inflammation and disease.* Nutr Rev, 2007. 65(12 Pt 2): p. S140-6.
145. Moldoveanu, B., et al., *Inflammatory mechanisms in the lung.* J Inflamm Res, 2009. 2: p. 1-11.
146. Sherwood, E.R. and T. Toliver-Kinsky, *Mechanisms of the inflammatory response.* Best Pract Res Clin Anaesthesiol, 2004. 18(3): p. 385-405.

147. Chen, G.Y. and G. Nunez, *Sterile inflammation: sensing and reacting to damage.* Nat Rev Immunol, 2010. 10(12): p. 826-37.
148. Mogensen, T.H., *Pathogen recognition and inflammatory signaling in innate immune defenses.* Clin Microbiol Rev, 2009. 22(2): p. 240-73, Table of Contents.
149. Turvey, S.E. and D.H. Broide, *Innate immunity.* J Allergy Clin Immunol, 2010. 125(2 Suppl 2): p. S24-32.
150. Krieger, M., *The other side of scavenger receptors: pattern recognition for host defense.* Curr Opin Lipidol, 1997. 8(5): p. 275-80.
151. Hansson, G.K. and K. Edfeldt, *Toll to be paid at the gateway to the vessel wall.* Arterioscler Thromb Vasc Biol, 2005. 25(6): p. 1085-7.
152. Brubaker, S.W., et al., *Innate immune pattern recognition: a cell biological perspective.* Annu Rev Immunol, 2015. 33: p. 257-90.
153. Guermonprez, P., et al., *Antigen presentation and T cell stimulation by dendritic cells.* Annu Rev Immunol, 2002. 20: p. 621-67.
154. Heijink, I.H., et al., *Cigarette smoke-induced damage-associated molecular pattern release from necrotic neutrophils triggers proinflammatory mediator release.* Am J Respir Cell Mol Biol, 2015. 52(5): p. 554-62.
155. Pouwels, S.D., et al., *Susceptibility for cigarette smoke-induced DAMP release and DAMP-induced inflammation in COPD.* Am J Physiol Lung Cell Mol Physiol, 2016. 311(5): p. L881-L892.
156. Pouwels, S.D., et al., *Cigarette smoke-induced necroptosis and DAMP release trigger neutrophilic airway inflammation in mice.* Am J Physiol Lung Cell Mol Physiol, 2016. 310(4): p. L377-86.
157. Babelova, A., et al., *Biglycan, a danger signal that activates the NLRP3 inflammasome via toll-like and P2X receptors.* J Biol Chem, 2009. 284(36): p. 24035-48.
158. Shapiro, S.D., et al., *Neutrophil elastase contributes to cigarette smoke-induced emphysema in mice.* Am J Pathol, 2003. 163(6): p. 2329-35.
159. Lukens, J.R., J.M. Gross, and T.D. Kanneganti, *IL-1 family cytokines trigger sterile inflammatory disease.* Front Immunol, 2012. 3: p. 315.
160. Mbitikon-Kobo, F.M., et al., *Characterization of a CD44/CD122int memory CD8 T cell subset generated under sterile inflammatory conditions.* J Immunol, 2009. 182(6): p. 3846-54.
161. Eigenbrod, T., et al., *Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells.* J Immunol, 2008. 181(12): p. 8194-8.
162. Chen, C.J., et al., *Identification of a key pathway required for the sterile inflammatory response triggered by dying cells.* Nat Med, 2007. 13(7): p. 851-6.
163. Botelho, F.M., et al., *IL-1alpha/IL-1R1 expression in chronic obstructive pulmonary disease and mechanistic relevance to smoke-induced neutrophilia in mice.* PLoS One, 2011. 6(12): p. e28457.
164. U.S. Department of Health, E., and Welfare., *Smoking and Health: Report of the Advisory Committee to the Surgeon General of the United States*. 1964.
165. Cohen, A. and O. George, *Animal models of nicotine exposure: relevance to second-hand smoking, electronic cigarette use, and compulsive smoking.* Front Psychiatry, 2013. 4: p. 41.
166. Audrain-McGovern, J. and N.L. Benowitz, *Cigarette smoking, nicotine, and body weight.* Clin Pharmacol Ther, 2011. 90(1): p. 164-8.
167. Bernstein, I.M., et al., *Maternal smoking and its association with birth weight.* Obstet Gynecol, 2005. 106(5 Pt 1): p. 986-91.
168. Jaddoe, V.W., et al., *Active and passive maternal smoking during pregnancy and the risks of low birthweight and preterm birth: the Generation R Study.* Paediatr Perinat Epidemiol, 2008. 22(2): p. 162-71.
169. Bruin, J.E., H.C. Gerstein, and A.C. Holloway, *Long-term consequences of fetal and neonatal nicotine exposure: a critical review.* Toxicol Sci, 2010. 116(2): p. 364-74.
170. Dwyer, J.B., S.C. McQuown, and F.M. Leslie, *The dynamic effects of nicotine on the developing brain.* Pharmacol Ther, 2009. 122(2): p. 125-39.

171. Pauly, J.R. and T.A. Slotkin, *Maternal tobacco smoking, nicotine replacement and neurobehavioural development.* Acta Paediatr, 2008. 97(10): p. 1331-7.
172. Pauly, J.R., *Gender differences in tobacco smoking dynamics and the neuropharmacological actions of nicotine.* Front Biosci, 2008. 13: p. 505-16.
173. Winzer-Serhan, U.H., *Long-term consequences of maternal smoking and developmental chronic nicotine exposure.* Front Biosci, 2008. 13: p. 636-49.
174. Wright, J.L., M. Cosio, and A. Churg, *Animal models of chronic obstructive pulmonary disease.* Am J Physiol Lung Cell Mol Physiol, 2008. 295(1): p. L1-15.
175. Eltom, S., C. Stevenson, and M.A. Birrell, *Cigarette smoke exposure as a model of inflammation associated with COPD.* Curr Protoc Pharmacol, 2013. Chapter 5: p. Unit5 64.
176. Vlahos, R., et al., *Modelling COPD in mice.* Pulm Pharmacol Ther, 2006. 19(1): p. 12-7.
177. Groneberg, D.A. and K.F. Chung, *Models of chronic obstructive pulmonary disease.* Respir Res, 2004. 5: p. 18.
178. Ghorani, V., et al., *Experimental animal models for COPD: a methodological review.* Tob Induc Dis, 2017. 15: p. 25.
179. Mahadeva, R. and S.D. Shapiro, *Chronic obstructive pulmonary disease * 3: Experimental animal models of pulmonary emphysema.* Thorax, 2002. 57(10): p. 908-14.
180. Botelho, F.M., et al., *Innate immune processes are sufficient for driving cigarette smoke-induced inflammation in mice.* Am J Respir Cell Mol Biol, 2010. 42(4): p. 394-403.
181. Morissette, M.C., et al., *Disruption of pulmonary lipid homeostasis drives cigarette smoke-induced lung inflammation in mice.* Eur Respir J, 2015.
182. Vanoirbeek, J.A., et al., *Noninvasive and invasive pulmonary function in mouse models of obstructive and restrictive respiratory diseases.* Am J Respir Cell Mol Biol, 2010. 42(1): p. 96-104.
183. Morissette, M.C., et al., *Persistence of pulmonary tertiary lymphoid tissues and anti-nuclear antibodies following cessation of cigarette smoke exposure.* Respir Res, 2014. 15: p. 49.
184. van der Vaart, H., et al., *Acute effects of cigarette smoke on inflammation and oxidative stress: a review.* Thorax, 2004. 59(8): p. 713-21.
185. Montuschi, P., et al., *Exhaled 8-isoprostane as an in vivo biomarker of lung oxidative stress in patients with COPD and healthy smokers.* Am J Respir Crit Care Med, 2000. 162(3 Pt 1): p. 1175-7.
186. Guatura, S.B., et al., *Increased exhalation of hydrogen peroxide in healthy subjects following cigarette consumption.* Sao Paulo Med J, 2000. 118(4): p. 93-8.
187. Cotgreave, I.A., et al., *The effect of acute cigarette smoke inhalation on pulmonary and systemic cysteine and glutathione redox states in the rat.* Toxicology, 1987. 45(2): p. 203-12.
188. Bilimoria, M.H. and D.J. Ecobichon, *Protective antioxidant mechanisms in rat and guinea pig tissues challenged by acute exposure to cigarette smoke.* Toxicology, 1992. 72(2): p. 131-44.
189. Aoshiba, K., et al., *Immunohistochemical evaluation of oxidative stress in murine lungs after cigarette smoke exposure.* Inhal Toxicol, 2003. 15(10): p. 1029-38.
190. Wright, J.L., et al., *Effects of cigarette smoke on nitric oxide synthase expression in the rat lung.* Lab Invest, 1999. 79(8): p. 975-83.
191. Traves, S.L., et al., *Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD.* Thorax, 2002. 57(7): p. 590-5.
192. Komiyama, M., et al., *Association between monocyte chemoattractant protein-1 and blood pressure in smokers.* J Int Med Res, 2018. 46(3): p. 965-974.
193. Kubo, S., et al., *Cytokine and chemokine expression in cigarette smoke-induced lung injury in guinea pigs.* Eur Respir J, 2005. 26(6): p. 993-1001.
194. Nouailles, G., et al., *CXCL5-secreting pulmonary epithelial cells drive destructive neutrophilic inflammation in tuberculosis.* J Clin Invest, 2014. 124(3): p. 1268-82.
195. Morissette, M.C., et al., *Impacts of peroxisome proliferator-activated receptor-gamma activation on cigarette smoke-induced exacerbated response to bacteria.* Eur Respir J, 2015. 45(1): p. 191-200.
196. Hogg, J.C., et al., *The nature of small-airway obstruction in chronic obstructive pulmonary disease.* N Engl J Med, 2004. 350(26): p. 2645-53.

197. Kuschner, W.G., et al., *Dose-dependent cigarette smoking-related inflammatory responses in healthy adults.* Eur Respir J, 1996. 9(10): p. 1989-94.
198. Hunninghake, G.W. and R.G. Crystal, *Cigarette smoking and lung destruction. Accumulation of neutrophils in the lungs of cigarette smokers.* Am Rev Respir Dis, 1983. 128(5): p. 833-8.
199. van Eeden, S.F. and J.C. Hogg, *The response of human bone marrow to chronic cigarette smoking.* Eur Respir J, 2000. 15(5): p. 915-21.
200. D'Hulst A, I., et al., *Time course of cigarette smoke-induced pulmonary inflammation in mice.* Eur Respir J, 2005. 26(2): p. 204-13.
201. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. 13(3): p. 159-75.
202. Belaaouaj, A., K.S. Kim, and S.D. Shapiro, *Degradation of outer membrane protein A in Escherichia coli killing by neutrophil elastase.* Science, 2000. 289(5482): p. 1185-8.
203. Weitz, J.I., et al., *Increased neutrophil elastase activity in cigarette smokers.* Ann Intern Med, 1987. 107(5): p. 680-2.
204. Botelho, F.M., et al., *Cigarette smoke-induced accumulation of lung dendritic cells is interleukin-1alpha-dependent in mice.* Respir Res, 2012. 13: p. 81.
205. Givi, M.E., et al., *Cigarette smoke differentially modulates dendritic cell maturation and function in time.* Respir Res, 2015. 16: p. 131.
206. Motz, G.T., et al., *Chronic cigarette smoke exposure primes NK cell activation in a mouse model of chronic obstructive pulmonary disease.* J Immunol, 2010. 184(8): p. 4460-9.
207. Phillips, B., et al., *Effect of smoking on human natural killer cell activity.* Cancer, 1985. 56(12): p. 2789-92.
208. Dubar, V., et al., *In vitro acute effects of tobacco smoke on tumor necrosis factor alpha and interleukin-6 production by alveolar macrophages.* Exp Lung Res, 1993. 19(3): p. 345-59.
209. Tsantikos, E., et al., *Granulocyte-CSF links destructive inflammation and comorbidities in obstructive lung disease.* J Clin Invest, 2018. 128(6): p. 2406-2418.
210. Morrison, D., et al., *Epithelial permeability, inflammation, and oxidant stress in the air spaces of smokers.* Am J Respir Crit Care Med, 1999. 159(2): p. 473-9.
211. Jones, J.G., et al., *Increased alveolar epithelial permeability in cigarette smokers.* Lancet, 1980. 1(8159): p. 66-8.
212. Witten, M.L., et al., *Acute cigarette smoke exposure increases alveolar permeability in rabbits.* Am Rev Respir Dis, 1985. 132(2): p. 321-5.
213. Burns, A.R., et al., *Respiratory epithelial permeability after cigarette smoke exposure in guinea pigs.* J Appl Physiol (1985), 1989. 66(5): p. 2109-16.
214. Maunders, H., et al., *Human bronchial epithelial cell transcriptome: gene expression changes following acute exposure to whole cigarette smoke in vitro.* Am J Physiol Lung Cell Mol Physiol, 2007. 292(5): p. L1248-56.
215. Guerassimov, A., et al., *The development of emphysema in cigarette smoke-exposed mice is strain dependent.* Am J Respir Crit Care Med, 2004. 170(9): p. 974-80.
216. van der Strate, B.W., et al., *Cigarette smoke-induced emphysema: A role for the B cell?* Am J Respir Crit Care Med, 2006. 173(7): p. 751-8.
217. Cosio, M.G., M. Saetta, and A. Agusti, *Immunologic aspects of chronic obstructive pulmonary disease.* N Engl J Med, 2009. 360(23): p. 2445-54.
218. Braber, S., et al., *Cigarette smoke-induced lung emphysema in mice is associated with prolyl endopeptidase, an enzyme involved in collagen breakdown.* Am J Physiol Lung Cell Mol Physiol, 2011. 300(2): p. L255-65.
219. Atkinson, J.J., et al., *The role of matrix metalloproteinase-9 in cigarette smoke-induced emphysema.* Am J Respir Crit Care Med, 2011. 183(7): p. 876-84.
220. Churg, A., et al., *Late intervention with a myeloperoxidase inhibitor stops progression of experimental chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 2012. 185(1): p. 34-43.

221. Churg, A., S. Zhou, and J.L. Wright, *Series "matrix metalloproteinases in lung health and disease": Matrix metalloproteinases in COPD.* Eur Respir J, 2012. 39(1): p. 197-209.
222. Phillips, B., et al., *A 7-month cigarette smoke inhalation study in C57BL/6 mice demonstrates reduced lung inflammation and emphysema following smoking cessation or aerosol exposure from a prototypic modified risk tobacco product.* Food Chem Toxicol, 2015. 80: p. 328-45.
223. Ansari, S., et al., *Comprehensive systems biology analysis of a 7-month cigarette smoke inhalation study in C57BL/6 mice.* Sci Data, 2016. 3: p. 150077.
224. Rinaldi, M., et al., *Long-term nose-only cigarette smoke exposure induces emphysema and mild skeletal muscle dysfunction in mice.* Dis Model Mech, 2012. 5(3): p. 333-41.
225. Dunnill, M.S., *Quantitative methods in the study of pulmonary pathology.* Thorax, 1962. 17(4): p. 320-28.
226. McDonough, J.E., et al., *Small-airway obstruction and emphysema in chronic obstructive pulmonary disease.* N Engl J Med, 2011. 365(17): p. 1567-75.
227. Cosio, M.G. and M.G. Cosio Piqueras, *Pathology of emphysema in chronic obstructive pulmonary disease.* Monaldi Arch Chest Dis, 2000. 55(2): p. 124-9.
228. Robbins, C.S., et al., *Cigarette smoke decreases pulmonary dendritic cells and impacts antiviral immune responsiveness.* Am J Respir Cell Mol Biol, 2004. 30(2): p. 202-11.
229. Robbins, C.S., et al., *Cigarette smoke exposure impairs dendritic cell maturation and T cell proliferation in thoracic lymph nodes of mice.* J Immunol, 2008. 180(10): p. 6623-8.
230. Motz, G.T., et al., *Chronic cigarette smoke exposure generates pathogenic T cells capable of driving COPD-like disease in Rag2-/- mice.* Am J Respir Crit Care Med, 2010. 181(11): p. 1223-33.
231. Podolin, P.L., et al., *T cell depletion protects against alveolar destruction due to chronic cigarette smoke exposure in mice.* Am J Physiol Lung Cell Mol Physiol, 2013. 304(5): p. L312-23.
232. John-Schuster, G., et al., *Cigarette smoke-induced iBALT mediates macrophage activation in a B cell-dependent manner in COPD.* Am J Physiol Lung Cell Mol Physiol, 2014. 307(9): p. L692-706.
233. Conlon, *Immuno-Metabolomic Regulation of B Cell Guided iBALT Positioning Drives Cigarette Smoke-Induced COPD*, in *American Thoracic Society*. 2018: San Diego.
234. Nunez, B., et al., *Anti-tissue antibodies are related to lung function in chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 2011. 183(8): p. 1025-31.
235. Thayaparan, D., et al., *Induction of pulmonary antibodies against oxidized lipids in mice exposed to cigarette smoke.* Respir Res, 2016. 17(1): p. 97.
236. Karayama, M., et al., *Antiendothelial Cell Antibodies in Patients With COPD.* Chest, 2010. 138(6): p. 1303-8.
237. Talbot, M., et al., *Impact of immunization against OxLDL on the pulmonary response to cigarette smoke exposure in mice.* Respir Res, 2018. 19(1): p. 131.
238. Rinaldi, M., et al., *Antielastin B-cell and T-cell immunity in patients with chronic obstructive pulmonary disease.* Thorax, 2012. 67(8): p. 694-700.
239. Thatcher, T.H., et al., *Aryl hydrocarbon receptor-deficient mice develop heightened inflammatory responses to cigarette smoke and endotoxin associated with rapid loss of the nuclear factor-kappaB component RelB.* Am J Pathol, 2007. 170(3): p. 855-64.
240. Rico de Souza, A., et al., *Genetic ablation of the aryl hydrocarbon receptor causes cigarette smoke-induced mitochondrial dysfunction and apoptosis.* J Biol Chem, 2011. 286(50): p. 43214-28.
241. Agundez, J.A., *Cytochrome P450 gene polymorphism and cancer.* Curr Drug Metab, 2004. 5(3): p. 211-24.
242. Korashy, H.M. and A.O. El-Kadi, *The role of aryl hydrocarbon receptor in the pathogenesis of cardiovascular diseases.* Drug Metab Rev, 2006. 38(3): p. 411-50.
243. Churg, A., et al., *Macrophage metalloelastase mediates acute cigarette smoke-induced inflammation via tumor necrosis factor-alpha release.* Am J Respir Crit Care Med, 2003. 167(8): p. 1083-9.
244. Lagente, V., C. Le Quement, and E. Boichot, *Macrophage metalloelastase (MMP-12) as a target for inflammatory respiratory diseases.* Expert Opin Ther Targets, 2009. 13(3): p. 287-95.

245. Lagente, V. and E. Boichot, *Role of matrix metalloproteinases in the inflammatory process of respiratory diseases.* J Mol Cell Cardiol, 2010. 48(3): p. 440-4.
246. Molet, S., et al., *Increase in macrophage elastase (MMP-12) in lungs from patients with chronic obstructive pulmonary disease.* Inflamm Res, 2005. 54(1): p. 31-6.
247. Morissette, M.C., et al., *Impact of cigarette smoke on the human and mouse lungs: a gene-expression comparison study.* PLoS One, 2014. 9(3): p. e92498.
248. Byrne, A.J., et al., *Pulmonary macrophages: key players in the innate defence of the airways.* Thorax, 2015. 70(12): p. 1189-96.
249. Maus, U.A., et al., *Resident alveolar macrophages are replaced by recruited monocytes in response to endotoxin-induced lung inflammation.* Am J Respir Cell Mol Biol, 2006. 35(2): p. 227-35.
250. Janssen, W.J., et al., *Fas determines differential fates of resident and recruited macrophages during resolution of acute lung injury.* Am J Respir Crit Care Med, 2011. 184(5): p. 547-60.
251. Rubins, J.B., *Alveolar macrophages: wielding the double-edged sword of inflammation.* Am J Respir Crit Care Med, 2003. 167(2): p. 103-4.
252. Bowdish, D.M. and S. Gordon, *Conserved domains of the class A scavenger receptors: evolution and function.* Immunol Rev, 2009. 227(1): p. 19-31.
253. Guilliams, M., et al., *Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF.* J Exp Med, 2013. 210(10): p. 1977-92.
254. Yona, S., et al., *Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis.* Immunity, 2013. 38(1): p. 79-91.
255. Hashimoto, D., et al., *Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes.* Immunity, 2013. 38(4): p. 792-804.
256. Hussell, T. and T.J. Bell, *Alveolar macrophages: plasticity in a tissue-specific context.* Nat Rev Immunol, 2014. 14(2): p. 81-93.
257. Grashoff, W.F., et al., *Chronic obstructive pulmonary disease: role of bronchiolar mast cells and macrophages.* Am J Pathol, 1997. 151(6): p. 1785-90.
258. Wallace, W.A., M. Gillooly, and D. Lamb, *Intra-alveolar macrophage numbers in current smokers and non-smokers: a morphometric study of tissue sections.* Thorax, 1992. 47(6): p. 437-40.
259. Chen, K., et al., *IL-17RA is required for CCL2 expression, macrophage recruitment, and emphysema in response to cigarette smoke.* PLoS One, 2011. 6(5): p. e20333.
260. Murray, P.J. and T.A. Wynn, *Protective and pathogenic functions of macrophage subsets.* Nat Rev Immunol, 2011. 11(11): p. 723-37.
261. Aoshiba, K., J. Tamaoki, and A. Nagai, *Acute cigarette smoke exposure induces apoptosis of alveolar macrophages.* Am J Physiol Lung Cell Mol Physiol, 2001. 281(6): p. L1392-401.
262. Sanders, C.L., et al., *Distribution of inhaled metal oxide particles in pulmonary alveoli.* Arch Intern Med, 1971. 127(6): p. 1085-9.
263. Green, G.M. and D. Carolin, *The depressant effect of cigarette smoke on the in vitro antibacterial activity of alveolar macrophages.* N Engl J Med, 1967. 276(8): p. 421-7.
264. Holt, P.G. and D. Keast, *Acute effects of cigarette smoke on murine macrophages.* Arch Environ Health, 1973. 26(6): p. 300-4.
265. Green, G.M. and E.H. Kass, *The Role of the Alveolar Macrophage in the Clearance of Bacteria from the Lung.* J Exp Med, 1964. 119: p. 167-76.
266. Yeager, H., Jr., *Alveolar cells: depression effect of cigarette smoke on protein synthesis.* Proc Soc Exp Biol Med, 1969. 131(1): p. 247-50.
267. Holt, P.G. and D. Keast, *The effect of tobacco smoke on protein synthesis in macrophages.* Proc Soc Exp Biol Med, 1973. 142(4): p. 1243-7.
268. Hirama, N., et al., *Increased surfactant protein-D and foamy macrophages in smoking-induced mouse emphysema.* Respirology, 2007. 12(2): p. 191-201.
269. Martin, R.R., *Altered morphology and increased acid hydrolase content of pulmonary macrophages from cigarette smokers.* Am Rev Respir Dis, 1973. 107(4): p. 596-601.

270. Pratt, S.A., et al., *A comparison of alveolar macrophages and pulmonary surfactant(?) obtained from the lungs of human smokers and nonsmokers by endobronchial lavage.* Anat Rec, 1969. 163(4): p. 497-507.
271. Wilson, A.M., et al., *Lipid and smoker's inclusions in sputum macrophages in patients with airway diseases.* Respir Med, 2011. 105(11): p. 1691-5.
272. Romero, F., et al., *A pneumocyte-macrophage paracrine lipid axis drives the lung toward fibrosis.* Am J Respir Cell Mol Biol, 2015. 53(1): p. 74-86.
273. Schoch, O.D., et al., *BAL findings in a patient with pulmonary alveolar proteinosis successfully treated with GM-CSF.* Thorax, 2002. 57(3): p. 277-80.
274. Gibeon, D., et al., *Lipid-laden bronchoalveolar macrophages in asthma and chronic cough.* Respir Med, 2014. 108(1): p. 71-7.
275. Rossi, G., et al., *The role of macrophages in interstitial lung diseases: Number 3 in the Series "Pathology for the clinician" Edited by Peter Dorfmuller and Alberto Cavazza.* Eur Respir Rev, 2017. 26(145).
276. Raghavan, S., et al., *Protein Kinase Ctheta Via Activating Transcription Factor 2-Mediated CD36 Expression and Foam Cell Formation of Ly6C(hi) Cells Contributes to Atherosclerosis.* Circulation, 2018. 138(21): p. 2395-2412.
277. Trapnell, B.C., et al., *Pulmonary alveolar proteinosis, a primary immunodeficiency of impaired GM-CSF stimulation of macrophages.* Curr Opin Immunol, 2009. 21(5): p. 514-21.
278. Hsia, C.C., D.M. Hyde, and E.R. Weibel, *Lung Structure and the Intrinsic Challenges of Gas Exchange.* Compr Physiol, 2016. 6(2): p. 827-95.
279. Glasser, J.R. and R.K. Mallampalli, *Surfactant and its role in the pathobiology of pulmonary infection.* Microbes Infect, 2012. 14(1): p. 17-25.
280. Wright, J.R., *Immunoregulatory functions of surfactant proteins.* Nat Rev Immunol, 2005. 5(1): p. 58-68.
281. Perez-Gil, J., *Structure of pulmonary surfactant membranes and films: the role of proteins and lipid-protein interactions.* Biochim Biophys Acta, 2008. 1778(7-8): p. 1676-95.
282. Pattle, R.E., *Properties, function and origin of the alveolar lining layer.* Nature, 1955. 175(4469): p. 1125-6.
283. Griese, M., *Pulmonary surfactant in health and human lung diseases: state of the art.* Eur Respir J, 1999. 13(6): p. 1455-76.
284. Fessler, M.B. and R.S. Summer, *Surfactant Lipids at the Host-Environment Interface. Metabolic Sensors, Suppressors, and Effectors of Inflammatory Lung Disease.* Am J Respir Cell Mol Biol, 2016. 54(5): p. 624-35.
285. Goerke, J., *Pulmonary surfactant: functions and molecular composition.* Biochim Biophys Acta, 1998. 1408(2-3): p. 79-89.
286. Wright, J.R. and J.A. Clements, *Metabolism and turnover of lung surfactant.* Am Rev Respir Dis, 1987. 136(2): p. 426-44.
287. Whitsett, J.A., S.E. Wert, and T.E. Weaver, *Diseases of pulmonary surfactant homeostasis.* Annu Rev Pathol, 2015. 10: p. 371-93.
288. Wright, J.R., *Pulmonary surfactant: a front line of lung host defense.* J Clin Invest, 2003. 111(10): p. 1453-5.
289. Johansson, J. and T. Curstedt, *Molecular structures and interactions of pulmonary surfactant components.* Eur J Biochem, 1997. 244(3): p. 675-93.
290. Wright, J.R., *Immunomodulatory functions of surfactant.* Physiol Rev, 1997. 77(4): p. 931-62.
291. Wright, J.R., *Clearance and recycling of pulmonary surfactant.* Am J Physiol, 1990. 259(2 Pt 1): p. L1-12.
292. Griese, M., L.I. Gobran, and S.A. Rooney, *Surfactant lipid uptake and secretion in type II cells in response to lectins and secretagogues.* Am J Physiol, 1991. 261(6 Pt 1): p. L434-42.
293. Nogee, L.M., *Alterations in SP-B and SP-C expression in neonatal lung disease.* Annu Rev Physiol, 2004. 66: p. 601-23.

294. Glasser, S.W., et al., *Altered stability of pulmonary surfactant in SP-C-deficient mice.* Proc Natl Acad Sci U S A, 2001. 98(11): p. 6366-71.
295. Clark, H. and L.S. Clark, *The genetics of neonatal respiratory disease.* Semin Fetal Neonatal Med, 2005. 10(3): p. 271-82.
296. Dunbar, A.E., 3rd, et al., *Prolonged survival in hereditary surfactant protein B (SP-B) deficiency associated with a novel splicing mutation.* Pediatr Res, 2000. 48(3): p. 275-82.
297. Nogee, L.M., et al., *A mutation in the surfactant protein B gene responsible for fatal neonatal respiratory disease in multiple kindreds.* J Clin Invest, 1994. 93(4): p. 1860-3.
298. Almlen, A., et al., *Synthetic surfactant based on analogues of SP-B and SP-C is superior to single-peptide surfactants in ventilated premature rabbits.* Neonatology, 2010. 98(1): p. 91-9.
299. Nogee, L.M., *Genetic mechanisms of surfactant deficiency.* Biol Neonate, 2004. 85(4): p. 314-8.
300. Anandarajan, M., S. Paulraj, and R. Tubman, *ABCA3 Deficiency: an unusual cause of respiratory distress in the newborn.* Ulster Med J, 2009. 78(1): p. 51-2.
301. Nouraeyan, N., et al., *Surfactant administration in neonates: A review of delivery methods.* Can J Respir Ther, 2014. 50(3): p. 91-5.
302. Zuo, Y.Y., et al., *Current perspectives in pulmonary surfactant--inhibition, enhancement and evaluation.* Biochim Biophys Acta, 2008. 1778(10): p. 1947-77.
303. Zhou, J., et al., *Upregulation of surfactant synthesis triggers ABCA1-mediated basolateral phospholipid efflux.* J Lipid Res, 2004. 45(9): p. 1758-67.
304. Beers, M.F. and S. Mulugeta, *The biology of the ABCA3 lipid transporter in lung health and disease.* Cell Tissue Res, 2017. 367(3): p. 481-493.
305. Beers, M.F., et al., *Aberrant lung remodeling in a mouse model of surfactant dysregulation induced by modulation of the Abca3 gene.* Ann Anat, 2017. 210: p. 135-146.
306. Whitsett, J.A., S.E. Wert, and B.C. Trapnell, *Genetic disorders influencing lung formation and function at birth.* Hum Mol Genet, 2004. 13 Spec No 2: p. R207-15.
307. Reis, A. and C.M. Spickett, *Chemistry of phospholipid oxidation.* Biochim Biophys Acta, 2012. 1818(10): p. 2374-87.
308. Gurel, O., et al., *Macrophage and type II cell catabolism of SP-A and saturated phosphatidylcholine in mouse lungs.* Am J Physiol Lung Cell Mol Physiol, 2001. 280(6): p. L1266-72.
309. Ikegami, M. and A.H. Jobe, *Surfactant protein metabolism in vivo.* Biochim Biophys Acta, 1998. 1408(2-3): p. 218-25.
310. Wright, J.R. and L.G. Dobbs, *Regulation of pulmonary surfactant secretion and clearance.* Annu Rev Physiol, 1991. 53: p. 395-414.
311. Quintero, O.A. and J.R. Wright, *Clearance of surfactant lipids by neutrophils and macrophages isolated from the acutely inflamed lung.* Am J Physiol Lung Cell Mol Physiol, 2002. 282(2): p. L330-9.
312. Wofford, J.A. and J.R. Wright, *Surfactant protein A regulates IgG-mediated phagocytosis in inflammatory neutrophils.* Am J Physiol Lung Cell Mol Physiol, 2007. 293(6): p. L1437-43.
313. Baritussio, A.G., et al., *Precursor-product relationship between rabbit type II cell lamellar bodies and alveolar surface-active material. Surfactant turnover time.* Biochim Biophys Acta, 1981. 666(3): p. 382-93.
314. Nicholas, T.E., J.H. Power, and H.A. Barr, *Surfactant homeostasis in the rat lung during swimming exercise.* J Appl Physiol Respir Environ Exerc Physiol, 1982. 53(6): p. 1521-8.
315. Ward, H.E. and T.E. Nicholas, *Effect of artificial ventilation and anaesthesia on surfactant turnover in rats.* Respir Physiol, 1992. 87(1): p. 115-29.
316. Finley, T.N. and A.J. Ladman, *Low yield of pulmonary surfactant in cigarette smokers.* N Engl J Med, 1972. 286(5): p. 223-7.
317. Lusuardi, M., et al., *Role of surfactant in chronic obstructive pulmonary disease: therapeutic implications.* Respiration, 1992. 59 Suppl 1: p. 28-32.
318. Schmekel, B., et al., *Integrity of the alveolar-capillary barrier and alveolar surfactant system in smokers.* Thorax, 1992. 47(8): p. 603-8.
319. Sachs, D.P., *Smoking and pulmonary macrophages.* N Engl J Med, 1972. 286(14): p. 788-9.

320. Clements, J.A., *Smoking and pulmonary surfactant.* N Engl J Med, 1972. 286(5): p. 261-2.
321. Webb, W.R., et al., *Cigarette smoke and surfactant.* Am Rev Respir Dis, 1967. 95(2): p. 244-7.
322. Cook, W.A. and W.R. Webb, *Surfactant in chronic smokers.* Ann Thorac Surg, 1966. 2(3): p. 327-33.
323. Higenbottam, T., *Lung lipids and disease.* Respiration, 1989. 55 Suppl 1: p. 14-27.
324. Subramaniam, S., P. Bummer, and C.G. Gairola, *Biochemical and biophysical characterization of pulmonary surfactant in rats exposed chronically to cigarette smoke.* Fundam Appl Toxicol, 1995. 27(1): p. 63-9.
325. Wirtz, H.R. and M. Schmidt, *Acute influence of cigarette smoke on secretion of pulmonary surfactant in rat alveolar type II cells in culture.* Eur Respir J, 1996. 9(1): p. 24-32.
326. Jha, *Cigarette Smoke Exposure Generates a Unique Peroxidized Phosphatidylcholine Fingerprint in the Lung*. 2017.
327. Duong, C., et al., *Glutathione peroxidase-1 protects against cigarette smoke-induced lung inflammation in mice.* Am J Physiol Lung Cell Mol Physiol, 2010. 299(3): p. L425-33.
328. Tarling, E.J., T.Q. de Aguiar Vallim, and P.A. Edwards, *Role of ABC transporters in lipid transport and human disease.* Trends Endocrinol Metab, 2013. 24(7): p. 342-50.
329. Goldstein, J.L., et al., *Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition.* Proc Natl Acad Sci U S A, 1979. 76(1): p. 333-7.
330. Goldstein, J.L. and M.S. Brown, *Familial hypercholesterolemia: pathogenesis of a receptor disease.* Johns Hopkins Med J, 1978. 143(1): p. 8-16.
331. Goldstein, J.L. and M.S. Brown, *The LDL receptor.* Arterioscler Thromb Vasc Biol, 2009. 29(4): p. 431-8.
332. Taylor, P.R., et al., *Macrophage receptors and immune recognition.* Annu Rev Immunol, 2005. 23: p. 901-44.
333. Park, Y.M., *CD36, a scavenger receptor implicated in atherosclerosis.* Exp Mol Med, 2014. 46: p. e99.
334. Kawano, Y. and D.E. Cohen, *Mechanisms of hepatic triglyceride accumulation in non-alcoholic fatty liver disease.* J Gastroenterol, 2013. 48(4): p. 434-41.
335. Silverstein, R.L. and M. Febbraio, *CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior.* Sci Signal, 2009. 2(72): p. re3.
336. Koonen, D.P., et al., *Increased hepatic CD36 expression contributes to dyslipidemia associated with diet-induced obesity.* Diabetes, 2007. 56(12): p. 2863-71.
337. Cooper, G.E., et al., *Viral Inhibition of Bacterial Phagocytosis by Human Macrophages: Redundant Role of CD36.* PLoS One, 2016. 11(10): p. e0163889.
338. Wang, J., et al., *Innate immune response of human alveolar macrophages during influenza A infection.* PLoS One, 2012. 7(3): p. e29879.
339. Dodd, C.E., et al., *CD36-Mediated Uptake of Surfactant Lipids by Human Macrophages Promotes Intracellular Growth of Mycobacterium tuberculosis.* J Immunol, 2016. 197(12): p. 4727-4735.
340. Yehualaeshet, T., et al., *A CD36 synthetic peptide inhibits bleomycin-induced pulmonary inflammation and connective tissue synthesis in the rat.* Am J Respir Cell Mol Biol, 2000. 23(2): p. 204-12.
341. Asada, K., et al., *Antiinflammatory roles of peroxisome proliferator-activated receptor gamma in human alveolar macrophages.* Am J Respir Crit Care Med, 2004. 169(2): p. 195-200.
342. Yoshimi, N., et al., *Oxidized phosphatidylcholine in alveolar macrophages in idiopathic interstitial pneumonias.* Lung, 2005. 183(2): p. 109-21.
343. Pons, A.R., et al., *Phenotypic characterisation of alveolar macrophages and peripheral blood monocytes in COPD.* Eur Respir J, 2005. 25(4): p. 647-52.
344. Taylor, A.E., et al., *Defective macrophage phagocytosis of bacteria in COPD.* Eur Respir J, 2010. 35(5): p. 1039-47.
345. Noguera, A., et al., *An investigation of the resolution of inflammation (catabasis) in COPD.* Respir Res, 2012. 13: p. 101.
346. Kelley, J.L., et al., *Scavenger receptor-A (CD204): a two-edged sword in health and disease.* Crit Rev Immunol, 2014. 34(3): p. 241-61.

347. Zani, I.A., et al., *Scavenger receptor structure and function in health and disease.* Cells, 2015. 4(2): p. 178-201.
348. Arredouani, M.S., et al., *Scavenger Receptors SR-AI/II and MARCO limit pulmonary dendritic cell migration and allergic airway inflammation.* J Immunol, 2007. 178(9): p. 5912-20.
349. Stahl, M., et al., *Lung collagens perpetuate pulmonary fibrosis via CD204 and M2 macrophage activation.* PLoS One, 2013. 8(11): p. e81382.
350. Kobayashi, H., et al., *Class A scavenger receptor (CD204) attenuates hyperoxia-induced lung injury by reducing oxidative stress.* J Pathol, 2007. 212(1): p. 38-46.
351. Thakur, S.A., R.F. Hamilton, Jr., and A. Holian, *Role of scavenger receptor a family in lung inflammation from exposure to environmental particles.* J Immunotoxicol, 2008. 5(2): p. 151-7.
352. Beamer, C.A. and A. Holian, *Scavenger receptor class A type I/II (CD204) null mice fail to develop fibrosis following silica exposure.* Am J Physiol Lung Cell Mol Physiol, 2005. 289(2): p. L186-95.
353. Poole, J.A., et al., *Pattern recognition scavenger receptor A/CD204 regulates airway inflammatory homeostasis following organic dust extract exposures.* J Immunotoxicol, 2015. 12(1): p. 64-73.
354. Wong, C.K., et al., *Aging Impairs Alveolar Macrophage Phagocytosis and Increases Influenza-Induced Mortality in Mice.* J Immunol, 2017. 199(3): p. 1060-1068.
355. Hollifield, M., et al., *Scavenger receptor A dampens induction of inflammation in response to the fungal pathogen Pneumocystis carinii.* Infect Immun, 2007. 75(8): p. 3999-4005.
356. Fitzgerald, M.L., et al., *Lipopolysaccharide induces scavenger receptor A expression in mouse macrophages: a divergent response relative to human THP-1 monocyte/macrophages.* J Immunol, 2000. 164(5): p. 2692-700.
357. Yamamoto, T., et al., *Expression of scavenger receptor class A and CD14 in lipopolysaccharide-induced lung injury.* Pathol Int, 1999. 49(11): p. 983-92.
358. Ganesan, S., et al., *Elastase/LPS-exposed mice exhibit impaired innate immune responses to bacterial challenge: role of scavenger receptor A.* Am J Pathol, 2012. 180(1): p. 61-72.
359. Kuronuma, K., et al., *Pulmonary surfactant protein A augments the phagocytosis of Streptococcus pneumoniae by alveolar macrophages through a casein kinase 2-dependent increase of cell surface localization of scavenger receptor A.* J Biol Chem, 2004. 279(20): p. 21421-30.
360. Sever-Chroneos, Z., et al., *Surfactant protein A (SP-A)-mediated clearance of Staphylococcus aureus involves binding of SP-A to the staphylococcal adhesin eap and the macrophage receptors SP-A receptor 210 and scavenger receptor class A.* J Biol Chem, 2011. 286(6): p. 4854-70.
361. Wyatt, T.A., et al., *Malondialdehyde-acetaldehyde-adducted protein inhalation causes lung injury.* Alcohol, 2012. 46(1): p. 51-9.
362. Sapkota, M., et al., *Malondialdehyde-acetaldehyde (MAA) adducted surfactant protein induced lung inflammation is mediated through scavenger receptor a (SR-A1).* Respir Res, 2017. 18(1): p. 36.
363. Kramer, B.W., A.H. Jobe, and M. Ikegami, *Exogenous surfactant changes the phenotype of alveolar macrophages in mice.* Am J Physiol Lung Cell Mol Physiol, 2001. 280(4): p. L689-94.
364. Gille, C., et al., *Differential effect of surfactant and its saturated phosphatidylcholines on human blood macrophages.* J Lipid Res, 2007. 48(2): p. 307-17.
365. Neyen, C., et al., *Macrophage scavenger receptor a promotes tumor progression in murine models of ovarian and pancreatic cancer.* J Immunol, 2013. 190(7): p. 3798-805.
366. Zhang, Y., et al., *Scavenger Receptor A1 Prevents Metastasis of Non-Small Cell Lung Cancer via Suppression of Macrophage Serum Amyloid A1.* Cancer Res, 2017. 77(7): p. 1586-1598.
367. Xie, L., et al., *Function of macrophage scavenger receptor 1 gene polymorphisms in chronic obstructive pulmonary disease with and without lung cancer in China.* Oncol Lett, 2018. 15(5): p. 8046-8052.
368. Ohar, J.A., et al., *COPD is associated with a macrophage scavenger receptor-1 gene sequence variation.* Chest, 2010. 137(5): p. 1098-107.
369. Thomsen, M., et al., *Scavenger receptor AI/II truncation, lung function and COPD: a large population-based study.* J Intern Med, 2011. 269(3): p. 340-8.
370. Thomsen, M., *Genetic variations in scavenger and beta(2)-adrenergic receptors and risk of pulmonary disease.* Dan Med J, 2014. 61(9): p. B4910.

371. Kaku, Y., et al., *Overexpression of CD163, CD204 and CD206 on alveolar macrophages in the lungs of patients with severe chronic obstructive pulmonary disease.* PLoS One, 2014. 9(1): p. e87400.
372. Dahl, M., et al., *Protection against inhaled oxidants through scavenging of oxidized lipids by macrophage receptors MARCO and SR-AI/II.* J Clin Invest, 2007. 117(3): p. 757-64.
373. Acton, S.L., et al., *Expression cloning of SR-BI, a CD36-related class B scavenger receptor.* J Biol Chem, 1994. 269(33): p. 21003-9.
374. Shen, W.J., et al., *Scavenger receptor B type 1: expression, molecular regulation, and cholesterol transport function.* J Lipid Res, 2018. 59(7): p. 1114-1131.
375. Shen, W.J., S. Azhar, and F.B. Kraemer, *SR-B1: A Unique Multifunctional Receptor for Cholesterol Influx and Efflux.* Annu Rev Physiol, 2018. 80: p. 95-116.
376. Valacchi, G., et al., *Scavenger receptor class B type I: a multifunctional receptor.* Ann N Y Acad Sci, 2011. 1229: p. E1-7.
377. Zhang, Y., et al., *Hepatic expression of scavenger receptor class B type I (SR-BI) is a positive regulator of macrophage reverse cholesterol transport in vivo.* J Clin Invest, 2005. 115(10): p. 2870-4.
378. Phillips, M.C., *Molecular mechanisms of cellular cholesterol efflux.* J Biol Chem, 2014. 289(35): p. 24020-9.
379. Van Eck, M., et al., *Increased oxidative stress in scavenger receptor BI knockout mice with dysfunctional HDL.* Arterioscler Thromb Vasc Biol, 2007. 27(11): p. 2413-9.
380. Navab, M., et al., *HDL and cardiovascular disease: atherogenic and atheroprotective mechanisms.* Nat Rev Cardiol, 2011. 8(4): p. 222-32.
381. Tsugita, M., et al., *SR-B1 Is a Silica Receptor that Mediates Canonical Inflammasome Activation.* Cell Rep, 2017. 18(5): p. 1298-1311.
382. Gowdy, K.M., et al., *Key role for scavenger receptor B-I in the integrative physiology of host defense during bacterial pneumonia.* Mucosal Immunol, 2015. 8(3): p. 559-71.
383. Baranova, I.N., et al., *Class B scavenger receptor types I and II and CD36 mediate bacterial recognition and proinflammatory signaling induced by Escherichia coli, lipopolysaccharide, and cytosolic chaperonin 60.* J Immunol, 2012. 188(3): p. 1371-80.
384. Baranova, I.N., et al., *Human SR-BI and SR-BII Potentiate Lipopolysaccharide-Induced Inflammation and Acute Liver and Kidney Injury in Mice.* J Immunol, 2016. 196(7): p. 3135-47.
385. Schafer, G., et al., *The role of scavenger receptor B1 in infection with Mycobacterium tuberculosis in a murine model.* PLoS One, 2009. 4(12): p. e8448.
386. Westhaus, S., et al., *Scavenger receptor class B member 1 (SCARB1) variants modulate hepatitis C virus replication cycle and viral load.* J Hepatol, 2017. 67(2): p. 237-245.
387. Kolleck, I., et al., *HDL and vitamin E in plasma and the expression of SR-BI on lung cells during rat perinatal development.* Lung, 2000. 178(4): p. 191-200.
388. Kolleck, I., et al., *HDL is the major source of vitamin E for type II pneumocytes.* Free Radic Biol Med, 1999. 27(7-8): p. 882-90.
389. Kolleck, I., P. Sinha, and B. Rustow, *Vitamin E as an antioxidant of the lung: mechanisms of vitamin E delivery to alveolar type II cells.* Am J Respir Crit Care Med, 2002. 166(12 Pt 2): p. S62-6.
390. Mooberry, L.K., et al., *Targeting the SR-B1 Receptor as a Gateway for Cancer Therapy and Imaging.* Front Pharmacol, 2016. 7: p. 466.
391. Feng, H., et al., *High scavenger receptor class B type I expression is related to tumor aggressiveness and poor prognosis in lung adenocarcinoma: A STROBE compliant article.* Medicine (Baltimore), 2018. 97(13): p. e0203.
392. Vasquez, M., et al., *Exploiting scavenger receptors in cancer immunotherapy: Lessons from CD5 and SR-B1.* Eur J Immunol, 2017. 47(7): p. 1108-1118.
393. Valacchi, G., et al., *Exploring the link between scavenger receptor B1 expression and chronic obstructive pulmonary disease pathogenesis.* Ann N Y Acad Sci, 2015. 1340: p. 47-54.
394. Sticozzi, C., et al., *Cigarette smoke affects keratinocytes SRB1 expression and localization via H2O2 production and HNE protein adducts formation.* PLoS One, 2012. 7(3): p. e33592.

395. Sticozzi, C., et al., *Resveratrol protects SR-B1 levels in keratinocytes exposed to cigarette smoke.* Free Radic Biol Med, 2014. 69: p. 50-7.
396. Janoff, A., et al., *Cigarette smoke inhalation decreases alpha 1-antitrypsin activity in rat lung.* Science, 1979. 206(4424): p. 1313-4.
397. Janciauskiene, S. and T. Welte, *Well-Known and Less Well-Known Functions of Alpha-1 Antitrypsin. Its Role in Chronic Obstructive Pulmonary Disease and Other Disease Developments.* Ann Am Thorac Soc, 2016. 13 Suppl 4: p. S280-8.
398. Lockett, A.D., et al., *Scavenger receptor class B, type I-mediated uptake of A1AT by pulmonary endothelial cells.* Am J Physiol Lung Cell Mol Physiol, 2015. 309(4): p. L425-34.
399. Elomaa, O., et al., *Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages.* Cell, 1995. 80(4): p. 603-9.
400. Elshourbagy, N.A., et al., *Molecular characterization of a human scavenger receptor, human MARCO.* Eur J Biochem, 2000. 267(3): p. 919-26.
401. Granucci, F., et al., *The scavenger receptor MARCO mediates cytoskeleton rearrangements in dendritic cells and microglia.* Blood, 2003. 102(8): p. 2940-7.
402. Palecanda, A., et al., *Role of the scavenger receptor MARCO in alveolar macrophage binding of unopsonized environmental particles.* J Exp Med, 1999. 189(9): p. 1497-506.
403. Kzhyshkowska, J., C. Neyen, and S. Gordon, *Role of macrophage scavenger receptors in atherosclerosis.* Immunobiology, 2012. 217(5): p. 492-502.
404. Kraal, G., et al., *The macrophage receptor MARCO.* Microbes Infect, 2000. 2(3): p. 313-6.
405. Jing, J., et al., *Role of macrophage receptor with collagenous structure in innate immune tolerance.* J Immunol, 2013. 190(12): p. 6360-7.
406. Thakur, S.A., et al., *Differential binding of inorganic particles to MARCO.* Toxicol Sci, 2009. 107(1): p. 238-46.
407. Thakur, S.A., et al., *Critical role of MARCO in crystalline silica-induced pulmonary inflammation.* Toxicol Sci, 2009. 108(2): p. 462-71.
408. Murthy, S., et al., *Alternative activation of macrophages and pulmonary fibrosis are modulated by scavenger receptor, macrophage receptor with collagenous structure.* FASEB J, 2015. 29(8): p. 3527-36.
409. Bin, L.H., et al., *Identification of uteroglobin-related protein 1 and macrophage scavenger receptor with collagenous structure as a lung-specific ligand-receptor pair.* J Immunol, 2003. 171(2): p. 924-30.
410. Arredouani, M., et al., *The scavenger receptor MARCO is required for lung defense against pneumococcal pneumonia and inhaled particles.* J Exp Med, 2004. 200(2): p. 267-72.
411. Arredouani, M.S., et al., *MARCO is the major binding receptor for unopsonized particles and bacteria on human alveolar macrophages.* J Immunol, 2005. 175(9): p. 6058-64.
412. Dorrington, M.G., et al., *MARCO is required for TLR2- and Nod2-mediated responses to Streptococcus pneumoniae and clearance of pneumococcal colonization in the murine nasopharynx.* J Immunol, 2013. 190(1): p. 250-8.
413. Jager, J., et al., *Human lung tissue explants reveal novel interactions during Legionella pneumophila infections.* Infect Immun, 2014. 82(1): p. 275-85.
414. Bowdish, D.M., et al., *Genetic variants of MARCO are associated with susceptibility to pulmonary tuberculosis in a Gambian population.* BMC Med Genet, 2013. 14: p. 47.
415. Martinez, N., et al., *Impaired Recognition of Mycobacterium tuberculosis by Alveolar Macrophages From Diabetic Mice.* J Infect Dis, 2016. 214(11): p. 1629-1637.
416. Wright, A.K., et al., *Pivotal Advance: Expansion of small sputum macrophages in CF: failure to express MARCO and mannose receptors.* J Leukoc Biol, 2009. 86(3): p. 479-89.
417. Maler, M.D., et al., *Key Role of the Scavenger Receptor MARCO in Mediating Adenovirus Infection and Subsequent Innate Responses of Macrophages.* MBio, 2017. 8(4).
418. Ghosh, S., et al., *MARCO regulates early inflammatory responses against influenza: a useful macrophage function with adverse outcome.* Am J Respir Cell Mol Biol, 2011. 45(5): p. 1036-44.

419. Sever-Chroneos, Z., et al., *GM-CSF modulates pulmonary resistance to influenza A infection.* Antiviral Res, 2011. 92(2): p. 319-28.
420. Stichling, N., et al., *Lung macrophage scavenger receptor SR-A6 (MARCO) is an adenovirus type-specific virus entry receptor.* PLoS Pathog, 2018. 14(3): p. e1006914.
421. Sun, K. and D.W. Metzger, *Inhibition of pulmonary antibacterial defense by interferon-gamma during recovery from influenza infection.* Nat Med, 2008. 14(5): p. 558-64.
422. Ordija, C.M., et al., *Free actin impairs macrophage bacterial defenses via scavenger receptor MARCO interaction with reversal by plasma gelsolin.* Am J Physiol Lung Cell Mol Physiol, 2017. 312(6): p. L1018-L1028.
423. Wu, M., et al., *Immunomodulators targeting MARCO expression improve resistance to postinfluenza bacterial pneumonia.* Am J Physiol Lung Cell Mol Physiol, 2017. 313(1): p. L138-L153.
424. Xu, J., et al., *Exploitation of Scavenger Receptor, Macrophage Receptor with Collagenous Structure, by Cryptococcus neoformans Promotes Alternative Activation of Pulmonary Lymph Node CD11b(+) Conventional Dendritic Cells and Non-Protective Th2 Bias.* Front Immunol, 2017. 8: p. 1231.
425. Xu, J., et al., *Scavenger Receptor MARCO Orchestrates Early Defenses and Contributes to Fungal Containment during Cryptococcal Infection.* J Immunol, 2017. 198(9): p. 3548-3557.
426. La Fleur, L., et al., *Expression of scavenger receptor MARCO defines a targetable tumor-associated macrophage subset in non-small cell lung cancer.* Int J Cancer, 2018.
427. Matsushita, N., et al., *Targeting MARCO can lead to enhanced dendritic cell motility and anti-melanoma activity.* Cancer Immunol Immunother, 2010. 59(6): p. 875-84.
428. Harvey, C.J., et al., *Targeting Nrf2 signaling improves bacterial clearance by alveolar macrophages in patients with COPD and in a mouse model.* Sci Transl Med, 2011. 3(78): p. 78ra32.
429. Baqir, M., et al., *Cigarette smoke decreases MARCO expression in macrophages: implication in Mycoplasma pneumoniae infection.* Respir Med, 2008. 102(11): p. 1604-10.
430. Thomsen, M., et al., *Genetic variation in the scavenger receptor MARCO and its association with chronic obstructive pulmonary disease and lung infection in 10,604 individuals.* Respiration, 2013. 85(2): p. 144-53.
431. Matthaus, C., et al., *Noninvasive imaging of intracellular lipid metabolism in macrophages by Raman microscopy in combination with stable isotopic labeling.* Anal Chem, 2012. 84(20): p. 8549-56.
432. Walther, T.C. and R.V. Farese, Jr., *Lipid droplets and cellular lipid metabolism.* Annu Rev Biochem, 2012. 81: p. 687-714.
433. Plakkal Ayyappan, J., A. Paul, and Y.H. Goo, *Lipid droplet-associated proteins in atherosclerosis (Review).* Mol Med Rep, 2016. 13(6): p. 4527-34.
434. Thiam, A.R., R.V. Farese, Jr., and T.C. Walther, *The biophysics and cell biology of lipid droplets.* Nat Rev Mol Cell Biol, 2013. 14(12): p. 775-86.
435. Liu, P., et al., *Chinese hamster ovary K2 cell lipid droplets appear to be metabolic organelles involved in membrane traffic.* J Biol Chem, 2004. 279(5): p. 3787-92.
436. Walther, T.C. and R.V. Farese, Jr., *The life of lipid droplets.* Biochim Biophys Acta, 2009. 1791(6): p. 459-66.
437. Bartz, R., et al., *Dynamic activity of lipid droplets: protein phosphorylation and GTP-mediated protein translocation.* J Proteome Res, 2007. 6(8): p. 3256-65.
438. Bartz, R., et al., *Lipidomics reveals that adiposomes store ether lipids and mediate phospholipid traffic.* J Lipid Res, 2007. 48(4): p. 837-47.
439. Tabas, I., *Consequences of cellular cholesterol accumulation: basic concepts and physiological implications.* J Clin Invest, 2002. 110(7): p. 905-11.
440. Tabas, I., *Cholesterol in health and disease.* J Clin Invest, 2002. 110(5): p. 583-90.
441. Pineau, L., et al., *Lipid-induced ER stress: synergistic effects of sterols and saturated fatty acids.* Traffic, 2009. 10(6): p. 673-90.
442. Steck, T.L. and Y. Lange, *Cell cholesterol homeostasis: mediation by active cholesterol.* Trends Cell Biol, 2010. 20(11): p. 680-7.

443. Paul, A., L. Chan, and P.E. Bickel, *The PAT family of lipid droplet proteins in heart and vascular cells.* Curr Hypertens Rep, 2008. 10(6): p. 461-6.
444. Paul, A., et al., *Deficiency of adipose differentiation-related protein impairs foam cell formation and protects against atherosclerosis.* Circ Res, 2008. 102(12): p. 1492-501.
445. Buers, I., et al., *Lipid droplet associated proteins: an emerging role in atherogenesis.* Histol Histopathol, 2011. 26(5): p. 631-42.
446. Itabe, H., et al., *Perilipins: a diversity of intracellular lipid droplet proteins.* Lipids Health Dis, 2017. 16(1): p. 83.
447. Masuda, Y., et al., *ADRP/adipophilin is degraded through the proteasome-dependent pathway during regression of lipid-storing cells.* J Lipid Res, 2006. 47(1): p. 87-98.
448. Zhao, X., et al., *Perilipin1 deficiency in whole body or bone marrow-derived cells attenuates lesions in atherosclerosis-prone mice.* PLoS One, 2015. 10(4): p. e0123738.
449. Ouimet, M. and Y.L. Marcel, *Regulation of lipid droplet cholesterol efflux from macrophage foam cells.* Arterioscler Thromb Vasc Biol, 2012. 32(3): p. 575-81.
450. Ouimet, M., et al., *Autophagy regulates cholesterol efflux from macrophage foam cells via lysosomal acid lipase.* Cell Metab, 2011. 13(6): p. 655-67.
451. Li, A.C. and C.K. Glass, *PPAR- and LXR-dependent pathways controlling lipid metabolism and the development of atherosclerosis.* J Lipid Res, 2004. 45(12): p. 2161-73.
452. Chinetti-Gbaguidi, G. and B. Staels, *Lipid ligand-activated transcription factors regulating lipid storage and release in human macrophages.* Biochim Biophys Acta, 2009. 1791(6): p. 486-93.
453. Keshamouni, V.G., S. Han, and J. Roman, *Peroxisome proliferator-activated receptors in lung cancer.* PPAR Res, 2007. 2007: p. 90289.
454. Robinson-Rechavi, M., et al., *How many nuclear hormone receptors are there in the human genome?* Trends Genet, 2001. 17(10): p. 554-6.
455. Smith, M.R., T.J. Standiford, and R.C. Reddy, *PPARs in alveolar macrophage biology.* PPAR Res, 2007. 2007: p. 23812.
456. Tyagi, S., et al., *The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases.* J Adv Pharm Technol Res, 2011. 2(4): p. 236-40.
457. Issemann, I. and S. Green, *Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators.* Nature, 1990. 347(6294): p. 645-50.
458. Braissant, O., et al., *Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat.* Endocrinology, 1996. 137(1): p. 354-66.
459. Sher, T., et al., *cDNA cloning, chromosomal mapping, and functional characterization of the human peroxisome proliferator activated receptor.* Biochemistry, 1993. 32(21): p. 5598-604.
460. Genovese, T., et al., *Effect of rosiglitazone and 15-deoxy-Delta12,14-prostaglandin J2 on bleomycin-induced lung injury.* Eur Respir J, 2005. 25(2): p. 225-34.
461. Tan, N.S., et al., *Peroxisome proliferator-activated receptor (PPAR)-beta as a target for wound healing drugs: what is possible?* Am J Clin Dermatol, 2003. 4(8): p. 523-30.
462. Michalik, L., et al., *Impaired skin wound healing in peroxisome proliferator-activated receptor (PPAR)alpha and PPARbeta mutant mice.* J Cell Biol, 2001. 154(4): p. 799-814.
463. Tan, N.S., et al., *Peroxisome proliferator-activated receptor-beta as a target for wound healing drugs.* Expert Opin Ther Targets, 2004. 8(1): p. 39-48.
464. Michalik, L. and W. Wahli, *Involvement of PPAR nuclear receptors in tissue injury and wound repair.* J Clin Invest, 2006. 116(3): p. 598-606.
465. Baker, A.D., et al., *PPARgamma regulates the expression of cholesterol metabolism genes in alveolar macrophages.* Biochem Biophys Res Commun, 2010. 393(4): p. 682-7.
466. Baker, A.D., et al., *Targeted PPAR{gamma} deficiency in alveolar macrophages disrupts surfactant catabolism.* J Lipid Res, 2010. 51(6): p. 1325-31.
467. Croasdell, A., et al., *PPARgamma and the Innate Immune System Mediate the Resolution of Inflammation.* PPAR Res, 2015. 2015: p. 549691.

468. Lu, X., et al., *PPAR{gamma} regulates hypoxia-induced Nox4 expression in human pulmonary artery smooth muscle cells through NF-{kappa}B.* Am J Physiol Lung Cell Mol Physiol, 2010. 299(4): p. L559-66.
469. Gong, K., et al., *Hypoxia induces downregulation of PPAR-gamma in isolated pulmonary arterial smooth muscle cells and in rat lung via transforming growth factor-beta signaling.* Am J Physiol Lung Cell Mol Physiol, 2011. 301(6): p. L899-907.
470. Harmon, G.S., et al., *Pharmacological correction of a defect in PPAR-gamma signaling ameliorates disease severity in Cftr-deficient mice.* Nat Med, 2010. 16(3): p. 313-8.
471. Cho, H.Y., et al., *Nrf2-regulated PPAR{gamma} expression is critical to protection against acute lung injury in mice.* Am J Respir Crit Care Med, 2010. 182(2): p. 170-82.
472. Reddy, R.C. and T.J. Standiford, *Nrf2 and PPAR{gamma}: PPARtnering against oxidant-induced lung injury.* Am J Respir Crit Care Med, 2010. 182(2): p. 134-5.
473. Khandekar, M.J., et al., *Noncanonical agonist PPARgamma ligands modulate the response to DNA damage and sensitize cancer cells to cytotoxic chemotherapy.* Proc Natl Acad Sci U S A, 2018. 115(3): p. 561-566.
474. Solleti, S.K., et al., *Airway epithelial cell PPARgamma modulates cigarette smoke-induced chemokine expression and emphysema susceptibility in mice.* Am J Physiol Lung Cell Mol Physiol, 2015. 309(3): p. L293-304.
475. Kelly, N.J. and S.D. Shapiro, *PPARgamma in emphysema: blunts the damage and triggers repair?* J Clin Invest, 2014. 124(3): p. 978-80.
476. Shan, M., et al., *Agonistic induction of PPARgamma reverses cigarette smoke-induced emphysema.* J Clin Invest, 2014. 124(3): p. 1371-81.
477. Hong, C. and P. Tontonoz, *Liver X receptors in lipid metabolism: opportunities for drug discovery.* Nat Rev Drug Discov, 2014. 13(6): p. 433-44.
478. Chen, J.D. and R.M. Evans, *A transcriptional co-repressor that interacts with nuclear hormone receptors.* Nature, 1995. 377(6548): p. 454-7.
479. Zelcer, N. and P. Tontonoz, *Liver X receptors as integrators of metabolic and inflammatory signaling.* J Clin Invest, 2006. 116(3): p. 607-14.
480. Baranowski, M., *Biological role of liver X receptors.* J Physiol Pharmacol, 2008. 59 Suppl 7: p. 31-55.
481. Peet, D.J., B.A. Janowski, and D.J. Mangelsdorf, *The LXRs: a new class of oxysterol receptors.* Curr Opin Genet Dev, 1998. 8(5): p. 571-5.
482. Peet, D.J., et al., *Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha.* Cell, 1998. 93(5): p. 693-704.
483. Alberti, S., et al., *Hepatic cholesterol metabolism and resistance to dietary cholesterol in LXRbeta-deficient mice.* J Clin Invest, 2001. 107(5): p. 565-73.
484. Sabol, S.L., H.B. Brewer, Jr., and S. Santamarina-Fojo, *The human ABCG1 gene: identification of LXR response elements that modulate expression in macrophages and liver.* J Lipid Res, 2005. 46(10): p. 2151-67.
485. Zelcer, N., et al., *LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor.* Science, 2009. 325(5936): p. 100-4.
486. Wang, Q., et al., *Activation of liver X receptor inhibits the development of pulmonary carcinomas induced by 3-methylcholanthrene and butylated hydroxytoluene in BALB/c mice.* Sci Rep, 2016. 6: p. 27295.
487. Cao, H., et al., *Liver X receptor agonist T0901317 reverses resistance of A549 human lung cancer cells to EGFR-TKI treatment.* FEBS Open Bio, 2017. 7(1): p. 35-43.
488. Chen, L., et al., *25-Hydroxycholesterol promotes migration and invasion of lung adenocarcinoma cells.* Biochem Biophys Res Commun, 2017. 484(4): p. 857-863.
489. Wairagu, P.M., et al., *Combined therapeutic potential of nuclear receptors with receptor tyrosine kinase inhibitors in lung cancer.* Biochem Biophys Res Commun, 2014. 447(3): p. 490-5.
490. Ma, X., et al., *Inhibition of tumor growth by U0126 is associated with induction of interferon-gamma production.* Int J Cancer, 2015. 136(4): p. 771-83.

491. Kashiwagi, K., et al., *Expression of liver X receptors in normal and refractory carcinoma tissues of the human lung and pancreas.* Histol Histopathol, 2018. 33(5): p. 497-505.
492. Birrell, M.A., et al., *Novel role for the liver X nuclear receptor in the suppression of lung inflammatory responses.* J Biol Chem, 2007. 282(44): p. 31882-90.
493. Smoak, K., et al., *Effects of liver X receptor agonist treatment on pulmonary inflammation and host defense.* J Immunol, 2008. 180(5): p. 3305-12.
494. Gong, H., et al., *Activation of the liver X receptor prevents lipopolysaccharide-induced lung injury.* J Biol Chem, 2009. 284(44): p. 30113-21.
495. Korf, H., et al., *Liver X receptors contribute to the protective immune response against Mycobacterium tuberculosis in mice.* J Clin Invest, 2009. 119(6): p. 1626-37.
496. Crisafulli, C., et al., *Effects of Liver x receptor agonist treatment on signal transduction pathways in acute lung inflammation.* Respir Res, 2010. 11: p. 19.
497. Wang, D., et al., *Synthetic LXR agonist T0901317 attenuates lipopolysaccharide-induced acute lung injury in rats.* Int Immunopharmacol, 2011. 11(12): p. 2098-103.
498. Delvecchio, C.J., et al., *Liver X receptor stimulates cholesterol efflux and inhibits expression of proinflammatory mediators in human airway smooth muscle cells.* Mol Endocrinol, 2007. 21(6): p. 1324-34.
499. Delvecchio, C.J., et al., *LXR-induced reverse cholesterol transport in human airway smooth muscle is mediated exclusively by ABCA1.* Am J Physiol Lung Cell Mol Physiol, 2008. 295(5): p. L949-57.
500. Birrell, M.A., et al., *Liver X receptor agonists increase airway reactivity in a model of asthma via increasing airway smooth muscle growth.* J Immunol, 2008. 181(6): p. 4265-71.
501. Shi, Y., et al., *A liver-X-receptor ligand, T0901317, attenuates IgE production and airway remodeling in chronic asthma model of mice.* PLoS One, 2014. 9(3): p. e92668.
502. Smet, M., et al., *Cholesterol-sensing liver X receptors stimulate Th2-driven allergic eosinophilic asthma in mice.* Immun Inflamm Dis, 2016. 4(3): p. 350-61.
503. Shi, Y., et al., *The effect of a liver-X-receptor ligand on bleomycin induced pulmonary fibrosis in mice.* Int Immunopharmacol, 2016. 41: p. 116-121.
504. Venteclef, N. and P. Ferre, *Liver X receptor: from metabolism to cancer.* Biochem J, 2014. 459(2): p. e1-3.
505. Dai, Y.B., et al., *Ablation of Liver X receptors alpha and beta leads to spontaneous peripheral squamous cell lung cancer in mice.* Proc Natl Acad Sci U S A, 2016. 113(27): p. 7614-9.
506. Higham, A., et al., *The role of the liver X receptor in chronic obstructive pulmonary disease.* Respir Res, 2013. 14: p. 106.
507. Wang, X., et al., *Macrophage ABCA1 and ABCG1, but not SR-BI, promote macrophage reverse cholesterol transport in vivo.* J Clin Invest, 2007. 117(8): p. 2216-24.
508. Cavelier, C., et al., *Lipid efflux by the ATP-binding cassette transporters ABCA1 and ABCG1.* Biochim Biophys Acta, 2006. 1761(7): p. 655-66.
509. Tarling, E.J. and P.A. Edwards, *ATP binding cassette transporter G1 (ABCG1) is an intracellular sterol transporter.* Proc Natl Acad Sci U S A, 2011. 108(49): p. 19719-24.
510. Santamarina-Fojo, S., et al., *Regulation and intracellular trafficking of the ABCA1 transporter.* J Lipid Res, 2001. 42(9): p. 1339-45.
511. Oram, J.F., *ATP-binding cassette transporter A1 and cholesterol trafficking.* Curr Opin Lipidol, 2002. 13(4): p. 373-81.
512. Santamarina-Fojo, S., et al., *Complete genomic sequence of the human ABCA1 gene: analysis of the human and mouse ATP-binding cassette A promoter.* Proc Natl Acad Sci U S A, 2000. 97(14): p. 7987-92.
513. Brunham, L.R., et al., *Clinical, Biochemical, and Molecular Characterization of Novel Mutations in ABCA1 in Families with Tangier Disease.* JIMD Rep, 2015. 18: p. 51-62.
514. Lawn, R.M., et al., *The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway.* J Clin Invest, 1999. 104(8): p. R25-31.

515. Remaley, A.T., et al., *Human ATP-binding cassette transporter 1 (ABC1): genomic organization and identification of the genetic defect in the original Tangier disease kindred.* Proc Natl Acad Sci U S A, 1999. 96(22): p. 12685-90.
516. Yvan-Charvet, L., et al., *ATP-binding cassette transporters and HDL suppress hematopoietic stem cell proliferation.* Science, 2010. 328(5986): p. 1689-93.
517. Yvan-Charvet, L., N. Wang, and A.R. Tall, *Role of HDL, ABCA1, and ABCG1 transporters in cholesterol efflux and immune responses.* Arterioscler Thromb Vasc Biol, 2010. 30(2): p. 139-43.
518. Attie, A.D., J.P. Kastelein, and M.R. Hayden, *Pivotal role of ABCA1 in reverse cholesterol transport influencing HDL levels and susceptibility to atherosclerosis.* J Lipid Res, 2001. 42(11): p. 1717-26.
519. Oram, J.F. and R.M. Lawn, *ABCA1. The gatekeeper for eliminating excess tissue cholesterol.* J Lipid Res, 2001. 42(8): p. 1173-9.
520. Takahashi, Y. and J.D. Smith, *Cholesterol efflux to apolipoprotein AI involves endocytosis and resecretion in a calcium-dependent pathway.* Proc Natl Acad Sci U S A, 1999. 96(20): p. 11358-63.
521. Denis, M., Y.D. Landry, and X. Zha, *ATP-binding cassette A1-mediated lipidation of apolipoprotein A-I occurs at the plasma membrane and not in the endocytic compartments.* J Biol Chem, 2008. 283(23): p. 16178-86.
522. Bates, S.R., et al., *Expression and biological activity of ABCA1 in alveolar epithelial cells.* Am J Respir Cell Mol Biol, 2008. 38(3): p. 283-92.
523. Bortnick, A.E., et al., *Identification and characterization of rodent ABCA1 in isolated type II pneumocytes.* Am J Physiol Lung Cell Mol Physiol, 2003. 285(4): p. L869-78.
524. Soumian, S., et al., *ABCA1 and atherosclerosis.* Vasc Med, 2005. 10(2): p. 109-19.
525. Chai, A.B., A.J. Ammit, and I.C. Gelissen, *Examining the role of ABC lipid transporters in pulmonary lipid homeostasis and inflammation.* Respir Res, 2017. 18(1): p. 41.
526. Agassandian, M., et al., *Oxysterols trigger ABCA1-mediated basolateral surfactant efflux.* Am J Respir Cell Mol Biol, 2004. 31(2): p. 227-33.
527. Bates, S.R., et al., *Pulmonary abnormalities due to ABCA1 deficiency in mice.* Am J Physiol Lung Cell Mol Physiol, 2005. 289(6): p. L980-9.
528. Dai, C., et al., *ATP-binding cassette transporter 1 attenuates ovalbumin-induced neutrophilic airway inflammation.* Am J Respir Cell Mol Biol, 2014. 51(5): p. 626-36.
529. Kusuhara, H. and Y. Sugiyama, *ATP-binding cassette, subfamily G (ABCG family).* Pflugers Arch, 2007. 453(5): p. 735-44.
530. Schmitz, G., T. Langmann, and S. Heimerl, *Role of ABCG1 and other ABCG family members in lipid metabolism.* J Lipid Res, 2001. 42(10): p. 1513-20.
531. Kennedy, M.A., et al., *ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation.* Cell Metab, 2005. 1(2): p. 121-31.
532. Baldan, A., et al., *Deletion of the transmembrane transporter ABCG1 results in progressive pulmonary lipidosis.* J Biol Chem, 2006. 281(39): p. 29401-10.
533. Baldan, A., et al., *ATP-binding cassette transporter G1 and lipid homeostasis.* Curr Opin Lipidol, 2006. 17(3): p. 227-32.
534. Out, R., et al., *Macrophage ABCG1 deletion disrupts lipid homeostasis in alveolar macrophages and moderately influences atherosclerotic lesion development in LDL receptor-deficient mice.* Arterioscler Thromb Vasc Biol, 2006. 26(10): p. 2295-300.
535. de Aguiar Vallim, T.Q., et al., *ABCG1 regulates pulmonary surfactant metabolism in mice and men.* J Lipid Res, 2017.
536. Baldan, A., et al., *Loss of ABCG1 results in chronic pulmonary inflammation.* J Immunol, 2008. 180(5): p. 3560-8.
537. Tarling, E.J. and P.A. Edwards, *Dancing with the sterols: critical roles for ABCG1, ABCA1, miRNAs, and nuclear and cell surface receptors in controlling cellular sterol homeostasis.* Biochim Biophys Acta, 2012. 1821(3): p. 386-95.
538. Thomassen, M.J., et al., *ABCG1 is deficient in alveolar macrophages of GM-CSF knockout mice and patients with pulmonary alveolar proteinosis.* J Lipid Res, 2007. 48(12): p. 2762-8.

539. Shah, P.L., et al., *Pulmonary alveolar proteinosis: clinical aspects and current concepts on pathogenesis.* Thorax, 2000. 55(1): p. 67-77.
540. Uchida, K., et al., *GM-CSF autoantibodies and neutrophil dysfunction in pulmonary alveolar proteinosis.* N Engl J Med, 2007. 356(6): p. 567-79.
541. Wojcik, A.J., et al., *A critical role for ABCG1 in macrophage inflammation and lung homeostasis.* J Immunol, 2008. 180(6): p. 4273-82.
542. Draper, D.W., et al., *ATP-binding cassette transporter G1 deficiency dysregulates host defense in the lung.* Am J Respir Crit Care Med, 2010. 182(3): p. 404-12.
543. Draper, D.W., et al., *ATP binding cassette transporter G1 deletion induces IL-17-dependent dysregulation of pulmonary adaptive immunity.* J Immunol, 2012. 188(11): p. 5327-36.
544. Baldan, A., et al., *ABCG1 is required for pulmonary B-1 B cell and natural antibody homeostasis.* J Immunol, 2014. 193(11): p. 5637-48.
545. Cheng, H.Y., et al., *Loss of ABCG1 influences regulatory T cell differentiation and atherosclerosis.* J Clin Invest, 2016. 126(9): p. 3236-46.
546. Wang, Y., et al., *Genetic variants in ABCG1 are associated with survival of nonsmall-cell lung cancer patients.* Int J Cancer, 2016. 138(11): p. 2592-601.
547. Tian, C., et al., *ABCG1 as a potential oncogene in lung cancer.* Exp Ther Med, 2017. 13(6): p. 3189-3194.
548. Duong, M., et al., *Relative contributions of ABCA1 and SR-BI to cholesterol efflux to serum from fibroblasts and macrophages.* Arterioscler Thromb Vasc Biol, 2006. 26(3): p. 541-7.
549. Rothblat, G.H., et al., *Cell cholesterol efflux: integration of old and new observations provides new insights.* J Lipid Res, 1999. 40(5): p. 781-96.
550. Yancey, P.G., et al., *In vivo modulation of HDL phospholipid has opposing effects on SR-BI- and ABCA1-mediated cholesterol efflux.* J Lipid Res, 2004. 45(2): p. 337-46.
551. Johnson, W.J., et al., *Cholesterol transport between cells and high-density lipoproteins.* Biochim Biophys Acta, 1991. 1085(3): p. 273-98.
552. Maxfield, F.R. and D. Wustner, *Intracellular cholesterol transport.* J Clin Invest, 2002. 110(7): p. 891-8.
553. Feingold, K.R. and C. Grunfeld, *Introduction to Lipids and Lipoproteins*, in *Endotext*, K.R. Feingold, et al., Editors. 2000: South Dartmouth (MA).
554. Daniels, T.F., et al., *Lipoproteins, cholesterol homeostasis and cardiac health.* Int J Biol Sci, 2009. 5(5): p. 474-88.
555. Daniels, S.R., *Can lipid and lipoprotein concentrations in childhood predict adult atherosclerosis?* J Am Coll Cardiol, 2009. 53(10): p. 870-1.
556. Olivecrona, G., *Role of lipoprotein lipase in lipid metabolism.* Curr Opin Lipidol, 2016. 27(3): p. 233-41.
557. Kobayashi, J., et al., *Hepatic Lipase: a Comprehensive View of its Role on Plasma Lipid and Lipoprotein Metabolism.* J Atheroscler Thromb, 2015. 22(10): p. 1001-11.
558. Mabuchi, H., A. Nohara, and A. Inazu, *Cholesteryl ester transfer protein (CETP) deficiency and CETP inhibitors.* Mol Cells, 2014. 37(11): p. 777-84.
559. Burstein, M., H.R. Scholnick, and R. Morfin, *Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions.* J Lipid Res, 1970. 11(6): p. 583-95.
560. Rader, D.J., *Molecular regulation of HDL metabolism and function: implications for novel therapies.* J Clin Invest, 2006. 116(12): p. 3090-100.
561. Vickers, K.C. and A.T. Remaley, *HDL and cholesterol: life after the divorce?* J Lipid Res, 2014. 55(1): p. 4-12.
562. Kontush, A., Chapman, M. J.,, *High-Density Lipoproteins structure, metabolism, function, and therapeutics*. 2012: John wiley & Sons.
563. Glomset, J.A., *The plasma lecithins:cholesterol acyltransferase reaction.* J Lipid Res, 1968. 9(2): p. 155-67.
564. Ross, R. and J.A. Glomset, *Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis.* Science, 1973. 180(4093): p. 1332-9.

565. Williamson, R., et al., *Marked reduction of high density lipoprotein cholesterol in mice genetically modified to lack apolipoprotein A-I.* Proc Natl Acad Sci U S A, 1992. 89(15): p. 7134-8.
566. Plump, A., *Atherosclerosis and the mouse: a decade of experience.* Ann Med, 1997. 29(3): p. 193-8.
567. Plump, A.S., et al., *ApoA-I knockout mice: characterization of HDL metabolism in homozygotes and identification of a post-RNA mechanism of apoA-I up-regulation in heterozygotes.* J Lipid Res, 1997. 38(5): p. 1033-47.
568. Huang, Y., et al., *Effects of genotype and diet on cholesterol efflux into plasma and lipoproteins of normal, apolipoprotein A-I-, and apolipoprotein E-deficient mice.* Arterioscler Thromb Vasc Biol, 1997. 17(10): p. 2010-9.
569. Wang, W., et al., *Genetic deletion of apolipoprotein A-I increases airway hyperresponsiveness, inflammation, and collagen deposition in the lung.* J Lipid Res, 2010. 51(9): p. 2560-70.
570. Madenspacher, J.H., et al., *Apolipoproteins and apolipoprotein mimetic peptides modulate phagocyte trafficking through chemotactic activity.* J Biol Chem, 2012. 287(52): p. 43730-40.
571. Yao, X., et al., *Emerging Roles of Apolipoprotein E and Apolipoprotein A-I in the Pathogenesis and Treatment of Lung Disease.* Am J Respir Cell Mol Biol, 2016. 55(2): p. 159-69.
572. Mahley, R.W., K.H. Weisgraber, and Y. Huang, *Apolipoprotein E: structure determines function, from atherosclerosis to Alzheimer's disease to AIDS.* J Lipid Res, 2009. 50 Suppl: p. S183-8.
573. Vedhachalam, C., et al., *The C-terminal lipid-binding domain of apolipoprotein E is a highly efficient mediator of ABCA1-dependent cholesterol efflux that promotes the assembly of high-density lipoproteins.* Biochemistry, 2007. 46(10): p. 2583-93.
574. Greenow, K., N.J. Pearce, and D.P. Ramji, *The key role of apolipoprotein E in atherosclerosis.* J Mol Med (Berl), 2005. 83(5): p. 329-42.
575. Mahley, R.W., Y. Huang, and K.H. Weisgraber, *Putting cholesterol in its place: apoE and reverse cholesterol transport.* J Clin Invest, 2006. 116(5): p. 1226-9.
576. Liu, C.C., et al., *Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy.* Nat Rev Neurol, 2013. 9(2): p. 106-18.
577. Mahley, R.W., *Apolipoprotein E: cholesterol transport protein with expanding role in cell biology.* Science, 1988. 240(4852): p. 622-30.
578. Mahley, R.W. and S.C. Rall, Jr., *Apolipoprotein E: far more than a lipid transport protein.* Annu Rev Genomics Hum Genet, 2000. 1: p. 507-37.
579. Suri, S., et al., *The forgotten APOE allele: a review of the evidence and suggested mechanisms for the protective effect of APOE varepsilon2.* Neurosci Biobehav Rev, 2013. 37(10 Pt 2): p. 2878-86.
580. Bekris, L.M., et al., *Genetics of Alzheimer disease.* J Geriatr Psychiatry Neurol, 2010. 23(4): p. 213-27.
581. Wang, H.Y., et al., *Increased Abeta42-alpha7-like nicotinic acetylcholine receptor complex level in lymphocytes is associated with apolipoprotein E4-driven Alzheimer's disease pathogenesis.* Alzheimers Res Ther, 2017. 9(1): p. 54.
582. Curtiss, L.K., *ApoE in atherosclerosis : a protein with multiple hats.* Arterioscler Thromb Vasc Biol, 2000. 20(8): p. 1852-3.
583. Curtiss, L.K. and W.A. Boisvert, *Apolipoprotein E and atherosclerosis.* Curr Opin Lipidol, 2000. 11(3): p. 243-51.
584. Choy, P.C., et al., *Lipids and atherosclerosis.* Biochem Cell Biol, 2004. 82(1): p. 212-24.
585. Tabas, I., G. Garcia-Cardena, and G.K. Owens, *Recent insights into the cellular biology of atherosclerosis.* J Cell Biol, 2015. 209(1): p. 13-22.
586. Schaefer, E.J., et al., *Genetic high density lipoprotein deficiency states and atherosclerosis.* Adv Exp Med Biol, 1986. 201: p. 1-15.
587. Ghiselli, G., et al., *Type III hyperlipoproteinemia associated with apolipoprotein E deficiency.* Science, 1981. 214(4526): p. 1239-41.
588. Plump, A.S., et al., *Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells.* Cell, 1992. 71(2): p. 343-53.
589. Zhang, S.H., et al., *Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E.* Science, 1992. 258(5081): p. 468-71.

590. Jawien, J., *The role of an experimental model of atherosclerosis: apoE-knockout mice in developing new drugs against atherogenesis.* Curr Pharm Biotechnol, 2012. 13(13): p. 2435-9.
591. Breslow, J.L., *Mouse models of atherosclerosis.* Science, 1996. 272(5262): p. 685-8.
592. Desurmont, C., et al., *Complete atherosclerosis regression after human ApoE gene transfer in ApoE-deficient/nude mice.* Arterioscler Thromb Vasc Biol, 2000. 20(2): p. 435-42.
593. Shimano, H., et al., *Overexpression of apolipoprotein E in transgenic mice: marked reduction in plasma lipoproteins except high density lipoprotein and resistance against diet-induced hypercholesterolemia.* Proc Natl Acad Sci U S A, 1992. 89(5): p. 1750-4.
594. Nikoulin, I.R. and L.K. Curtiss, *An apolipoprotein E synthetic peptide targets to lipoproteins in plasma and mediates both cellular lipoprotein interactions in vitro and acute clearance of cholesterol-rich lipoproteins in vivo.* J Clin Invest, 1998. 101(1): p. 223-34.
595. Massaro, D. and G.D. Massaro, *Apoetm1Unc mice have impaired alveologenesis, low lung function, and rapid loss of lung function.* Am J Physiol Lung Cell Mol Physiol, 2008. 294(5): p. L991-7.
596. Massaro, D. and G.D. Massaro, *Developmental alveologenesis: new roles for ApoE and LDL receptor.* Pediatr Res, 2011. 70(5): p. 458-61.
597. Ryan, A.J., et al., *Maternal loading with very low-density lipoproteins stimulates fetal surfactant synthesis.* Am J Physiol Lung Cell Mol Physiol, 2002. 283(2): p. L310-8.
598. Yao, X., et al., *Apolipoprotein E negatively regulates house dust mite-induced asthma via a low-density lipoprotein receptor-mediated pathway.* Am J Respir Crit Care Med, 2010. 182(10): p. 1228-38.
599. van den Elzen, P., et al., *Apolipoprotein-mediated pathways of lipid antigen presentation.* Nature, 2005. 437(7060): p. 906-10.
600. Gerard, H.C., et al., *Apolipoprotein E4 enhances attachment of Chlamydophila (Chlamydia) pneumoniae elementary bodies to host cells.* Microb Pathog, 2008. 44(4): p. 279-85.
601. Van Oosten, M., et al., *Apolipoprotein E protects against bacterial lipopolysaccharide-induced lethality. A new therapeutic approach to treat gram-negative sepsis.* J Biol Chem, 2001. 276(12): p. 8820-4.
602. de Bont, N., et al., *Apolipoprotein E knock-out mice are highly susceptible to endotoxemia and Klebsiella pneumoniae infection.* J Lipid Res, 1999. 40(4): p. 680-5.
603. Jacobsen, N.R., et al., *Lung inflammation and genotoxicity following pulmonary exposure to nanoparticles in ApoE-/- mice.* Part Fibre Toxicol, 2009. 6: p. 2.
604. Venosa, A., et al., *Characterization of Distinct Macrophage Subpopulations during Nitrogen Mustard-Induced Lung Injury and Fibrosis.* Am J Respir Cell Mol Biol, 2016. 54(3): p. 436-46.
605. Yamashita, C.M., et al., *Apolipoprotein E-deficient mice are susceptible to the development of acute lung injury.* Respiration, 2014. 87(5): p. 416-27.
606. Su, W.P., et al., *Apolipoprotein E expression promotes lung adenocarcinoma proliferation and migration and as a potential survival marker in lung cancer.* Lung Cancer, 2011. 71(1): p. 28-33.
607. Wang, Y., et al., *The diagnostic value of apolipoprotein E in malignant pleural effusion associated with non-small cell lung cancer.* Clin Chim Acta, 2013. 421: p. 230-5.
608. Arunachalam, G., et al., *Emphysema is associated with increased inflammation in lungs of atherosclerosis-prone mice by cigarette smoke: implications in comorbidities of COPD.* J Inflamm (Lond), 2010. 7: p. 34.
609. Lo Sasso, G., et al., *The Apoe(-/-) mouse model: a suitable model to study cardiovascular and respiratory diseases in the context of cigarette smoke exposure and harm reduction.* J Transl Med, 2016. 14(1): p. 146.
610. Rice, S.J., et al., *Proteomic profiling of human plasma identifies apolipoprotein E (APOE) as being associated with smoking and a marker for squamous metaplasia of the lung.* Proteomics, 2015.
611. Miyazaki, O., *Preβ1.HDL, a key element of reverse cholesterol transport: its potential as a biomarker.* Clinical Lipidology, 2010. 5:3.
612. Mulligan, J.J., et al., *Cigarette smoking impairs hepatic uptake of high density lipoproteins.* Biochem Biophys Res Commun, 1983. 112(3): p. 843-50.
613. Aseem, O., et al., *Cubilin maintains blood levels of HDL and albumin.* J Am Soc Nephrol, 2014. 25(5): p. 1028-36.

614. Levels, J.H., et al., *Lipopolysaccharide is transferred from high-density to low-density lipoproteins by lipopolysaccharide-binding protein and phospholipid transfer protein.* Infect Immun, 2005. 73(4): p. 2321-6.
615. Terasaka, N., et al., *High-density lipoprotein protects macrophages from oxidized low-density lipoprotein-induced apoptosis by promoting efflux of 7-ketocholesterol via ABCG1.* Proc Natl Acad Sci U S A, 2007. 104(38): p. 15093-8.
616. Negre-Salvayre, A., et al., *Antioxidant and cytoprotective properties of high-density lipoproteins in vascular cells.* Free Radic Biol Med, 2006. 41(7): p. 1031-40.
617. Rye, K.A., et al., *The metabolism and anti-atherogenic properties of HDL.* J Lipid Res, 2009. 50 Suppl: p. S195-200.
618. Van Lenten, B.J., et al., *Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response. Loss of protective effect of HDL against LDL oxidation in aortic wall cell cocultures.* J Clin Invest, 1995. 96(6): p. 2758-67.
619. Navab, M., et al., *Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: steps 2 and 3.* J Lipid Res, 2000. 41(9): p. 1495-508.
620. Navab, M., et al., *Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: step 1.* J Lipid Res, 2000. 41(9): p. 1481-94.
621. Bergt, C., et al., *The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport.* Proc Natl Acad Sci U S A, 2004. 101(35): p. 13032-7.
622. Bergt, C., et al., *Lysine residues direct the chlorination of tyrosines in YXXK motifs of apolipoprotein A-I when hypochlorous acid oxidizes high density lipoprotein.* J Biol Chem, 2004. 279(9): p. 7856-66.
623. Shao, B., et al., *Pathways for oxidation of high-density lipoprotein in human cardiovascular disease.* Curr Opin Mol Ther, 2006. 8(3): p. 198-205.
624. Shao, B., et al., *Myeloperoxidase: an inflammatory enzyme for generating dysfunctional high density lipoprotein.* Curr Opin Cardiol, 2006. 21(4): p. 322-8.
625. Shao, B., et al., *Myeloperoxidase impairs ABCA1-dependent cholesterol efflux through methionine oxidation and site-specific tyrosine chlorination of apolipoprotein A-I.* J Biol Chem, 2006. 281(14): p. 9001-4.
626. Bridges, R.B., M.C. Fu, and S.R. Rehm, *Increased neutrophil myeloperoxidase activity associated with cigarette smoking.* Eur J Respir Dis, 1985. 67(2): p. 84-93.
627. Garrison, R.J., et al., *Cigarette smoking and HDL cholesterol: the Framingham offspring study.* Atherosclerosis, 1978. 30(1): p. 17-25.
628. Sigurdsson, G., Jr., et al., *Interaction between a polymorphism of the apo A-I promoter region and smoking determines plasma levels of HDL and apo A-I.* Arterioscler Thromb, 1992. 12(9): p. 1017-22.
629. Batic-Mujanovic, O., et al., *[The effect of cigarette smoking on HDL-cholesterol level].* Med Arh, 2006. 60(6 Suppl 2): p. 90-2.
630. Kim, C., et al., *Attenuation of Cigarette Smoke-Induced Emphysema in Mice by Apolipoprotein A-1 Overexpression.* Am J Respir Cell Mol Biol, 2016. 54(1): p. 91-102.
631. Nicholas, B.L., et al., *Identification of lipocalin and apolipoprotein A1 as biomarkers of chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 2010. 181(10): p. 1049-60.
632. Moffatt, R.J., B.A. Stamford, and K.D. Biggerstaff, *Influence of worksite environmental tobacco smoke on serum lipoprotein profiles of female nonsmokers.* Metabolism, 1995. 44(12): p. 1536-9.
633. Neufeld, E.J., et al., *Passive cigarette smoking and reduced HDL cholesterol levels in children with high-risk lipid profiles.* Circulation, 1997. 96(5): p. 1403-7.
634. He, B.M., S.P. Zhao, and Z.Y. Peng, *Effects of cigarette smoking on HDL quantity and function: implications for atherosclerosis.* J Cell Biochem, 2013. 114(11): p. 2431-6.
635. Richard, F., et al., *Effect of smoking cessation on lipoprotein A-I and lipoprotein A-I:A-II levels.* Metabolism, 1997. 46(6): p. 711-5.
636. Forey, B.A., et al., *The effect of quitting smoking on HDL-cholesterol - a review based on within-subject changes.* Biomark Res, 2013. 1(1): p. 26.

637. Badimon, J.J., L. Badimon, and V. Fuster, *Regression of atherosclerotic lesions by high density lipoprotein plasma fraction in the cholesterol-fed rabbit.* J Clin Invest, 1990. 85(4): p. 1234-41.
638. Kaul, S., et al., *Intramural delivery of recombinant apolipoprotein A-IMilano/phospholipid complex (ETC-216) inhibits in-stent stenosis in porcine coronary arteries.* Circulation, 2003. 107(20): p. 2551-4.
639. Nanjee, M.N., et al., *Effects of intravenous infusion of lipid-free apo A-I in humans.* Arterioscler Thromb Vasc Biol, 1996. 16(9): p. 1203-14.
640. Eriksson, M., et al., *Stimulation of fecal steroid excretion after infusion of recombinant proapolipoprotein A-I. Potential reverse cholesterol transport in humans.* Circulation, 1999. 100(6): p. 594-8.
641. Remaley, A.T., M. Amar, and D. Sviridov, *HDL-replacement therapy: mechanism of action, types of agents and potential clinical indications.* Expert Rev Cardiovasc Ther, 2008. 6(9): p. 1203-15.
642. Krause, B.R. and A.T. Remaley, *Reconstituted HDL for the acute treatment of acute coronary syndrome.* Curr Opin Lipidol, 2013. 24(6): p. 480-6.
643. Tardif, J.C., *Emerging high-density lipoprotein infusion therapies: fulfilling the promise of epidemiology?* J Clin Lipidol, 2010. 4(5): p. 399-404.
644. Tardif, J.C., et al., *Effects of reconstituted high-density lipoprotein infusions on coronary atherosclerosis: a randomized controlled trial.* JAMA, 2007. 297(15): p. 1675-82.
645. Calkin, A.C., et al., *Reconstituted high-density lipoprotein attenuates platelet function in individuals with type 2 diabetes mellitus by promoting cholesterol efflux.* Circulation, 2009. 120(21): p. 2095-104.
646. Patel, S., et al., *Reconstituted high-density lipoprotein increases plasma high-density lipoprotein anti-inflammatory properties and cholesterol efflux capacity in patients with type 2 diabetes.* J Am Coll Cardiol, 2009. 53(11): p. 962-71.
647. Chen, Z., et al., *Reconstituted HDL elicits marked changes in plasma lipids following single-dose injection in C57Bl/6 mice.* J Cardiovasc Pharmacol Ther, 2012. 17(3): p. 315-23.
648. Gebhard, C., et al., *Beneficial Effects of Reconstituted High-Density Lipoprotein (rHDL) on Circulating CD34+ Cells in Patients after an Acute Coronary Syndrome.* PLoS One, 2017. 12(1): p. e0168448.
649. Franceschini, G., et al., *A-IMilano apoprotein. Decreased high density lipoprotein cholesterol levels with significant lipoprotein modifications and without clinical atherosclerosis in an Italian family.* J Clin Invest, 1980. 66(5): p. 892-900.
650. Weisgraber, K.H., et al., *Apolipoprotein A-IMilano. Detection of normal A-I in affected subjects and evidence for a cysteine for arginine substitution in the variant A-I.* J Biol Chem, 1983. 258(4): p. 2508-13.
651. Gualandri, V., et al., *AIMilano apoprotein identification of the complete kindred and evidence of a dominant genetic transmission.* Am J Hum Genet, 1985. 37(6): p. 1083-97.
652. Franceschini, G., et al., *Relationship of the phenotypic expression of the A-IMilano apoprotein with plasma lipid and lipoprotein patterns.* Atherosclerosis, 1985. 58(1-3): p. 159-74.
653. Sirtori, C.R., et al., *Cardiovascular status of carriers of the apolipoprotein A-I(Milano) mutant: the Limone sul Garda study.* Circulation, 2001. 103(15): p. 1949-54.
654. Nissen, S.E., et al., *Effect of recombinant ApoA-I Milano on coronary atherosclerosis in patients with acute coronary syndromes: a randomized controlled trial.* JAMA, 2003. 290(17): p. 2292-300.
655. Nicholls, S.J., et al., *Relationship between atheroma regression and change in lumen size after infusion of apolipoprotein A-I Milano.* J Am Coll Cardiol, 2006. 47(5): p. 992-7.
656. Parolini, C., et al., *Dose-related effects of repeated ETC-216 (recombinant apolipoprotein A-I Milano/1-palmitoyl-2-oleoyl phosphatidylcholine complexes) administrations on rabbit lipid-rich soft plaques: in vivo assessment by intravascular ultrasound and magnetic resonance imaging.* J Am Coll Cardiol, 2008. 51(11): p. 1098-103.
657. Kempen, H.J., et al., *Effect of repeated apoA-IMilano/POPC infusion on lipids, (apo)lipoproteins, and serum cholesterol efflux capacity in cynomolgus monkeys.* J Lipid Res, 2013. 54(9): p. 2341-53.
658. Kempen, H.J., et al., *Incubation of MDCO-216 (ApoA-IMilano/POPC) with Human Serum Potentiates ABCA1-Mediated Cholesterol Efflux Capacity, Generates New Prebeta-1 HDL, and Causes an Increase in HDL Size.* J Lipids, 2014. 2014: p. 923903.

659. Kempen, H.J., et al., *High-Density Lipoprotein Subfractions and Cholesterol Efflux Capacities After Infusion of MDCO-216 (Apolipoprotein A-IMilano/Palmitoyl-Oleoyl-Phosphatidylcholine) in Healthy Volunteers and Stable Coronary Artery Disease Patients.* Arterioscler Thromb Vasc Biol, 2016. 36(4): p. 736-42.
660. Kempen, H.J., et al., *Persistent changes in lipoprotein lipids after a single infusion of ascending doses of MDCO-216 (apoA-IMilano/POPC) in healthy volunteers and stable coronary artery disease patients.* Atherosclerosis, 2016. 255: p. 17-24.
661. Kallend, D.G., et al., *A single infusion of MDCO-216 (ApoA-1 Milano/POPC) increases ABCA1-mediated cholesterol efflux and pre-beta 1 HDL in healthy volunteers and patients with stable coronary artery disease.* Eur Heart J Cardiovasc Pharmacother, 2016. 2(1): p. 23-9.
662. Reijers, J.A.A., et al., *MDCO-216 Does Not Induce Adverse Immunostimulation, in Contrast to Its Predecessor ETC-216.* Cardiovasc Drugs Ther, 2017. 31(4): p. 381-389.
663. Tardif, J.C., et al., *Effects of the high-density lipoprotein mimetic agent CER-001 on coronary atherosclerosis in patients with acute coronary syndromes: a randomized trial.* Eur Heart J, 2014. 35(46): p. 3277-86.
664. Andrews, J., et al., *Effect of serial infusions of reconstituted high-density lipoprotein (CER-001) on coronary atherosclerosis: rationale and design of the CARAT study.* Cardiovasc Diagn Ther, 2017. 7(1): p. 45-51.
665. Graversen, J.H., et al., *Trimerization of apolipoprotein A-I retards plasma clearance and preserves antiatherosclerotic properties.* J Cardiovasc Pharmacol, 2008. 51(2): p. 170-7.
666. Yao, X., et al., *The A's Have It: Developing Apolipoprotein A-I Mimetic Peptides Into a Novel Treatment for Asthma.* Chest, 2016. 150(2): p. 283-8.
667. Kim, T.H., et al., *Role of lung apolipoprotein A-I in idiopathic pulmonary fibrosis: antiinflammatory and antifibrotic effect on experimental lung injury and fibrosis.* Am J Respir Crit Care Med, 2010. 182(5): p. 633-42.
668. Lee, E., et al., *Overexpression of apolipoprotein A1 in the lung abrogates fibrosis in experimental silicosis.* PLoS One, 2013. 8(2): p. e55827.
669. Park, S.W., et al., *Apolipoprotein A1 potentiates lipoxin A4 synthesis and recovery of allergen-induced disrupted tight junctions in the airway epithelium.* Clin Exp Allergy, 2013. 43(8): p. 914-27.
670. Zamanian-Daryoush, M., et al., *The cardioprotective protein apolipoprotein A1 promotes potent anti-tumorigenic effects.* J Biol Chem, 2013. 288(29): p. 21237-52.
671. Navab, M., et al., *Apolipoprotein A-I mimetic peptides and their role in atherosclerosis prevention.* Nat Clin Pract Cardiovasc Med, 2006. 3(10): p. 540-7.
672. Navab, M., et al., *Oral administration of an Apo A-I mimetic Peptide synthesized from D-amino acids dramatically reduces atherosclerosis in mice independent of plasma cholesterol.* Circulation, 2002. 105(3): p. 290-2.
673. Navab, M., et al., *Oral D-4F causes formation of pre-beta high-density lipoprotein and improves high-density lipoprotein-mediated cholesterol efflux and reverse cholesterol transport from macrophages in apolipoprotein E-null mice.* Circulation, 2004. 109(25): p. 3215-20.
674. Bloedon, L.T., et al., *Safety, pharmacokinetics, and pharmacodynamics of oral apoA-I mimetic peptide D-4F in high-risk cardiovascular patients.* J Lipid Res, 2008. 49(6): p. 1344-52.
675. Garber, D.W., et al., *A new synthetic class A amphipathic peptide analogue protects mice from diet-induced atherosclerosis.* J Lipid Res, 2001. 42(4): p. 545-52.
676. Sethi, A.A., et al., *Asymmetry in the lipid affinity of bihelical amphipathic peptides. A structural determinant for the specificity of ABCA1-dependent cholesterol efflux by peptides.* J Biol Chem, 2008. 283(47): p. 32273-82.
677. Sharma, S., et al., *Apolipoprotein A-I mimetic peptide 4F rescues pulmonary hypertension by inducing microRNA-193-3p.* Circulation, 2014. 130(9): p. 776-85.
678. Dai, C., et al., *Apolipoprotein A-I attenuates ovalbumin-induced neutrophilic airway inflammation via a granulocyte colony-stimulating factor-dependent mechanism.* Am J Respir Cell Mol Biol, 2012. 47(2): p. 186-95.

679. Yao, X., A.T. Remaley, and S.J. Levine, *New kids on the block: the emerging role of apolipoproteins in the pathogenesis and treatment of asthma.* Chest, 2011. 140(4): p. 1048-1054.
680. Yao, X., et al., *5A, an apolipoprotein A-I mimetic peptide, attenuates the induction of house dust mite-induced asthma.* J Immunol, 2011. 186(1): p. 576-83.
681. Van Lenten, B.J., et al., *Influenza infection promotes macrophage traffic into arteries of mice that is prevented by D-4F, an apolipoprotein A-I mimetic peptide.* Circulation, 2002. 106(9): p. 1127-32.
682. Nandedkar, S.D., et al., *D-4F, an apoA-1 mimetic, decreases airway hyperresponsiveness, inflammation, and oxidative stress in a murine model of asthma.* J Lipid Res, 2011. 52(3): p. 499-508.
683. Lynch, J.R., et al., *APOE genotype and an ApoE-mimetic peptide modify the systemic and central nervous system inflammatory response.* J Biol Chem, 2003. 278(49): p. 48529-33.
684. Wang, H., et al., *APOE genotype affects outcome in a murine model of sepsis: implications for a new treatment strategy.* Anaesth Intensive Care, 2009. 37(1): p. 38-45.
685. Naura, A.S., et al., *High-fat diet induces lung remodeling in ApoE-deficient mice: an association with an increase in circulatory and lung inflammatory factors.* Lab Invest, 2009. 89(11): p. 1243-51.
686. Brewer, H.B., Jr., *Focus on high-density lipoproteins in reducing cardiovascular risk.* Am Heart J, 2004. 148(1 Suppl): p. S14-8.
687. Sacks, F.M., et al., *Selective delipidation of plasma HDL enhances reverse cholesterol transport in vivo.* J Lipid Res, 2009. 50(5): p. 894-907.
688. Pownall, H.J., *Detergent-mediated phospholipidation of plasma lipoproteins increases HDL cholesterophilicity and cholesterol efflux via SR-BI.* Biochemistry, 2006. 45(38): p. 11514-22.
689. Pownall, H.J. and C. Ehnholm, *The unique role of apolipoprotein A-I in HDL remodeling and metabolism.* Curr Opin Lipidol, 2006. 17(3): p. 209-13.
690. Kingwell, B.A., et al., *HDL-targeted therapies: progress, failures and future.* Nat Rev Drug Discov, 2014. 13(6): p. 445-64.
691. Ogata, M., et al., *On the mechanism for PPAR agonists to enhance ABCA1 gene expression.* Atherosclerosis, 2009. 205(2): p. 413-9.
692. Naik, S.U., et al., *Pharmacological activation of liver X receptors promotes reverse cholesterol transport in vivo.* Circulation, 2006. 113(1): p. 90-7.
693. Joseph, S.B., et al., *Synthetic LXR ligand inhibits the development of atherosclerosis in mice.* Proc Natl Acad Sci U S A, 2002. 99(11): p. 7604-9.
694. Terasaka, N., et al., *T-0901317, a synthetic liver X receptor ligand, inhibits development of atherosclerosis in LDL receptor-deficient mice.* FEBS Lett, 2003. 536(1-3): p. 6-11.
695. Verschuren, L., et al., *LXR agonist suppresses atherosclerotic lesion growth and promotes lesion regression in apoE*3Leiden mice: time course and mechanisms.* J Lipid Res, 2009. 50(2): p. 301-11.
696. Levin, N., et al., *Macrophage liver X receptor is required for antiatherogenic activity of LXR agonists.* Arterioscler Thromb Vasc Biol, 2005. 25(1): p. 135-42.
697. Im, S.S. and T.F. Osborne, *Liver x receptors in atherosclerosis and inflammation.* Circ Res, 2011. 108(8): p. 996-1001.
698. Beltowski, J., *Liver X receptors (LXR) as therapeutic targets in dyslipidemia.* Cardiovasc Ther, 2008. 26(4): p. 297-316.
699. Kratzer, A., et al., *Synthetic LXR agonist attenuates plaque formation in apoE-/- mice without inducing liver steatosis and hypertriglyceridemia.* J Lipid Res, 2009. 50(2): p. 312-26.
700. Getz, G.S. and C.A. Reardon, *Nutrition and cardiovascular disease.* Arterioscler Thromb Vasc Biol, 2007. 27(12): p. 2499-506.
701. Pitsavos, C., et al., *Diet, exercise and the metabolic syndrome.* Rev Diabet Stud, 2006. 3(3): p. 118-26.
702. Odegaard, A.O., et al., *Western-style fast food intake and cardiometabolic risk in an Eastern country.* Circulation, 2012. 126(2): p. 182-8.
703. Hayek, T., et al., *Dietary fat increases high density lipoprotein (HDL) levels both by increasing the transport rates and decreasing the fractional catabolic rates of HDL cholesterol ester and apolipoprotein*

(Apo) A-I. Presentation of a new animal model and mechanistic studies in human Apo A-I transgenic and control mice. J Clin Invest, 1993. 91(4): p. 1665-71.
704. Brinton, E.A., S. Eisenberg, and J.L. Breslow, *A low-fat diet decreases high density lipoprotein (HDL) cholesterol levels by decreasing HDL apolipoprotein transport rates.* J Clin Invest, 1990. 85(1): p. 144-51.
705. Sridhar, M.K., *Nutrition and lung health.* BMJ, 1995. 310(6972): p. 75-6.
706. Shahar, E., et al., *Dietary n-3 polyunsaturated fatty acids and smoking-related chronic obstructive pulmonary disease. Atherosclerosis Risk in Communities Study Investigators.* N Engl J Med, 1994. 331(4): p. 228-33.
707. Strachan, D.P., et al., *Ventilatory function and winter fresh fruit consumption in a random sample of British adults.* Thorax, 1991. 46(9): p. 624-9.
708. Britton, J.R., et al., *Dietary antioxidant vitamin intake and lung function in the general population.* Am J Respir Crit Care Med, 1995. 151(5): p. 1383-7.
709. Schwartz, J. and S.T. Weiss, *Dietary factors and their relation to respiratory symptoms. The Second National Health and Nutrition Examination Survey.* Am J Epidemiol, 1990. 132(1): p. 67-76.
710. Grievink, L., et al., *Dietary intake of antioxidant (pro)-vitamins, respiratory symptoms and pulmonary function: the MORGEN study.* Thorax, 1998. 53(3): p. 166-71.
711. Kallner, A.B., D. Hartmann, and D.H. Hornig, *On the requirements of ascorbic acid in man: steady-state turnover and body pool in smokers.* Am J Clin Nutr, 1981. 34(7): p. 1347-55.
712. Menon, D.K., P.J. Bacon, and J.G. Jones, *Nutrition and lung health.* BMJ, 1995. 310(6982): p. 805.
713. Kelly, Y., A. Sacker, and M. Marmot, *Nutrition and respiratory health in adults: findings from the health survey for Scotland.* Eur Respir J, 2003. 21(4): p. 664-71.
714. Wood, L.G., M.L. Garg, and P.G. Gibson, *A high-fat challenge increases airway inflammation and impairs bronchodilator recovery in asthma.* J Allergy Clin Immunol, 2011. 127(5): p. 1133-40.
715. Varraso, R., et al., *Dietary patterns and asthma in the E3N study.* Eur Respir J, 2009. 33(1): p. 33-41.
716. Varraso, R., et al., *Prospective study of cured meats consumption and risk of chronic obstructive pulmonary disease in men.* Am J Epidemiol, 2007. 166(12): p. 1438-45.
717. Varraso, R., et al., *Prospective study of dietary patterns and chronic obstructive pulmonary disease among US women.* Am J Clin Nutr, 2007. 86(2): p. 488-95.
718. Varraso, R., et al., *Prospective study of dietary patterns and chronic obstructive pulmonary disease among US men.* Thorax, 2007. 62(9): p. 786-91.
719. Vardavas, C.I., et al., *Does adherence to the Mediterranean diet have a protective effect against active and passive smoking?* Public Health, 2011. 125(3): p. 121-8.
720. Berthon, B.S. and L.G. Wood, *Nutrition and respiratory health--feature review.* Nutrients, 2015. 7(3): p. 1618-43.
721. Whyand, T., et al., *Pollution and respiratory disease: can diet or supplements help? A review.* Respir Res, 2018. 19(1): p. 79.
722. Ng, T.P., et al., *Curcumins-rich curry diet and pulmonary function in Asian older adults.* PLoS One, 2012. 7(12): p. e51753.
723. Yost, R.W., et al., *Synthesis of phosphatidylcholine by rat lung during choline deficiency.* J Appl Physiol (1985), 1986. 61(6): p. 2040-4.
724. Yost, R.W., et al., *Stimulation of the methylation pathway for phosphatidylcholine synthesis in rat lungs by choline deficiency.* Biochim Biophys Acta, 1986. 875(1): p. 122-5.
725. Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, O.B.V., and Choline,, *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline.* 1998.
726. Picciotto, M.R., M.J. Higley, and Y.S. Mineur, *Acetylcholine as a neuromodulator: cholinergic signaling shapes nervous system function and behavior.* Neuron, 2012. 76(1): p. 116-29.
727. Zeisel, S.H. and K.A. da Costa, *Choline: an essential nutrient for public health.* Nutr Rev, 2009. 67(11): p. 615-23.

728. Yao, Z.M. and D.E. Vance, *The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes.* J Biol Chem, 1988. 263(6): p. 2998-3004.
729. Penry, J.T. and M.M. Manore, *Choline: an important micronutrient for maximal endurance-exercise performance?* Int J Sport Nutr Exerc Metab, 2008. 18(2): p. 191-203.
730. Sanders, L.M. and S.H. Zeisel, *Choline: Dietary Requirements and Role in Brain Development.* Nutr Today, 2007. 42(4): p. 181-186.
731. da Costa, K.A., et al., *Common genetic polymorphisms affect the human requirement for the nutrient choline.* FASEB J, 2006. 20(9): p. 1336-44.
732. da Costa, K.A., et al., *Choline deficiency increases lymphocyte apoptosis and DNA damage in humans.* Am J Clin Nutr, 2006. 84(1): p. 88-94.
733. Konstantinova, S.V., et al., *Dietary patterns, food groups, and nutrients as predictors of plasma choline and betaine in middle-aged and elderly men and women.* Am J Clin Nutr, 2008. 88(6): p. 1663-9.
734. Konstantinova, S.V., et al., *Divergent associations of plasma choline and betaine with components of metabolic syndrome in middle age and elderly men and women.* J Nutr, 2008. 138(5): p. 914-20.
735. Oyen, J., et al., *Plasma choline, nicotine exposure, and risk of low bone mineral density and hip fracture: the Hordaland health study.* J Bone Miner Res, 2014. 29(1): p. 242-50.
736. Rinella, M.E. and R.M. Green, *The methionine-choline deficient dietary model of steatohepatitis does not exhibit insulin resistance.* J Hepatol, 2004. 40(1): p. 47-51.
737. Stankovic, M.N., et al., *Time-dependent changes and association between liver free fatty acids, serum lipid profile and histological features in mice model of nonalcoholic fatty liver disease.* Arch Med Res, 2014. 45(2): p. 116-24.
738. Larter, C.Z., et al., *MCD-induced steatohepatitis is associated with hepatic adiponectin resistance and adipogenic transformation of hepatocytes.* J Hepatol, 2008. 49(3): p. 407-16.
739. Takahashi, Y., Y. Soejima, and T. Fukusato, *Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis.* World J Gastroenterol, 2012. 18(19): p. 2300-8.
740. Bottiglieri, T., *S-Adenosyl-L-methionine (SAMe): from the bench to the bedside--molecular basis of a pleiotrophic molecule.* Am J Clin Nutr, 2002. 76(5): p. 1151S-7S.
741. National Research Council (US) Subcommittee on the Tenth Edition of the Recommended Dietary Allowances, *Recommended Dietary Allowances: 10th Edition.* 1989.
742. Tessari, P., A. Lante, and G. Mosca, *Essential amino acids: master regulators of nutrition and environmental footprint?* Sci Rep, 2016. 6: p. 26074.
743. Cheng, X. and R.J. Roberts, *AdoMet-dependent methylation, DNA methyltransferases and base flipping.* Nucleic Acids Res, 2001. 29(18): p. 3784-95.
744. Lushchak, V.I., *Free Radicals, Reactive Oxygen Species, Oxidative Stresses and Their Classifications.* Ukr Biochem J, 2015. 87(6): p. 11-8.
745. Lushchak, V.I., *Glutathione homeostasis and functions: potential targets for medical interventions.* J Amino Acids, 2012. 2012: p. 736837.
746. Rizki, G., et al., *Mice fed a lipogenic methionine-choline-deficient diet develop hypermetabolism coincident with hepatic suppression of SCD-1.* J Lipid Res, 2006. 47(10): p. 2280-90.
747. Caballero, F., et al., *Specific contribution of methionine and choline in nutritional nonalcoholic steatohepatitis: impact on mitochondrial S-adenosyl-L-methionine and glutathione.* J Biol Chem, 2010. 285(24): p. 18528-36.
748. Buchman, A.L., et al., *Choline deficiency: a cause of hepatic steatosis during parenteral nutrition that can be reversed with intravenous choline supplementation.* Hepatology, 1995. 22(5): p. 1399-403.
749. Savendahl, L., et al., *Prolonged fasting in humans results in diminished plasma choline concentrations but does not cause liver dysfunction.* Am J Clin Nutr, 1997. 66(3): p. 622-5.
750. Zeisel, S.H., et al., *Choline, an essential nutrient for humans.* FASEB J, 1991. 5(7): p. 2093-8.
751. Gordon, E.M., et al., *High-density Lipoproteins and Apolipoprotein A-I: Potential New Players in the Prevention and Treatment of Lung Disease.* Front Pharmacol, 2016. 7: p. 323.
752. Sonett, J., et al., *A critical role for ABC transporters in persistent lung inflammation in the development of emphysema after smoke exposure.* FASEB J, 2018: p. fj201701381.

753. von Scheidt, M., et al., *Applications and Limitations of Mouse Models for Understanding Human Atherosclerosis.* Cell Metab, 2017. 25(2): p. 248-261.
754. Hogarth, C.A., A. Roy, and D.L. Ebert, *Genomic evidence for the absence of a functional cholesteryl ester transfer protein gene in mice and rats.* Comp Biochem Physiol B Biochem Mol Biol, 2003. 135(2): p. 219-29.
755. Agellon, L.B., et al., *Reduced high density lipoprotein cholesterol in human cholesteryl ester transfer protein transgenic mice.* J Biol Chem, 1991. 266(17): p. 10796-801.
756. Marotti, K.R., et al., *Severe atherosclerosis in transgenic mice expressing simian cholesteryl ester transfer protein.* Nature, 1993. 364(6432): p. 73-5.
757. Kim, C., et al., *Attenuation of Cigarette Smoke-Induced Emphysema in Mice by Apolipoprotein A-1 Overexpression.* Am J Respir Cell Mol Biol, 2016. 54(1): p. 91-102.
758. Dong, C., J.R. Nevins, and P.J. Goldschmidt-Clermont, *ABCA1 single nucleotide polymorphisms. Snipping at the pathogenesis of atherosclerosis.* Circ Res, 2001. 88(9): p. 855-7.
759. Akao, H., et al., *ABCA1 gene variation and heart disease risk reduction in the elderly during pravastatin treatment.* Atherosclerosis, 2014. 235(1): p. 176-81.
760. Hardy, L.M., E. Frisdal, and W. Le Goff, *Critical Role of the Human ATP-Binding Cassette G1 Transporter in Cardiometabolic Diseases.* Int J Mol Sci, 2017. 18(9).
761. Iida, A., et al., *Catalog of 605 single-nucleotide polymorphisms (SNPs) among 13 genes encoding human ATP-binding cassette transporters: ABCA4, ABCA7, ABCA8, ABCD1, ABCD3, ABCD4, ABCE1, ABCF1, ABCG1, ABCG2, ABCG4, ABCG5, and ABCG8.* J Hum Genet, 2002. 47(6): p. 285-310.
762. Sano, R. and J.C. Reed, *ER stress-induced cell death mechanisms.* Biochim Biophys Acta, 2013. 1833(12): p. 3460-3470.
763. Xu, W., I.G. Charles, and S. Moncada, *Nitric oxide: orchestrating hypoxia regulation through mitochondrial respiration and the endoplasmic reticulum stress response.* Cell Res, 2005. 15(1): p. 63-5.
764. Karalis, I. and J.W. Jukema, *HDL Mimetics Infusion and Regression of Atherosclerosis: Is It Still Considered a Valid Therapeutic Option?* Curr Cardiol Rep, 2018. 20(8): p. 66.
765. Nicholls, S.J., et al., *Effect of Serial Infusions of CER-001, a Pre-beta High-Density Lipoprotein Mimetic, on Coronary Atherosclerosis in Patients Following Acute Coronary Syndromes in the CER-001 Atherosclerosis Regression Acute Coronary Syndrome Trial: A Randomized Clinical Trial.* JAMA Cardiol, 2018. 3(9): p. 815-822.
766. Nicholls, S.J., et al., *Effect of Infusion of High-Density Lipoprotein Mimetic Containing Recombinant Apolipoprotein A-I Milano on Coronary Disease in Patients With an Acute Coronary Syndrome in the MILANO-PILOT Trial: A Randomized Clinical Trial.* JAMA Cardiol, 2018. 3(9): p. 806-814.
767. Rader, D.J., *Apolipoprotein A-I Infusion Therapies for Coronary Disease: Two Outs in the Ninth Inning and Swinging for the Fences.* JAMA Cardiol, 2018. 3(9): p. 799-801.
768. Guedes, A. and J.C. Tardif, *Intravascular ultrasound assessment of atherosclerosis.* Curr Atheroscler Rep, 2004. 6(3): p. 219-24.
769. Caron, M.A., et al., *Alterations in skeletal muscle cell homeostasis in a mouse model of cigarette smoke exposure.* PLoS One, 2013. 8(6): p. e66433.
770. Lehti, M., et al., *High-density lipoprotein maintains skeletal muscle function by modulating cellular respiration in mice.* Circulation, 2013. 128(22): p. 2364-71.
771. Grefhorst, A., et al., *Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles.* J Biol Chem, 2002. 277(37): p. 34182-90.
772. Kirchgessner, T.G., et al., *Beneficial and Adverse Effects of an LXR Agonist on Human Lipid and Lipoprotein Metabolism and Circulating Neutrophils.* Cell Metab, 2016. 24(2): p. 223-33.
773. Magida, J.A. and R.M. Evans, *Rational application of macrophage-specific LXR agonists avoids the pitfalls of SREBP-induced lipogenesis.* Proc Natl Acad Sci U S A, 2018. 115(20): p. 5051-5053.

774. Muse, E.D., et al., *Cell-specific discrimination of desmosterol and desmosterol mimetics confers selective regulation of LXR and SREBP in macrophages.* Proc Natl Acad Sci U S A, 2018. 115(20): p. E4680-E4689.
775. Yu, S., et al., *Dissociated sterol-based liver X receptor agonists as therapeutics for chronic inflammatory diseases.* FASEB J, 2016. 30(7): p. 2570-9.
776. Millar, C.L., Q. Duclos, and C.N. Blesso, *Effects of Dietary Flavonoids on Reverse Cholesterol Transport, HDL Metabolism, and HDL Function.* Adv Nutr, 2017. 8(2): p. 226-239.
777. Marques, L.R., et al., *Reverse Cholesterol Transport: Molecular Mechanisms and the Non-medical Approach to Enhance HDL Cholesterol.* Front Physiol, 2018. 9: p. 526.
778. Basu, A., et al., *Acute Cocoa Supplementation Increases Postprandial HDL Cholesterol and Insulin in Obese Adults with Type 2 Diabetes after Consumption of a High-Fat Breakfast.* J Nutr, 2015. 145(10): p. 2325-32.
779. Rondanelli, M., et al., *MediterrAsian Diet Products That Could Raise HDL-Cholesterol: A Systematic Review.* Biomed Res Int, 2016. 2016: p. 2025687.
780. Mooradian, A.D. and M.J. Haas, *The effect of nutritional supplements on serum high-density lipoprotein cholesterol and apolipoprotein A-I.* Am J Cardiovasc Drugs, 2014. 14(4): p. 253-74.
781. Gonzalez, R., et al., *Effects of flavonoids and other polyphenols on inflammation.* Crit Rev Food Sci Nutr, 2011. 51(4): p. 331-62.
782. Pandey, K.B. and S.I. Rizvi, *Plant polyphenols as dietary antioxidants in human health and disease.* Oxid Med Cell Longev, 2009. 2(5): p. 270-8.
783. Schartum-Hansen, H., et al., *Plasma choline, smoking, and long-term prognosis in patients with stable angina pectoris.* Eur J Prev Cardiol, 2015. 22(5): p. 606-14.
784. Tall, A.R. and D.J. Rader, *Trials and Tribulations of CETP Inhibitors.* Circ Res, 2018. 122(1): p. 106-112.
785. Barter, P.J. and K.A. Rye, *Cholesteryl ester transfer protein inhibition as a strategy to reduce cardiovascular risk.* J Lipid Res, 2012. 53(9): p. 1755-66.
786. Lincoff, A.M., et al., *Evacetrapib and Cardiovascular Outcomes in High-Risk Vascular Disease.* N Engl J Med, 2017. 376(20): p. 1933-1942.
787. Group, H.T.R.C., et al., *Effects of Anacetrapib in Patients with Atherosclerotic Vascular Disease.* N Engl J Med, 2017. 377(13): p. 1217-1227.
788. Ohashi, R., et al., *Reverse cholesterol transport and cholesterol efflux in atherosclerosis.* QJM, 2005. 98(12): p. 845-56.
789. Guerin, M., et al., *Dose-dependent action of atorvastatin in type IIB hyperlipidemia: preferential and progressive reduction of atherogenic apoB-containing lipoprotein subclasses (VLDL-2, IDL, small dense LDL) and stimulation of cellular cholesterol efflux.* Atherosclerosis, 2002. 163(2): p. 287-96.
790. Nissen, S.E., et al., *Effect of intensive compared with moderate lipid-lowering therapy on progression of coronary atherosclerosis: a randomized controlled trial.* JAMA, 2004. 291(9): p. 1071-80.
791. Nissen, S.E., *Effect of intensive lipid lowering on progression of coronary atherosclerosis: evidence for an early benefit from the Reversal of Atherosclerosis with Aggressive Lipid Lowering (REVERSAL) trial.* Am J Cardiol, 2005. 96(5A): p. 61F-68F.
792. Nissen, S.E., et al., *Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial.* JAMA, 2006. 295(13): p. 1556-65.
793. Luo, Y. and A.R. Tall, *Sterol upregulation of human CETP expression in vitro and in transgenic mice by an LXR element.* J Clin Invest, 2000. 105(4): p. 513-20.
794. Groot, P.H., et al., *Synthetic LXR agonists increase LDL in CETP species.* J Lipid Res, 2005. 46(10): p. 2182-91.
795. Bagaitkar, J., D.R. Demuth, and D.A. Scott, *Tobacco use increases susceptibility to bacterial infection.* Tob Induc Dis, 2008. 4: p. 12.
796. Arcavi, L. and N.L. Benowitz, *Cigarette smoking and infection.* Arch Intern Med, 2004. 164(20): p. 2206-16.

797. Vreugdenhil, A.C., et al., *LPS-binding protein circulates in association with apoB-containing lipoproteins and enhances endotoxin-LDL/VLDL interaction.* J Clin Invest, 2001. 107(2): p. 225-34.
798. Lu, Y.C., W.C. Yeh, and P.S. Ohashi, *LPS/TLR4 signal transduction pathway.* Cytokine, 2008. 42(2): p. 145-151.
799. Wurfel, M.M. and S.D. Wright, *Lipopolysaccharide (LPS) binding protein catalyzes binding of LPS to lipoproteins.* Prog Clin Biol Res, 1995. 392: p. 287-95.
800. Miao, B., et al., *Raising HDL cholesterol without inducing hepatic steatosis and hypertriglyceridemia by a selective LXR modulator.* J Lipid Res, 2004. 45(8): p. 1410-7.
801. Mason, R.J., *Biology of alveolar type II cells.* Respirology, 2006. 11 Suppl: p. S12-5.
802. Zhao, C.Z., et al., *Involvement of type II pneumocytes in the pathogenesis of chronic obstructive pulmonary disease.* Respir Med, 2010. 104(10): p. 1391-5.
803. Maniscalco, W.M., J.N. Finkelstein, and A.B. Parkhurst, *Effects of exogenous fatty acids and inhibition of de novo fatty acid synthesis on disaturated phosphatidylcholine production by fetal lung cells and adult type II cells.* Exp Lung Res, 1989. 15(3): p. 473-89.
804. Arigliani, M., et al., *Nutrition and Lung Growth.* Nutrients, 2018. 10(7).
805. Massaro, D. and G.D. Massaro, *Lung development, lung function, and retinoids.* N Engl J Med, 2010. 362(19): p. 1829-31.
806. Chen, L., et al., *Identification of vitamin D sensitive pathways during lung development.* Respir Res, 2016. 17: p. 47.
807. Lykkedegn, S., et al., *Vitamin D Depletion in Pregnancy Decreases Survival Time, Oxygen Saturation, Lung Weight and Body Weight in Preterm Rat Offspring.* PLoS One, 2016. 11(8): p. e0155203.
808. Mehta, A.K., et al., *Acute toxicity assessment of choline by inhalation, intraperitoneal and oral routes in Balb/c mice.* Regul Toxicol Pharmacol, 2009. 54(3): p. 282-6.
809. Mehta, A.K., et al., *Choline supplementation reduces oxidative stress in mouse model of allergic airway disease.* Eur J Clin Invest, 2009. 39(10): p. 934-41.
810. Mehta, A.K., et al., *Choline attenuates immune inflammation and suppresses oxidative stress in patients with asthma.* Immunobiology, 2010. 215(7): p. 527-34.
811. Detopoulou, P., et al., *Dietary choline and betaine intakes in relation to concentrations of inflammatory markers in healthy adults: the ATTICA study.* Am J Clin Nutr, 2008. 87(2): p. 424-30.
812. Schall, J.I., et al., *Choline Supplementation With a Structured Lipid in Children With Cystic Fibrosis: A Randomized Placebo-Controlled Trial.* J Pediatr Gastroenterol Nutr, 2016. 62(4): p. 618-26.