GHENT UNIVERSITY

FACULTY OF MEDICINE AND HEALTH SCIENCES

ROLE OF MICRORNAS IN THE PATHOGENESIS OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

Griet Conickx

2017

Promotor: Prof. Dr. Guy Brusselle

Co-promotor: Prof. Dr. Ken Bracke

Thesis submitted to fulfill the requirements for the degree of

"Doctor in Health Sciences"

Financial support: the Concerted Research Action of the Ghent University (BOF/GOA, 01G02714), the Fund for Scientific Research in Flanders (FWO Vlaanderen, G.0A99.13N) and the Interuniversity Attraction Poles program (IUAP, P7/30).

ISBN: 978-94-6197-562-1

Department of Respiratory Medicine
Laboratory for Translational Research in Obstructive Pulmonary Diseases
Ghent University Hospital
De Pintelaan 185
9000 Ghent
BELGIUM

Table of Contents

ABBREVIATIONS

AGO	Argonaute
Ahr	Arylhydrocarbonreceptor
AP	Alkaline phosphatase
ASO	Antisense oligonucleotide
BAL	Bronchoalveolar lavage
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BEGM	Bronchial epithelial growth medium
BSA	Bovine serum albumin
CAT	COPD assessment test
CCL	Chemokine (C-C motif) ligand
cDNA	Complement DNA
ceRNA	Competing endogenous RNA
CLIP	Crosslinking immunoprecipitation
COPD	Chronic obstructive pulmonary disease
CRISPR	Clustered regularly interspaced short palindromic repeat
CS	Cigarette smoke
CSC	Cigarette smoke condensate
CSE	Cigarette smoke extract
CXCL	Chemokine (C-X-C motif) ligand
DAB	3,3'-diaminobenzidine
DC	Dendritic cell
DGCR8	DiGeorge Syndrome Critical Region 8
DIG	Digoxigenin
DLCO	Carbon monoxide diffusing capacity
DRD1	Dopamine D1 receptor
DUSP5	Dual specificity phosphatase 5
ECS	Environmental cigarette smoke
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eQTL	Expression quantitative trait loci
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FC	Fold change
FDR	False discovery rate
FEV_1	Forced expiratory volume in 1 second
FFPE	Formalin fixated paraffin embedded
FKBP1A	FK506 binding protein 1A
FVC	Forced vital capacity
GalNAc	N-Acetylgalactosamine
GOLD	Global initiative for obstructive lung diseases
GSEA	Gene set enrichment analysis
GWAS	Genome wide association study
HBECs	Human bronchial epithelial cells
HBSS	Hanks' Balanced Salt Solution
HCV	Hepatitis C virus
HDAC	Histone deacetylases
HIF-1α	Hypoxia inducible factor 1 alpha
HLA-DQA2	Major histocompatibility complex, class II, DQ alpha 2
HMOX1	Heme oxygenase 1

ICS	Inhaled corticosteroids
IGFBP3	Insulin-like growth factor-binding protein 3
IRF8	Interferon regulatory factor 8
ISH	*in situ* hybridization
IL	Interleukin
ILC	Innate lymphoid cell
IL-xR	Interleukin-x Receptor
KCO	DLCO/alveolar volume
KEGG	Kyoto Encyclopedia of Genes and Genomes
LABA	Long-acting β2-agonist
LAMA	Long-acting muscarinic antagonist
LNA	Locked nucleic acid
lncRNA	Long non-coding ribonucleic acid
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein 1
MHC	Major-histocompatibility-complex
miRNA	microRNA
miRQC study	microRNA quality control study
mRNA	messenger ribonucleic acid
MMP	Matrix metalloproteinase
mMRC	modified British Medical Research Counsil
MRE	MiRNA response element
NBT	Nitro-blue tetrazolium chloride
NFκB	Nuclear factor κB
NGS	Next generation sequencing
NHBE	Normal human bronchial epithelial
NRF2	Nuclear factor erythroid 2–related factor 2
NSCLC	Non-small-cell lung carcinoma
P-bodies	Processing bodies
PBS	Phosphate-buffered saline
PDE4	phosphodiesterase-4
PI3K	Phosphoinositide 3-kinase
PPARγ	Peroxisome proliferator-activated receptor-γ
PRR	Pattern recognition receptor
RISC	RNA-induced silencing complex
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SLIT2	Slit Homolog 2
SNP	Single Nucleotide Polymorphism
TGF-β	Transforming growth factor β
Th	T helper
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TRBP	Transactivation-responsive RNA-binding protein
Treg	Regulatory T
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
6MWD	6-minutes walking distance

I. GENERAL INTRODUCTION

CHAPTER 1: COPD

1.1 Definition

According to the Global Initiative for Obstructive Lung diseases (GOLD), Chronic Obstructive Pulmonary Disease (COPD) is defined as:

" *a common preventable and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases*" [1].

Common: Despite the fact that the term COPD doesn't sound familiar to the lay public, COPD (GOLD stage II-IV) has a high worldwide prevalence of 9-10% among adults aged 40 years and older [2]. Important differences in prevalence have been noted between countries ranging from 4-22% [3]. Most likely, this prevalence is an underestimation [4].

Preventable: COPD is often caused by repeated inhalation of noxious particles or gases (e.g. cigarette smoke). Preventive measures to avoid such exposures can block the onset or slow down the progression of COPD [5,6].

Treatable: The treatment of COPD is largely symptom driven, only smoking cessation is able to slow down the accelerated decline in lung function [7]. At present, the disease cannot be cured since no drugs can considerably halt disease progression or mortality [8].

Persistent airflow limitation: Patients with COPD typically present with persistent not-fully reversible airflow limitation which is measured by a standardized lung function test, i.e. spirometry. Generally, a ratio of post-bronchodilator Forced Expiratory Volume in 1 second (FEV_1) to Forced Vital Capacity (FVC) below 0.70 identifies airflow limitation.

Airway and/or alveolar abnormalities: Inhalation of harmful particles or gases induces an abnormal inflammatory reaction in airways and lungs of patients with COPD, resulting in pathological changes (see **1.4**) [9].

Noxious particles or gases: Mainly cigarette smoking is an initiating factor for COPD, but not exclusively. Other important causes are occupational exposure to harmful dusts and gases, and exposure to in- and outdoor pollution [6,10].

1.2 Burden

Several factors contribute to the high burden of COPD as COPD is a prevalent disease that has major implications on the patient's life and is associated with considerable costs for the society. Worldwide, COPD ranks as the 4th leading cause of death after ischemic heart diseases, stroke and lower respiratory infections [11,12]. In Europe, COPD was reported to cause the death of 150,000 people in 2010 (**Figure 1**) [13].

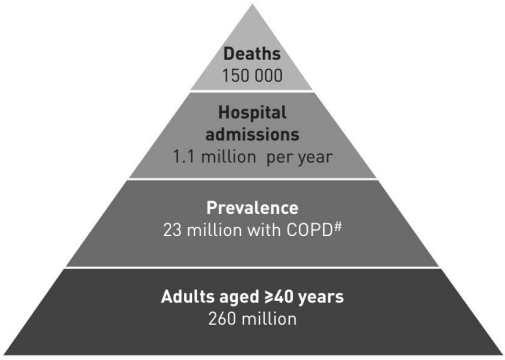


Figure 1. The burden of COPD in older adults in Europe in 2010. #: Global Initiative for Chronic Obstructive Lung Disease stages II–IV. An additional 17 million adults aged ≥40 years had stage I chronic obstructive pulmonary disease (COPD). Figure adapted from the European Lung White Book, Respiratory Health and Disease in Europe [13].

The worldwide prevalence of COPD (stage II-IV) in adults older than 40 is 9-10% [2,3]. In the Rotterdam study and another population study in the Netherlands, the overall prevalence of COPD increased with age, was higher in men and higher in ever-smokers compared to non-smokers [14,15]. Also, the mortality increased with COPD severity and was substantially higher in COPD patients compared to non-COPD patients of the same age, accentuating the burden of the disease [15].

COPD has a significant impact on the daily life of persons confronted with this disease. Impaired exercise performance and the presence of daily symptoms, commonly combined with anxiety, depression and the resulting social isolation contribute to the morbidity [5].

COPD is a growing burden on healthcare systems as well. Across the globe, the mean annual direct medical cost for COPD per patient varied from 431 to 34,101 USD [16]. Additionally, indirect costs of the disease such as lost or impaired productivity at work have a detrimental impact on the national income [5,16].

1.3 Clinical features and risk factors

COPD is a progressive disease that causes lung function decline. Patients typically present with **symptoms** such as coughing, sputum production and dyspnea. These symptoms vary over time and are worse in the morning [17]. The earliest, but non alarming, symptom is coughing. Mostly, the patients seek medical help when experiencing (exertional) dyspnea since this causes disability and anxiety [1]. This breathlessness during activity is persistent and worsens over time and is commonly accompanied in severe ill patients by fatigue and weight loss [1]. To assess dyspnea, the modified British Medical Research Counsil (mMRC) Questionnaire was developed [18]. Beyond assessing dyspnea, a more comprehensive questionnaire such as the COPD Assessment Test (CAT) which measures the symptomatic impact of COPD is recommended [19,20]. Regular sputum production during ≥ 3 months in two consecutive years is the classical definition of chronic bronchitis, however this does not reflect disease severity [1,21].

Diagnosis is confirmed based on patient's history, presence of characteristic symptoms and standardized lung-function measurements that reveal a not-fully-reversible airflow limitation ($FEV_1/FVC < 0.70$). FEV_1 is the maximum volume of air exhaled in the first second of a forced expiration started from a full inspiration. FVC is the maximum volume of air exhaled with maximally forced effort started from a maximal inspiration. Both parameters are measured during spirometry. Spirometry is assessed in comparison with reference values based on age, height, sex and race. Currently, spirometry is the cornerstone for COPD diagnosis, staging, response to therapy and follow-up [9]. It outputs a reproducible and objective measurement which is also noninvasive. Classification of COPD severity in GOLD stages (1-4) is based on post-bronchodilator FEV_1 (**Figure 3**). However, the degree of airflow limitation in COPD is only loosely related to disease severity [5].

Exacerbations and comorbidities contribute to the overall severity in individual patients. **Exacerbations**, acute worsening of respiratory symptoms that is beyond the normal day-to-day variation and that may warrant a change in regular medication, influence the progression of COPD and are the main cause of morbidity/mortality, particularly in those patients requiring hospitalization. An initial exacerbation increases the susceptibility to a new exacerbation [22]. The most frequent causes of exacerbations are viral (in 15-25% of all infective exacerbations) or bacterial infections, or both and account for 60-80% of all exacerbations [2].

COPD involves a complex pathogenesis that not only affects the lungs but is also often associated with other chronic **comorbidities**. Therefore patients do not always die from respiratory causes. Comorbid

diseases that may accompany COPD encompass lung cancer, cardiovascular disease, osteoporosis, metabolic syndrome, skeletal muscle weakness, diabetes, anxiety, depression and cognitive dysfunction [23]. The presence of comorbidities is only poorly related to the severity of airflow obstruction [24]. However, in patients with COPD, the presence of comorbidities increases the risk of hospitalization and mortality independently [25]. Therefore, they do require specific treatment.

COPD results from the interplay between genetic susceptibility and environmental stimuli [2]. The most important and best studied **risk factor** is cigarette smoking. The amount and duration of cigarette smoking contributes to the severity. Generally, multiple exposures over decades are needed to accelerate the decline in FEV_1 and to consequently develop COPD. Importantly, it is estimated that only 15-20% of smokers develop COPD, suggesting variability in the susceptibility to cigarette smoke (CS) [5]. Nevertheless, this may be an underestimation since far more than 15% of smokers have some amount of respiratory impairment/symptoms, yet do not fall within the COPD classification [26].

Genetic and epigenetic factors have been put forward to be responsible for this difference in susceptibility. Genetic factors that influence disease susceptibility are alpha1-antitrypsin deficiency (1-3% of COPD patients) and gene polymorphisms [27-29]. Smoking also induces reversible and irreversible epigenetic changes [30-34] (**see 1.5**).

In addition, a subpopulation of patients with COPD have never smoked [35], meaning that other factors such as occupational exposure to harmful gases, dusts, particulate matter, exposure to in- and outdoor pollution, poor socio-economic status, asthma and ageing can increase the risk or contribute to the development of COPD [5,6,36,37]. Recently, it was postulated that different trajectories of lung function exist, all resulting in COPD diagnosis. About one half of the patients have an accelerated decline in lung function while the other half has a normal lung function decline, starting from an already impaired lung function at the age of 20-40 years [38]. Processes that affect lung growth or development reduce the maximal attained lung function capacity, putting these individuals at risk for COPD [38]. These processes encompass events occurring during gestation, birth and childhood such as intrauterine growth retardation, maternal smoking during pregnancy, history of pulmonary tuberculosis and early life exposures to infectious and non-infectious agents [35,39-45].

The overall course and changes in FEV_1 over lifetime were already clearly presented in the well-known Fletcher and Peto curve [46]. Current insights have additionally accentuated the importance of impaired lung development at an early age. Various parameters such as inter-person variations in susceptibility, differences in level of decline in lung function and an impaired attainment of the normal spirometric plateau contribute to the onset of COPD (**Figure 2**).

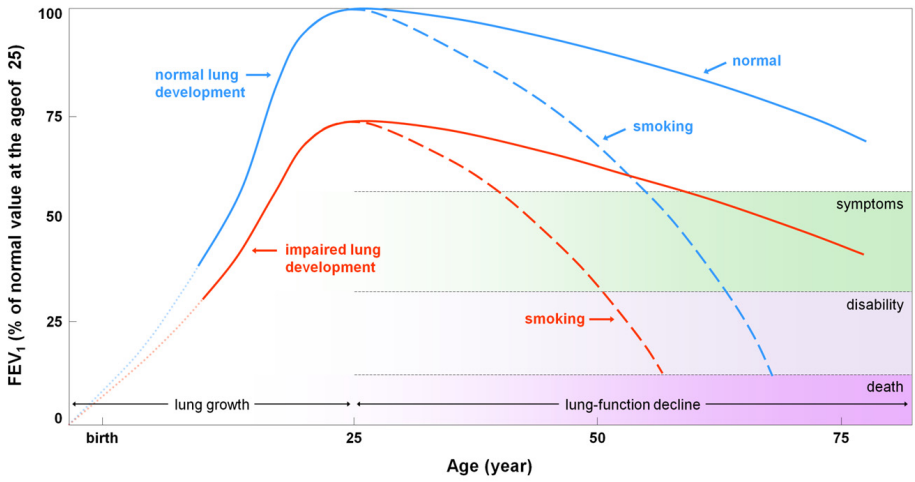


Figure 2: Adapted Fletcher and Peto Curve. The course of FEV_1 over time in smokers with or without airflow limitation. Both processes affecting lung development early in life as an accelerated lung function decline due to susceptibility to smoking put persons at risk for developing COPD.
Adapted from Brusselle GG., N Engl J Med 2009;361(27):2664-2665 [47].
FEV_1: forced expiratory volume in 1 second; COPD: chronic obstructive pulmonary disease

The degree of airflow limitation is assessed by spirometry, based on post-bronchodilator FEV_1 and according to the GOLD criteria [48]. However, there is only a weak correlation between FEV_1 and the experienced symptoms and health status impairment [49]. For this reason, COPD assessment also considers, besides the level of airflow limitation, the impact on the patient's health status, presence of comorbidities and estimates the risk of future events such as exacerbations, hospital admissions or mortality [9]. A new approach has been proposed which revises the original ABCD assessment tool of the GOLD update in 2011 [9]. This assessment tool separates the level of airflow limitation from the patient's perceived symptoms and exacerbation history and is illustrated in **Figure 3**. A patient is then classified with a GOLD grade number (1-4) and a letter (A-D) which will aid in (pharmaco) therapeutic decision making [1].

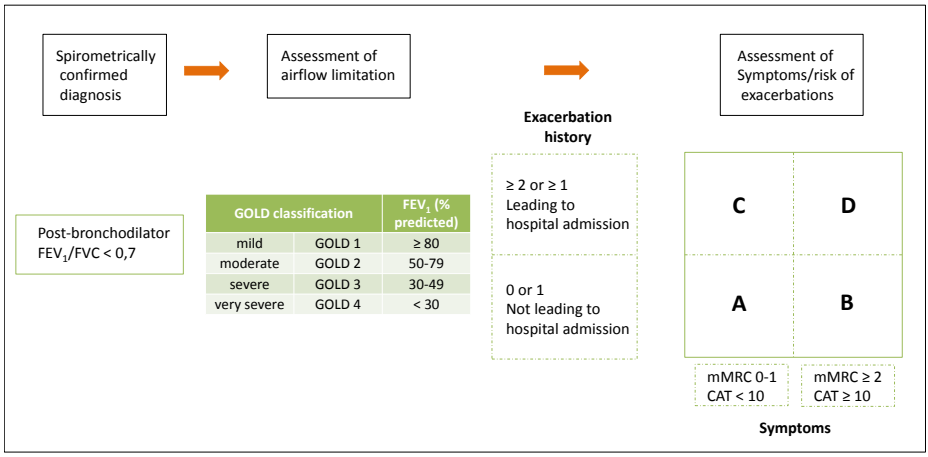


Figure 3 . The refined ABCD assessment tool. Figure adapted from [1]. This tool separates the level of airflow limitation (spirometry) from the patient's perceived symptoms (questionnaire: mMRC or CAT) and exacerbation history, resulting in a classification with a GOLD grade number (1-4) and a letter (A-D).
FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; GOLD: global initiative for obstructive lung diseases; CAT: COPD assessment test; COPD: chronic obstructive pulmonary disease; mMRC: modified British Medical Research Counsil

1.4 Pathology

The pattern of pathological changes depends on the underlying disease processes (chronic bronchitis, obstructive bronchiolitis, emphysema) complemented with individual susceptibility and disease severity [2].

Inhalation of harmful particles (e.g. CS) or gases causes an exaggerated chronic inflammatory response in patients with COPD. This chronic inflammatory state gives rise to the airflow limitation that is characteristic for COPD. The pathology is caused by a mixture of small airway disease (e.g. obstructive bronchiolitis) and parenchymal destruction (emphysema) (**Figure 4**). Their relative contribution varies from person to person and over time [1]. Intriguingly, in COPD patients, the inflammation persists following smoking cessation [50-52].

In the central airways (> 2mm in internal diameter), CS induces abnormalities in the airway epithelium including goblet cell metaplasia, enlarged mucous glands, airway epithelial cell hyperplasia, ciliary dysfunction and thickening of the bronchial walls [53]. Increased numbers of inflammatory cells infiltrate and populate the (sub)epithelial area [21]. All these features contribute to the pathological diagnosis of **chronic bronchitis** which is mainly recognized by symptoms as coughing and sputum production. Yet, not all patients with chronic bronchitis develop airflow limitation [54].

The small conducting airways (< 2mm in internal diameter) are considered the major site of obstruction in patients with COPD [55]. Persistently inhaled toxic particulates deposit in this region, initiating a chronic inflammatory immune cell infiltration besides repair and remodeling processes [56,57]. As a consequence, the airway wall is thickened (remodeling) by airway smooth muscle hypertrophy and fibrotic processes, narrowing the airway and thus increasing the resistance (**obstructive bronchiolitis**) [56]. Infiltration of neutrophils, macrophages, B and T lymphocytes is observed in these small airways, which further increases as COPD progresses [58]. Lymphocytes aggregated into lymphoid follicles are mainly present in the later stages of COPD [54,58].

In the alveoli of patients with **emphysema**, the lung parenchyma is progressively destroyed and airspaces enlarged. The centrilobular pattern of emphysematous destruction (predominantly in the upper zones) results from dilatation and destruction of the terminal and respiratory bronchioles and is most closely associated with cigarette smoking [54]. The panacinar (destruction of the whole acinus) pattern is more common in the lower lobes and is associated with α1 antitrypsin deficiency [54]. Of interest, it is observed by CT imaging that the widespread loss of terminal bronchioles preceeds the onset of emphysematous destruction [56,59].

Early changes in the **pulmonary vasculature** comprise thickening of the intima and endothelial dysfunction [60-62]. In a later phase, other pathological transformations are observed in patients with

COPD such as hypertrophy of vascular smooth muscles, deposition of collagen (remodeling), destruction of the pulmonary capillary bed and development of pulmonary hypertension [63].

The histological visible thickened airway wall (remodeling) and the loss of elastic recoil (emphysema) of the parenchyma contribute to the decreased FEV_1 and hyperinflation [2].

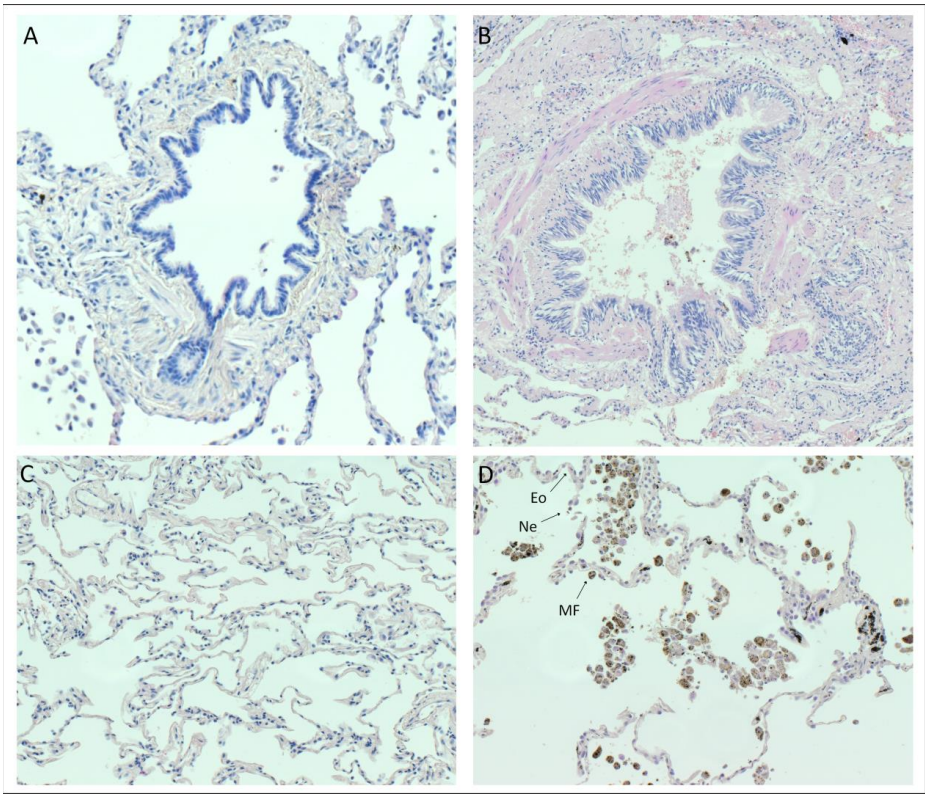


Figure 4. Lung histology on hematoxylin and eosin staining of (A, C) a never-smoker and (B,D) a patient with COPD.

(A,B) airways and (C,D) parenchyma. (B) airway remodeling and (D) emphysema with infiltration of inflammatory cells with macrophages (MF) constituting the predominant cell type. Less frequent are the neutrophils (Ne) and smaller cells are indicative for lymphocytes. Also 1 eosinophil (Eo) could be observed.

1.5 Complexity of COPD

COPD is a complex and heterogeneous disease since it encompasses more than only airflow limitation. COPD is complex since its pathology cannot be attributed to 1 single determinant or cause. Secondly, COPD is a heterogeneous disease meaning that different patients can have different (extra)pulmonary manifestations of the disease which can vary over time, still all patients are classified as 'COPD patients' [64]. Therefore, the use of multidimensional assessment indices such as the BODE (body mass index, FEV_1, dyspnea and exercise capacity) index [65], ADO (age, dyspnea, FEV_1) index [66] and DOSE (dyspnea, FEV_1, smoking status and frequency of exacerbations) index [67] are more accurate to capture the complexity of COPD. The BODE index gives a better prediction of survival than any single component it comprises [65].

To understand the pathogenesis of COPD, we will focus on 4 different, but interrelated levels of the complexity of COPD (**Figure 5**). The main goal of studying the pathogenesis of COPD is to understand the underlying disease process but also to identify novel molecular drivers of disease in an attempt to discover useful biomarkers for diagnosis and therapy.

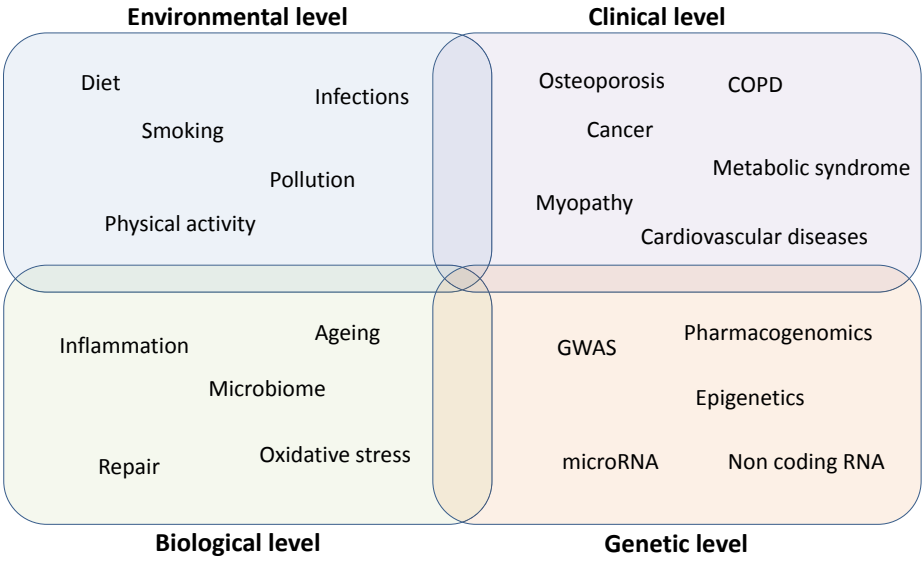


Figure 5. Diagram illustrating the different levels of complexity of COPD.
Adapted from Agusti A. and Vestbo J. [68].
GWAS: genome wide association study

1.5.1 Environmental level

A whole plethora of factors affect the initiation and/or course of COPD which are often related to life-style and thus, can be avoided or changed. Exposure of the lungs to **CS** and in- or outdoor pollution causes an exaggerated inflammatory response in the lungs of patients with COPD. Therefore, urgent measures are needed including anti-tobacco campaigns, prohibition of indoor use of biomass fuels and strict rules concerning lower vehicle and factory emissions in residential areas, ensuring the reduction of risk factors for the development of airway diseases.

Regarding the **activity** level, patients with COPD are often extremely inactive. The fitness of the patient, as measured by the 6-min walking distance (6MWD), is a good predictor of mortality [23]. Hence, it is advisable to stimulate patients with COPD to be more active [69]. Moreover, some degree of **malnutrition** is common in about a third of patients with COPD and may be severe in advanced COPD [70]. In a review summarizing 17 studies investigating the effect of nutritional supplementation, they found growing evidence that supplementation had a positive effect on body weight, muscle strength, performance of the 6MWD and quality of life, especially in malnourished patients [70]. Patients with COPD are also considered at risk for vitamin D deficiency due to faster skin ageing (from smoking), possible treatment with corticosteroids, less outdoor activity and less food intake [71]. Vitamin D deficiency has negative consequences throughout the body, including the immune system [72] and has been linked to many chronic illnesses. In COPD, the level of vitamin D is associated with disease severity (as measured by FEV_1) and consequently, vitamin D supplementation is warranted, especially in patients with severe COPD [71,73]. Further, high-fiber diets have been linked to reductions in lung function decline, COPD incidence and respiratory mortality by attenuating the innate immune-mediated systemic and pulmonary inflammation [74]. Viral and bacterial **infections** are the predominant cause of exacerbations and are estimated to actively contribute to the pathogenesis of COPD [75,76].

1.5.2 Biological level

A good functioning innate defense machinery encompasses adequate mucociliary clearance, an intact epithelial barrier, humoral factors and immune cells initiating an appropriate immune response, followed by removal and resolution of the inflammation. Inflammation is present in the lungs, especially the small airways, of all people who smoke. However, in patients with COPD, the response to chronic CS exposure leads to an amplified inflammatory response, impairment of defense mechanisms, tissue destruction and disruption of repair. In general, these inflammatory and structural airway changes aggravate with disease severity and persist even after smoking cessation [50,51,77].

Aside from nicotine, lipopolysaccharide (LPS), heavy metals and carcinogens, CS contains a whole mixture of oxidants [78]. Exposure to CS constituents causes an immediate pulmonary inflammatory reaction within minutes to hours [79] by activating several pattern recognition receptors (PRRs). PRRs

constitutively expressed on airway epithelial cells, alveolar macrophages and dendritic cells can be activated directly by substances present in CS and infectious agents via pathogen-associated molecular patterns (PAMPs) or indirectly by the damage that is induced to the airway epithelium by CS, giving rise to the release of damage-associated molecular patterns (DAMPs) [80-82]. Generally, this leads to a non-specific innate immune response with the release of pro-inflammatory cytokines and chemokines, reactive oxygen species and proteolytic enzymes [82]. The CS-induced release of pro-inflammatory cytokines and chemokines initiates the recruitment of neutrophils, macrophages and dendritic cells. As an example, interleukin-8 (IL-8), whose levels are increased in bronchial epithelium, sputum and plasma of patients with COPD compared to controls, plays a pivotal role in the activation and chemotaxis of neutrophils [83-85]. In addition, Chemokine (C-C motif) ligand 20 (CCL20), produced by the inflamed bronchial epithelium and increased in airways of patients with COPD, functions as one of the most potent recruiters of immature DCs via interaction with C-C Motif Chemokine Receptor 6 (CCR6) [86,87].

Increased numbers of neutrophils and macrophages cause lung destruction by releasing oxygen radicals and proteolytic enzymes such as neutrophil elastase and matrix metalloproteinase (MMP)-12. If these proteolytic enzymes and oxidative stress are not sufficiently counterbalanced with anti-proteases and anti-oxidants, the net result is further damage [2]. Also, inflammatory cells may change their phenotype in a later phase of disease. For instance, macrophages may switch towards an M2 phenotype, potentially limiting the inflammation and propagating fibrosis with less antibacterial capacity [88]. Recently, interest is raised in innate lymphoid cells (ILC) for their role in the pathogenesis of lung diseases. In lungs of COPD patients compared to controls, a tendency towards more natural cytotoxicity receptor-negative ILC3s was reported [89].

Immature dendritic cells (DCs), specialized antigen-presenting cells that link the innate with the adaptive immune response, are recruited towards the airway epithelium, take up the antigen, migrate to the draining lymph nodes and present the antigen to naïve T lymphocytes via expression of the major-histocompatibility-complex (MHC) proteins [87,90]. MHC class I-restricted DCs present antigens to $CD8^+$ T lymphocytes, whereas MHC class II-restricted DCs drive the differentiation of naïve $CD4^+$ T helper (Th) lymphocytes towards Th 1, 2, 17 or regulatory T (Treg) lymphocytes [82].

In stable COPD, mostly Th1 and Th17 lymphocytes are accumulating [91]. When activated, antigen-specific CD8+ T lymphocytes secrete proteolytic enzymes such as perforin and granzymes [92]. Th1 cells migrate to the site of injury and govern the adaptive immune response primarily by interferon-γ release with subsequent activation of other immune cells such as priming macrophages for efficient killing [93]. However, this mechanism is hampered in COPD [93]. Pro-inflammatory Th17 cells produce IL-17A and IL-17F which mediate defense against extracellular pathogens, aid in lymphoid follicle formation and promote neutrophil and macrophage accumulation at the site of injury [94-96]. Th17 cells

are also involved in the development of autoimmunity and elevated expression of IL-17A is demonstrated in (end-stage) COPD [96-99]. As a counterbalance, Treg cells dampen the inflammatory reaction by interacting with DCs and T lymphocytes or by producing the anti-inflammatory IL-10 [100]. Contradictorily, the number of Treg cells is increased or decreased in COPD, depending on the anatomical location or methodology used. In addition, B cell numbers are increased in patients with COPD [58]. Different B cell subsets are identified such as plasma cells (antigen-specific production of antibodies) and memory B cells [101]. As the disease progresses towards its severe state, an aggregation of B, T cells and follicular DCs into lymphoid follicles is frequently observed near the airways and in the parenchyma [58,101] (**Figure 6**).

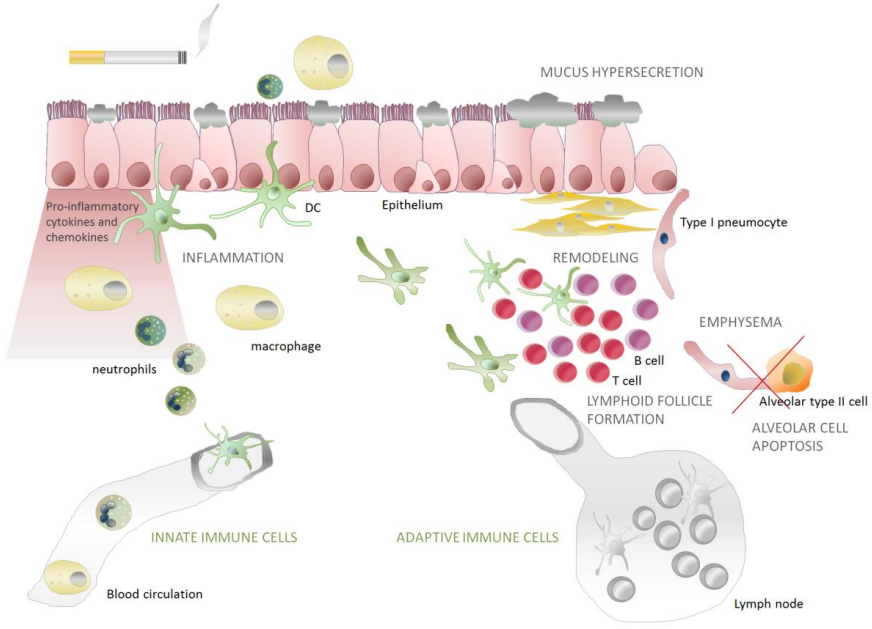


Figure 6. Innate and adaptive immune reaction following cigarette smoke exposure in the pathogenesis of COPD. Cigarette smoke causes damage to the airway epithelium and leads to recruitment of innate immune cells such as macrophages and neutrophils towards the epithelium. Following activation, DCs migrate to the lymph nodes and initiate adaptive B and T cell immune responses. Chronic inflammation gives rise to destruction of alveolar walls, enhanced mucus secretion, remodeling processes and formation of lymphoid follicles.
DC: dendritic cell

Apart from this immunologic reaction, several other endogenous processes may underlie the pathogenesis of COPD (**Figure 7**). The pathogenesis of COPD is centered around an excess of **oxidative stress**, from both endogenous or exogenous origin, contributing to many of the pathogenic events such as the imbalance between proteases and anti-proteases, propagation of the inflammation leading

to tissue destruction, fibrosis and remodeling [102,103]. Nuclear factor erythroid 2–related factor 2 (Nrf2) is an antioxidant transcription factor that is implicated in both the initiation and in the progression of the CS-induced injury. Nrf2 controls more than 100 genes involved in antioxidant defenses, detoxification and cellular physiology [104], is a known key player in the susceptibility to emphysema and is decreased in lungs of patients with COPD, probably through reduced histone deacetylase 2 (HDAC2) activity resulting in increased acetylation of Nrf2 [105-107]. Mice lacking Nrf2 show increased susceptibility to lung inflammation, alveolar cell apoptosis and emphysema following CS exposure [106,108].

Some evidence suggests shared features between pulmonary emphysema and lung **ageing** [109,110]. Cell senescence, a non-proliferative state in which cells are metabolically active but apoptosis-resistant, can be caused by cigarette smoke and oxidative stress [111]. Intriguingly, several animal models of ageing have concomitant emphysema [112,113]. To maintain lung integrity, alveolar cell apoptosis and matrix destruction is compensated by cell renewal and **repair** mechanisms. The inflammation induced by chronic CS exposure is amplified in COPD by limited alveolar repair and enhanced apoptosis [80]. Skeletal muscle wasting and decreased physical activity, major comorbidities of COPD, have been linked to both apoptosis and less vascular regeneration [114,115]. In addition, phagocytosis of apoptotic cells and bacteria by alveolar macrophages is impaired in COPD which can contribute to chronic bacterial colonization and to acute infectious exacerbations [116-118]. Dysregulated repair mechanisms involve abnormal transforming growth factor (TGF)-β signaling, leading to fibrosis and remodeling. Destruction of the extracellular matrix accelerates the development of emphysema.

Due to less oxygen perfusion, some regions in the lungs become **hypoxic**. In hypoxic conditions, HIF-1α is transcribed which activates transcription of other pro-inflammatory genes and which prolongs the life-span of neutrophils, enhancing the breakdown of extracellular matrix [119]. When mice were instilled with LPS and elastase, pulmonary microbiota were found to promote pulmonary inflammation through stimulation of IL-17A production, providing evidence for a host-**microbiome** cross-talk [120]. In patients with COPD GOLD IV, the diversity of the lung microbiome declined versus controls and was associated with emphysematous destruction, remodeling and infiltration by CD4+ T cells [121]. Furthermore, dynamic changes have been observed in lung microbiota following exacerbations or pharmacological treatment [122]. Sputum IL-8 levels, alongside other serum and sputum biomarkers, correlated with the structure and diversity of the lung microbiome.

In those patients who develop COPD, the inflammatory response fails to resolve after quitting smoking. Several mechanisms that have been mentioned earlier might contribute to the perpetuation of the inflammation including impaired clearance, chronic colonization and infection of the lower airways, oxidative stress, autoimmunity, impaired and excessive innate immune responses, tissue hypoxia, airway wall remodeling, lung ageing, genetic susceptibility and epigenetic changes [82,123].

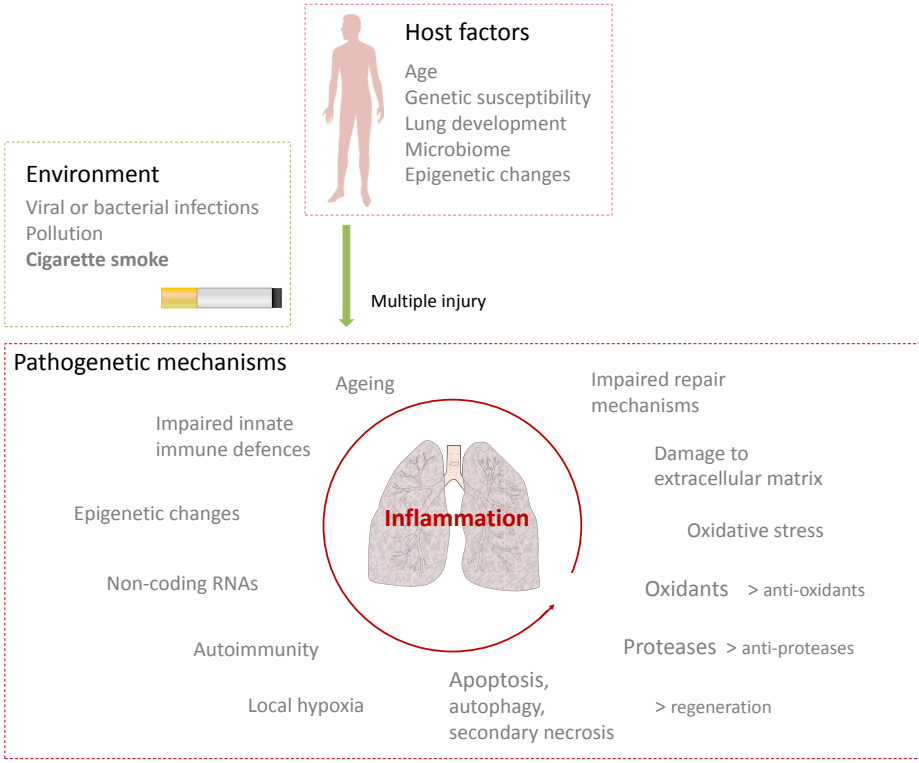


Figure 7. Mechanisms underlying the pathogenesis of COPD. Host factors form the basis for susceptibility to environmental triggers such as cigarette smoke. The relentless lung injury due to oxidative stress, alongside the impairment of protective and repair responses from the lung, ultimately lead to prolonged inflammation and the development of COPD.

1.5.3 Genetic level

An altered (epi)genome could be responsible for different disease susceptibility and can hence be of interest for evaluating persons at risk. Twin studies provided help in estimating the **genetic** component, as opposed to the impact of the environment, in the risk of developing COPD [124]. Gene polymorphisms are allelic variations or point mutations in the DNA, including single-nucleotide polymorphisms (SNPs). Over the past years, several gene polymorphisms have been reported to be linked with COPD susceptibility such as microsomal epoxide hydrolase, heme oxygenase-1, a disintegrin and metalloproteinase 33 (ADAM33) and tumor necrosis factor α (TNFα), although consistent data are still lacking, especially when studying a different ethnic origin [27,29,124,125]. The first established polymorphism related to COPD susceptibility was the Z allele of the alpha1-antitrypsin gene, encoded by the highly polymorphic SERPINA1, resulting in different levels of α1-antitrypsin. Carrying two copies of the susceptibility allele was suggested as a genetic risk factor, severely predisposing smoking subjects of developing early-onset emphysema [126].

The Rotterdam study, an ongoing prospective cohort study enrolling persons aged 45 years or more, is a member of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium [127], which was initiated to facilitate genome-wide association studies (GWAS), meta-analyses and replication studies. GWAS are based on genotyping epidemiological cohorts with high density SNP arrays. Thus far, GWAS have identified thousands of genetic loci that contribute to the susceptibility for a variety of diseases [128-130]. For COPD, GWAS and genome-wide joint meta-analyses have identified genetic loci associated with lung function and emphysema distribution [131-139]. Meta-analyses of GWAS for airway obstruction, in which the Rotterdam study collaborated, confirmed an association with the nicotinic acetylcholine receptor, subunits alpha 5 and alpha 3 (CHRNA5/3), located in the intriguing region on chromosome 15q25.1, and with 5-hydroxytryptamine receptor 4 (HTR4) [138,140]. However, our lab could not demonstrate the involvement of 5-HT4 receptor, HTR4, in bronchial hyper responsiveness to serotonin in CS-exposed mice [141]. A genetic variant in the CHRNA3/5 region, also containing the IREB2 gene which may confer COPD susceptibility, has been associated with smoking intensity and smoking addiction, although the association with smoking behavior remains controversial [142]. By integrating the largest published GWAS on FEV_1 and FEV_1/FVC with a lung tissue expression quantitative trait loci (eQTL) study, tissue-specific genes were identified that are involved in developmental (e.g. HHIP) and inflammatory pathways, thereby pinpointing genes that were more likely to be responsible for the GWAS signal [143].

Despite the elucidating effect of GWAS where genetic determinants of human complex diseases were uncovered, a substantial proportion remains unexplained. It is therefore of interest to explore how non-genetic variations, including **epigenetic** factors, can influence disease etiology [144]. The epigenome is dynamic and changes in response to the environment, diet, disease and ageing [145]. The three main classes of epigenetic marks – defined as mechanisms other than changes in the underlying DNA sequence that cause changes in gene expression – are DNA methylation, modification of histone tails and non-coding RNAs. Several studies demonstrate a clear association between exposure to CS and changes in epigenetic marks, although proving causality remains uncertain. In children, a global reduction in DNA methylation was linked to *in utero* CS exposure [146]. In human airway epithelial cells, CS condensate (CSC) time- and dose-dependently induced changes in histone modifications [147]. In lung tissue of patients with increasing severity of COPD, graded reduction in HDAC2 expression was reported combined with an increase in IL-8 mRNA and histone-4 acetylation at the Nuclear Factor κB (NFκB) binding site of the IL-8 promoter, shifting the balance towards histone acetylation [33,148]. An imbalance between histone deacetylation and acetylation in favor of acetylation may contribute to the persistent inflammation present in smokers susceptible to developing COPD [148]. In human bronchial epithelium and in lungs of mice and rats exposed to CS, a predominant down-regulation of the majority of microRNAs (miRNAs) was observed following CS exposure [149-151]. By combining expression data from

messenger RNA (mRNA) and miRNA in lung tissue of smokers with and without airflow limitation, TGF-β, Wnt and focal adhesion pathways were identified as potential pathways in which miRNAs may be relevant to the pathogenesis of COPD [152]. Moreover, in lungs or sputum of patients with COPD, miRNA expression was significantly altered [152-155]. We will further discuss the role of miRNAs in **chapter 2**. Also, a long non-coding RNA (lncRNA) called SCAL1 was up-regulated upon CS extract exposure *in vitro* [156]. In addition, several long non-coding RNAs were differentially expressed in lung tissue of smokers with COPD versus never-smokers and smokers without airflow limitation [157].

Dynamic changes in the **transcriptome** can reveal biological pathways that are associated with disease activity or with smoking. A clear smoking signature has been reported in non-tumorous lung tissue [158] and in lung cancer tissue [159-161]. In non-tumorous lung, the gene expression signature consistently segregated never- from current-smokers. The majority of altered genes reverted following smoking cessation, whereas a minority of genes did not return to baseline levels such as SERPIND1. These slowly reversible genes may be of importance in understanding the initial and persistent processes leading to lung diseases [158]. Transcriptome analysis in lungs of smokers or COPD patients revealed major altered processes between COPD and control smokers such as signal transduction, receptor function, growth factor, adhesion and cytoskeleton and metabolism [162] while another study provides evidence for genes involved in tissue remodeling and repair [163]. Interestingly, active smoking substantially altered the pulmonary gene expression in COPD in comparison with smokers and never-smokers [148]. COPD pathology can be distinguished between the bronchiolitis and the emphysema phenotype, although both often co-exist in the same patient. Transcriptome analysis comparing the differential gene expression between ex-smoking patients with bronchiolitis and emphysema revealed an enrichment in B-cell related genes in emphysema [164].

The airway epithelium is important in the first encounter with pathogens and inhaled particles. It is therefore not surprising that smoking-induced gene expression changes are reflected in the 'normal' airway epithelium as in the airway epithelium of patients with COPD [149,165-171]. Also here, reversible and permanent gene expression alterations have been noted [172]. Intriguingly, the smoking-induced alterations in airway gene expression are already reflected in the epithelium from nose and mouth, including genes related to detoxification, oxidative stress, and wound healing [173,174]. Further, treatment with inhaled corticosteroids (ICS) dynamically affected airway gene expression [175]. Similarly, other transcriptome studies demonstrate significant gene modulation by smoking in other cell types such as alveolar macrophages [176] and lymphocytes [177].

Yet, a complex disease is never the consequence of 1 single gene but merely a reflection of various perturbations in inter- and intracellular networks, all contributing to the observed phenotype [178,179]. (Epi)genetic research aims to identify the responsible gene/molecular pathway for the development of COPD, however the results are not straightforward. [162,165,180]. There is limited overlap between

studies which can be explained by differences in patient selection criteria, patient characteristics, sample acquisition, detection platform used, heterogeneity in lung tissue pathology and data analysis. Although proving causality and understanding the underlying regulatory mechanism remains a challenge, these insights from GWAS and transcriptome analyses have broad and promising implications for screening and treatment.

1.5.4 Clinical level

It is increasingly recognized that COPD is not only limited to the pulmonary compartment. Other diseases often coexist with COPD (i.e. **comorbidities**) independent of the severity of COPD [2]. Systemic inflammation is the mainstay of most manifestations. Increased concentrations of cytokines (TNF-α, IL-6), acute phase proteins, chemokines and adipokines in the circulation may lead to **cachexia and skeletal muscle weakness** [23]. As COPD progresses, exercise capacity decreases due to ventilatory limitation. **Impaired exercise capacity** negatively impacts bone density besides other risk factors responsible for the high prevalence of osteoporosis in COPD such as aging, smoking, malnutrition, low body-mass index (BMI) and vitamin D deficiency [181]. In addition, inflammatory mediators in the circulation stimulate osteoclasts, reinforcing the development of osteoporosis in COPD patients [23].

Besides sharing risk factors such as smoking and ageing, COPD and **cardiovascular disease** are each associated with an increased systemic inflammation, oxidative stress and sedentarism. Damage to the endothelium, vascular remodeling, elevated plasma fibrinogen levels and an increase in pro-coagulant activity are central to the pathogenesis of the associated cardiovascular events. Patients with COPD are particularly susceptible to vascular events after an exacerbation, when systemic inflammation is more present [182].

COPD and **lung cancer** are closely related diseases, occurring as co-morbidities at a higher rate than if they were independently triggered by smoking. Chronic exposure to pro-inflammatory cytokines and increased oxidative stress may accelerate the growth of lung cancer [183].

The causes of **anemia** in patients with COPD are probably multifactorial and include nutritional deficits, carboxyhemoglobin effects of cigarette smoking but most importantly, the chronic inflammatory nature of COPD [184]. In patients with COPD, there is an increased prevalence of **diabetes and metabolic syndrome**, possibly mediated by the augmented plasma concentrations of inflammatory markers [23].

For sure, these (often multiple) comorbidities have a major impact on the patient's quality of life and survival. Thus, treatment of these comorbidities is a key element in the management of COPD and may have a beneficial effect in COPD [9,23].

In patients with COPD, different underlying mechanisms are responsible for the diversity in pulmonary pathology. Therefore, a subdivision of COPD patients in **COPD phenotypes** (e.g. chronic bronchitis,

never-smoker with COPD, α1-antitrypsin deficiency, emphysema, frequent exacerbator,..) is justified [185-188] and a personalized or precision medicine is warranted [68].

1.6 Treatment options in COPD

In developed countries where cigarette smoking generally comprises the main risk factor for developing COPD, the key therapeutic intervention is smoking cessation. For this, pharmacological support (bupropion, varenicline and nortryptyline) or nicotine replacement therapy in combination with professional counseling can provide help. Recently, the advent of e-cigarettes has opened new perspectives, however, the efficacy and safety of e-cigarettes is uncertain and will be investigated further [189].

The current **pharmacological therapy** of COPD is largely symptom-driven and takes the risk for future exacerbations into account. For the past couple of decades, inhaled medication is the backbone of COPD management. The GOLD guidelines recommend the use of long-acting bronchodilators (Long-acting β2-agonists (LABAs) and/or Long-acting muscarinic antagonists (LAMAs)) as first-line maintenance therapy (**Figure 8**). In mild COPD, monotherapy with a bronchodilator is first choice. When symptom relief appears not to be sufficient, combination therapy with both LAMA and LABA provides additional benefit (additive effect) with lower risk of side-effects and a greater stabilization of airway tone [64]. Both LAMA and LABA facilitate bronchodilation by inducing smooth muscle relaxation through binding to different receptors. They significantly improve lung function, dyspnea, health status and reduce exacerbation rates. Short acting bronchodilators can be inhaled for quick symptom relief [1].

For patients with moderate to severe COPD and recurrent exacerbations, first choice remains combination therapy with both LAMA and LABA. If not sufficient, second choice is the combination of a bronchodilator with ICS. However, ICS use can cause side-effects such as oral candidiasis, skin bruising, higher risk for pneumonia and reduction of bone density [64,190-192]. Evidence exists for a superiority of LABA/LAMA (e.g. indacaterol/glycopyrrolate) over LABA/ICS, which was studied across different severities of exacerbations [193]. Yet, LABA/ICS therapy may be first choice in patients with a history suggestive of asthma-COPD overlap and/or high blood eosinophils [194-196].

Mucolytic and antioxidant agents can improve health status and may reduce exacerbations [197].

Recently, anti-inflammatory agents were added to the therapeutic scheme of the pulmonologists. Patients with severe to very severe COPD, chronic bronchitis and a history of exacerbations, the phosphodiesterase-4 (PDE4) inhibitor roflumilast can be given orally since this molecule reduces the exacerbation rate, probably through inhibition of cellular trafficking and cytokine and chemokine release from immune cells [198-201]. PDE4 is the predominant phosphodiesterase expressed in neutrophils, T cells and macrophages. Nonetheless, orally administered PDE4 inhibitors have numerous side-effects. PDE3 inhibitors relax airway smooth muscles. The development of inhaled dual

PDE3/PDE4 inhibitors that combine bronchodilation and anti-inflammatory activity shows great potential in treating patients with COPD [202,203].

To reduce the exacerbation rate, especially in older patients and ex-smokers, long-term use of macrolide antibiotics such as azithromycin and erythromycin is recommended. Macrolides have immunomodulatory and antibacterial effects. The beneficial effects are possibly due to an improvement in phagocytosis by alveolar macrophages, an inhibition of IL-17 production by T lymphocytes and an inhibition of the release of chemokine (C-X-C motif) ligand (CXCL)-8 and Granulocyte-macrophage colony-stimulating factor (GM-CSF) by epithelial cells [204]. However, treatment with azithromycin is associated with an increase in the incidence of bacterial resistance and hearing problems.

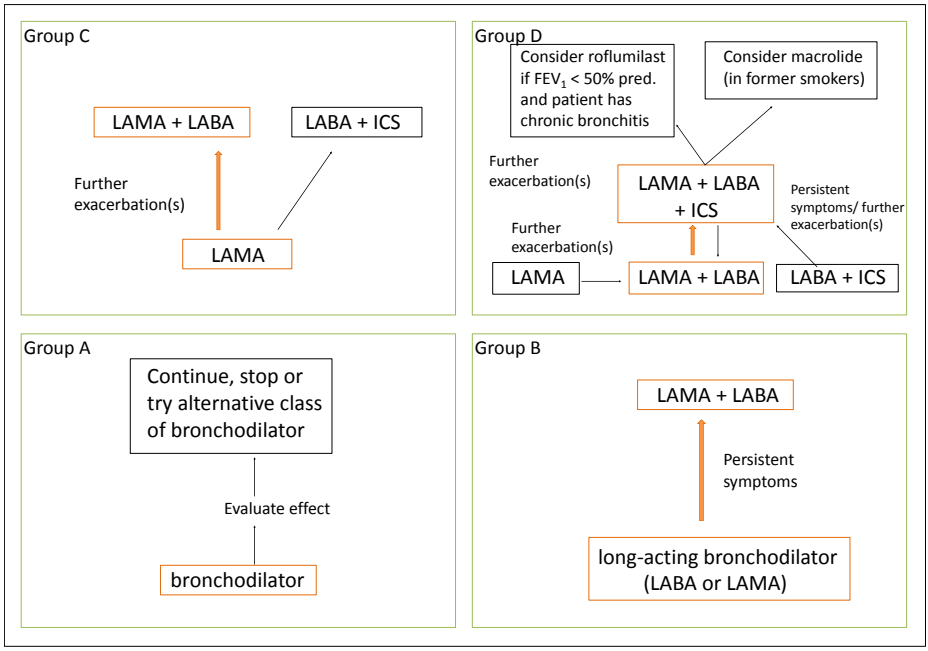


Figure 8. Pharmacological treatment algorithms by GOLD Grade. Boxes and arrows in orange indicate preferred treatment strategy. Figure adapted from [1].
LAMA: Long-acting muscarinic antagonist; LABA: Long-acting β2-agonist; ICS: inhaled corticosteroids; FEV_1: forced expiratory volume in 1 second

Since COPD is often associated with co-morbidities with systemic inflammation as the underlying driver, the use of the anti-inflammatory anti-TNFα antibody (infliximab) in the setting of COPD was thought to be promising. Unfortunately, clinical trials with infliximab showed no benefit and even raised major safety concerns [205]. Further, statins (3-hydroxy-3-methylglutaryl coenzyme A reductase

inhibitors), a class of drugs used to treat hypercholesterolemia and to prevent cardiovascular event, may reduce morbidity and mortality in COPD patients but does not affect the risk of exacerbations [206]. More specifically, long-term statin use is associated with a beneficial effect on all-cause mortality in COPD patients with a high level of systemic inflammation, as measured by high-sensitivity C-reactive protein levels above 3mg/L [207]. In COPD patients with coexistent heart failure or post-myocardial infarction, cardioselective β-blockers may be indicated [208].

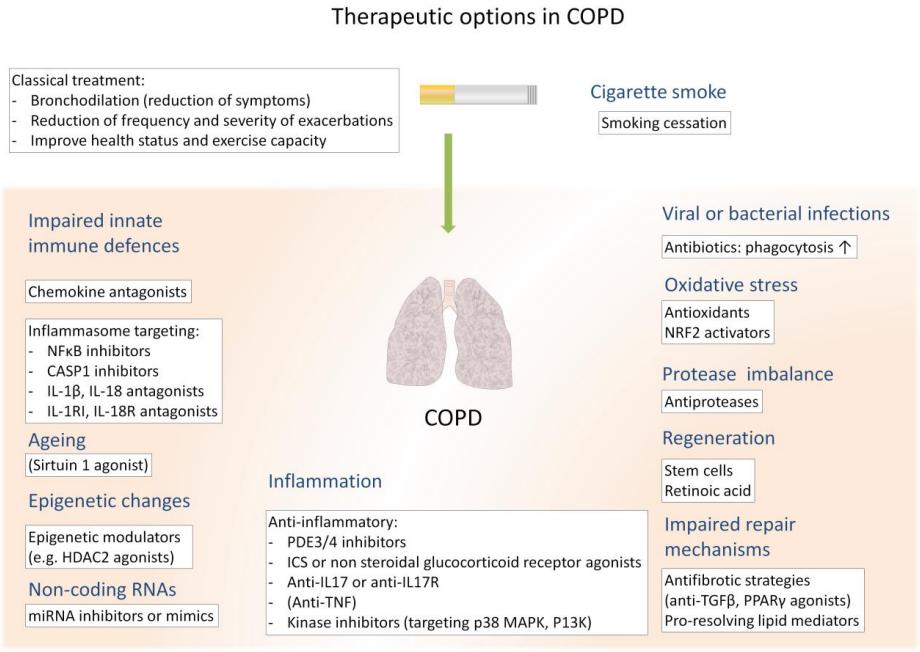


Figure 9. Treatment options in COPD
NFκB: Nuclear factor κB; CASP1: Caspase-1; IL-1β: interleukin-1β; IL-1RI: interleukin-1 receptor type I; HDAC2: Histone deacetylase 2; PDE3/4: phosphodiesterase-3/4; ICS: inhaled corticosteroids; TNF: tumor necrosis factor; MAPK: Mitogen-activated protein kinase; PI3K: Phosphoinositide 3-kinase; NRF2: Nuclear factor erythroid 2–related factor 2; TGF-β: Transforming growth factor β; PPARγ: peroxisome proliferator-activated receptor-γ

In the future, development of medication targeting crucial drivers of disease should be promoted. Since COPD is a heterogeneous disease with differing underlying immunopathological processes, a targeted or precision medicine is warranted (**Figure 9**). Several barriers to the development of an effective anti-inflammatory treatment of COPD need to be overcome [8]. First, it remains uncertain which are the main underlying inflammatory drivers of disease. Second, ICS can't be used as a golden standard to compare with new treatments, as is the case in asthma. Third, clinical trials to assess the efficacy and safety of medication in patients with COPD are very time-consuming. Fourth, the treatment of COPD requires a targeted precision medicine that integrates the biological endotype and

the clinical phenotype in order to maximize the benefit-versus-risk ratio . Fifth, suitable biomarkers to follow-up the therapeutic response are still lacking.

Numerous small molecules (most chemical drugs with low molecular weight) or biologics (larger and more heterogeneous such as antibodies) are under investigation for COPD with a disease-modifying purpose. Since levels of IL-1β are increased in the lung and sputum of patients with COPD, targeting the inflammasome (necessary for the activation of IL-1β and IL-18) seems interesting [209-211]. Therefore, blocking of IL-1β, IL-18 and its receptors IL-1RI, IL-18R and caspase-1 is being evaluated, as is the inhibition of NFκB, a crucial protein complex central in propagating inflammation and the immune response upon stimuli [8,212]. Further, restoring α1-antitrypsin activity can be introduced in the current therapy [213,214] and several monoclonal antibodies are under investigation: anti-IL-17 or anti-IL-17R, anti-IL-5 (reslizumab, mepolizumab) or anti-IL-5R (benralizumab), anti-IL-13 (lebrikizumab), anti-B cell therapies such as anti-CD20 (rituximab), anti-CD22 and anti-BAFF [212,215]. Unfortunately, targeting leukotriene B4, IL-1β (canakinumab), IL-1RI (MEDI8968) and neutrophil elastase failed to show efficacy in patients with COPD [8,215,216]. A monoclonal antibody against IL-8 (ABX-IL8) was only slightly effective at reducing the severity of dyspnea. Another neutrophil targeting approach by antagonizing the CXCR2 receptor showed significant improvement on FEV_1 and reduction in sputum neutrophil count in a phase II study. However, a too drastic reduction in absolute neutrophil count led to discontinuation of this study by some participants. Whilst effective in patients with rheumatoid arthritis, the antibody against IL-6R (tocilizumab) has not been tested in COPD [216]. Although a very promising molecule, Sulforaphane, an NRF2 activator, did not stimulate the expression of NRF2 target genes nor had an effect on inflammatory markers in patients with COPD [217]. Kinase inhibitors such as inhibitors of Mitogen-activated protein kinase (MAPK) and Phosphoinositide 3-kinase (PI3K) are being evaluated in clinical trials [212]. Thus far, an epidermal growth factor kinase inhibitor was poorly tolerated and did not exhibit efficacy in decreasing epithelial mucin stores [218]. Although there is no evidence that fibrosis can be reversed in COPD, anti-fibrotic strategies include anti-TGF-β1, endothelin receptor antagonists and peroxisome proliferator-activated receptor-γ (PPARγ) activators. Other interesting challenges are the evaluation of non-antibiotic macrolides, the identification of nonsteroidal glucocorticoid receptor agonists, the reversal of corticoid resistance, the use of stem cells and the combat against accelerated ageing [8,212,219].

Special attention is also being focused on enhancing patient compliance by combining 3 substances (e.g. LAMA, LABA and ICS) in 1 inhaler device (i.e. triple therapy) and by favoring inhaled (local effect) and once-daily medication [220]. In addition, alternative formulations to minimize the interaction between molecules and newly developed delivery technologies are being tested [212]. Bi-functional (or dual pharmacophore) muscarinic β2-agonist (MABA) agents have been developed. This is a novel

approach to “dual” bronchodilator therapy by combining muscarinic antagonism and β2-agonism in a single molecule [221].

Non-pharmacological treatment for all COPD patients includes smoking cessation, but also reduction of all personal exposures to harmful particles or gases, influenza vaccination, stimulation of a healthy life-style encompassing a healthy diet and physical activity training. Pneumococcal vaccination is only recommended in patients > 65 years of age or younger persons with significant comorbid conditions [222]. Patients presenting with symptoms and risk for exacerbations (group B,C and D) should follow a pulmonary rehabilitation program [223]. In patients with stable very severe COPD, oxygen therapy can be given and interventional therapy such as bronchoscopy, lung volume reduction surgery or lung transplantation can be considered [1].

CHAPTER 2: microRNAs

2.1 Introduction

On top of the information obtained from the human genome sequence and associated diseases, there is another interesting layer of information contained in tissue-specific epigenomic marks. It is well understood that epigenetic modifications, such as DNA methylation and posttranslational modifications of the various histone tails, are essential for normal development [224,225]. Characterization of how these epigenetic processes contribute to human biology and disease is an interesting field. Interestingly, the earlier called 'junk DNA' constitutes practically 98 percent of the 3 billion base pairs in the human genome, yet they do not code for proteins. Scientists used to believe that DNA sequences not coding for proteins were simply a waste of space, but these sequences may actually play a significant role in disease progression, as 50 to 75% of them are transcribed into RNA similar to their protein-coding neighbors, named non-coding RNAs (ncRNAs) [226,227]. ncRNAs represent an ever-growing and ubiquitous class of regulatory RNAs that are principally involved in control of many cellular processes such as development and cell cycle regulation. In fact, the number of ncRNAs in a species reflects the organism complexity in contrast to the number of coding genes which remains relatively unaltered across different species (**Figure 10**) [228].

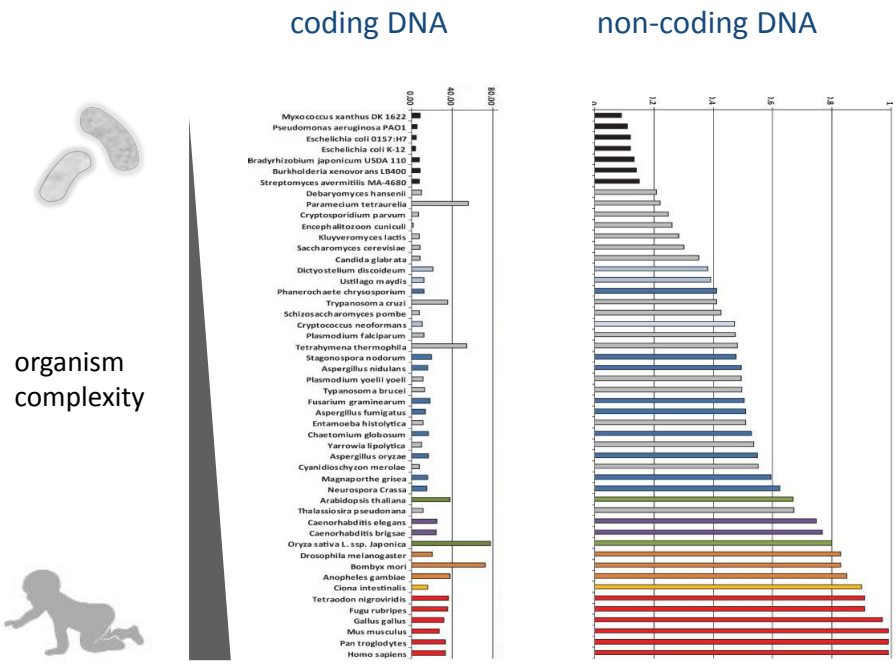


Figure 10. The non-coding portion of the genome reflects organism complexity (adapted from[228])**.**

Mechanisms of their function are not yet comprehensively understood. However, it is now clear that they also play considerable roles in pathology [155,228,229].

miRNAs constitute a large class of highly conserved small non-coding RNAs that have emerged as key post-transcriptional regulators of gene expression. miRNAs control a wide spectrum of biological functions, primarily to maintain and to protect the organ tissues. However, when dysregulated, their impact in diseases should not be underestimated, since one single miRNA can interfere with multiple targets within several biological pathways [230,231].

miRNA nomenclature is built on a few principles: miRNAs are named using the 'miR' prefix and a sequentially assigned number. Identical miRNAs across different species are numbered equally and a reference to the species is built in the miRNA name (e.g. hsa for human, mmu for mouse). The mature miRNA sequences are designated 'miR', whereas the precursor hairpin sequences are labeled 'mir'. The suffixes -3 and -5p refer to the arm from which the mature miRNA originates. Highly related miRNA sequences, only differing in 1 or 2 nucleotides, are given lettered suffixes, such as miR-135a and miR-135b, both members of the miR-135 family. Distinct hairpin loci that give rise to the same mature miRNA have a numbered suffix, such as mir-218-1 and mir-218-2.

2.2 miRNA biogenesis

MicroRNAs are small non-coding RNAs of 19-23 nucleotides long. The genes from where they are transcribed are mainly located in introns of non-coding or coding genes but some miRNAs are encoded by exonic regions as well [232-234]. Some miRNA genes are organized in clusters in the genome and are hence often co-transcribed, linking their genomic localization with a similar functional role [235,236].

The biogenesis of miRNAs is a multi-step and tightly regulated process that starts in the nucleus. Most miRNAs follow the canonical biogenesis pathway while only a minority of miRNAs are generated through alternative processing.

In the canonical miRNA biogenesis (**Figure 11**), the miRNA genes are transcribed by RNA polymerase II into a primary miRNA transcript (pri-miRNA). Subsequently, this transcript is cleaved into a 60-70 nucleotide long hairpin structure by the combined action of the Microprocessor constituting the enzymes Drosha and its cofactor DiGeorge Syndrome Critical Region 8 (DGCR8) [237]. Drosha is the subunit that cuts the strand through recognition of the key features of the pri-miRNA, more specifically the basal junction. DGCR8 recognizes the apical motif and enhances the accuracy and efficiency of the processing [233]. The resulting precursor miRNA (pre-miRNA) is then transported to the cytoplasm by means of exportin-5, associated with Ran GTP, a GTPase that moves RNA through the nuclear pore [234].

In the cytoplasm, the pre-miRNA is further processed by a complex containing Dicer and transactivation-responsive RNA-binding protein (TRBP) into an asymmetrical miRNA duplex (miR-3p/miR-5p), corresponding to the two sides of the stem. This duplex associates with an Argonaute (AGO) protein within the precursor RNA-induced silencing complex (pre-RISC). Release of the passenger strand after its cleavage converts pre-RISC to RISC: only one single stranded guide RNA will be retained in the mature RISC [235]. The choice of the strand is not strict and depends usually on the thermodynamic stability with a preference for the least thermodynamic stable 5' end, but strand fate can also be determined by the nucleotide sequence, tissue type or environmental conditions resulting in incorporation of the other strand as well [238-240].

Loaded in the RISC, the now mature miRNA guides the RISC to its target mRNA resulting in inhibition of protein translation and/or mRNA degradation [241]. miRNA-silenced mRNA is then directed to processing bodies (P-bodies), i.e. discrete and highly dynamic cytoplasmic foci that are enriched in proteins involved in mRNA catabolism and translational repression [242,243]. Remarkably, under certain conditions or in specific cells, the mRNA is released from these P-bodies and recruited to polysomes [243].

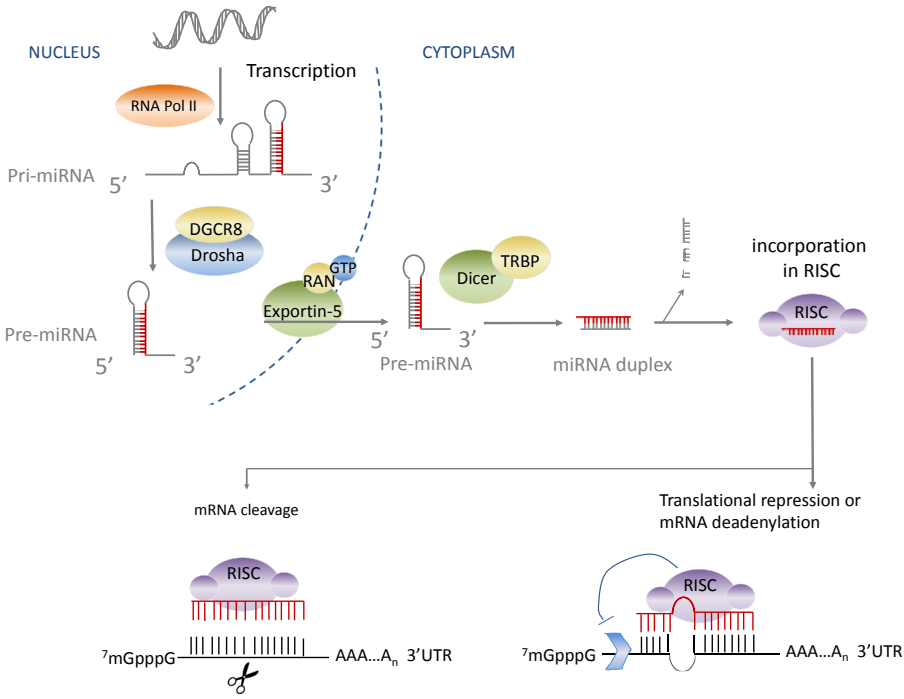


Figure 11. canonical miRNA biogenesis. Schematic representation of microRNA transcription by RNA polymerase, nuclear processing by the microprocessor complex comprising Drosha and DGCR8 and nuclear export by exportin-5. The microRNA biogenesis is then pursued in the cytoplasm by Dicer-mediated processing and RISC loading, after which the mature microRNA strand is guided to its target mRNA, predominantly resulting in inhibition of translation and/or mRNA degradation.
RNA Pol II: RNA polymerase II; DGCR8: DiGeorge Syndrome Critical Region 8; TRBP: transactivation-responsive RNA-binding protein; RISC: RNA-induced silencing complex; 3'UTR: untranslated region

Alternatively, besides the generation of the canonical mature miRNA sequence, as described by these different biogenesis mechanisms, a single miRNA gene can give rise to multiple transcripts, the so called isomiRs [244]. Additionally, miRNA precursors can be potential disease markers as well. Of interest, the measured amount of the precursor doesn't necessarily correlate with the amount of mature miRNA [245].

Importantly, interference is possible at different levels throughout the entire miRNA biogenesis pathway (transcription, processing, RNA editing, AGO loading, RNA decay), affecting miRNA expression and function [233]. Genetic and epigenetic alterations can result in aberrant expression of miRNAs, a mechanism that is frequently encountered during cancer and disease [246]. Transcription factors such as p53 or MYC can positively or negatively affect miRNA expression by interfering with RNA Polymerase II [233,247]. Interference with the expression or the function of Drosha or Dicer is also frequently reported. In response to DNA damage, p53 promotes Drosha-mediated processing of certain miRNAs that are involved in regulating cell cycle or cell proliferation [247]. Dicer sumoylation is described in macrophages

of smokers [248] but also other mechanisms can affect Dicer expression such as hypoxia-inducible epigenetic regulation of Dicer expression [249] or another miRNA such as let-7, miR-630 or miR-103/107 that lowers the expression of its target DICER1 [250-252]. Of note, it is also suggested that restricted Dicer cleavage could contribute to the tissue- or cell-specific expression of miRNAs [253].

When the mature miRNA is present in the cytoplasm, different mechanisms can affect the mature miRNA level as well. One such mechanism is the transport of the mature miRNA back to the nucleus, (e.g. by Exportin 1 or Importin 8) giving rise to nuclear miRNAs [254]. Also, RNA-binding proteins [233,255] and the sequestration of miRNAs by endogenous sponges such as competing endogenous RNAs (ceRNAs) can alter mature miRNA levels [256,257].

Cell communication can occur through cell contact, soluble mediators and exosomes, defined as small cell-derived vesicles whose cargo contains stable small RNAs including miRNA [258]. As such, miRNAs are stably present in plasma and other body fluids [259]. Active processing and secretion of mature miRNAs to neighboring cells in exosomes (40nm-100nm) or microvesicles (100nm-1µm) contributes to intercellular communication and target regulation in distant recipient cells, directing biological processes under normal physiological as well as under pathological conditions. Although not quite similar regarding their characteristics and biogenesis, exosomes and microvesicles are often grouped as extracellular vesicles. These extracellular vesicles have been demonstrated to be involved in activation or suppression of immune responses, in tissue repair, but also in tumor biology [260]. Aberrant presence or expression of these miRNAs in body fluids, bound to proteins or within microvesicles or exosomes, can be used as a predictive or diagnostic marker of disease [261]. Moreover, targeting these extracellular vesicles, as well as utilizing them as therapeutic drug delivery vehicle or therapeutic agent may open new avenues in – but not only – miRNA therapy.

2.3 Target regulation by miRNAs

More than 60% of the human protein-coding genes contain at least one conserved miRNA-binding site [262]. Considering that also non-conserved sites exist, most protein-coding genes might be controlled by miRNAs.

The mechanisms by which a miRNA regulates its mRNA target, depend on the specific AGO protein in which the miRNA is loaded and the extent of complementarity between the miRNA and mRNA [235,263]. First, AGO proteins constitute an essential component of the miRISC. They enhance the speed of target finding, especially when no mismatch is present in the seed sequence, while protecting the guide from degradation and ensuring a stable binding [264]. While miRNAs function as the guide, AGO proteins function as effectors by recruiting factors that induce translational repression, mRNA deadenylation and mRNA decay [233]. Second, the vast majority of miRNAs form partial duplexes with the 3'untranslated region (UTR) of the target. Generally, a miRNA can associate with 4 types of canonical sites located in the 3'UTR of its target gene, a 6mer, 7mer-1A, 7mer-m8 and 8mer site (**Figure 12**) [235,265]. Perfect pairing is pursued between nucleotides 2 and 7 at the 5' end of the miRNA ('the seed') and the target site.

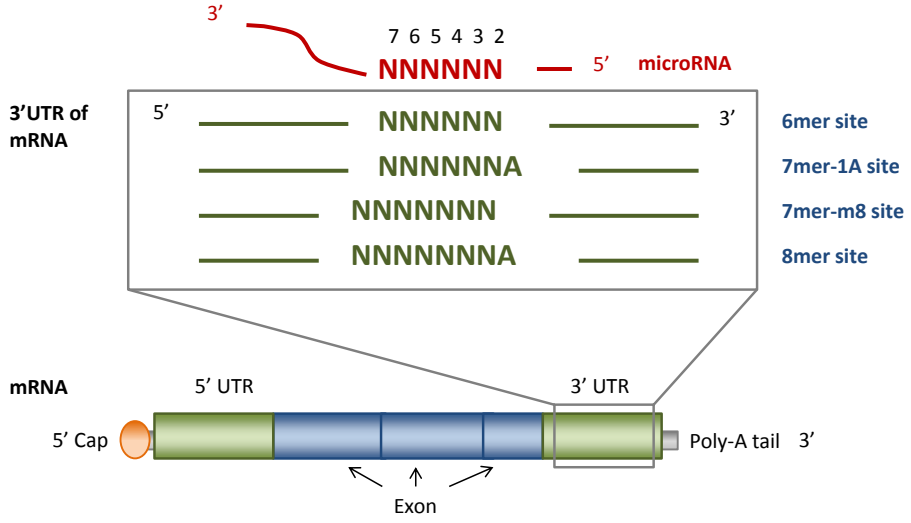


Figure 12. Different types of canonical miRNA target sites [265]. The vast majority of microRNAs form partial duplexes with microRNA recognition elements within the 3'UTR of the target mRNA transcript. Based on the characteristics of the base pairing between the microRNA seed and the target, 4 types of miRNA target sites can be distinguished.

Flexibility to this global 'seed'-rule has been described repeatedly [243,266,267]. Imperfect base pairing with the seed can be compensated by extensive 3' end pairing or base pairing to nucleotides 13-16 of the miRNA. 'Centred sites' where the middle sequence of the miRNA forms base pairs with the target are

also reported [268]. Factors contributing to miRNA-target binding are proximity to sites for co-expressed miRNAs or an AU-rich nucleotide area near the site. Other important requisites that mediate miRNA-target efficiency are presence of *in vivo* concentrations of miRNA and target, positioning away from the center of long UTRs or orienting within the 3'UTR at least 15 nucleotides away from the stop codon or the poly(A) tail [243,269]. In general, the repression of a miRNA target is less efficient when the seed has only a 6mer site match compared to a 8mer site. Furthermore, additional base pairing adds to the stability of the binding [265]. For effective repression, multiple sites for the same or for different co-operating miRNAs are required (synergistic effect) [243].

Interestingly, one target often contains multiple miRNA binding sites so that many miRNAs are able to regulate the expression of that target. On the other hand, a single miRNA can regulate several mRNA targets [262]. First, the cellular concentration of the miRNA dictates the protein output of its target. Second, miRNAs rather fine-tune protein expression levels or establish a threshold which potentially leads to substantial biological consequences [265]. Third, although a miRNA is able to repress hundreds of proteins, only a few of these proteins may be critical for a particular biological process [270].

Although their primary mode of action is inhibition of translation and mRNA degradation, a few groups have also reported that miRNAs can induce mRNA or protein expression [271,272]. Cell cycle phase, stress conditions or SNPs in miRNA genes or miRNA response elements (MREs) can alter the functionality of miRNAs. As an example, miR369-3 oscillates between repression and activation in coordination with the cell cycle. In G_1/G_0 arrest, it directs association of proteins with the AU-rich elements (AREs) in TNF-α to activate translation [273]. Adding a level of complexity, targets can reciprocally control the level and function of miRNAs as well [274].

In addition, crosstalk between miRNAs and other ncRNAs has been described repeatedly with competition for the same substrate, shared effector proteins and cross-regulation of each other [275-277], as well as miRNAs affecting the epigenetic state [278,279]. RNA-binding proteins can cooperate with miRNAs in the down-regulation of the shared target or protect the target from miRNA binding [255]. For miRNAs present in the nucleus, there is a lack of clarity regarding their function. Studies have suggested functions such as regulating gene and non-coding RNA expression [272,280,281], controlling the biogenesis of other miRNAs [282], affecting chromatin state and fine-tuning the expression of mRNA expression in the cytoplasm [254,283-285].

Another interesting fact that needs further investigation is the fate of miRNAs following mRNA targeting. It has been shown that miRNAs can be recycled after target recognition and thus can participate in multiple rounds of targeting. During this process, the 3'end of the miRNA can be modified, accelerating the rate of miRNA decay [286].

2.4 miRNAs and the immune system

miRNAs are intricately connected to most biological processes including normal homeostasis and inflammation in a tissue specific and even time-specific manner [287]. Early evidence for their wide-spread regulatory role comes from loss of Drosha, Dgcr8, Dicer or miRNA-associated Argonaute protein studies which resulted in severe developmental defects or even early death [288-290]. Targeting or deletion of Dicer in specific cell-types provided more information on the, often vital, role of miRNAs in cell development and function [252,291,292]. Through lung-specific, targeted deletion of Dicer, a global reduction in miRNA processing resulted in abnormal embryonic lung development, manifested by apoptosis and abnormal airway branching [293].

miRNAs are involved in the tight regulation of components of Toll-like receptor (TLR) signaling and in **innate** immune pathways, therefore being well placed to function as adequate immunomodulators [294]. TLRs are a family of PRRs that play an essential role in innate immunity. Both TLRs and miRNAs are restrictedly expressed in particular immune cells and epithelial cells, which enables miRNAs to control these cells' reaction to infection or injury, to dampen excessive inflammation and to allow the cells to return to homeostasis. An interesting perspective is that relatively few conserved miRNA binding sites are confirmed on common TLR signaling mediators, enabling a selected group of miRNAs to target these mediators upon TLR activation, thereby avoiding excessive pro-inflammatory responses. In return, TLR activation itself can give rise to a sequential early or late induction, or decrease, in miRNA levels that then controls the strength and the longevity of the inflammatory response [295]. Both miR-155 and miR-146a, 2 important regulators of inflammation, are induced upon TLR4 stimulation with LPS in monocytes. However, miR-146a functions as a brake on the inflammatory response by negatively regulating NFκB signaling through targeting of both IRAK1 and TRAF6, while miR-155 enhances the inflammation [295-297]. miR-21 is induced following TLR activation of macrophages and acts as a molecular switch between the pro-inflammatory (NFκB) and the anti-inflammatory response (IL-10) via its target programmed cell death 4 (PDCD4), resulting in positively influencing IL-10 while negatively regulating NFκB [298,299].

Myeloid-specific miR-223 negatively regulates myeloid progenitor proliferation and granulocyte differentiation and activation [300]. MiR-223 mutant mice develop lung pathology with an exaggerated tissue destruction and a pro-inflammatory phenotype following LPS injection [301,302].

At steady state, miRNAs influence immune cell development and function, and balance hematopoietic output by negatively regulating key immune development genes alongside important nodes in the regulatory circuit [303,304]. An integral role for miRNAs in the **adaptive** immune response is extensively studied [298]. As an example, miR-150 and its target c-Myb are critical for the transition from pro- to pre-

B cell, with a high expression of miR-150 in mature B and T cells but not in their progenitors, whereas c-Myb is highly expressed in lymphocyte progenitors and down-regulated on maturation [270,291]. Further, both B and T cells rely on miR-155 for adaptive immune responses [305]. For instance, absence of miR-155 in B cells resulted in an attenuated production of IgG1 antibodies [306]. Besides a regulatory role in T cell differentiation and function, miRNAs are also required for the maintenance of the naïve T cell state [307]. miR-155 promotes T-cell mediated inflammation through the regulation of both Th1 and Th17 responses while miR-146a seemes to have an intrinsic impact on the function of Treg cells [307]. Besides exceptionally high levels in Treg cells, the expression of miR-146a, a negative regulator of the immune response and widely expressed across the hematopoietic system, generally increases with maturation and activation [297]. A microRNA profiling study focusing on Treg cells, revealed that miR-199a-5p was repressed in Treg cells of patients with COPD compared to controls. Additional *in vitro* experiments showed an involvement of miR-199a-5p in TGF-β signaling, suggesting an influence of this miRNA on the TGF-β-induced Treg differentiation and the modulation of the adaptive immune balance towards Th1 and Th17 responses [308]. In addition, DCs from *bic*/miR-155-deficient mice fail to present antigen to T cells following endotoxin challenge [309].

Although the cell builds in a plethora of protective measurements, dysregulation of one or more crucial miRNAs can lead to an aberrant cell development or immune function [303] and can facilitate development of disease [310-312].

2.5 miRNAs and smoking

Cigarette smoking is the predominant risk factor for developing COPD in the western world. CS clearly reduced the overall miRNA profile in murine lungs as well as in human airway epithelial cells and alveolar macrophages [149,150,313]. Among the subset of decreased miRNAs in lungs of CS-exposed mice, many had functions in stress response, cell proliferation and apoptosis [150]. miR-218, which was strongly down-regulated in airway epithelium of smokers, contributed to the induction of a number of smoking-related genes [149]. In human alveolar macrophages, the decrease in miRNA abundance was related to the number of pack years smoked, hereby establishing a link with cumulative smoking history [313]. Of note, alterations in miRNA levels are an early event following CS exposure [314].

The effect of long-term smoking on the miRNA profile in plasma was also assessed. Of the differentially expressed miRNAs, 43 of 44 miRNAs were higher expressed in plasma of smokers compared to never-smokers. Notably, 24 of the 44 differentially expressed miRNAs were previously reported as potential biomarkers of diseases, suggesting that smoking history should be taken into account when assessing disease risk in blood. In return, smoking cessation restored the plasma microRNome to resemble that of never-smokers [315].

When mice deficient or heterozygous for the Arylhydrocarbonreceptor (Ahr), a suppressor of inflammation, oxidative stress and apoptosis in response to constituents in CS, were exposed to CS, more miRNAs were up-regulated in lung tissue of $Ahr^{-/-}$ mice compared to $Ahr^{+/-}$ mice including miR-96 [316]. Moreover, miR-101 and miR-144, 2 miRNAs induced upon cigarette smoke extract (CSE) exposure *in vitro*, targeted the cystic fibrosis transmembrane conductance regulator, a chloride channel important for epithelial fluid homeostasis [317].

The mechanism by which CS alters miRNA levels remains to be elucidated and many hypotheses have been put forward. First, CS is highly toxic and contains free radicals and oxidative compounds that cause oxidative stress and are mutagenic, thereby establishing a pivotal connection between CS and carcinogenesis. In addition, genes for miRNAs often lie in 'fragile sites' in the genome. Second, CS might be linked to an altered epigenetic state that consequently affects miRNA gene transcription. As an example, miR-218 and its host gene Slit Homolog 2 (SLIT2) are down-regulated in the bronchial epithelium of smokers compared to never-smokers, probably via promoter hypermethylation. Third, CS can affect the global processing of miRNAs by post-translationally modifying the processing enzymes such as Dicer [248] or by affecting the stability of the RISC complex [318]. Fourth, CS is suggested to increase miRNA degradation or target miRNAs to stress granules or P-bodies.

An interesting question is whether CS can influence miRNA function in the brain and can alter the neurobehavioral circuitry. It has previously been reported that the dopamine D1 receptor gene (DRD1) is associated with nicotine dependence and additionally that two alleles (A and G) of polymorphism

rs686 in the 3'UTR of DRD1 gave rise to a differentially expressed dopamine receptor in the brain, thereby mediating dopamine action. Polymorphism rs686A of DRD1 was complementary to the seed sequence of miR-504 and differential targeting by miR-504 may explain nicotine dependence [319].

2.6 miRNAs in COPD

miRNA research in COPD has opened new avenues for a better understanding of COPD pathogenesis. It is increasingly recognized that miRNAs, besides other factors, are entangled in many biological pathways such as repair and inflammation that are important in COPD pathogenesis, as well as in COPD pathology such as emphysema. Altered miRNA expression has been observed in human lungs of patients with COPD compared to control smokers as well as in other compartments. Further, *in vitro* or *in vivo* mechanistic studies highlight the impact of several miRNAs in the pathogenesis of COPD.

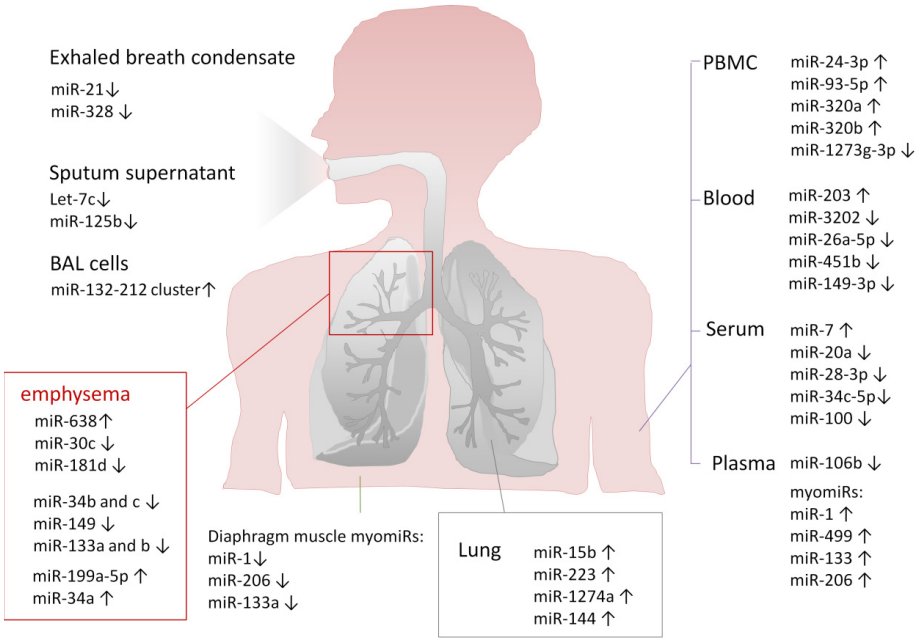


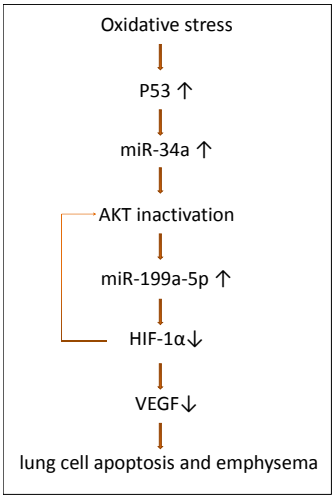
Figure 13. Overview of microRNA dysregulation in COPD patients compared to control. Differentially expressed microRNAs are represented according to compartment, anatomical location or disease phenotype. The arrow ↑ indicates that this microRNA is up-regulated in patients with COPD compared to controls.
PBMC: peripheral blood mononuclear cells

In order to elucidate the pathogenesis of COPD, mRNA and miRNA profiles were studied in **lung** tissue, revealing 70 differentially expressed miRNAs between smokers with or without airflow limitation of which miR-223, miR-1274a and miR-144 showed the strongest up-regulation and miR-923, miR-937 and miR-422a the strongest down-regulation in COPD patients [152]. In another study, miRNAs were detected in lung tissue and in blood of patients with COPD. Intriguingly, miR-203 was lower expressed in lung tissue while higher expressed in blood of COPD patients compared to never-smokers (**Figure 13**) [320]. Moreover, in the cell fraction of bronchoalveolar lavage (**BAL)** from 87 patients with either

adenocarcinoma or COPD or both, 66 miRNAs were differentially expressed. In COPD patients, the miR-132-212 cluster was up-regulated and was negatively correlated with α1-antitrypsin mRNA [321]. Further, in **blood** samples of smokers with COPD compared to controls, 56 miRNAs were differentially expressed. Besides others, the expression of miR-3202, miR-26a-5p, miR-451b and miR-149-3p was significantly reduced in smokers with or without COPD compared to never-smokers. miR-149-3p was shown to regulate the TLR4/NFκB pathway in murine monocytic THP-1 cells [322]. Next, in **serum** of COPD patients and controls, 72 miRNAs were examined by RT-qPCR array. Although the rather small pool of miRNAs investigated, 5 miRNAs were found to be significantly dysregulated with miR-20a, miR-28-3p, miR-34c-5p and miR-100 being down-regulated and miR-7 being up-regulated in COPD compared to controls [323]. In **plasma**, 9 miRNAs were significantly lower expressed between current smokers with and without airflow limitation as detected by low-density array screening of which miR-106b was further validated by RT-qPCR [324]. In **peripheral blood mononuclear cells** from COPD patients versus smokers without airflow limitation, 8 miRNAs were up-regulated (e.g. miR-24-3p, miR-93-5p, miR-320a and miR-320b) and 3 miRNAs were down-regulated (e.g. miR-1273g-3p) of which 5 were validated. Regulatory network analysis on integrated miRNA and mRNA expression data revealed NOD and TLR as most enriched pathways [325].

Our research group showed that let-7c and miR-125b were robustly decreased in **sputum supernatant** of smokers with COPD compared to never-smokers. In addition, let-7c was associated with FEV_1/FVC and inversely correlated to its predicted target, the soluble TNF Receptor II [153]. Using another non-invasive method such as **exhaled breath condensate**, miRNA expression was quantified in patients with asthma and COPD compared to healthy controls. miR-21 and miR-328 were significantly lower expressed in COPD compared to controls [326].

Several miRNAs may be involved in the pathogenesis of **emphysema**. Interestingly, Christenson and colleagues integrated miRNA and mRNA data from lung tissue of patients with COPD GOLD IV versus controls at eight different locations of varying degree of emphysema. Expression levels of 63 microRNAs were altered with regional emphysema, including miR-638, miR-30c, and miR-181d. Genes correlated with these miRNAs were enriched in pathways associated with emphysema pathophysiology such as oxidative stress and accelerated aging [327]. Moreover, miR-34c, miR-34b, miR-149, miR-133a and miR-133b were significantly down-regulated in lungs from patients with moderate compared to mild emphysema. These authors reported the strongest correlation of miR-34c with its target SERPINE1, a protease inhibitor, which highlighted the disturbed protease/anti-protease balance, important in emphysema [328].



Up-regulation of miR-199a-5p and miR-34a was noted in lungs of COPD patients compared to never-smokers alongside a lower expression of vascular endothelial growth factor (VEGF) and hypoxia inducible factor (HIF-1α). The proposed mechanism involved an increase in p53 expression in answer to the burden of oxidative stress (**Figure 14**). P53 then enhanced the transcription of miR-34a which mediated the increase in miR-199a-5p, possibly through AKT inactivation. Consecutively, increased levels of miR-199a-5p targeted HIF-1α which could result in lung cell apoptosis and emphysema through lower VEGF expression [329] since VEGF receptor signaling is required for maintenance of the alveolar structures, as shown in a rat model by blocking VEGF receptor signaling [330].

Figure 14. proposed mechanism in lungs of COPD patients involving miR-34a and miR-199a-5p. Adapted from Mizuno S. et al. Chest 2012; 143 (3):663-672 [329].

Differentially expressed miRNAs in lung tissue of smokers with or without airflow limitation revealed biological pathways that are relevant for the pathogenesis of COPD such as **TGF-β signaling**, Wnt and focal adhesion pathways. As an example, miR-15b, up-regulated in patients with COPD compared to control smokers and expressed in bronchial epithelium and alveolar type II cells, altered TGF-β signaling by affecting the levels of SMAD7, SMURF2 and decorin [152]. In TGF-β-responsive airway smooth muscle cells, miR-145 negatively regulated pro-inflammatory cytokine release in COPD by targeting SMAD3 [331].

Skeletal **muscle** weakness is a predictor of mortality in patients with moderate to severe COPD. Detection of muscle-specific miRNAs (myomiRs) in plasma of stable COPD patients revealed elevated levels of these myomiRs as a result of increased muscle wasting and turnover [332]. In contrast, in diaphragm muscle biopsies from sedentary persons with or without airflow limitation, lower expression of myomiRs was detected in patients with COPD, besides an up-regulation of HDAC4 and the muscle-related transcription factor MEF2C [333].

More information regarding miRNAs in COPD has been published in some excellent reviews [155,312,334-338].

2.7 miRNA therapy

In every cell, a highly complicated web of interactions exists between DNA, RNA and proteins. Over the last couple of decades, researchers have taken the challenge to identify important interacting 'nodes'. One of these breakthroughs was the discovery of the RNA interference mechanism where double-stranded RNA triggers suppression of gene activity in a homology-dependent manner by Andrew Fire and Craig Mello. Their work was rewarded with the Nobel Prize in 2006. Soon after, another principle of gene regulation was revealed by small non-coding RNAs, including miRNAs, that added to the complexity. Dysregulation of miRNAs in disease and infection meant that these could be manipulated, making them a very appealing target or therapeutic (**Figure 15**).

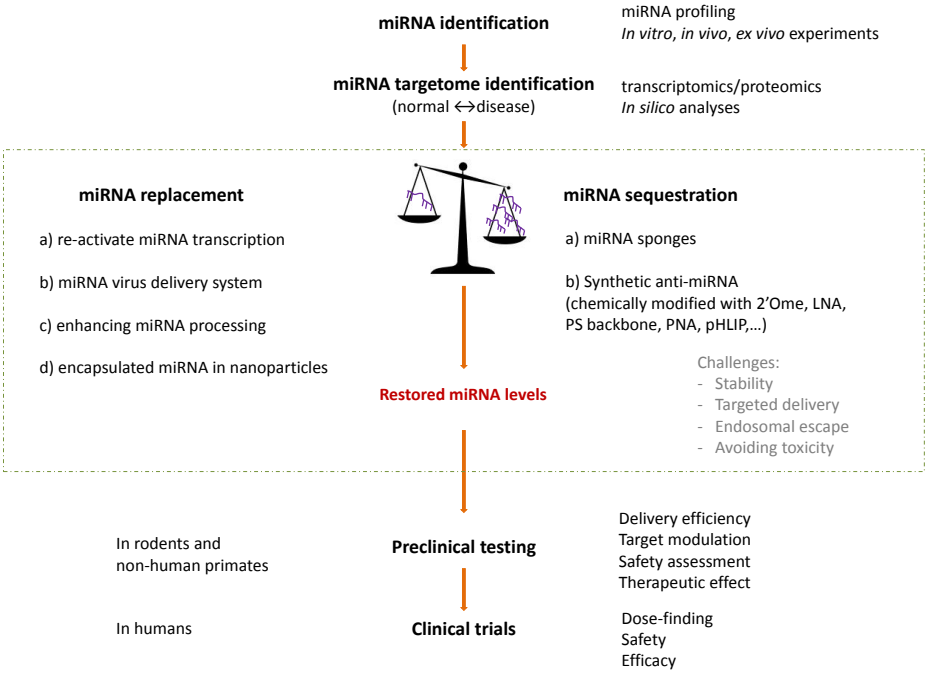


Figure 15. Therapy modalities for targeting miRNAs in human disease. To develop a microRNA therapeutic, the first step is to identify a microRNA candidate, relevant to the disease of interest. Depending on the altered expression profile in disease, restoring microRNA levels should be aimed for. This can be achieved through different replacement or sequestration technologies which bears many challenges. If promising, preclinical testing and careful evaluations through the different stages of clinical trials finally bring the microRNA therapeutic to the market.

Interestingly, miRNAs have several assets for being ideal as a therapy or therapeutic target. First, miRNAs are small in size and have a conserved sequence. Second, targeting a single miRNA can affect multiple pathways, which is preferable above a mixture of components. However, besides the

advantages of miRNA-based therapeutics, miRNA therapy bears some challenges as well. Modulation of the miRNA expression can be beneficial in one tissue while harmful in the other, stressing the need for targeted delivery. In addition, the small sequence of a miRNA is subject to nuclease degradation, requiring extra modifications or formulations. These chemical modifications, conjugations to carrier molecules or formulations, intend to ameliorate tissue uptake, to delay plasma clearance and to enhance the efficacy [339]. To effectively deliver the cargo from the endosome to the cytoplasm, and thus induce endosomal escape, several strategies can be relied on such as proton sponge effect, membrane fusion, pore formation and membrane disruption [340]. Another important issue is the toxicity risk as a result of off-target effects ~~or~~ such as potent immune activation or other unintended effects of miRNA mimics or antagomiRs [341]. Also, over- or under-dosing can adversely lead to hyper- or hypo-activation.

Various approaches are developed to re-establish appropriate miRNA levels. miRNA mimics or miRNA expression vectors can be administered to replenish the level of a specific miRNA, as well as drugs that enhance miRNA transcription and processing. Despite the excellent cell entry capacities of viral vectors, they can induce immune activation. Therefore, non-viral vectors with minimal toxicity and immunogenicity are being developed. Efficient delivery of miRNA mimics can be achieved by introducing chemical modifications to a synthetic RNA duplex (e.g. 2'F- or 2'OMe-modification, conjugation to cholesterol, methylation of the passenger strand, introduction of mismatches,..) to enhance the stability and cellular uptake, but also to favor RISC loading of the guide or antisense strand over the passenger strand [342]. Another solution is the encapsulation of the miRNA mimic into carrier systems such as liposomes and nanoparticles with differing charge, composition, addition of specific antibodies for targeted delivery or conjugates in order to protect the mimic and to allow endosomal escape [343]. Different nanoparticle technologies have already been designed. Intravenous injection of lipid-polymer nanocomposites have been reported for their successful and specific uptake in lung endothelium [344]. However, safe transportation and delivery of miRNA mimics to the lungs via inhalation and targeted delivery to the cell of interest remains a major challenge. Polymeric nanoparticles based on polyethyleneimine were successful in complexing the miRNA mimic and delivering an actively functioning miRNA to human F508del CFTR bronchial epithelial cells (CFBE41o-cells) *in vitro* [345]. Also, various *in vitro* experiments indicate that bio-inspired nanocomposites consisting of a pulmonary surfactant outer shell and a siRNA- or miRNA-loaded hydrogel core may be a promising candidate for inhalation therapy [346]. However, both nanoparticle formulations still need to prove their value in *in vivo* experiments.

On the other hand, the expression of a specific miRNA can be lowered by adding miRNA antagonists such as locked nucleic acid (LNA) anti-miRs, antagomiRs or miRNA sponges (competitive inhibitors of miRNA function). Also here, different modifications and (targeted) carrier systems can increase

stability, delivery and specificity for a certain environment. In the so-called first generation of single-stranded antisense oligonucleotides (ASOs), the phosphate backbone was substituted by a phosphorothioate backbone which improved solubility and membrane penetration, although their efficacy was poor. In the second generation ASOs, additional modifications were introduced to the ribose sugars (2'-O-Methyl, 2'-methoxy-ethyl, 2'-Fluoro) resulting in greater nuclease resistance and ameliorated binding affinities. The next generation was developed to increase the binding affinities (LNA, peptide nucleic acids and morpholinos). Addition of specific conjugates such as N-Acetylgalactosamine (GalNAc) ligands can further enhance potency for targets in hepatocytes. Targeting entire miRNA families can be accomplished by miRNA sponges and short seed-targeting LNAs [347]. Based on complementarity, these molecules sequester the endogenous miRNA, thereby preventing its function [343]. All these former mentioned modifications have significantly improved potency but *in vivo* delivery of nucleic acid based therapeutics still remains a major hurdle. To overcome the delivery problem, small molecules that target miRNA processing are under investigation. As an example, streptomycin functions as a miR-21 inhibitor by binding to the terminal loop region of pre-miR-21 which blocks Dicer processing. Inforna is a platform that assists in sequence-based prediction and design of small molecules targeting RNA [348].

At present, miRNAs are generally more used as biomarkers/diagnostics in clinical settings than as a therapeutic. However, the number of miRNA therapeutics is rising and only a small number of miRNA therapeutics have moved from bench to bedside [349]. Miravirsen (Santaris Pharma), a 15-nucleotide phosphorothioate DNA-LNA mixmer which sequesters mature miR-122, was the first successful miRNA-based therapy for treating hepatitis C virus (HCV) infections [350]. miR-122 is a highly conserved, liver-specific miRNA that positively regulates HCV replication by binding to the 5'UTR of HCV mRNA, resulting in protection of viral RNA from degradation [351]. However, miR-122 is also involved in lipid metabolism and acts as a tumor suppressor [352]. Until now (additional phase II clinical trials with long-term follow-up and multidrug combinations are ongoing), Miravirsen was well tolerated and short-term use provided long-lasting antiviral activity with only limited evidence of viral resistance. Recently, RG-101 (Regulus Therapeutics), another anti-miR-122 reached phase II clinical trials for chronic HCV infections [353]. RG-101 has a different chemistry GalNAC-conjugated antimiR-122 oligonucleotide) than Miravirsen to enhance uptake by the hepatocytes and to increase its potency. However, after treatment of patients with a combination of RG-101 and a direct-acting antiviral, the trial was put on hold due to a second case of jaundice. Apart from these cases, no important adverse effects have been reported yet in several animal studies [339,354,355] and clinical trials. Nonetheless, long-term anti-miR-122 therapy might be inadvisable due to its tumor suppressor function, as mir-122 KO mice develop hepatocellular carcinoma [356].

In several animal models of non-small-cell lung carcinoma (NSCLC), delivery of miRNA mimics or antagomiRs showed promising effects on tumor outcome and safety [357,358]. MRX34 (Mirna Therapeutics), a miR-34 mimic encapsulated in a lipid carrier acquiring a positive charge under low pH conditions, has entered a multicenter phase I trial in patients with NSCLC or other tumors. Although the initial results were promising, some immune-related toxicities were observed, terminating the trial until the cause has been identified. Another mimic for miR-16 enrolled phase I clinical trials for mesothelioma and NSCLC, delivered in an EnGenIC Delivery Vehicle nanocell (also called TargomiR). These are bacterium-derived particles coated on the surface with epidermal growth factor receptor (EGFR)-specific antibodies for targeted delivery [343].

Furthermore, a multi-target anti-miRNA antisense oligodeoxyribonucleotide (MTg-AMO) approach has been designed to target miR-21, miR-155 and miR-17-5p in cancer, resulting in increased inhibition of cancer growth [359].

Promising candidates in COPD pathogenesis, provided that targeted delivery can be achieved, could be mimics for let-7 family members, miR-146a, miR-34 family members, miR-218, and antagomiRs for miR-21, miR-155, miR-15b and miR-135b. In this dissertation, we focus on miR-218-5p which will be further discussed in part II (Research work, **chapter 5**).

CHAPTER 3: Translational research in COPD: materials and methods

3.1 Rationale

Translational research is an integrated approach with as ultimate goal translating findings from basic research to the clinic ('from bench to bedside') (**Figure 16**).

An illustrative example of a translational approach is the research into the role of miR-122 in hepatitis. Basic researchers found that miR-122 was highly up-regulated in hepatitis compared to control liver and tested the therapeutic potential of an anti-miR-122 in in vitro and in vivo experimental models (bench). The success story was picked up by industry and miravirsen was developed by Santaris Pharma. Miravirsen is now vigorously tested in clinical trials, hopefully ending as a clinically approved therapeutic product that fights hepatitis (bedside).

Basic research aims to elucidate a basic research question such as the role of miRNAs in COPD. Therefore, a wide range of *in vitro* and *in vivo* tools, which will be discussed in detail in this chapter, are used. Knowledge obtained by basic research will then be validated on *ex vivo* human lung tissue from patients and in that way translated to the human situation, as a first step towards clinical research. Clinical research investigates the safety and effectiveness of medication, biomarkers, treatment regimens etc., intended for use in humans.

Another aspect of translational research is that findings from the clinic at the patient level can be investigated in large population studies. As such, the contribution of, for instance, co-morbidities and treatments on COPD outcomes can be investigated at the population level (**Figure 16**).

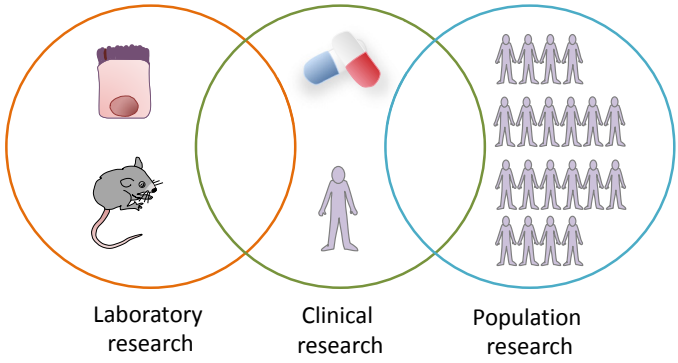


Figure 16. Translational research (both intersections). Translational research focuses on the integrated approach where laboratory research findings from *in vitro* cell culture systems or *in vivo* animal models are translated into patient-oriented therapy development (clinical research). The therapeutic interventions in the clinical research can then give rise to new insights regarding therapy and disease conditions in large population studies.

3.2 *In vitro* culture of normal human bronchial epithelial (NHBE) cells

Airway epithelial cells are important cell types in the encounter with inhaled toxic substances and initiation of inflammatory signaling. Stimulation of these cells with CS(E) or other substances can provide important information on the inflammatory reaction *in vitro*.

Submerged primary NHBE cells (supplied by Lonza, **Figure 17**) are cultured using Bronchial Epithelial Growth Medium (BEGM), supplemented with necessary additives (BEGM SingleQuots kit, Lonza), according to the manufacturers' instructions. Cells are grown in cell culture flasks and plates with Nunclon™ delta coating. Experiments are conducted at passage 2 and 3. Cell survival is evaluated with flow cytometry and Trypan blue, and a lactate dehydrogenase (LDH) test (**Figure 18**) provides information regarding cellular damage when the cells are stimulated with a compound such as CSE. Cells are harvested by trypsinization or directly lyzed in Qiazol solution (Qiagen) for RNA extraction. Cell supernatant is stored for protein analysis by enzyme-linked immunosorbent assay (ELISA).

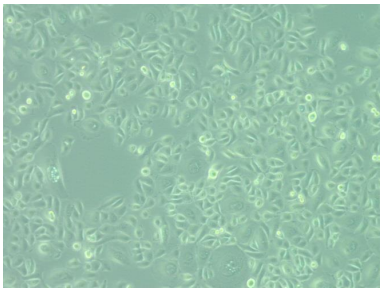


Figure 17. Normal human bronchial epithelial cells. Submerged culture of primary NHBE cells grown in a 6-well plate after passage 2 that were untreated (air-exposed and not transfected).

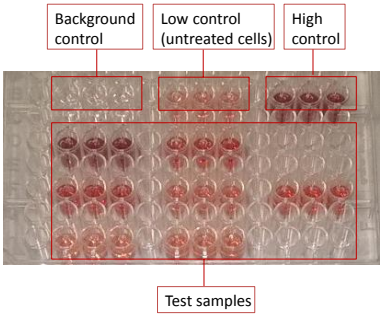


Figure 18. Lactate dehydrogenase (LDH) test. Example of a colorimetric assay were the release of the enzyme lactate dehydrogenase activity is measured as an indicator for cellular damage in NHBE cell supernatant of cells exposed to increasing doses of cigarette smoke extract ranging from 1% to 100%. For the high control, cell lysis was induced with Triton-X.

However, one single cell type cannot reflect the complex reactional cascade following a stimulus in the human body which consists of a crosstalk between different cell types and organs. To overcome this limitation, experimental animal models are used.

3.3 Murine model of COPD

Our lab has developed a CS-induced mouse model [360] that mimics most of the pathological features of COPD. In this dissertation, mice are exposed to 1 week (acute), 4 weeks (subacute) and 24 weeks (chronic) of CS. The acute and subacute exposures are ideal to investigate the CS-induced inflammation. Instead, the chronic CS exposure is required for investigating the hallmarks of COPD pathology such as emphysema, airway remodeling and presence of lymphoid follicles.

Mice are in several aspects suited for research since they are cheap, easy to breed and their anatomy, biology and immune system is extensively studied [361]. In addition, we use the C57BL/6 mouse strain which is moderately sensitive to CS and of which a multitude of knock-in and knock-out models exist [362,363]. However, certain differences must be taken into account when translating research findings from mice to humans [364].

For example, the protein-coding regions of mouse and human genomes are on average 85% identical. Some genes are 99% identical while others are only 60% identical. Many regions are evolutionarily conserved because they are required for essential functions. In contrast, the non-coding regions are much less similar (only 50% or less). However, miRNAs form an exception since these small RNAs are highly conserved between species, making translations from miRNA research in mice relevant in humans. Nonetheless, the 3'UTRs and MREs in some target genes are known to differ between humans and mice [365].

Thus, this CS-induced mouse model enables us to answer specific (miRNA-related) research questions in the pathogenesis of COPD.

3.3.1 Protocol of smoke exposure

Male C57BL/6 mice, purchased from Charles River (L'Arbresle Cedex, France), are housed under a 12h light-dark cycle in autoclaved cages and bedding, with unlimited access to water and food. Using a smoking apparatus and a smoke chamber, groups of C57BL/6 mice are exposed whole body to the tobacco smoke of five 3R4F reference cigarettes (without filter, University of Kentucky, Lexington, KY), 4 times a day with 30 minutes smoke-free intervals. The mice are exposed for 5 days per week, for 1 week, for 4 weeks or 24 weeks. An optimal smoke-to-air ratio of 1:6 is maintained and control groups are exposed to air.

Twenty-four hours after the last exposure, the mice are weighted and sacrificed with an overdose of pentobarbital. Next, (lung) tissue and BAL is collected.

The ethics committee for animal experimentation of the faculty of Medicine and Health Sciences (Ghent University) approved all *in vivo* manipulations.

3.3.2 Harvesting BAL and lung tissue

By performing BAL, immune cells are retrieved, giving an insight into the cellular content within alveolar spaces and airways. Total cell counting on BAL cells is performed using a Bürker chamber and differential cell counts are obtained through stained cytospins (May-Grünwald-Giemsa staining) (**Figure 19**) and flow cytometry (**see 3.6.5**).

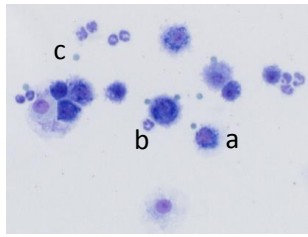


Figure 19. Cytospin of BAL fluid (a. macrophage; b. neutrophil; c. lymphocyte)

After rinsing the pulmonary and systemic circulation, lung tissue is collected. First, the left lung is fixated and paraffin embedded. Next, slices of the left lung are used for immunohistochemistry (**see 3.6.3**) or *in situ* hybridization (ISH) (**see 3.6.4**). Second, a single cell suspension is made from the largest lobe of the right lung. These cells are then labeled for flow cytometry. Finally, the rest of the right lung is used for RNA extraction and subsequent RT-qPCR (**see 3.6.1**) (**Figure 20**).

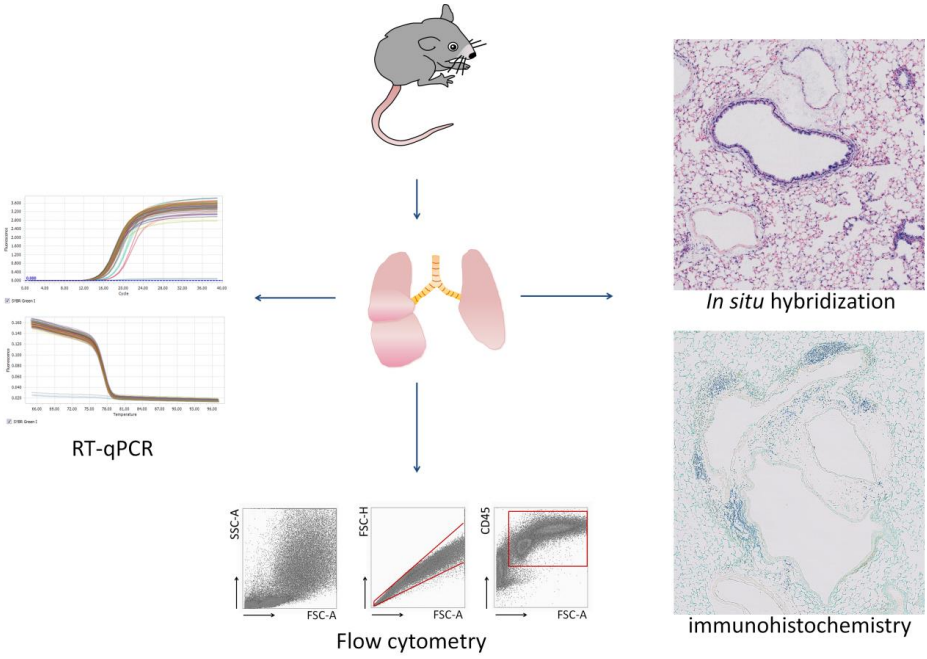


Figure 20. Overview of murine lung tissue sample collection and analysis. The day after the last smoke or air exposure, murine lung tissue is collected. Further treatment with enzymes for digestion or fixative allows flow cytometric analysis and microscopic evaluation of stained lung slices, respectively. The rest of the lung is stored for later analyses such as RT-qPCR.

3.4 Human lung tissue

To validate our findings from the *in vitro* and murine experiments, our lab collects *ex vivo* lung tissue from patients with COPD or controls to investigate gene and protein expression.

Specifically, lung tissue is obtained from patients undergoing lobectomy or pneumectomy for mostly lung tumors. Patients are defined as having COPD when the post-bronchodilator FEV_1/FVC ratio is below 0.70. All patients with COPD have stable disease since patients with recent respiratory tract infections and exacerbations are excluded. Other exclusion criteria are receiving chemotherapy, radiotherapy or diagnosis of asthma and mesothelioma. Patients are considered ex-smokers when they quitted smoking for more than 1 year. Our study is approved by the medical ethics committee of the Ghent University Hospital and only subjects that provide written informed consent are included.

Lung tissue is chosen by the pathologist as far from the lesion as possible. To obtain maximal information from this tissue, a part is sampled for immunohistochemistry (**see 3.6.3**) and ISH (**see 3.6.4**), and a part is sampled for RNA extraction (and subsequent RT-qPCR) (**see 3.6.1**).

3.5 miRNA research

3.5.1 miRNA detection and quantification

miRNA research starts with the detection of (mature and/or precursor) miRNAs [366]. To ensure large-scale parallel detection of mature and/or precursor miRNA in collected cells or tissues, the chosen platform (Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) [367], (microarray) hybridization [368] or next generation sequencing (NGS) [369]) will depend on the research question and experimental set-up [370]. Different platforms have been tested in terms of reproducibility, sensitivity, specificity, accuracy and concordance of differential expression in the miRQC study [371]. Overall, substantial interplatform differences were noted (**Table 1**).

Firstly, **microarray** platforms allow detection of miRNA expression by hybridization of the miRNAs in a sample with pre-designed labeled probes, complementary to the miRNA sequences. Microarrays rather serve to report a relative change in expression such as in a diseased versus a non-diseased state, or to determine the presence of a certain miRNA, followed by a validation, usually by RT-qPCR. Microarray platforms can vary in surface chemistry, probe design, labeling techniques and input sample requirements. Microarrays are usually designed to detect the canonical miRNA. The probe design (variability in melting temperatures) and detection stringency largely define how well a distinction can be made between family members and isoforms [372]. A limitation of the technique is that a false negative result remains possible when the hybridization signal doesn't exceed the background noise [373]. It is also important to consider that some microarray platforms do not include precursor probe sets or that the detection of miRNA precursors can interfere with the detection of mature miRNAs [372]. In addition, preferential hybridization of certain sequences remains an issue.

Secondly, to detect relative abundancies of mature miRNAs with great sensitivity, especially for a low amount of input RNA, **qPCR** platforms generally perform better (**see 3.6.1**) [374]. Briefly, RT-qPCR is a technique used to detect, in this case, specific miRNAs in a sample. Therefore, RNA is reverse transcribed into complement DNA (cDNA) which will then undergo a sequence-specific amplification. Subsequently, the amplified product is detected and quantified. This technology can also be applied to pre-miRNA and pri-miRNA profiling. To generate cDNA, a unique sequence-specific primer can be used or a universal tailing primer. Although faster and easier to use, the latter can induce false positive results. To enable a simple RT step with higher sensitivity, a mixture of sequence-specific stem-loop primers was introduced by Applied Biosystems. qPCR efficiency can be affected by sample concentration, degradation or nonspecific amplification products, and presence of PCR inhibitors or enhancers. Ideally, the efficiency is 100%, corresponding to a doubling of the PCR product during each cycle.

Thirdly, to discover new miRNAs and to detect isoforms, small RNA **sequencing** is the platform of choice, as probe-based methodologies can only detect previously known sequences [372]. As miRNAs are directly sequenced, sequence variations or information on posttranscriptional RNA editing becomes available as well. Another advantage is that precursors, as well as primary and mature miRNAs can be identified in a high-throughput mode by NGS. However, NGS is rather laborious and expensive, and technical bias can be introduced during ligation and amplification. Also, handling sequencing data requires several analysis steps (read mapping, counts computation, counts normalization and differential expression analysis) and bears substantial bioinformatic challenges [375]. Challenges in analyzing RNA-seq data are often introduced by low sample sizes (due to the high cost) and based on the direction of the differential expression, presence of outliers and the degree of overdispersion. Awareness of these characteristics will result in a balanced and informed choice of the test used for differential expression analysis such as DESeq and edgeR [376]. Nevertheless, each platform has its strengths and weaknesses [377].

	qPCR	**Microarray**	**Sequencing**
Time (including data analysis)	< 6 hours	± 2 days	< 1 weeks
Sample input (total RNA)	50-500 ng	100-1000ng	500-5000ng
Estimated cost	++	+	++(+)
Dynamic range	6 orders of magnitude	4 orders of magnitude	5 or more orders of magnitude
Infrastructure and technical requirements	+	++	+++

Table 1. Platform comparison for microRNA profiling [245,370,371]

Although fairly time-consuming, **northern blotting** enables visualization of the mature as well as the precursor miRNA. Both radioactive and non-radioactive labeled miRNA probes can be used. However, cross-reactivity between related sequences can occur due to lower specificity. Practically, northern blotting is used to provide evidence of the sequestration of the miRNA by an anti-miR. Another technically challenging tool to visualize tissue- or cell-specific miRNA expression patterns in cryosections or paraffin-embedded tissue is **ISH** (**see 3.6.4**) [366,378].

Since 2002, miRBase is the reference database for miRNA annotation. Importantly, miRNA annotation has evolved over time, due to revisiting insights in miRNA biology followed by adaptations in miRBase. As a result, the same miRNAs were sometimes named differently throughout many publications over the last decade. To avoid misinterpreting research results, miRBase tracker has been developed, an

online database (http://mellfire.ugent.be/public/miRBaseTracker/) that enables finding historical and current miRNA annotations [379]. Most research nowadays still centers on the canonical miRNA with a well-defined annotation in miRBase and clear-cut detection assays. However, much information might be missed because in several diseases the biogenesis might favor a certain isomiR over the canonical miRNA. Importantly, miRNA profiling results can be drastically affected by small sequence variations in miRNAs. Further, the stability of the miRNA can be altered by modifications, protein complex formation and by exposure to nucleases.

3.5.2 miRNA target genes

The initial step in keeping track of the biological relevance of miRNAs, is identification of the target genes. **Target prediction databases** facilitate the search for likely miRNA targets based on sequence complementarity (sometimes allowing seed G-U wobbles and mismatches), thermodynamics, absence of complicated secondary structures, evolutionary conservation, location in 3'UTR, local A-U content and experimental validation. Mostly, a combination of factors is taken into account. Examples of target prediction sites are miRDB and Targetscan. A few years back, the miRNA body map, a free accessible web-based tool (http://mellfire.ugent.be/public/body_map/), was designed to facilitate the selection of tissue-specific miRNA targets by integrating data from 8 target prediction databases (thereby augmenting the specificity), data from corresponding miRNA and mRNA gene expression, and mechanistic models for gene network regulation [302]. An extra asset to miRNA target predictions are databases that show an update of validated target genes, such as miRWalk and miRTarBase [380]. With their slightly distinct sequence, isomiRs can affect other targets, requiring target prediction databases based on the isomiR/miRNA sequence and specific isomiR detection assays [244,381].

Second, it is important that both the miRNA and its target must be co-expressed under the same conditions in the cell type or tissue of interest, making the *in silico* predictions physiologically relevant. Third, to find out whether a certain gene of interest is subject to miRNA regulation, wet-lab methods are developed to ascertain biological miRNA binding such as a luciferase reporter assay, a miRNAome-wide 3'UTR miRNA library screen or the introduction of a Target Site Blocker (Exiqon) or a Target Protector (Qiagen). These techniques ensure validation of the **miRNA-target interaction**, although often not under physiological circumstances. Alternatively, identification of miRNA-bound targets in a cell- and context-dependent way can be obtained by biochemical pulldown assays such as high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP). HITS-CLIP enables identification of RNAs in RISC after immunoprecipitation of participating proteins, usually AGO2, and subsequent sequencing of the miRNA and the fragments of the target sites derived from the mRNAs [382]. Another method, cross-linking ligation and sequencing of hybrids (CLASH), generates

cross-linked miRNA-target site chimeras, followed by sequencing. Minor drawbacks are the relatively low efficiency of hybrid capture and the inability to provide quantitative information concerning the strength of the interactions [266]. Recently, a new approach has been developed, i.e. miR-CLIP. This is a biochemically pulldown assay of a specific miRNA and associated mRNA targets followed by sequencing. Additionally, the miR-CLIP capture technique can also identify novel miRNA-lncRNA interactions [383]. The recently updated databases miRTarBase and starBase have a repository of CLIP-seq data involving miRNAs [380,384]. On the other hand, an experimental approach has been developed starting from a specific mRNA. This approach detects the attached miRNAs using a capture affinity assay involving a biotinylated DNA antisense oligonucleotide, antisense to the full length of the mRNA [385].

Fourth, although technically challenging and time-consuming, is the identification of physiologically active miRNA regulatory elements (MREs) to really show that this MRE contributes to the phenotype. **Site-specific miRNA-MRE interactions** can be demonstrated *in vivo* or *in vitro* using the transcription activator-like effector nuclease (TALEN) or clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 technology which can help in discriminating between direct or indirect miRNA-mediated regulation [386].

3.5.3 miRNA function

To elucidate the **function** of a miRNA, several strategies for gain- and loss-of-function studies have been developed. Functional validation of miRNAs can be performed by overexpressing or inhibiting the miRNA *in vitro* or *in vivo* (**see 3.6.6**). Genetically modified mice can exhibit inducible or constitutive overexpression of a miRNA, or are deficient for a certain miRNA. To affect most miRNAs, conditional deletion of Dicer (or another miRNA processing enzyme) leads to an overall down-regulation of miRNA expression.

For specific miRNA inhibition, miRNA sponges, miRNA erasers or chemically modified anti-miRNAs can be used. Overexpressing miRNAs can be addressed by a viral vector, a plasmid containing a constitutive promoter to overexpress a pri-miRNA or a chemically synthesized precursor. To increase (targeted) delivery and stability, the miRNA mimic is often encapsulated. In case of *in vitro* transfections, these experiments should ideally be performed in the cell type of interest. Detection of the *in vivo* distribution of an anti-miR or mimic can be performed through ISH, northern blotting or radioactive labeling. To find key regulatory miRNAs in a disease-specific context, genome-wide functional screens were developed using miRNA mimics or inhibitors [387]. Detection of candidate target mRNA or protein, for instance after miRNA perturbation, can be obtained by gene expression microarray (**see 3.6.1**) or stable isotope labeling using amino acids in cell culture (SILAC), respectively.

As such, interesting insights can be obtained by combining the differential miRNA expression with gene expression (e.g. gene set enrichment analysis, **see 3.6.2**) or protein expression data. The resulting change in phenotype, perturbation of pathways or the shift in the transcriptome or proteome can then be attributed to the direct or indirect effect of the miRNA. Overall, gain- and loss-of-function studies are an interesting tool to shed light on the biological function of the miRNA, although often supra-physiological levels are reached.

3.6 Experimental techniques used in this dissertation

3.6.1 Measurement of mRNA and miRNA expression

Currently, qPCR is considered the gold standard for **single** gene expression measurement.

The workflow of RT-qPCR in this thesis is described briefly. RNA from lung tissue or cells is extracted using the miRNeasy Mini kit (Qiagen) and cDNA is prepared using the miScript II RT kit (Qiagen) according to the manufacturer's instructions (**Figure 21**). The expression of the gene of interest is detected using TaqMan Gene Expression assays (Applied Biosystems). All reactions are set up in duplicate and a 2-step PCR approach was used. The amplification conditions are 10 minutes at 95°C and 50 cycles of 95°C for 10 seconds followed by 15 seconds at 60°C, using the LightCycler 96 system (Roche). A standard curve derived from serial dilutions of pooled samples is generated and the expression of the gene of interest is normalized to at least 3 stably expressed reference genes [388].

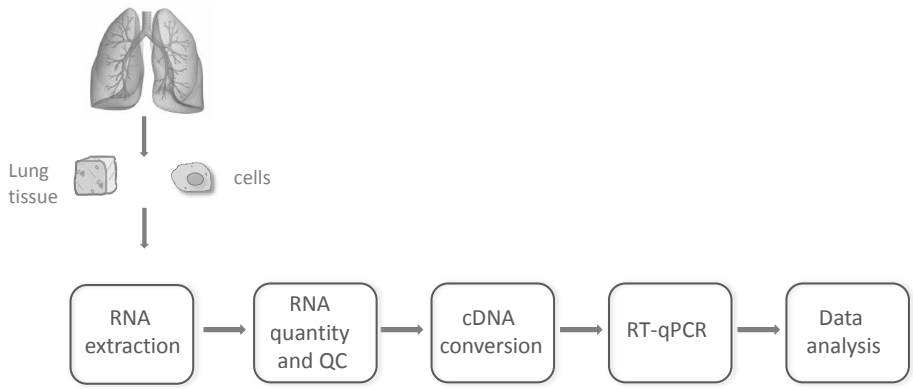


Figure 21. workflow of RT-qPCR. Starting material for RT-qPCR is good quality RNA, either from lung tissue or cells, that is converted into cDNA, followed by qPCR and data analysis. QC: quality control; cDNA: complement DNA; RT-qPCR: reverse transcription-quantitative polymerase chain reaction

Gene expression profiling enables simultaneous comparison of expression levels of many genes between two or more sample types. Whole transcriptome or large-scale gene expression profiling can be performed with NGS, RT-qPCR or microarray. Currently, RNA sequencing is the method of choice. In this dissertation, mRNA expression is quantified using a microarray and NGS. On human lung, mRNA is quantified by applying a custom microarray based on the Agilent Sureprint 8x60K platform. The RNA is labeled using the Agilent's Low Input Quick Amp Labeling Kit and hybridized to the custom array. After scanning, the raw intensities are background corrected and quantile normalized using the limma package in R. On RNA from transfected NHBE cells, we performed RNA sequencing. For this, RNA

libraries are prepared using the TruSeq stranded mRNA library prep kit (Illumina) according to the manufacturer's instructions. Next, amplified cDNA libraries are sequenced (single end 75 bp reads) on a NextSeq 500 instrument (Illumina) generating an average of 20 million reads per sample. Reads are then mapped and gene expression is quantified using the RNA Express app (Illumina Basespace). Differentially expressed genes are identified using DESeq2 in R. Among the negative binomial tests, DESeq generally is a conservative test method, even in the presence of outliers, except when sample sizes are extremely small [376].

For **miRNA detection**, there are two priming methods used among commercially available qPCR-based platforms. Some use unique, sequence-specific RT primers (e.g. TaqMan, Applied Biosystems) while others make cDNA using universal tailing primers (e.g. miScript, Qiagen). The miScript system adds a poly-A tail to all miRNAs, followed by reverse transcription and addition of an adapter to the 3' end. Amplification of specific miRNAs occurs by using a forward primer specific to the miRNA, and a reverse (universal) primer complementary to the 3' adapter. For detection, SYBR Green can be used which binds to double-stranded DNA. The TaqMan system is more specific since a miRNA-specific stem-loop reverse transcription is followed by qPCR with 2 specific primers for each miRNA complemented with a TaqMan probe. The TaqMan probe which bears a reporter and a quencher dye hybridizes to the miRNA sequence. Amplification of the target sequence separates the fluorophore from a quencher fluorophore, generating the fluorescent detection signal.
A major challenge in miRNA research is proper normalization. Ideally, standard reference miRNA genes should be used [388]. Since none are available, stably expressed (mi)RNAs must be found in the tissue or cells of interest. Most researchers use genes for small RNAs that might not be transcribed by the same RNA polymerase as the miRNA precursors, and are thus less representative of general miRNA regulation. Another proper normalization method can be chosen based on the mean expression of miRNAs in each or all samples when many miRNA expression profiles are generated [389,390]. The workflow of miRNA detection with RT-qPCR is similar to detecting gene expression (**Figure 21**). Enrichment for small RNAs is preferable since most of the RNA consists of ribosomal RNA. Also here, an effort should be done for proper data reporting according to the MIQE guidelines [391].

The **miRNA profiling** mentioned in this dissertation is based on the RT-qPCR technology using stem-loop primers and a pre-amplification of the cDNA [374,392]. A volcano plot or a heatmap are visually attractive tools to present miRNA (or mRNA, lncRNA,…) profiling data (**Figure 22**).

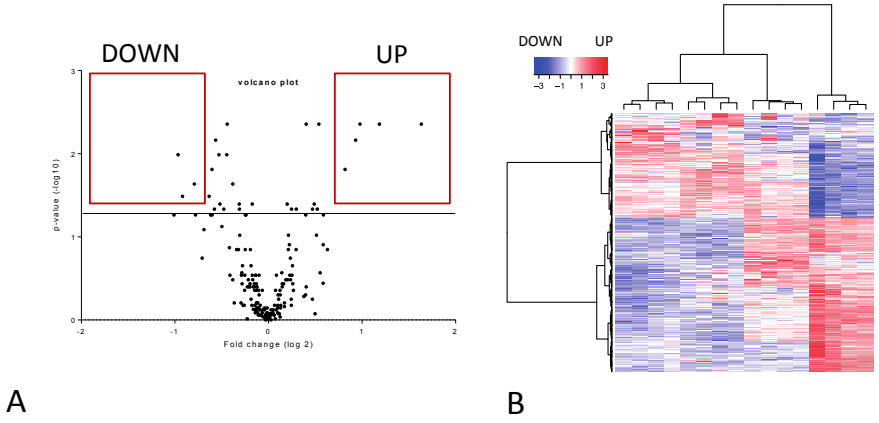


Figure 22. Volcano plot (A) and heatmap (B). (A) In the volcano plot, the data are represented as dots with in the X-axis, the fold change (log2) and in the Y-axis, the –log 10 value of the significance level. This means that the most interesting dots (significant and differentially expressed between the 2 conditions) lay in the red squares, above the 0.05 significance level (horizontal line). (B) In the heatmap, a clear clustering of patient samples can be observed. Each rows represents a gene and each column indicates a patient sample. Blue color indicates a reduced expression while the red color indicates a higher expression.

3.6.2 Gene set enrichment analysis (GSEA)

GSEA is an interesting bioinformatics tool to investigate functional enrichment of mRNA expression data. This tool can be used to infer putative biological pathways or functions for an identified miRNA through a guilt-by-association approach. Therefore, mRNA microarray expression data were used to create a list of spearman rank correlation coefficients for each mRNA with the miRNA of interest, generating a ranked list of mRNAs based on the correlation coefficient. (However, mRNA data can also be ranked via fold change, etcetera.) Genes that share a common biological function (Gene Ontology – Biological Process gene sets, downloaded from the Molecular Signatures Database v4.0, Broad Institute) or that are entangled within the same biological pathways (Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets) are grouped in gene sets. By correlating miRNA expression data with transcriptome data, positive or negative enrichment (if the majority of genes from a gene set appear on the top or on the bottom of the ranked list, respectively) can be detected for certain pathways or biological processes, suggesting a high likelihood of the miRNA being involved in these pathways or processes, built on the guilt-by-association principle [393]. Thus, the goal of GSEA is to determine whether a set of genes involved in a given pathway or biological process is found at the top or bottom of the ranked list of mRNA expression data, indicating a putative association, rather than being distributed randomly across the list. This results in an enrichment score, which reflects the degree to which a gene

set is overrepresented at the top or the bottom of a ranked list of genes. The leading edge subset of genes from this gene set are the genes that contribute most to the enrichment (**Figure 23**).

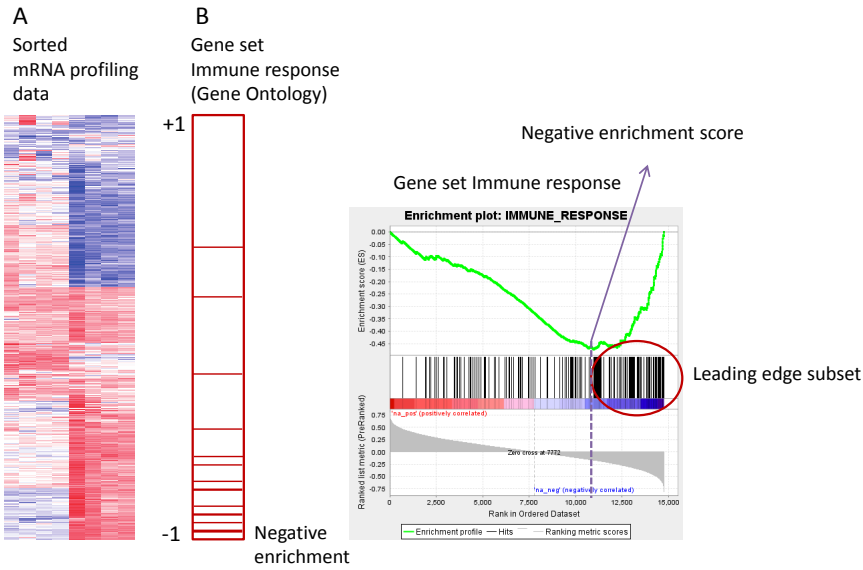


Figure 23. Principle of GSEA. A) Gene expression data set of 8 samples (in columns). The genes within this data set are shown in the rows. Red indicates higher expression and blue indicates lower expression. These genes will then be ranked according to the correlation with, for instance, the expression of a miRNA within the same samples. B) negative enrichment for the gene set 'immune response' is observed, with the red lines corresponding to genes within this gene set. Biological interpretation of this result means that there is enrichment of immune response genes that are negatively correlated to the miRNA expression in these samples. For instance, a lower expression of the miRNA is correlated with a higher expression of genes involved in the immune response.

3.6.3 Measurement of protein expression

ELISA enables quantification of protein levels in lung tissue, BAL or cell supernatant and is based on the detection of an antigen by an antibody followed by an enzyme-induced colorimetric reaction. Commercially available kits are used in this thesis and ELISAs are performed according to the manufacturer's instructions.

For tissue- or cell-specific information on protein expression in lungs, immunohistochemistry is an ideal technique. Starting from formalin fixated paraffin embedded (FFPE) tissue or cryosections, the tissue is pretreated and incubated with an antigen-specific antibody, finally resulting in a staining reaction complemented with a background staining and compared to an isotype control.

3.6.4 *in situ* hybridization (ISH)

Recent advances in ISH allow sensitive detection of miRNAs in heterogeneous tissues, defining cellular miRNA localization [394,395].

To guarantee RNase free conditions, all glassware must be baked for minimum 8h at 180°C, all other materials cleaned with RNase ZAP (Ambion, Life technologies) and all solutions must be RNAse free.

For miRNA detection in FFPE tissue, 6µm thick lung tissue is attached to slides and dried on a heating plate. After deparaffinization, lung tissue is pretreated with proteinase K (allowing access of the detection probe) and hybridized with a 5' and 3' digoxigenin (DIG)-labeled miRCURY LNA Detection probe (Exiqon). Multiple washes according to a stringency gradient at hybridization temperature ensures removal of not adhered excess probe. Detection of the probe can be achieved in different ways leading to a different staining color. First, miRNA detection can be carried out with sheep anti-DIG-Alkaline phosphatase (AP) using Nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT/BCIP) as a substrate leading to a blue color. Nuclear fast red serves as counterstaining which stains the nuclei red. Second, miRNA detection can be carried out with a goat anti-DIG antibody followed by incubation with anti-goat-Horseradish peroxidase (HRP), using 3,3'-diaminobenzidine (DAB) as a substrate leading to a brown color. Hematoxylin serves then as counterstaining which stains the tissue purple. When an antibody conjugated to HRP is used, an endogenous peroxidase removal step is added to the pretreatment. Omitting of the probe or an LNA scrambled probe serves as negative control and the LNA U6 small nuclear RNA probe serves as positive control (staining of the nuclei)(**Figure 24**).

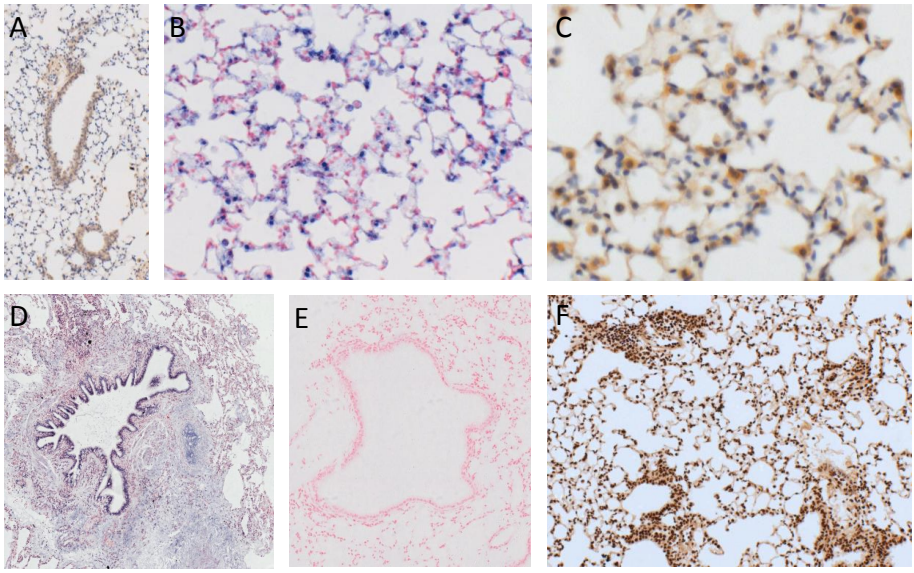


Figure 24. *in situ* hybridization for (A) miR-21-5p, (B-C) miR-135b-5p, (D) miR-218-5p, (E) scrambled probe (negative control), (F) U6 snRNA (positive control). B,D and E were stained with NBT/BCIP whereas A, C and F were stained with DAB. A-C, E-F represents staining on murine lung tissue while D represents staining on human lung tissue.

3.6.5 Flow cytometry

Flow cytometry is used to **enumerate or sort** different cell populations present in a single cell suspension. This technique makes separation of different cells possible based on cell-specific characteristics (size, granularity, antigens). Cells are labeled with (a combination of) antibodies, specific for antigens of interest, attached to different fluorophores. The fluidics system of the flow cytometer transports these cells in a stream to the lasers. The fluorophore on cells positive for this antigen absorb the light emitted by the lasers, transmit a fluorescent signal which is then measured by the detectors of the flow cytometer. Each fluorophore has a characteristic peak excitation and emission wavelength. Acquisition of these data allows quantification and identification of cells according to a gating strategy, using the FlowJo software (Tree Star Inc.).

3.6.5 *In vitro* and *in vivo* perturbation of miRNA levels

In this dissertation, a double stranded and chemically modified miRNA mimic is transfected ***in vitro*** to submerged NHBE cells using Lipofectamine RNAiMAX in comparison to a scrambled control, mock (only transfection reagents) and medium according to the manufacturer's instructions. Transfection efficiency as well as cell survival is assessed (**Figure 25**). Serum- and antibiotics-free conditions are maintained during transfection.

Intriguingly, investigating the influence of cell confluence on miRNA expression, the Mendell group showed that miRNA biogenesis is globally activated with increasing cellular density, leading to a stronger repression of target mRNAs [396].

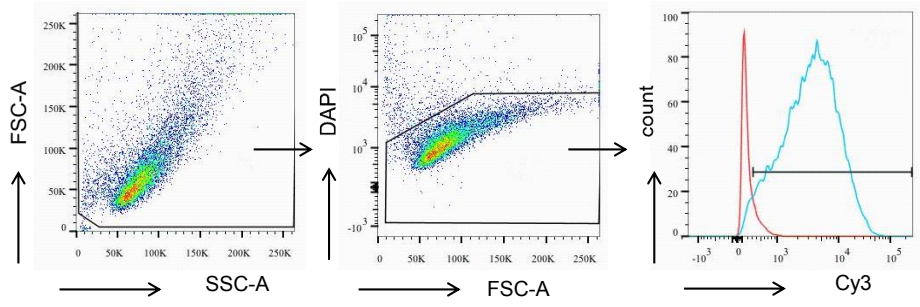


Figure 25. Evaluation of survival and transfection efficiency using flow cytometry. Trypsinized NHBE cells transfected with a Cy3-labeled scrambled sequence were analyzed by flow cytometry. 4',6-diamidino-2-phenylindole (DAPI) is a blue fluorescent nucleic acid stain that binds to double stranded DNA of dead cells and

is used as a viability marker. Viability was over 90%. After gating for the living cells, the transfection efficiency was evaluated. Uptake of the Cy3-labeled scrambled oligonucleotide (blue line) was compared with non-transfected cells (red line) and transfection efficiency was over 90%.

miRNA inhibition is also established ***in vivo*** by intranasal administration of an LNA-modified anti-miRNA probe to air- or CS-exposed mice, compared to a scrambled probe and PBS (solvent). A dose of 10mg/kg is administered twice during the 5 day exposure period. Cell populations in BAL and lung can then be investigated, as well as gene and protein expression.

II. RESEARCH WORK

CHAPTER 4: Research objectives

RESEARCH OBJECTIVES

COPD is hallmarked by an exaggerated inflammatory response in the lungs following inhalation of noxious particles or gases of which CS is the most important inciting agent. In this thesis, we focus on small regulatory RNA molecules, miRNAs, that recently emerged as important post-transcriptional gene regulators and that are implicated in many inflammatory signaling cascades. However, little is known about their impact in the disease COPD. The ultimate goal of this thesis is to put forward one or more miRNAs that can serve as a possible therapeutic target for this devastating disease.

Research question 1: which miRNAs are differentially expressed upon CS exposure and in COPD?

First, we aimed to **identify** aberrantly expressed miRNAs in **lungs** of **COPD patients** and in **lungs and BAL supernatant** of CS-exposed **mice** by performing miRNA profiling. Moreover, miRNAs are often highly conserved, enabling us to use a translational approach.
By concentrating on 2 respiratory relevant compartments (BAL and lung) in mice in association with data on inflammatory cell subsets, we highlight some miRNAs that may be of importance in the CS-induced inflammation and likely also in COPD.

Research question 2: what is the expression profile, localization and functional role of miR-218-5p in CS-induced inflammation and the pathogenesis of COPD?

Starting from the miRNA profiling data, we decided to focus on miR-218-5p. For this particular miRNA, we thoroughly investigated its expression profile, localization and functional role in the context of CS-induced inflammation and COPD.

miR-218-5p shows a high expression level in lung tissue compared to other tissue types. However, a reduced expression of this miRNA is often observed in disease. We hypothesized that miR-218-5p could be involved in the CS-induced inflammatory response in the lungs and in the pathogenesis of COPD. Therefore, we investigated the functional role of miR-218-5p in COPD *in vitro* and *in vivo*.

CHAPTER 5: microRNA profiling reveals a role for microRNA-218-5p in the pathogenesis of COPD

Dysregulation of microRNAs (miRNAs), critical post-transcriptional negative regulators of gene expression, contributes to disease pathogenesis. The role of miRNAs, and more specifically of miR-218-5p, in the pathogenesis of COPD is still unknown. Therefore, we aimed to thoroughly unravel the expression pattern, the localization and the functional role of miR-218-5p in this disease. We reached this purpose by analyzing different patient cohorts and murine samples, by including *in vitro* expression and transfection data and by performing an *in vivo* perturbation experiment, all this complemented with *in silico* bio-informatics analyses.

Conickx G, Mestdagh P, Avila Cobos F, Verhamme FM, Maes T, Vanaudenaerde BM, Seys LJ, Lahousse L, Kim RY, Hsu AC, Wark PA, Hansbro PM, Joos GF, Vandesompele J, Bracke KR, Brusselle GG.
MicroRNA Profiling Reveals a Role for MicroRNA-218-5p in the Pathogenesis of Chronic Obstructive Pulmonary Disease.
Am J Respir Crit Care Med 2017; 195: 43-56. [154]

Conickx G, Mestdagh P, Vandesompele J, Brusselle GG, Bracke KR.
Reply: Direct Detection of Circulating MicroRNAs Unveiled the Absence of MicroRNA-218-5p in Smoker Subjects.
Am J Respir Crit Care Med 2017; 196: 533. [397]

ABSTRACT

Rationale: Since aberrant expression of microRNAs (miRNAs) can have a detrimental role in disease pathogenesis, we aimed to identify dysregulated miRNAs in lung tissue of patients with COPD.

Methods: We performed miRNA and mRNA profiling – using high throughput stem-loop RT-qPCR and mRNA microarray, respectively – on lung tissue of 30 patients (screening cohort) encompassing 8 never smokers, 10 smokers without airflow limitation and 12 smokers with COPD. Differential expression of microRNA-218-5p (miR-218-5p) was validated by RT-qPCR in an independent cohort of 71 patients, an *in vivo* murine model of COPD, and primary human bronchial epithelial cells (HBECs). Localization of miR-218-5p was assessed by *in situ* hybridization. *In vitro* and *in vivo* perturbation of miR-218-5p combined with RNA sequencing and gene set enrichment analysis was used to elucidate its functional role in COPD pathogenesis.

Measurements and main results: Several miRNAs were differentially expressed among the different patient groups. Interestingly, miR-218-5p was significantly down-regulated in both smokers without airflow limitation and in patients with COPD, compared to never smokers. Decreased pulmonary expression of miR-218-5p was validated in an independent validation cohort, in cigarette smoke-exposed mice and in HBECs. Importantly, expression of miR-218-5p strongly correlated with airway obstruction. Furthermore, cellular localization of miR-218-5p in human and murine lung revealed highest expression of miR-218-5p in the bronchial airway epithelium. Perturbation experiments with a miR-218-5p mimic or inhibitor demonstrated a protective role of miR-218-5p in cigarette smoke-induced inflammation and COPD.

Conclusions: We highlight a role for miR-218-5p in the pathogenesis of COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is defined by a progressive airflow limitation due to an abnormal inflammatory response of the lung to noxious particles and gases [57]. The pathology of COPD is often associated with comorbidities, making COPD a major cause of morbidity and mortality worldwide [398]. Cigarette smoking is by far the most common risk factor for COPD, besides other risk factors such as genetic susceptibility and inhalation of occupational dusts, chemicals and pollutants [6]. Several genes involved in inflammation, tissue repair, proliferation and apoptosis are dysregulated in COPD [162,163]. Gene expression can be affected by epigenetic modulators such as microRNAs (miRNAs). miRNAs are small non-coding RNAs that negatively regulate their target messenger RNA (mRNA) by binding predominantly to the 3' untranslated region (3'UTR) with their short (6-8 nt) recognition sequence (the 'seed'), resulting in mRNA degradation or inhibition of translation. In that way, miRNAs are able to fine-tune protein output in a cell-type dependent manner. Considering the fact that one single miRNA can control up to hundreds of target genes, miRNAs may be involved in diverse biological pathways [230,262,265]. Besides their role in development and maintenance under normal conditions, dysregulation of miRNAs might affect the onset or progression of several diseases, including COPD.

miRNAs have been investigated in association with COPD or smoking. Our lab has reported differential miRNA expression in induced sputum supernatant of smokers with COPD compared to smokers without airflow limitation and never smokers [153]. Down-regulation of let-7c was inversely correlated with soluble TNFR-II, a receptor implicated in COPD pathogenesis. In human bronchial airway epithelium, miRNA expression was affected by smoking with the majority of miRNAs being down-regulated in current smokers [149]. miRNA expression was also altered in lung tissue of patients with COPD and smokers, with the majority of miRNAs being upregulated in COPD [152].

Here, we aimed for a comprehensive (RT-qPCR based) approach to quantify miRNA expression in the context of COPD on a large and well-characterized patient cohort across different patient groups. Therefore, we first performed miRNA and mRNA profiling on lung tissue of 30 patients (screening cohort) consisting of smokers with COPD, smokers without airflow limitation and never smokers. Based on the results of the miRNA profiling and previous reports in literature, we further investigated and validated the expression and localization of microRNA-218-5p (miR-218-5p) in human lung tissue as well as in lungs of mice that were exposed to air or cigarette smoke (CS) for 4 or 24 weeks. *In vitro* and *in vivo* perturbation of miR-218-5p and gene set enrichment analysis (GSEA) improved our understanding of the potential involvement of miR-218-5p in the response to CS and in COPD.

MATERIALS AND METHODS

Human lung tissue study population

In total, 101 patients participated in this study. Lung resection specimens were obtained from these 101 patients, of which 87 were from surgery for solitary pulmonary tumors (Ghent University Hospital, Ghent, Belgium) and 14 were from explant lungs of end-stage COPD patients undergoing lung transplantation (University Hospital Gasthuisberg, Leuven, Belgium). The initial screening cohort of 30 patients was divided into 3 subgroups: 8 never smokers, 10 current smokers without airflow limitation and 12 current smokers with COPD (GOLD stage II) (**Table 1**). In the validation cohort of 71 patients, we included also ex- smokers without COPD and ex-smokers with COPD (GOLD stage II and GOLD stage III- IV) (**Table 2**). Our study was approved by the medical ethics committee of the Ghent University Hospital (2011/114) and the University Hospital Gasthuisberg (S51577). All subjects provided written informed consent.

Table 1. Characteristics of the screening cohort.

	Never smokers	Smokers	COPD
Number	8	10	12
Gender (male/female)	2/6 ‡	7/3 ‡	12/0 ‡
Age (years)	60 (40-70)	58 (51-65)	64 (55-69)
BMI	25 (23-28)	23 (20-26)	24 (21-26)
Current-smoker/ex-smoker	-	10/0	12/0
Smoking history (pack years)	0 (0-0)	34 (22-46)*	45 (40-71)* †
FEV_1 post-bronchodilator (L)	2.6 (2.3-3.7)	3.1 (2.7-3.4)	2.2 (1.9-2.6) †
FEV_1 post-bronchodilator (% predicted)	108 (92-120)	100 (93-114)	72 (65-74)* †
FEV_1/FVC post-bronchodilator (%)	77 (75-79)	76 (73-81)	56 (45-57)* †
ICS (yes/no)	0/8 ‡	0/10 ‡	6/6 ‡
Lung resection for lung cancer (yes/no)	8/0	10/0	12/0

Footnote

Abbreviations: FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; ICS: inhaled corticosteroids.

Data are presented as median (IQR); p-values were determined by Mann-Whitney U test: * $p < 0.05$ versus never smokers; † $p < 0.05$ versus smokers or Fisher's exact test: ‡ $p < 0.001$

Lung tissue was collected at maximum distance of the tumor.

Cigarette smoke exposed mice

C57BL/6 mice were exposed whole body to CS, as described previously [360].

miRNA expression profiling and analysis (screening cohort)

To characterize the miRNA expression of 740 miRNAs and 15 endogenous controls in lung tissue of the screening cohort, RNA was extracted and reverse transcribed (using miRNA specific stem-loop primers) followed by a pre-amplification step and qPCR quantification (TaqMan miRNA assays). For generating the heatmap (Figure 2), differentially expressed miRNAs across the 3 groups were identified using a Kruskal-Wallis test (p-value < 0.05) and were hierarchically clustered using clustering method 'Ward'

and distance 'Manhattan'. Multiple testing correction was performed using the Benjamini-Hochberg algorithm (adj. p-value < 0.05). To identify differentially expressed miRNAs between 2 patients groups, an ANOVA test was chosen followed by the Tukey Post Hoc Test (adj. p-value < 0.05).

miRNA-218-5p expression analysis by RT-qPCR

miRNA expression was assessed with the Qiagen miScript PCR system. RNA was converted to cDNA using the miScript II RT kit and cDNA was amplified according to the miScript PCR protocol for miR-218-5p.

mRNA profiling (screening cohort)

For mRNA quantification on human lung (screening cohort), we applied a custom microarray based on the Agilent Sureprint 8x60K platform.

In situ hybridization

6µm thick formalin fixed and paraffin embedded lung tissue was attached to superfrost plus slides (Thermo scientific Menzel gläzer) and dried on a heating plate at 57°C. Every handling was performed in order to guarantee RNase free conditions. All glassware was baked for minimum 8h at 180°C, all other materials were cleaned with RNase ZAP (Ambion, Life technologies) and all solutions were RNAse free. Protocol was performed according to the manufacturer's guidelines (Exiqon, Denmark). Briefly, after deparaffinization, lung tissue was pretreated with proteinase K (5µg/ml for mouse lung, 15µg/ml for human lung) and hybridized with 5' and 3' DIG-labeled miRCURY LNA Detection probes. Detection of the probe was carried out with sheep anti-DIG-AP (Roche Diagnostics) using NBT/BCIP (Roche Diagnostics) as a substrate. Nuclear fast red (Sigma) served as counterstaining. For miR-218-5p, the hsa-miR-218-5p probe (18111-15, Exiqon, Denmark) was used. Omitting of the probe or an LNA scrambled probe served as a negative control and the LNA U6 small nuclear RNA probe served as a positive control.

Human bronchial biopsy study population

Bronchial biopsies from 19 patients, consisting of 9 never smokers and 10 patients with COPD (GOLD stage I and II), were obtained during bronchoscopy (John Hunter Hospital, Newcastle, Australia) (**Table S3**). None of the patients were diagnosed with lung cancer.

Human bronchial epithelial cells (HBECs)

Primary cells were obtained by enzymatic digestion from lung resection specimens of 5 anonymous donors. At passage 2, the cells were cultured on an air-liquid interface and after 14 days of culture, the cells were exposed to air (control) or CS as described previously [399]. Twenty-four hours after the exposure, RNA was harvested.

In vitro perturbation of miR-218-5p in normal human bronchial epithelial (NHBE) cells

NHBE cells (CC-2540, Lonza) were cultured in BEGM (CC-3170, Lonza) according to the manufacturers' guidelines. At passage 2, cells were seeded at 30-60% confluency in 6-well plates and 24h later, cells were transfected with 30nM of the mirVana miR-218-5p mimic (4464066, Life technologies) or the negative scrambled control (4464058, Life technologies) using Lipofectamine RNAiMAX (Invitrogen) or a lipofectamine control (mock). Culture medium was refreshed after 16 hours. Then, survival and transfection efficiency was confirmed and 24h after transfection (Fig. S7 A), medium with or without cigarette smoke extract (CSE) at a final concentration of 2.5% was added. At 48h after transfection, supernatant and RNA were harvested. RNA was used for either RNA-sequencing and subsequent analysis (Illumina) or for cDNA synthesis (miScript II RT kit, Qiagen) followed by RT-qPCR in duplicate using the TaqMan Gene Expression Assays for IGFBP3, HMOX1, FKBP1A, DUSP5, IL-8 and CCL20. mRNA levels were normalized to HPRT1, GAPDH and RPL13A. Protein levels of CCL20 and IL-8 (R&D systems, Minneapolis, USA) were measured in the supernatant by ELISA according to the manufacturer's instructions. Data were analyzed with an ANOVA test followed by the Tukey post hoc test.

Cigarette smoke extract (CSE)

To prepare CSE, the smoke of 10 3R4F reference cigarettes (without filter, University of Kentucky, Lexington, KY) was bubbled through 30ml of Opti-MEM (Thermo Fisher Scientific). The resulting suspension was filter-sterilized and considered to be 100%. This solution was then diluted with BEGM medium to a final concentration of 2.5%.

RNA-sequencing

RNA sequencing (Illumina) was performed on RNA from NHBE cells transfected with scrambled control (n=4) or miR-218-5p mimic (n=4), without CSE exposure.

In vivo perturbation of miR-218-5p in air or CS-exposed mice

miR-218-5p was inhibited in lungs of mice that were exposed to air or CS for 5 days. 10mg/kg of an LNA modified mmu-miR-218-5p inhibitor, a scrambled control or PBS (solvent) was intranasally administered on the first and the fourth exposure day, before the start of air or CS exposure. The day after the last air or CS exposure, the mice were sacrificed and bronchoalveolar lavage (BAL) fluid was investigated.

Bronchoalveolar lavage (BAL)

Via a tracheal cannula, lungs were first lavaged using 3 times 300 µl HBSS (free of Ca2+ and Mg2+ and supplemented with 1% BSA). Supernatant of this fraction was used for ELISA. Then, lungs were lavaged using 3 times 1 ml HBSS supplemented with 0.6 mM EDTA. The six lavage fractions were pooled, centrifuged, and the cell pellet was resuspended in 200 µl FACS buffer (PBS supplemented with 1 %

BSA, 5mM EDTA and 0.1 % sodium azide). Subsequently, total cell counts were obtained using a Bürker chamber and differential cell counts (on at least 400 cells) were performed on cytocentrifuged preparations after May-Grünwald-Giemsa staining. Further, BAL cells were used for flow cytometric analysis.

Flow cytometry

After FcR blockade, BAL cells were stained with the following anti-mouse antibodies: CD45-FITC (30-F11), Siglec-F-PE (E50-2440), Ly6G-PE-Cy7 (1A8), CD11c-APC (HL3), Ly6C-Alexa Fluor 700 (AL-21) and CD8-BV605 (53-6.7) from BD biosciences, CD11b-BV605 (M1/70), MHCII (I-A/I-E)-APC-Cy7 (M5/114.15.2), CD103-BV421 (2E7) and CD4-APC (GK1.5) from Biolegend and CD3-Alexa Fluor 700 (17A2) from eBiosciences. Analysis was performed on an LSR Fortessa (BD Biosciences, San Diego, USA) and data were analyzed with FlowJo software (Tree Star Inc., Ashland, USA). More in detail, after excluding doublets, the $CD45^+$ cells were retained. First, alveolar macrophages were gated out as $Siglec\ F^+$ and $CD11c^+$ cells. Of the remaining cell population, the dendritic cells (DCs) were defined as $MHCII^{hi}$ and $CD11c^{hi}$ with as subpopulations: $CD103^+$ DCs ($CD103^+$ and $CD11b^-$) and $CD11b^+$ DCs ($CD103^-$ and $CD11b^+$). After gating out the alveolar macrophages and the DCs, neutrophils ($CD11c^-$, $CD11b^+$, $Ly6C^{int}$ and $Ly6G^+$) and inflammatory monocytes ($CD11c^-$, $CD11b^+$, $Ly6G^-$ and $Ly6C^+$) were identified. From the $CD45^+$ cell population, T cells were identified as $CD3^+$ cells with the $CD8^+$ T cells and $CD4^+$ T cells as subpopulations.

In silico analysis

GSEA was performed using Gene Ontology – Biological Process and Kyoto Encyclopedia of Genes and Genomes (KEGG) gene sets [393].

Statistical analysis

The following statistical analyses were performed with R (version 3.1.1) or SPSS (SPSS Inc, Chicago, IL, USA): Kruskal-Wallis, Mann-Whitney U, Fisher's exact test, ANOVA, paired t-test, linear regression and Spearman correlation analysis. The Benjamini-Hochberg algorithm or Bonferroni correction was used for multiple testing correction. Reported values are expressed as mean ± SEM and p-values < 0.05 were considered statistically significant.

Table 2. Characteristics of the validation cohort.

	Never smokers	Current smokers	Ex-smokers	COPD II current smokers	COPD II ex-smokers	COPD III-IV ex-smokers
Number	12	8	13	12	12	14
Gender (male/female)	3/9 **	5/3 **	10/3 **	10/2 **	12/0 **	8/6 **
Age (years)	68 (63-72)	65 (56-71)	69 (59-72)	66 (58-68)	67 (63-71)	56 (54-60)* †‡ § II
BMI	27 (22-30)	24 (20-27)	27 (24-30)	20 (19-26)*	28 (25-30) §	21 (20-23)*‡ II
Smoking history (pack years)	0 (0-0)	39 (21-52)*	20 (12-60)*	45 (35-55)*	47 (30-60)*	30 (25-30)* § II
FEV_1 post-bronchodilator (L)	2.4 (2.0-3.0)	2.3 (1.7-2.4)	2.6 (2.3-3.5) †	2.1 (1.8-2.4) ‡	2.1 (1.8-2.4) *‡	0.7 (0.7-0.9)* †‡§ II
FEV_1 post-bronchodilator (% predicted)	104 (89-114)	93 (76-101)	96 (92-114)	70 (61-78)* †‡	65 (60-73)* †‡	26 (20-32)* †‡§ II
FEV_1/FVC post-bronchodilator (%)	81 (73-83)	75 (71-78)	77 (71-79)	55 (50-62)* †‡	56 (55-62)* †‡	32 (27-35)* †‡§ II
ICS (yes/no)	1/11 **	2/6 **	0/13 **	5/7 **	4/8 **	13/1 **
Lung resection for lung cancer (yes/no)	12/0 **	8/0**	12/1**	11/1**	12/0**	0/14**

Footnote

Abbreviations: FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity; ICS : use of inhaled corticosteroids.

Data are presented as median (IQR). Mann-Whitney U test: * p<0.05 vs never-smokers, † p <0.05 vs current-smokers, ‡ p <0.05 vs ex-smokers, § p <0.05 vs GOLD II current-smokers and II p <0.05 vs GOLD II ex-smokers ; Fisher's exact test: ** p <0.01

When a lesion was present, lung tissue was collected at maximum distance.

RESULTS

miRNA expression profiling in lung tissue

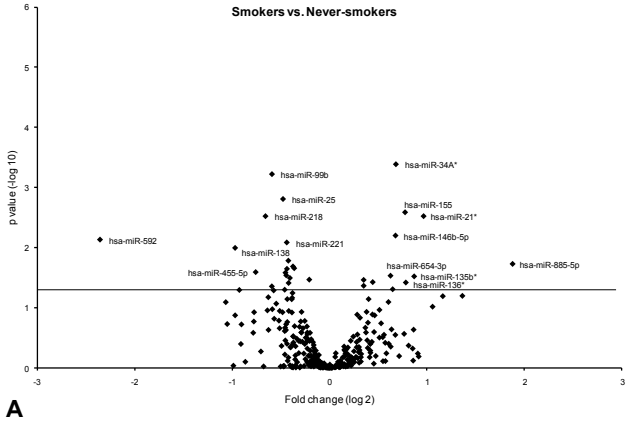
The screening cohort consisted of 8 never smokers, 10 current smokers without airflow limitation and 12 current smokers with stable COPD (GOLD stage II). Patient characteristics are shown in **Table 1**. Lung tissue of these 30 patients was used for miRNA profiling using the stem-loop RT-qPCR method [374]. Of the 740 miRNAs profiled, 377 were detected in lung tissue. 29 miRNAs were significantly differentially expressed between smokers without airflow limitation and never smokers (12 up-regulated and 17 down-regulated in smokers without airflow limitation) (**Figure 1A**). 59 miRNAs were significantly differentially expressed between patients with COPD and never smokers (15 up-regulated and 44 down-regulated in patients with COPD) (**Figure 1B**). Furthermore, 3 miRNAs showed significant differential expression between patients with COPD and smokers without airflow limitation of which 1 was up-regulated (**Figure 1C**). An overview of these differentially expressed miRNAs is represented in **Table S1**.

57 miRNAs were differentially expressed across the 3 patient groups (**Figure 2**). After correction for multiple testing, 5 miRNAs remained differentially expressed: hsa-miR-221-3p, hsa-miR-34a-3p, hsa-miR-92a-3p, hsa-miR-99b-5p and hsa-miR-218-5p (**Table 3**). Of note, a marked down-regulation of most miRNAs was observed in the COPD group (**Figure 1B & 2**).

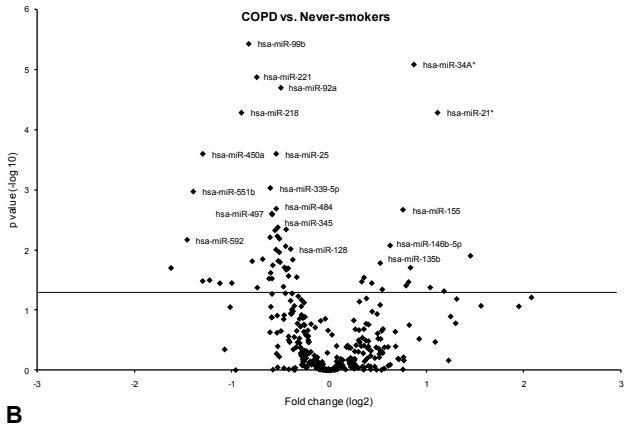
Table 3. Differentially expressed miRNAs across all patient groups

	miRBase release 21	Unadj. p-value	Adj. p-value
hsa-miR-221	hsa-miR-221-3p	0.000283	0.0356
hsa-miR-34Ax	hsa-miR-34a-3p	0.000342	0.0356
hsa-miR-92a	hsa-miR-92a-3p	0.000331	0.0356
hsa-miR-99b	hsa-miR-99b-5p	0.000415	0.0356
hsa-miR-218-5p	hsa-miR-218-5p	0.000685	0.0470

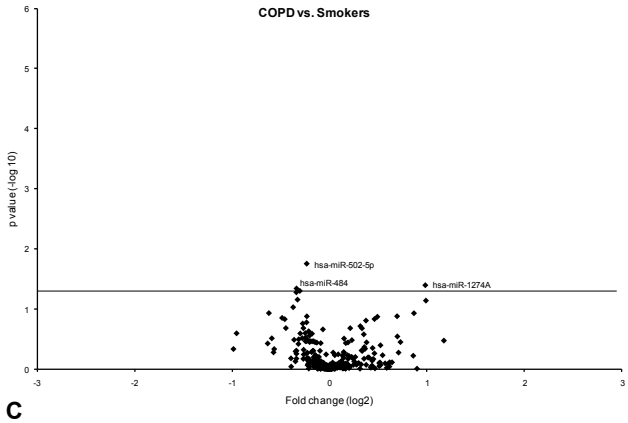
Footnote
First column: annotation of miRNA at the time of the miRNA profiling; Second column: annotation of miRNA according to miRBase release 21
Abbreviations: Unadj.: unadjusted ; Adj. p-value: p-value adjusted for multiple testing



A



B



C

Figure 1. miRNA profiling in lung tissue (screening cohort).

Volcano plots showing the differential miRNA expression (in fold change on X-axis) and significance level (-log 10 adjusted p-value on Y-axis). (A) Differential miRNA expression in smokers without airflow limitation (n=10) versus never smokers (n=8) (B) Differential miRNA expression in smokers with COPD (n=12) versus never smokers (n=8) (C) Differential miRNA expression in smokers with COPD (n=12) versus smokers without airflow limitation (n=10).

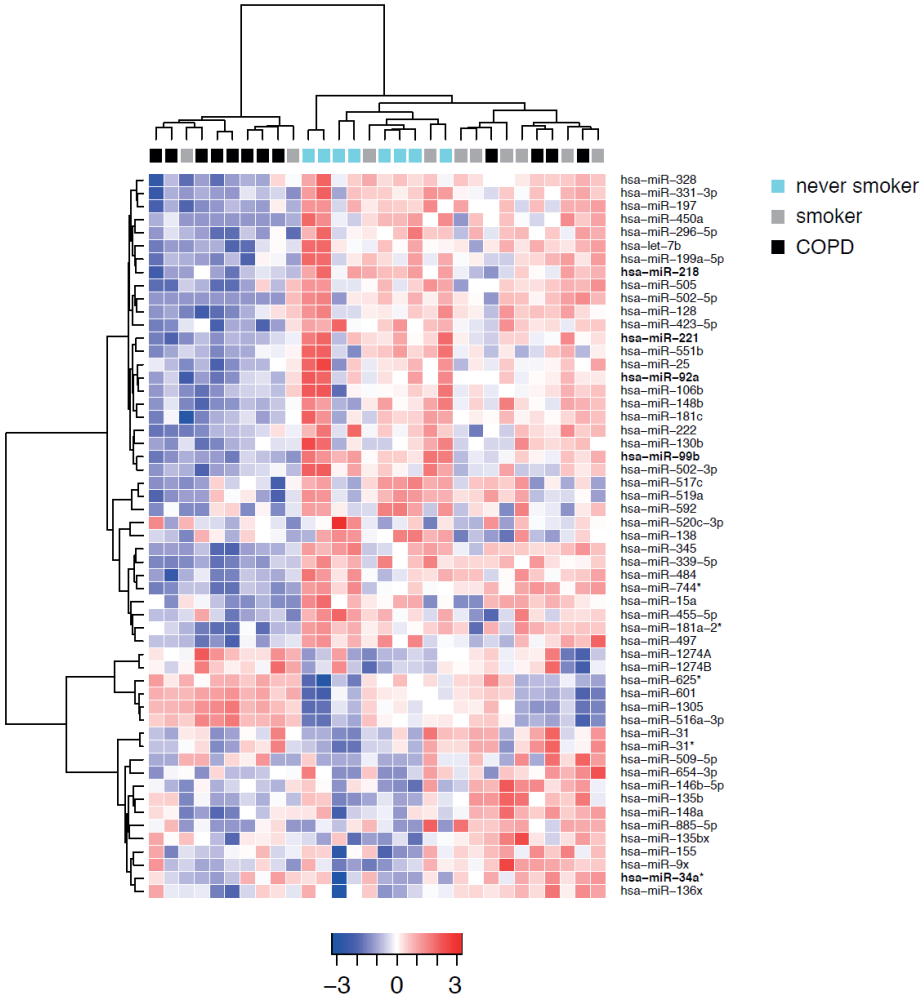


Figure 2. Hierarchical clustering of differentially expressed miRNAs in lung tissue (screening cohort) across the 3 patient groups. In this heatmap, all miRNAs are represented that are differentially expressed across the 3 patient groups. Each column represents a patient sample, each row represents a miRNA. The 5 miRNAs that remained differentially expressed after multiple testing correction (adj. $p < 0.05$) are represented in bold. The color code indicates expression level: red indicates higher expression, blue indicates lower expression.

Expression of miR-218-5p in lung tissue

We decided to focus on miR-218-5p since this miRNA showed a strong and significant down-regulation in smokers without airflow limitation (fold change=1.578, adj. p=0.003) and in patients with COPD (fold change=1.865, adj. p=5.24x10-5), compared to never smokers (**Figure S1 A**) in the screening cohort. In addition, involvement of miR-218-5p in smoking and COPD was suggested previously [34,149,153,321]. To validate the expression of miR-218-5p and to evaluate the effect of smoking cessation, we selected a

larger validation cohort that also included ex-smokers. The independent validation cohort consisted of 71 patients including 12 never smokers, 8 current smokers without COPD, 13 ex-smokers without COPD, and 38 subjects with COPD (12 current smokers with COPD GOLD stage II, 12 ex-smokers with COPD GOLD stage II and 14 ex-smokers with COPD GOLD stage III-IV). Patient characteristics are represented in **Table 2**. Similar to the screening cohort, expression analysis by RT-qPCR revealed a significant down-regulation of miR-218-5p in smokers (fold change=2.36, adj. p=0.0055) and in current smoking patients with COPD GOLD stage II (fold change=2.14, adj. p=0.0028) compared to never smokers (**Figure 3A**). In ex-smokers without airflow limitation, the expression of miR-218-5p manifested normal levels when smoking was quitted for at least 1 year (**Figure 3A**). However, in patients with COPD, the ability to reach normal miR-218-5p expression levels upon smoking cessation seemed to be hampered, especially in patients with severe COPD (**Figure 3A**). Overall, when combining all smokers without airflow limitation and all patients with COPD from the validation cohort, significant lower expression levels of miR-218-5p were observed among patients with COPD compared to never smokers and smokers without airflow limitation (**Figure S1 B**).

Correlation of pulmonary miR-218-5p expression with clinical parameters

We correlated the normalized expression of miR-218-5p in the validation cohort with parameters of disease severity i.e. % forced expiratory volume in 1 second (FEV_1) post-bronchodilator and its ratio to forced vital capacity (FVC): FEV_1/ FVC and smoking history, such as pack years. In addition, we correlated the expression of miR-218-5p with the diffusing capacity of the lungs: DLCO (carbon monoxide diffusing capacity) and KCO (DLCO/alveolar volume). miR-218-5p significantly correlated with % FEV_1 post-bronchodilator (Rs=0.576, p=2.729x10-7), FEV_1/FVC (Rs=0.604, p=1.582x10-7), DLCO (Rs=0.665, p=9.474x10-9) and KCO (Rs=0.528, p=2.9x10-5) (**Figure 3B-E**). There was only a weak correlation between miR-218-5p expression and pack years (Rs=-0.281, p=0.026) and no correlation at all when never smokers were omitted (Rs=0.015, p=0.918) (**Figure S2 E**). Importantly, the strong correlation of miR-218-5p with disease severity remained significant when never smokers were excluded (**Figure S2 A-D**) and when only patients with COPD were included (**Figure S3 A-D**). Linear regression analysis showed that severe COPD was associated with decreased miR-218-5p levels ($p < 0.001$) even after adjustment for covariates including current smoking and age (**Table S2**).

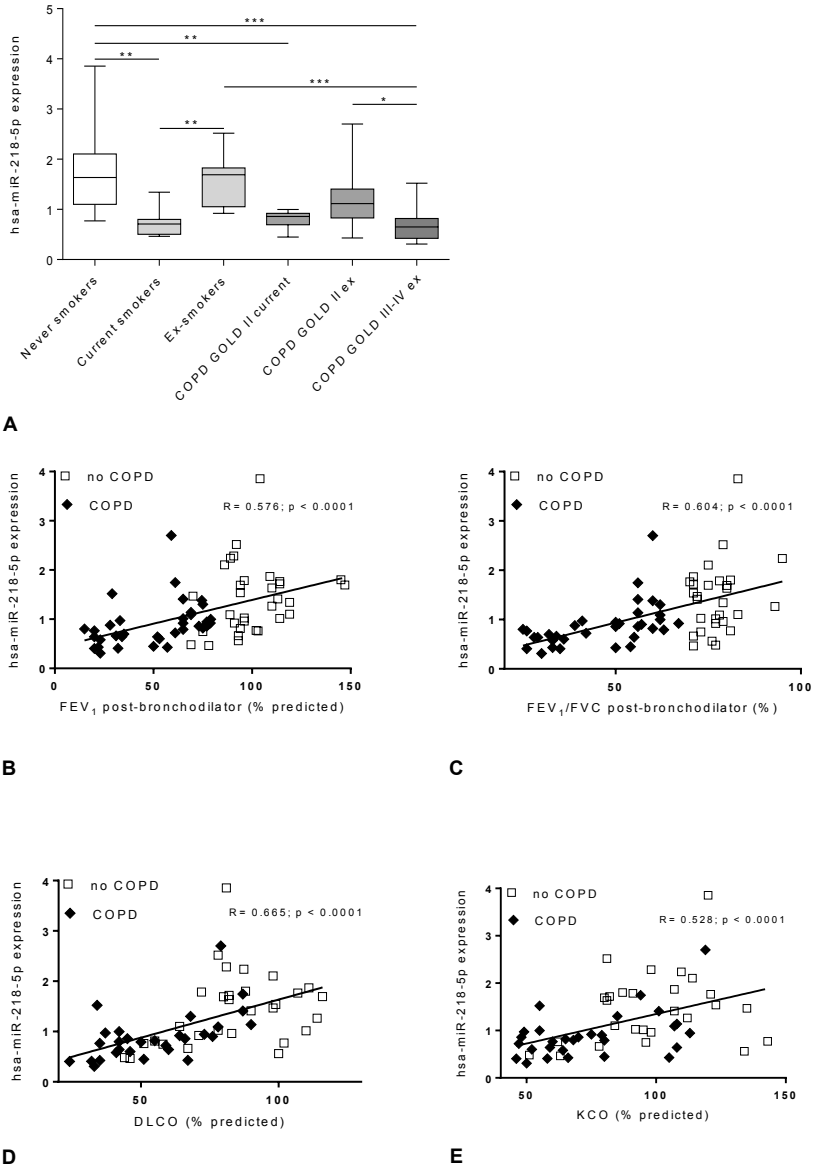


Figure 3. Expression of miR-218-5p in lung tissue (validation cohort). (A) Expression of miR-218-5p was assessed by RT-qPCR, normalized to the controls SNORD95, SNORD96A and SNORD68 and corrected for multiple comparisons with bonferroni correction. Data are expressed as normalized relative quantities. *adj. $p < 0.05$, **adj. $p < 0.01$, ***adj. $p < 0.001$. Spearman correlation between miR-218-5p and (B) % FEV_1 post-bronchodilator, (C) % FEV_1/FVC (D) DLCO and (E) KCO. Abbreviations: FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; DLCO: diffusing capacity of carbon monoxide; KCO: transfer coefficient of carbon monoxide (corrected for alveolar volume)

Expression of miR-218-5p in human bronchial biopsies

Additionally, we investigated the expression of miR-218-5p in bronchial biopsies of 9 never smokers and 10 patients with COPD GOLD stage I-II. Patient characteristics are represented in **Table S3**. In biopsies of patients with COPD GOLD stage I-II, the expression of miR-218-5p was significantly lower (0.67 ± 0.14) compared to the never smokers (2.5 ± 0.58, **Figure 4A**).

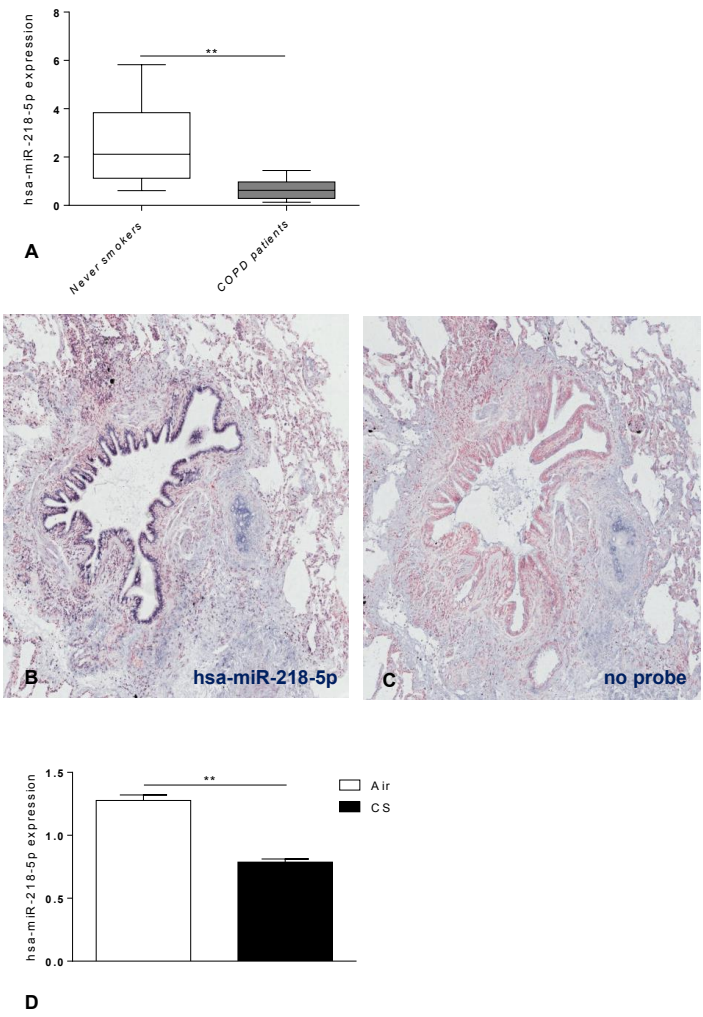
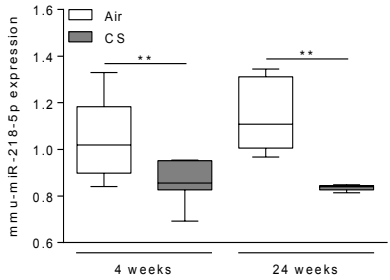


Figure 4. Expression of miR-218-5p in human bronchial epithelium. (A) Expression of miR-218-5p in human bronchial biopsies of 9 never smokers (controls) and 10 ex-smokers with COPD GOLD stage I and II assessed by RT-qPCR and normalized to the controls SNORD95, SNORD96A and SNORD68. Data are expressed as normalized relative quantities ± SEM. **adj. p < 0.01. (B) *In situ* hybridization of miR-218-5p on lung tissue of a never smoker. Blue staining indicates miR-218-5p expression. (C) *In situ* hybridization on lung tissue of the same never smoker when the miR-218-5p probe was omitted (negative control) (D) miR-218-5p expression in *in vitro* cultured HBECs (n=5) exposed to air or CS. RNA was extracted 24h after exposure. RT-qPCR was performed and the expression of miR-218-5p was normalized to the controls SNORD95, SNORD96A and SNORD68. **p < 0.01

High expression of miR-218-5p in human bronchial airway epithelium

To determine the localization of the expression of miR-218-5p in the lung, we performed *in situ* hybridization on human lung tissue (**Figure 4B-C**). Although expressed in various other cell types, highest expression of miR-218-5p was observed in the bronchial epithelium. In order to mimic the effect of CS exposure on human airway epithelium, we exposed primary HBECs, cultured on an air-liquid interface, to air or CS. We observed a significant down-regulation of miR-218-5p in HBECs 24h after CS exposure (0.79 ± 0.025), compared to air-exposed cells (1.28 ± 0.044, **Figure 4D**).



A

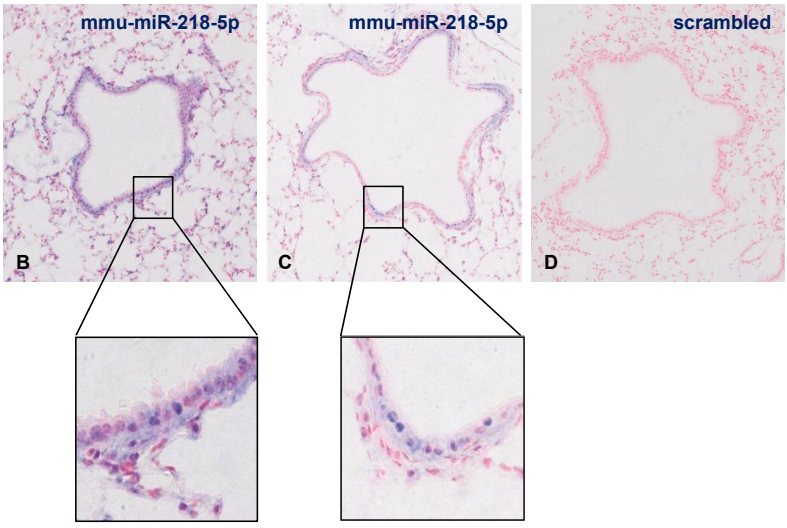


Figure 5. Expression of mmu-miR-218-5p in lungs of mice exposed to cigarette smoke. (A) Expression of mmu-miR-218-5p was assessed by RT-qPCR and normalized to the controls SNORD95, SNORD61 and SNORD68 in lungs of mice that were exposed to air or CS for 4 or 24 weeks. (n=5-8/group). Expression of mmu-miR-218-5p is expressed as a normalized relative quantity. **p < 0.01. (B-C) *In situ* hybridization of mmu-miR-218-5p in lung tissue of mice that were exposed to (B) air or (C) CS for 4 weeks. Blue staining indicates mmu-miR-218-5p expression. (D) *In situ* hybridization on mouse lung for the scrambled probe (negative control).

Expression of mmu-miR-218-5p in lung tissue of cigarette smoke-exposed mice

Since miR-218-5p is highly conserved across species, we analyzed its expression in lungs of mice that were exposed to air or CS for 4 weeks (subacute model) or 24 weeks (chronic model). In agreement with our findings in the human lung, we demonstrated that mmu-miR-218-5p was significantly down-regulated in mouse lung upon CS exposure (Air 4 weeks: 1.0 ± 0.058; CS 4 weeks: 0.86 ± 0.032; Air 24 weeks: 1.1 ± 0.071; CS 24 weeks: 0.84 ± 0.0059, **Figure 5A**). In the bronchial airway epithelium and also to a lesser extent in the lung parenchyma, *in situ* hybridization clearly revealed lower expression of mmu-miR-218-5p in CS-exposed mice compared to air-exposed mice (**Figure 5B-D**).

Correlation of pulmonary miR-218-5p expression with its predicted targets

We performed a mRNA microarray on lung tissue of patients from the screening cohort. 14,759 genes were expressed in this microarray. By coupling the miRNA profiling data with the mRNA microarray data in the screening cohort, we were able to determine whether down-regulation of miR-218-5p modulates the expression of its target genes in COPD. To facilitate selection of predicted targets for miR-218-5p, we used the miRNA bodymap [400] which summarizes data of eight different publically available databases for target prediction, i.e. miRDB, TargetScan, MicroCosm, DIANA, TarBase, PITA, RNA22 and miRecords. Putative targets for miR-218-5p were retained when they were predicted by at least three different databases. 2,106 genes were predicted to be targeted by miR-218-5p of which 1,710 were expressed on the mRNA microarray. We calculated the Spearman rank correlation coefficients for miR-218-5p with its 1,710 predicted targets and detected 307 significantly correlated target genes. Of these 307 significantly correlated target genes, 163 were negatively correlated with the expression of miR-218-5p. After correction for multiple testing, mRNA expression of 29 target genes was significantly negatively correlated while 33 target genes were significantly positively correlated with the expression of miR-218-5p (data not shown). mRNA expression of four target genes of miR-218-5p (CDC like kinase 3, Cytochrome P450 family subfamily B member 1, Leukemia inhibitory factor, Dual specificity phosphatase 5 [DUSP5]) in lung tissue is depicted in **Figure S4**.

Gene set enrichment analysis for miR-218-5p in the context of COPD

To explore pathways and functions associated with miR-218-5p activity in COPD, we performed GSEA using 14,759 genes expressed in lungs of patients with COPD [400]. Defense response, immune and inflammatory response and pathways involved in NOD-like receptor signaling and cytokine-cytokine-receptor interaction were found to be inversely correlated to miR-218-5p activity (Figure 6) (**Figure S5**). By exploring the genes belonging to the top ranked gene sets (as shown in Figure 6 and Figure S5), highest negative correlation with miR-218-5p activity is shown for genes that affect development and recruitment of immune cells towards the sites of inflammation (**Figure S6**).

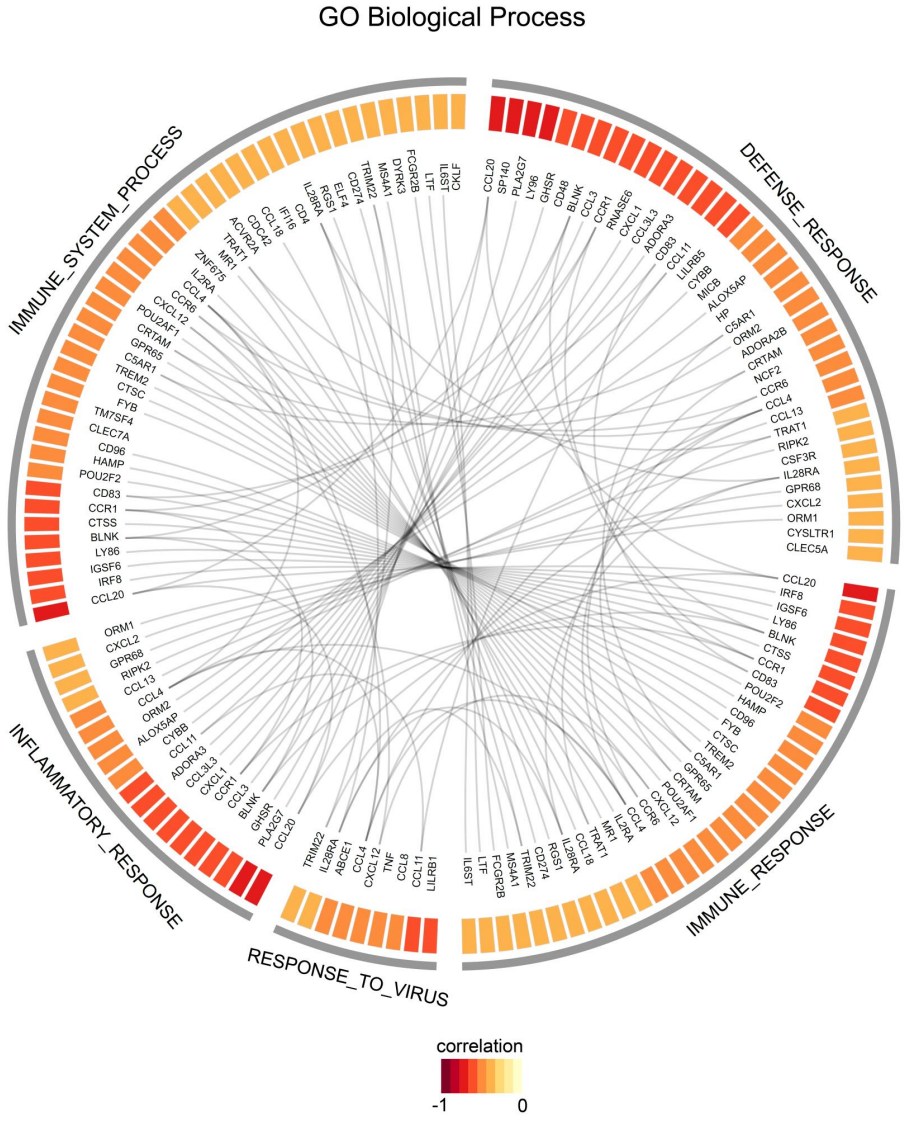


Figure 6. GSEA for miR-218-5p in lung tissue (Gene Ontology- biological process). Circos plot where the outer circle indicates the most significant gene sets (FDR < 0.001) that are inversely correlated with miR-218-5p activity. The inner circle shows a subset of the leading edge genes (those with r ≤ -0.4) and the underlying heatmap shows the correlation value (r) between the respective genes and miR-218-5p. Leading edge genes that are part of multiple gene sets are linked. Abbreviation: FDR: false discovery rate

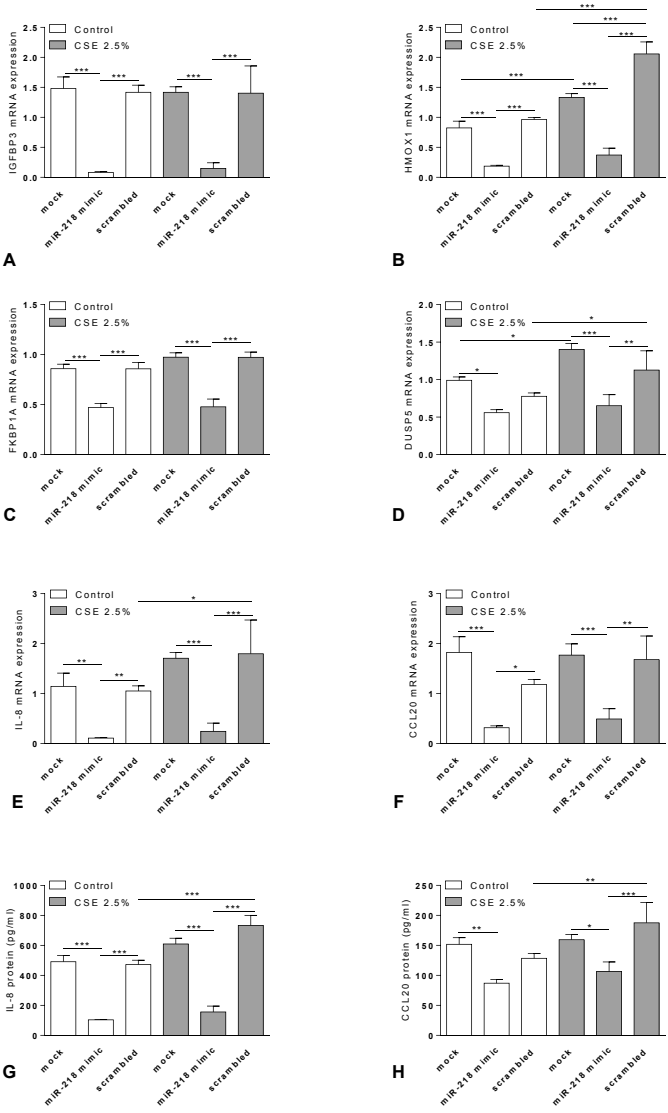


Figure 7. *In vitro* transfection of miR-218-5p mimic in NHBE cells. NHBE cells exposed to 2.5% CSE or control medium and transfected with miR-218-5p compared to scrambled control and mock (lipofectamine control). mRNA expression of (A) IGFBP3, (B) HMOX1, (C) FKBP1A and (D) DUSP5, 4 predicted target genes of miR-218-5p, as assessed by RT-qPCR and normalized to the controls HPRT1, GAPDH and RPL13A. mRNA expression of (E) IL-8 and (F) CCL20, as assessed by RT-qPCR and normalized to the controls HPRT1, GAPDH and RPL13A. Protein expression of (G) IL-8 and (H) CCL20 measured in cell supernatant by ELISA. mRNA data are expressed as normalized relative quantities. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Abbreviations: IGFBP3: Insulin-like growth factor-binding protein 3; HMOX1: heme oxygenase 1; FKBP1A: FK506 binding protein 1A; DUSP5: dual specificity phosphatase 5; IL-8: interleukin 8; CCL20: chemokine (C-C motif) ligand 20

In vitro overexpression of miR-218-5p in normal human bronchial epithelial cells

To elucidate the functional effect of miR-218-5p on predicted targets and markers of inflammation in a single cell type, we transfected NHBE cells *in vitro* with a miR-218-5p mimic, a scrambled negative control or a lipofectamine control (mock) and exposed these cells to 2.5% CSE or control medium. More than 90% of NHBE cells survived the transfection and the transfection efficiency was over 90% (**Figure S7 A**). The expression of miR-218-5p was significantly up-regulated in cells transfected with the mimic (**Figure S7 B**). RNA sequencing revealed 2,914 genes that were significantly differentially expressed in NHBE cells transfected with the miR-218-5p mimic, compared to the scrambled control (data not shown). Of these 2,914 genes, 600 were predicted target genes of miR-218-5p of which 509 were down-regulated upon miR-218-5p overexpression (data not shown). This indicated that ectopic overexpression of miR-218-5p had a significant effect on its predicted target genes, more than expected by chance ($p < 0.001$). Down-regulation of four miR-218-5p target genes (IGFBP3, HMOX1, FKBP1A, DUSP5) was validated by RT-qPCR (**Figure 7 A-D**). We also investigated the effect of the miR-218-5p mimic on CCL20 and IL-8, 2 important chemokines in the pathogenesis of COPD [84,85,87]. Both the mRNA and the protein levels of CCL20 and IL-8 were significantly lower in the cells transfected with the mimic, compared to the scrambled or mock controls (**Figure 7 E-H**).

GSEA on all 12,930 expressed genes, ranked according to fold change, revealed that overexpression of miR-218-5p was negatively associated with defense and inflammatory responses, regulation of the NFκB pathway, antigen processing and presentation, and chemokine signaling (data not shown).

In vivo inhibition of miR-218-5p in CS-exposed mice

To investigate the *in vivo* effect of miR-218-5p in lungs during CS exposure, we intranasally administered a miR-218-5p inhibitor, a scrambled control or PBS (solvent) to mice that were exposed to air or CS for 5 days. In BAL, exposure to CS significantly increased the number of total BAL cells, neutrophils, inflammatory monocytes, dendritic cells and CD4+ and CD8+ T cells (**Figure 8 A-F**) (**Figure S8 A-B**). Importantly, administration of the miR-218-5p inhibitor significantly aggravated the CS-induced increase in inflammatory cells in BAL. Moreover, protein levels of CCL2, a chemokine known to be up-regulated in patients with COPD, were higher in BAL fluid of CS-exposed mice treated with the miR-218-5p inhibitor (**Figure S8 C**) [401].

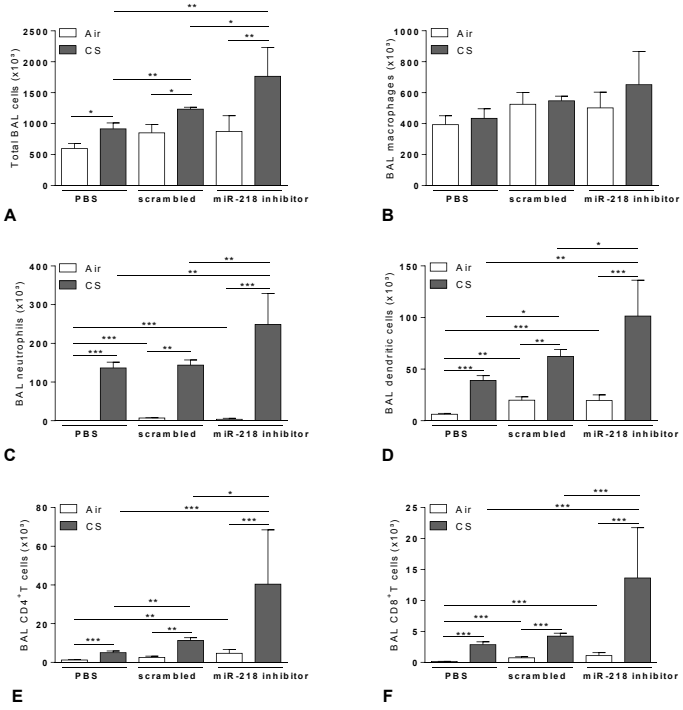


Figure 8. *In vivo* administration of a miR-218-5p inhibitor to air- or CS-exposed mice. Effect of a miR-218-5p inhibitor, a scrambled control or PBS (solvent) on total bronchoalveolar lavage (BAL) cells and cell differentiation in BAL of male wild type mice exposed to air or CS for 5 days. (A) Total BAL cells, (B) alveolar macrophages, (C) neutrophils, (D) dendritic cells, (E) CD4+ T cells, (F) CD8+ T cells, enumerated by flow cytometry. Results are expressed as mean ± SEM. N= 6-8 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001

DISCUSSION

Aberrant expression of miRNAs has been associated with several pulmonary disorders, suggesting their involvement in the pathogenesis of these diseases. In this study, we explored the expression and functional characteristics of miR-218-5p in lung tissue in the context of COPD. Starting from miRNA profiling on lung tissue of never smokers, smokers without airflow limitation and smokers with COPD, a significant down-regulation of miR-218-5p in patients with COPD and an association of miR-218-5p expression with airway obstruction and lung diffusing capacity was observed. Intriguingly, in ex-smokers without airflow limitation, miR-218-5p expression displayed normal levels whereas in ex-smoking patients with COPD, this was not the case, especially not for patients who suffered from severe COPD. Interestingly, we show, both in human and mouse lung, that miR-218-5p is strongly expressed in the bronchial epithelium. Finally, *in vitro* overexpression of miR-218-5p in NHBE cells and *in vivo* inhibition of miR-218-5p in CS-exposed mice provide functional data for a protective role of miR-218-5p in CS-induced inflammatory responses and COPD pathogenesis.

By performing miRNA profiling on lung tissue, we detected 29 differentially expressed miRNAs between smokers without airflow limitation and never smokers and 59 differentially expressed miRNAs between smokers with COPD and never smokers. The miRNA profiling revealed only 3 differentially expressed miRNAs between smokers with or without airflow limitation. In contrast, a previous study by Ezzie et al. reported 70 differentially expressed miRNAs in lung tissue of smokers without airflow limitation and patients with COPD [152]. This difference could be explained by the fact that in the latter study the majority of patients with COPD had severe disease (GOLD IV) whereas our patients with COPD only had moderate disease (GOLD II). By surveying the differentially expressed miRNA spectrum across the 3 patient groups of our screening cohort, the vast majority of miRNAs was down-regulated in the COPD group when compared to non-smoking controls, indicating that the overall suppressive effect of miRNAs on gene expression and translation is abrogated. Of the miRNAs that were down-regulated in the profiling, miR-218-5p is an interesting candidate since it is strongly down-regulated in patients with COPD versus never smokers and has already been reported in relation to smoking or COPD [34,149,402]. Down-regulation of miR-218-5p has previously been described upon exposure to cigarette smoke condensate or smoking in human bronchial and small airway epithelium [34,149] and in lung squamous cell carcinoma [402]. Down-regulation of miR-218-5p was also documented in other lung compartments. We previously showed a significant lower expression of miR-218-5p in induced sputum supernatant of smokers with or without airflow limitation versus never smokers [153]. In cells from bronchoalveolar lavage fluid of patients with lung cancer and/or COPD, miR-218-5p associated with the COPD group when miRNA expression profiles were quantified [321], thereby suggesting a greater involvement of miR-218-5p in COPD than in lung cancer. In addition, when the

expression profile of human miRNAs was evaluated in several tissues, highest expression of miR-218-5p was detected in lung tissue [403].

We confirmed decreased expression of miR-218-5p in smokers and patients with COPD in an independent validation cohort. By including ex-smokers in the validation cohort, we also obtained information on the effect of smoking cessation on miR-218-5p expression. A recent study shows that in the small airway epithelium of healthy smokers, miR-218-5p levels do not return to normal levels when smoking is quitted for 3 months, which is in contrast to most other miRNAs [34]. Our results demonstrate that, in lungs of smokers without airflow limitation, miR-218-5p shows normal expression levels after smoking cessation for at least 1 year, which was not the case for patients with COPD. The irreversible down-regulation of miR-218-5p in patients with COPD might contribute to the persistent systemic and pulmonary inflammation, irrespective of smoking.

By correlating the expression of miR-218-5p with physiological parameters of airway obstruction and emphysema, we demonstrate a strong association of miR-218-5p with COPD, which remained significant when excluding never smokers or when including only patients with COPD. Importantly, by performing linear regression analysis, we demonstrate an association of miR-218-5p with severe COPD, independent of smoking status.

By exploring the expression of mmu-miR-218-5p in lung tissue of mice that were subacutely or chronically exposed to air or CS, we underscore its biological relevance among a variety of species in smoking-induced disease. A consistent overlap between rodent miRNAs and their human homologs was shown by Izzotti and coworkers [150]. After 4 weeks of CS exposure, we observed a significant down-regulation of mmu-miR-218-5p which was more pronounced after chronic CS exposure. In agreement with our results, Izzotti et al. described a significant down-regulation of rno-miR-218-5p in lungs of rats after 4 weeks of exposure to environmental CS (ECS) and a borderline significant down-regulation of mmu-miR-218-5p in mice after 5 weeks of exposure to ECS [150,151].

In both human and murine lung, *in situ* hybridization (ISH) revealed the highest expression of miR-218-5p in the bronchial epithelium. In the murine lung, ISH clearly showed reduced expression of mmu-miR-218-5p in the bronchial airway epithelium upon CS exposure. To confirm direct or indirect effect of CS exposure on the epithelium, we analyzed the expression of miR-218-5p in primary HBECs that were grown on an air-liquid interface and were exposed to air or CS. Twenty-four hours after CS exposure, we observed a down-regulation of miR-218-5p which was in agreement with our expression data in lung and ISH. Similarly, Schembri et al. showed a down-regulation of miR-218-5p in normal cultured HBECs that were exposed to CS condensate [149].

To find out how miR-218-5p might be involved in pathways that are relevant to the pathogenesis of COPD, we correlated the mRNA expression profile of 14,759 expressed genes with the expression of miR-218-5p in lung tissue and performed GSEA. GSEA suggests a functional role for miR-218-5p in

immune, defense and inflammatory responses. Within the top ranked gene sets, some genes are highly negatively associated with miR-218-5p activity. More in detail, CCL20 is a chemokine that attracts dendritic cells (DCs) and is known to be elevated in lungs of patients with COPD [87], whereas CXCL1 attracts monocytes and neutrophils [404]. IRF8 is necessary for the development of antigen presenting cells (DCs, monocytes and B cells) [405,406] and miR-218-5p is also negatively correlated with MHCII molecules such as HLA-DQA2. These genes (and gene sets) emphasize the association of down-regulation of miR-218-5p activity with an accumulation and activation of immune cells, characteristic for the chronic inflammation in lungs of patients with COPD. However, as expected in a complex *in vivo* setting, mRNAs and miRNAs do not operate in isolation but rather in regulatory networks, which might explain why some predicted target genes were positively correlated with the expression of miR-218-5p [407,408].

To further strengthen our observations by mechanistic studies, we overexpressed miR-218-5p *in vitro* in NHBE cells, the cell type with the highest miR-218-5p expression. RNA sequencing showed down-regulation of 509 miR-218-5p target genes and GSEA revealed a negative association of miR-218-5p with inflammatory and defense pathways. These data support the protective or even therapeutic properties of miR-218-5p in CS-induced airway inflammation and COPD. Moreover, by RT-qPCR and ELISA, we confirmed diminished expression of the inflammatory chemokines IL-8 and CCL20, and of the miR-218-5p predicted target genes IGFBP3, HMOX1, FKBP1A and DUSP5. IGFBP3 is overexpressed in idiopathic pulmonary fibrosis, in allergic airway remodeling, during acute respiratory distress syndrome and its effect on pathological and cellular proliferation processes might be independent of insulin growth factors [409-411]. HMOX, a rapid inducible oxidative stress response gene, is reported to be higher expressed upon CS [412]. FKBP1A, a highly conserved immunophilin of the FK506-binding protein (FKBP) family, functions as a regulator of the TGF-β superfamily (TGF-β, activin) signal transduction [413]. Recent studies also provide evidence for participation of FKBPs in innate and adaptive immunity and in the development of immunopathologies [414]. Within the MAPK pathway, DUSP5 ensures the inactivation and nuclear translocation of ERK1/2 [415]. DUSP5 is up-regulated in the bronchial airway epithelium of patients with COPD and this up-regulation was modified by administration of inhaled corticosteroids [168].

Importantly, by inhibiting miR-218-5p in CS-exposed mice, we clearly demonstrate an aggravation of the CS-induced inflammation, leading to significant higher numbers of neutrophils, dendritic cells and T cells, all important cell types in the pathogenesis of COPD. These results indicate that reduced expression of miR-218-5p likely contributes to the CS-induced inflammation.

For this study, expression of miR-218-5p was investigated in-depth across different patient groups with a total sample size of 101 well-characterized subjects. This miR-218-5p expression was validated, not only using 2 independent cohorts, but also using 2 different RT-qPCR methods (TaqMan stem-loop RT-

qPCR technology (Life technologies) in the screening cohort versus miScript miRNA assay (Qiagen) in the validation cohort), which strengthen our findings [371]. In addition, we obtained concordant results in human bronchial epithelial cells, in *in vitro* cultured primary epithelial cells and in a COPD mouse model, underscoring the biological relevance of our observations. Integration of gene expression and miRNA data allowed us to unravel the potential functional role of miR-218-5p in the context of COPD in total lung. By perturbing the miR-218-5p levels *in vitro* and *in vivo*, we highlight the biological relevance of miR-218-5p in CS-induced inflammation and COPD pathogenesis.

However, there are also a few limitations to our study. First, we are aware of the gender imbalance in our screening cohort and tried to address this issue in the independent validation cohort. Second, a longitudinal cohort study of smokers who develop COPD would ideally be suited to further investigate the causal effect of miR-218-5p on COPD pathogenesis. Third, for this study, lung tissue was obtained mainly from patients who underwent surgery for solitary lung tumors. It has been described that the expression level of miR-218-5p changes from within the lung tumor (lowest expression) to the adjacent lung tissue (intermediate expression), to the tissue far away from the tumor (highest expression) [416]. To avoid any confounding effects of the tumor on our results of the screening and validation cohorts, we used lung tissue as far as possible from the tumor. Although it has previously been shown that miR-218-5p decreases in lung cancer, we observed the lowest miR-218-5p levels in severe COPD subjects without lung cancer. Additionally, we have validated the decreased expression of miR-218-5p in bronchial biopsies from patients with COPD compared to never smokers. Importantly, all patients were without diagnosis of lung cancer.

In conclusion, our results strongly suggest a role for miR-218-5p in the host response against CS exposure and in the pathogenesis of COPD. First, we demonstrate a significant down-regulation of miR-218-5p in patients with COPD versus never smokers, which is persistent upon smoking cessation. Second, miR-218-5p strongly correlates with disease severity. Third, we show that the highest expression of miR-218-5p in the lung is localized to the bronchial airway epithelium which is the first cell line of defense in combat against harm-causing agents such as CS. Fourth, GSEA points towards an involvement of miR-218-5p in defense and inflammatory responses. Finally, by overexpressing or inhibiting miR-218-5p *in vitro* and *in vivo*, we provide functional data of miR-218-5p impacting biological pathways that are relevant to COPD pathogenesis. In the future, more research is needed to investigate the regulatory mechanism that controls the expression of miR-218-5p in a disease state versus a non-disease state and to unravel the biological implications of down-regulation of miR-218-5p in COPD. A further understanding of its role in the host response against CS and the pathogenesis of COPD will be required to decide whether miR-218-5p can be put forward as a candidate for therapeutic interventions in COPD.

Supplementary Tables

Table S1. Differentially expressed miRNAs in screening cohort.

Up-regulated in COPD vs never smokers	miRBase release 21	FC	Adj. p-value
hsa-miR-31x	hsa-miR-31-3p	2.743	0.013
hsa-miR-31	hsa-miR-31-5p	2.273	0.049
hsa-miR-21x	hsa-miR-21-3p	2.170	<0.001
hsa-miR-1274A		2.060	0.042
hsa-miR-34Ax	hsa-miR-34a-3p	1.833	<0.001
hsa-miR-136x	hsa-miR-136-3p	1.789	0.020
hsa-miR-135bx	hsa-miR-135b-3p	1.765	0.034
hsa-miR-625x	hsa-miR-625-3p	1.735	0.039
hsa-miR-155	hsa-miR-155-5p	1.695	0.002
hsa-miR-146b-5p	hsa-miR-146b-5p	1.547	0.008
hsa-miR-625	hsa-miR-625-5p	1.463	0.046
hsa-miR-135b	hsa-miR-135b-5p	1.442	0.017
hsa-miR-425x	hsa-miR-425-3p	1.358	0.036
hsa-miR-148a	hsa-miR-148a-3p	1.285	0.029
hsa-miR-191	hsa-miR-191-5p	1.265	0.034

Up-regulated in smokers vs never smokers	miRBase release 21	FC	Adj. p-value
hsa-miR-885-5p	hsa-miR-885-5p	3.680	0.019
hsa-miR-21x	hsa-miR-511-5p	1.953	0.003
hsa-miR-135bx	hsa-miR-654-3p	1.826	0.030
hsa-miR-136x	hsa-miR-136-3p	1.721	0.039
hsa-miR-155	hsa-miR-155-5p	1.712	0.003
hsa-miR-34Ax	hsa-miR-34a-3p	1.604	<0.001
hsa-miR-146b-5p	hsa-miR-146b-5p	1.599	0.006
hsa-miR-511	hsa-miR-511-5p	1.567	0.050

hsa-miR-654-3p	hsa-miR-654-3p	1.543	0.030
hsa-miR-340x	hsa-miR-340-3p	1.360	0.038
hsa-miR-148a	hsa-miR-148a-3p	1.275	0.044
hsa-miR-191	hsa-miR-191-5p	1.275	0.035

Up-regulated in COPD vs smokers	miRBase release 21	FC	Adj. p-value
hsa-miR-1274A		1.980	0.040

Down-regulated in COPD vs never smokers	miRBase release 21	FC	Adj. p-value
hsa-miR-519a	hsa-miR-519a-3p	3.079	0.020
hsa-miR-592	hsa-miR-592	2.745	0.007
hsa-miR-551b	hsa-miR-551b-3p	2.628	0.001
hsa-miR-517c	hsa-miR-517c-3p	2.458	0.033
hsa-miR-450a	hsa-miR-450a-5p	2.457	<0.001
hsa-miR-455-3p	hsa-miR-455-3p	2.340	0.032
hsa-miR-744x	hsa-miR-744-3p	2.174	0.036
hsa-miR-517a	hsa-miR-517a-3p	1.997	0.036
hsa-miR-218	hsa-miR-218-5p	1.865	<0.001
hsa-miR-99b	hsa-miR-99b-5p	1.771	<0.001
hsa-miR-455-5p	hsa-miR-455-5p	1.727	0.015
hsa-miR-221	hsa-miR-221-3p	1.672	<0.001
hsa-miR-30dx	hsa-miR-30d-3p	1.665	0.043
hsa-miR-181c	hsa-miR-181c-5p	1.602	0.014
hsa-miR-125a-3p	hsa-miR-125a-3p	1.531	0.030
hsa-miR-296-5p	hsa-miR-296-5p	1.521	0.006
hsa-miR-339-5p	hsa-miR-339-5p	1.518	0.001
hsa-miR-149	hsa-miR-149-5p	1.514	0.024

hsa-miR-497	hsa-miR-497-5p	1.502	0.003
hsa-miR-181a-2x	hsa-miR-181a-2-3p	1.500	0.030
hsa-miR-502-5p	hsa-miR-502-5p	1.497	0.003
hsa-miR-197	hsa-miR-197-3p	1.491	0.018
hsa-miR-331-3p	hsa-miR-331-3p	1.467	0.005
hsa-miR-423-5p	hsa-miR-423-5p	1.457	0.010
hsa-miR-25	hsa-miR-25-3p	1.455	<0.001
hsa-miR-484	hsa-miR-484	1.455	0.002
hsa-miR-199a-5p	hsa-miR-199a-5p	1.443	0.006
hsa-miR-345	hsa-miR-345-5p	1.438	0.004
hsa-miR-328	hsa-miR-328-3p	1.435	0.015
hsa-miR-130b	hsa-miR-130b-3p	1.425	0.011
hsa-miR-106b	hsa-miR-106b-5p	1.422	0.007
hsa-miR-505	hsa-miR-505-3p	1.416	0.016
hsa-miR-92a	hsa-miR-92a-3p	1.408	<0.001
hsa-miR-30ax	hsa-miR-30a-5p/-3p	1.382	0.041
hsa-miR-222	hsa-miR-222-3p	1.372	0.020
hsa-let-7b	hsa-let-7b-5p	1.361	0.009
hsa-miR-502-3p	hsa-miR-502-3p	1.356	0.005
hsa-miR-193a-5p	hsa-miR-193a-5p	1.355	0.021
hsa-miR-148b	hsa-miR-148b-3p	1.348	0.021
hsa-miR-15a	hsa-miR-15a-5p	1.336	0.020
hsa-miR-99bx	hsa-miR-99b-3p	1.333	0.027
hsa-miR-128	hsa-miR-128-3p	1.313	0.010
hsa-miR-27b	hsa-miR-27b-3p	1.294	0.015
hsa-miR-93	hsa-miR-93-5p	1.257	0.028

Down-regulated in smokers vs never smokers	miRBase release 21	FC	Adj. p-value
hsa-miR-592	hsa-miR-592	5.125	0.007
hsa-miR-138	hsa-miR-138-5p	1.959	0.010
hsa-miR-455-5p	hsa-miR-455-5p	1.692	0.026
hsa-miR-218	hsa-miR-218-5p	1.578	0.003
hsa-miR-181a	hsa-miR-181a-5p	1.508	0.045
hsa-miR-99b	hsa-miR-99b-5p	1.507	0.001
hsa-miR-25	hsa-miR-25-3p	1.393	0.002
hsa-miR-222	hsa-miR-222-3p	1.369	0.026
hsa-miR-497	hsa-miR-497-5p	1.361	0.029
hsa-miR-221	hsa-miR-221-3p	1.356	0.008
hsa-miR-345	hsa-miR-345-5p	1.354	0.023
hsa-let-7b	hsa-let-7b-5p	1.341	0.017
hsa-miR-199a-5p	hsa-miR-199a-5p	1.338	0.039
hsa-miR-339-5p	hsa-miR-339-5p	1.326	0.032
hsa-miR-502-3p	hsa-miR-502-3p	1.297	0.021
hsa-miR-27b	hsa-miR-27b-3p	1.286	0.022
hsa-miR-15a	hsa-miR-15a-5p	1.155	0.035

Down-regulated in COPD vs smokers	miRBase release 21	FC	Adj. p-value
hsa-miR-484	hsa-miR-484	1.262	0.046
hsa-miR-502-5p	hsa-miR-502-5p	1.174	0.018

In total, 343 miRNAs were tested.
smokers: current smokers without airflow limitation; COPD: current smokers with COPD GOLD stage II;
Abbreviations: FC: fold change ; Adj. p-value: p-value adjusted for multiple testing (Benjamini-Hochberg)
First column: annotation of miRNA at the time of the miRNA profiling; Second column: annotation of miRNA according to miRBase release 21

Table S2. Linear regression analysis (validation cohort)

Parameter		B	Std. Error	t	p-value
Gender	male	-0.052	0.114	-0.454	0.651
	female	0a			
COPD status	COPD III-IV	-0.797	0.224	-3.560	<0.001
	COPD II	-0.069	0.126	-0.546	0.587
	no COPD	0a			
Current smoking	current smoker	-0.677	0.171	-3.973	<0.001
	ex-smoker	-0.122	0.161	-0.758	0.451
	never smoker	0a			
Inhaled steroids	inhaled steroids use	-0.146	0.138	-1.064	0.292
	no inhaled steroids use	0a			
BMI		0.004	0.009	0.475	0.636
Age		-0.017	0.007	-2.288	0.026

[a] reference parameter is set to 0.

The unstandardized coefficient (β) is the natural logarithm fold change in miR-218-5p expression (i.e. the increase in units of dependent variable) when increasing the predictor variable with 1 unit and holding the other variables constant.

Table S3. Patient characteristics (bronchial biopsies)

	Never smokers	COPD GOLD I-II
Number	9	10
Gender (male/female)	2/7	4/6
Age (years)	60 (52-62)	72 (63-80)
BMI (kg/m^2)	28 (24-30)	26 (23-36)
Current-smoker/ex-smoker	-	2/8
Smoking history (pack years)	0 (0-0)	40 (29-89)*
FEV_1 post-bronchodilator (% predicted)	93 (77-105)	82 (71-93)
FEV_1/FVC post-bronchodilator (%)	75 (74-78)	64 (62-69)*
ICS (yes/no)	0/9 †	4/6

Footnote

Abbreviations: FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; ICS: inhaled corticosteroids. Data are presented as median (IQR); p-values were determined by Mann-Whitney U test: * $P < 0.001$ versus never smokers or Fisher's exact test: † $P < 0.05$

Supplementary Figures

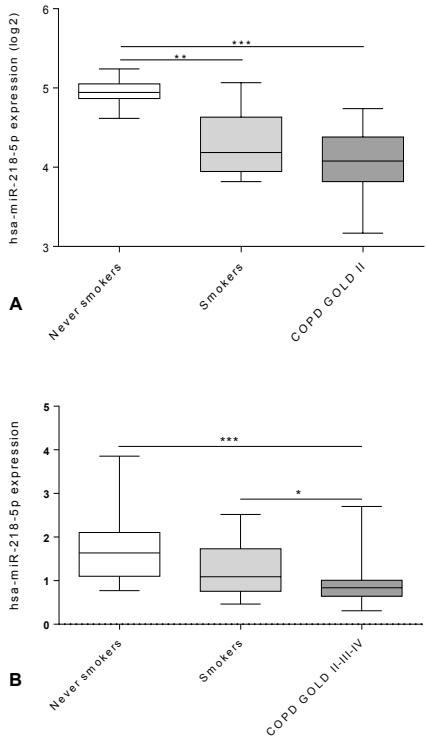


Figure S1. Expression of miR-218-5p in lung tissue (screening cohort). (A) Normalized expression of miR-218-5p as assessed by stem-loop RT-qPCR in lung tissue of 8 never smokers, 10 smokers without airflow limitation and 12 smokers with COPD (screening cohort). Normalization was achieved using the global mean on common targets. **adj. $p < 0.01$, ***adj. $p < 0.001$ (B) Normalized expression of miR-218-5p as assessed by RT-qPCR in lung tissue of 12 never smokers, 21 current and ex-smokers without airflow limitation and 38 patients with COPD GOLD II-III-IV (current and ex-smokers) (validation cohort). Data are expressed as normalized relative quantities, normalized to the controls SNORD95, SNORD96A and SNORD68. *$p < 0.05$, ***$p < 0.001$

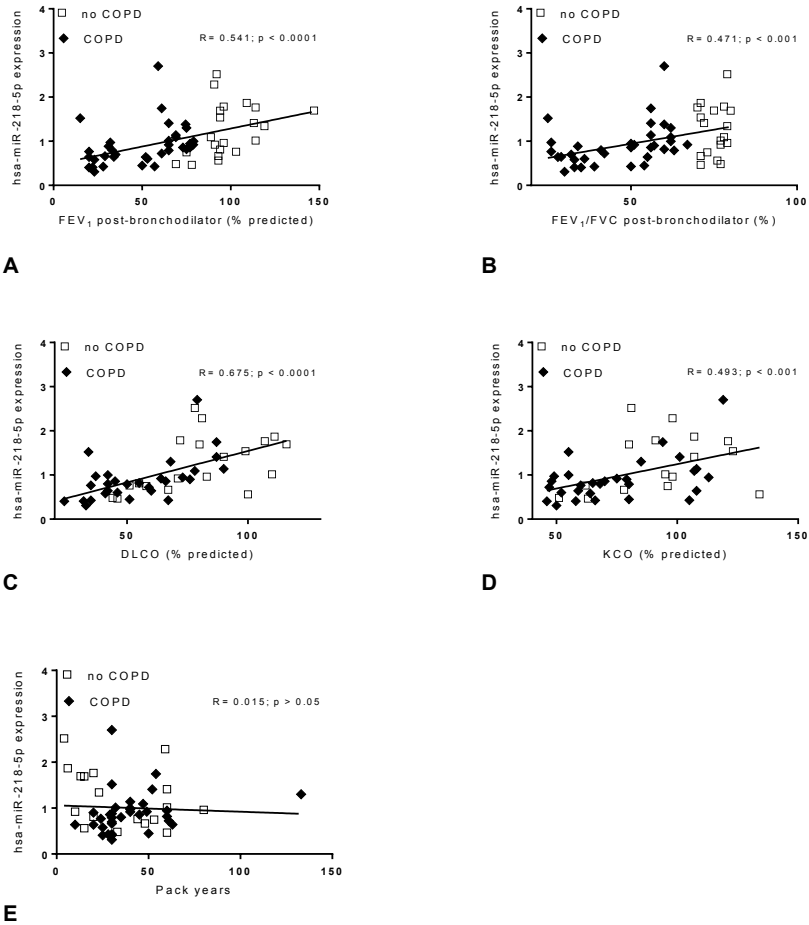


Figure S2. Spearman correlation between miR-218-5p and patient characteristics (validation cohort) when excluding never smokers. Spearman correlation between miR-218-5p and (A) % FEV_1 post-bronchodilator, (B) % FEV_1/FVC (C) DLCO, (D) KCO and (E) pack years in the validation cohort. Abbreviations: FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; DLCO: diffusing capacity of carbon monoxide; KCO: transfer coefficient of carbon monoxide (corrected for alveolar volume)

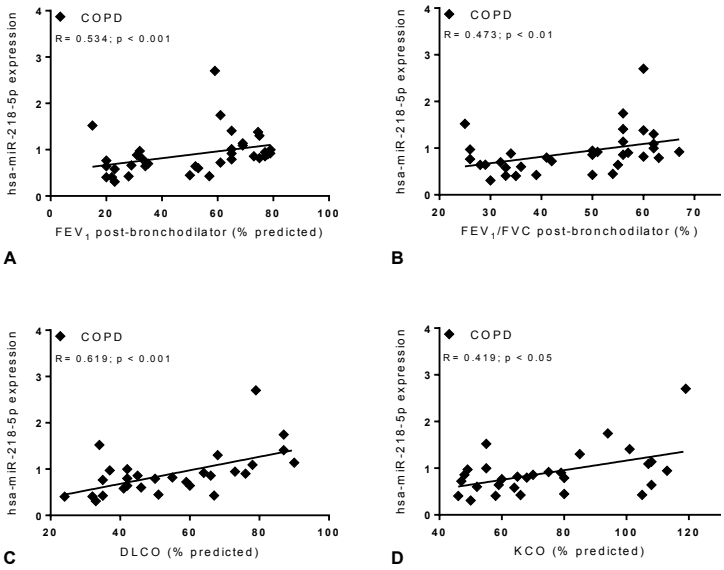


Figure S3. Spearman correlation between miR-218-5p and patient characteristics (validation cohort) in patients with COPD. Spearman correlation between miR-218-5p and (A) % FEV_1 post bronchodilator, (B) % FEV_1/FVC (C) DLCO and (D) KCO in the validation cohort. Abbreviations: FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; DLCO: diffusing capacity of carbon monoxide; KCO: transfer coefficient of carbon monoxide (corrected for alveolar volume)

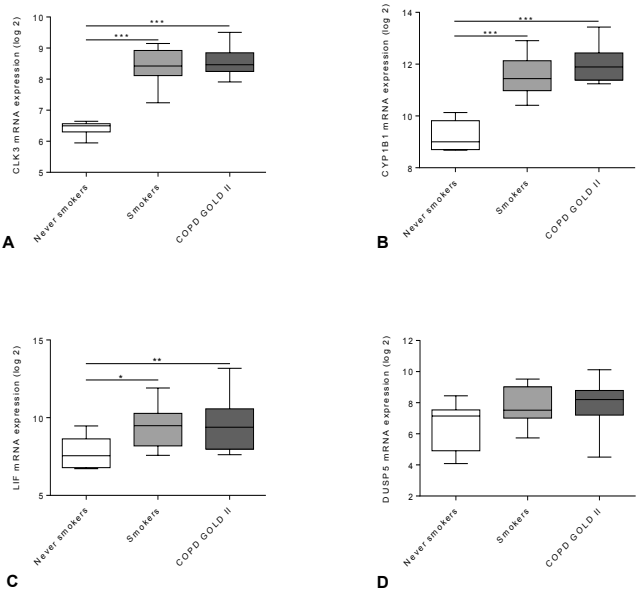


Figure S4. mRNA expression of predicted target genes of miR-218-5p. mRNA expression of (A) CLK3, (B) CYP1B1, (C) LIF, (D) DUSP5 in lung tissue of the screening cohort. mRNA data, assessed by mRNA microarray, are represented as background corrected and quantile normalized intensities. *p < 0.05, **p < 0.01, ***p < 0.001. Abbreviations: CLK3: CDC-like kinase 3; CYP1B1: cytochrome P450, family 1, subfamily B, polypeptide 1; LIF: leukemia inhibitory factor; DUSP5: dual specificity phosphatase 5

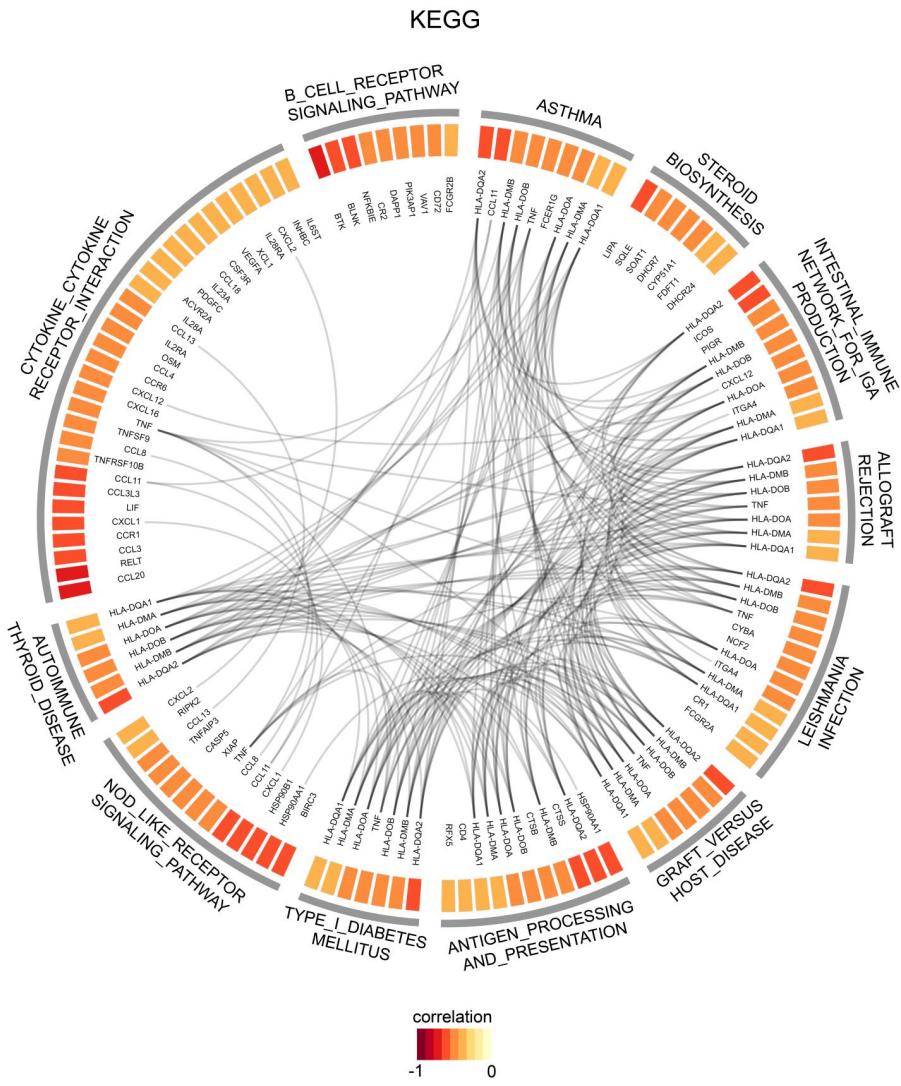


Figure S5. Gene set enrichment analysis (GSEA) for miR-218-5p in lung tissue (KEGG). Circos plot where the outer circle indicates the most significant gene sets (FDR < 0.001) that are inversely correlated with miR-218-5p activity. The inner circle shows a subset of the leading edge genes (those with r $\leqslant$ -0.4) and the underlying heatmap shows the correlation value (r) between the respective genes and miR-218-5p. Leading edge genes that are part of multiple gene sets are linked. Abbreviation: KEGG: Kyoto Encyclopedia of Genes and Genomes, FDR: false discovery rate

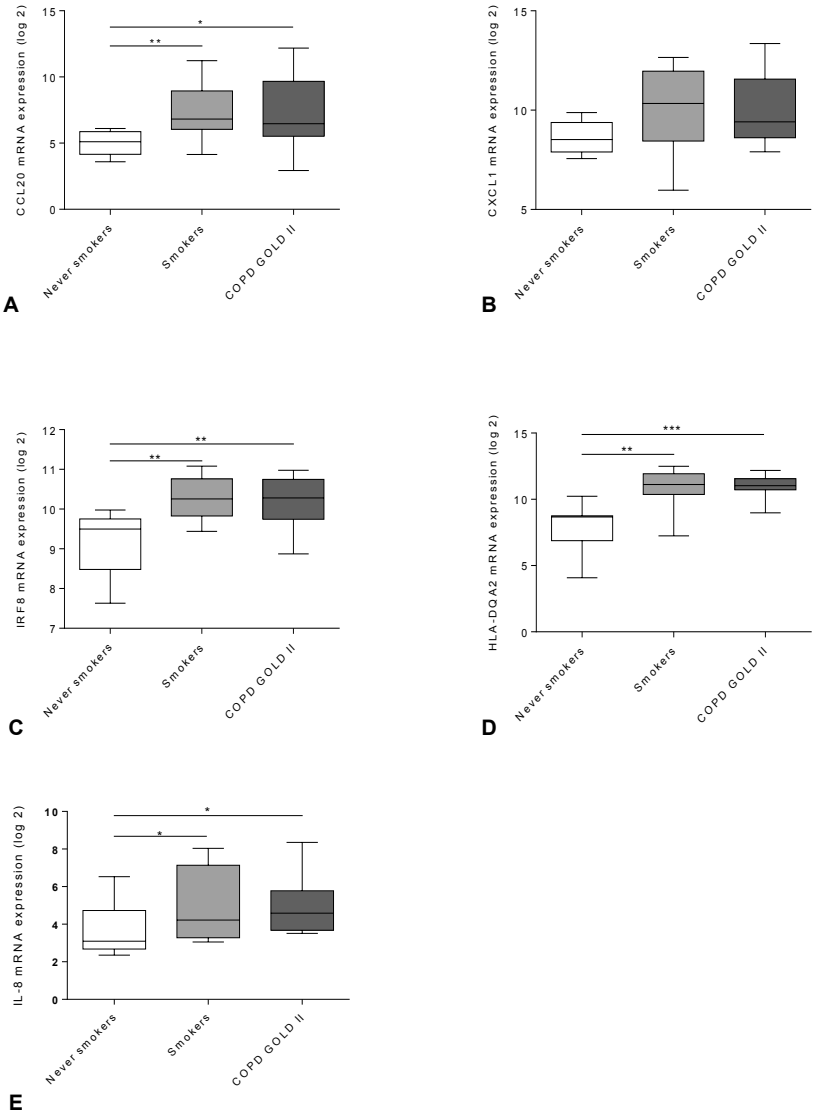
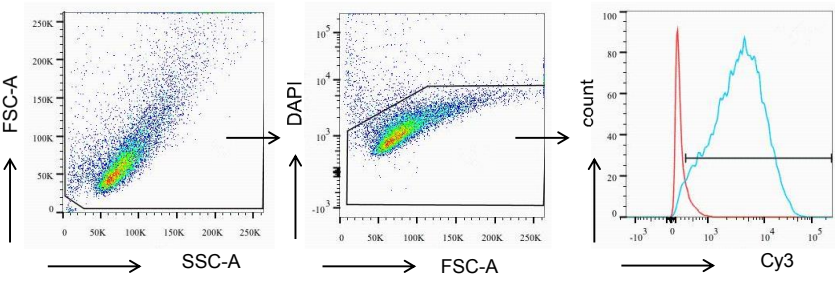
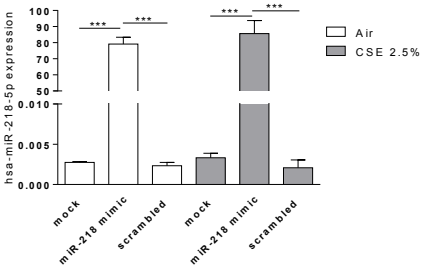


Figure S6. mRNA expression of genes inversely correlated to miR-218-5p activity. mRNA expression of (A) CCL20, (B) CXCL1, (C) IRF8, (D) HLA-DQA2 and (E) IL-8 in lung tissue of the screening cohort. mRNA data, assessed by mRNA microarray, are represented as background corrected and quantile normalized intensities. *p < 0.05, **p < 0.01, ***p < 0.001. Abbreviations: CCL20: chemokine (C-C motif) ligand 20 ; CXCL1: chemokine (C-X-C motif) ligand 1 ; IRF8 : interferon regulatory factor 8 ; HLA-DQA2: major histocompatibility complex, class II, DQ alpha 2; IL-8: interleukin 8

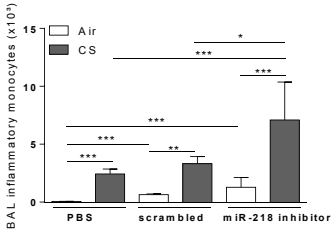


A

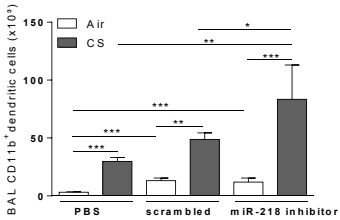


B

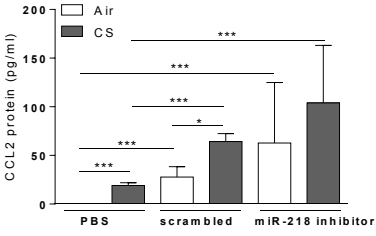
Figure S7. In vitro transfection of miR-218-5p mimic in NHBE cells. NHBE cells exposed to 2.5% CSE or control medium and transfected with miR-218-5p compared to scrambled control and mock. (A) Gating strategy to investigate cell survival and transfection efficiency. (B) miR-218-5p expression assessed by RT-qPCR and normalized to the controls SNORD95, and SNORD96A. Data are expressed as normalized relative quantities. ***p < 0.001



A



B



C

Figure S8. In vivo administration of a miR-218-5p inhibitor to air- or CS-exposed mice. Effect of a miR-218-5p inhibitor, a scrambled control or PBS (solvent) on cell differentiation in BAL and protein levels of cytokines in BAL fluid of male wild type mice exposed to air or CS for 5 days. (A) Total CD11b+Ly6C+ cells, (B) total CD11b+ dendritic cells, enumerated by flow cytometry and (E) CCL2 protein levels, measured in BAL fluid by ELISA. Results are expressed as mean ± SEM. N= 6-8 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001

Direct detection of circulating microRNAs unveiled the absence of miR 218-5p in smoker subjects

Erika Cione[1] and Luca Gallelli[2]

[1]Department of Pharmacy, Health and Nutritional Sciences, University of Calabria, Rende (CS), Italy

[2]Department of Health Science, School of Medicine, University of Catanzaro, Clinical Pharmacology Unit, Mater Domini University Hospital, Italy.

Dear Editor,

we have read with great interest the paper entitled "MicroRNA Profiling Reveals a Role for MicroRNA-218-5p in the Pathogenesis of Chronic Obstructive Pulmonary Disease" by Conickx et al [154]. In their study the authors evaluated miRNAs profile in 30 patients (8 never smoked, 10 were smokers without airflow limitation and 12 smokers with COPD), showing that the hsa-microRNA-218-5p in both COPD and smokers biopsies is downregulated. Therefore, the authors suggest a protective role of this miR in cigarette smoke-induced inflammation and COPD. Even if this study is of high interest, the cohort COPD patients evaluated are under pharmacological treatment and it is known that drugs, indirectly can perturb miR expression results [417].

At present, there is growing scientific interest on the role of miR in COPD patients, and "in vitro" studies documented that hsa-miR-1343 reduces the expression of both isoforms (type 1 and 2) of the TGF-beta receptor [418], and hsa-miR-145 negatively regulates the release of proinflammatory cytokines by airway smooth muscle cells [331]. An important feature of miRs is the stability in the blood-stream, thus they are designed as biomarkers of different diseases. For such reason, our research group has started a clinical study (https://clinicaltrials.gov/ct2/show/NCT02633280) that is still ongoing, in order to evaluate in smoking and non-smoking patients with and without COPD, the serum miRnome levels (HumanV3 miRNA Assay-Kit-12 assay). In our tests, we used the NanoString'snCounter Flex System, based on the direct hybridization of hundreds (up to 800 simultaneously) of different capture/miR probes containing biotin and an unique fluorescent designed as a 'molecular barcodes' for each target. The probe form hydrids with their corresponding target [419] and are then immobilized on a streptoavidin cartridge; the nonhybridized probes are removed in the prep station system and individual hybridized probes are visualized and counted with fluorescent microscopic imaging/scanning. The low background of this technique provides a very sensitive detection (LOD 10^{-15}) and the advantages of this platform are also the precision and since no amplification is required, the totally absence of retrotrascription bias. All the characteristics describe above, make this technology robust and

relatively cost effective compared to microarray in the field of translational medicine because it is able to analyze the entire signal transduction pathways in one reaction [420].

In our sets of serum patients tested (divided in 4 healthy non-smokers, 5 smokers and 3 ex-smokers) we highlighted that in smoker subjects' plasma hsa-miR-218-5p is not detected, compared to healthy ones supporting the concept that may progressive reduction in the plasma miR-218-5p level could be linked to lung health status. We did not detect also hsa-miR-218-5p in ex-smokers (who have quit for at least 2 years). At the moment we were not able to screen COPD naïve patients (for therapy) and we are still searching for such. In all the serum samples extracted we added two spike in (osa-miR434 and ath-miR159), in a known amount, in order to know the efficiency of extraction. Beside the excitement in miR studies for COPD patients with the discovery of microRNAs signature as predictive biomarkers of diseases (better if in serum/plasma samples) what is now challenging is the discovery of the target gene(s) of the miR-218-5p.

Reply to direct detection of circulating microRNAs unveiled the absence of miR-218-5p in smoker subjects

Griet Conickx[1], Pieter Mestdagh[2], Jo Vandesompele[2], Guy G Brusselle[1], Ken R Bracke[1]

[1]Laboratory for Translational Research in Obstructive Pulmonary Diseases, Department of Respiratory Medicine, Ghent University Hospital, Ghent, Belgium

[2]Center for Medical Genetics, Ghent University, Ghent, Belgium

From the Authors:

We thank Cione and colleagues for their interest in our work concerning the role of miR-218-5p in the pathogenesis of chronic obstructive pulmonary disease (COPD). We agree that investigating the contribution of microRNA (miRNA) to the pathogenesis of COPD is an attractive field [152,153,421]. For clarity, we did not investigate the miRNA profile in lung biopsies, but in lung tissue specimens from lobectomy or lung transplantation. Down-regulation of miR-218-5p in lung tissue from smokers and COPD patients was first detected in a screening cohort of 30 patients and subsequently validated in a validation cohort of 71 patients and in bronchial biopsies of 19 patients. To address the concern of Cione and colleagues on drugs affecting miR-218-5p expression, only 6 out of 30 patients in the screening cohort were using inhaled corticosteroids and linear regression analysis could not demonstrate an impact of the treatment on the expression of miR-218-5p.

For the quantification of miRNA expression in human lung tissue, we used the stem-loop RTqPCR method, as this approach has both high sensitivity and specificity [371,374]. While we did not investigate the expression of miR-218-5p in blood, several studies reported miR-218 levels in plasma or serum using RT-qPCR [422-424]. We regret that Cione and colleagues are not able to detect miR-218-5p levels in serum in their limited cohort of smokers. Since detection of miRNA levels is highly technology-dependent, it is crucial to evaluate the sensitivity of the proposed nCounter Flex System (NanoString), a hybridization-based miRNA quantification platform [372]. Recently, the performance of different miRNA quantification platforms has been extensively evaluated in the microRNA quality control (miRQC) study [371]. In this study, the sensitivity of the individual platforms was investigated by evaluating the number of detected miRNAs in serum RNA samples. Detection rates in serum RNA were highly variable among platforms, with qPCR platforms displaying higher sensitivity compared to hybridization-based platforms. While the nCounter Flex System was included in the miRQC study, Nanostring did not profile the serum samples and detection specificity and sensitivity of this platform in serum could therefore not be assessed.

Regarding the functional impact of miR-218-5p, *in vitro* and *in vivo* perturbation experiments demonstrated that a reduced expression of miR-218-5p likely contributes to the cigarette smoke-induced inflammation [154], providing novel insights into the pathogenesis of COPD.

CHAPTER 6: microRNA profiling in lung tissue and bronchoalveolar lavage of cigarette smoke-exposed mice and in COPD patients: a translational approach

miRNAs are generally highly conserved in mammals, indicating that murine models can be adequate models to identify relevant miRNAs. Our aim was to put forward interesting miRNAs for further research in the context of COPD. Therefore, we used our murine COPD model and detected miRNAs whose expression was altered following CS exposure in lung tissue and matched bronchoalveolar lavage (BAL) fluid, 2 different respiratory compartments, which was not yet investigated before. Moreover, we correlated with the inflammatory profile (i.e. altered inflammatory cell numbers and chemokine levels) in lung and BAL, and sought for overlap with lung tissue, bronchial biopsies and induced sputum of patients with COPD.

Conickx G[#], Avila Cobos F[#], van den Berge M, Faiz A, Timens W, Hiemstra PS, Joos GF, Brusselle GG, Mestdagh P*, Bracke KR*.
MicroRNA profiling in lung tissue and bronchoalveolar lavage supernatant of cigarette smoke-exposed mice and in COPD patients: a translational approach
Scientific Reports (revision submitted)

ABSTRACT

Chronic obstructive pulmonary disease (COPD) is characterized by a progressive airflow limitation and is associated with a chronic inflammatory response in the airways and the lungs. microRNAs (miRNAs) are often highly conserved between species and have an intricate role within homeostatic conditions and immune responses. Also, miRNAs are dysregulated in smoking-associated diseases. We investigated the miRNA profile of 523 miRNAs by stem-loop RT-qPCR in lung tissue and cell-free bronchoalveolar lavage (BAL) supernatant of mice exposed to air or cigarette smoke (CS) for 4 or 24 weeks. After 24 weeks of CS exposure, 31 miRNAs were differentially expressed in lung tissue and 78 in BAL supernatant. Next, we correlated the miRNA profiling data to inflammation in BAL and lung, obtained by flow cytometry or ELISA. In addition, we surveyed for overlap with newly assessed miRNA profiles in bronchial biopsies and with previously assessed miRNA profiles in lung tissue and induced sputum supernatant of smokers with COPD. Several miRNAs showed concordant differential expression between both species including miR-31*, miR-155, miR-218 and let-7c. Thus, investigating miRNA profiling data in different compartments and both species provided accumulating insights in miRNAs that may be relevant in CS-induced inflammation and the pathogenesis of COPD.

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is a debilitating respiratory condition which is characterized by a progressive and irreversible airflow limitation due to an abnormal inflammatory response to inhalation of noxious particles or gases [82,425,426]. The pathology comprises a mixture of small airway obstruction and destruction of lung parenchyma (emphysema); their relative contribution varying between patients and within the lung [59]. The main risk factor for developing COPD is cigarette smoking. However, only 20% of smokers develop COPD, suggesting that genetic susceptibility or alterations in the epigenetic machinery may be of importance in the development of COPD.

microRNAs (miRNAs), i.e. small non-coding RNAs, are key regulators in diverse biological pathways. One single miRNA can bind to target sequences in multiple mRNAs, typically resulting in mRNA degradation and/or translational inhibition [265]. By doing so, miRNAs embed a post-transcriptional control within multiple gene signaling cascades. Also, certain miRNAs are critically involved in immune cell development and function. Given this far-reaching influence, it is not surprising that altered miRNA levels contribute to disease pathogenesis [427].

Ideally, differentially expressed miRNAs in disease versus control can serve as biomarkers of disease initiation/progression or as therapeutic target. In lungs of patients with COPD, we have shown the involvement of down-regulated miRNA-218-5p in recruiting inflammatory cells towards the airways, thereby assisting in the sustained inflammation [154]. In sputum of patients with COPD, down-regulation of let-7c was inversely correlated with soluble TNFR-II, a receptor implicated in COPD pathogenesis [153]. The expression of miRNAs is described to be altered after cigarette smoke (CS) exposure in lungs of mice and patients with COPD [151,152,155,428]. Smoking can potentiate inflammatory processes by affecting the expression of miRNAs that play a key role in immune responses. To obtain full insights in the CS-induced alterations in murine miRNA levels and immune cell populations in the lung, we performed an RT-qPCR-based miRNA profiling in bronchoalveolar lavage (BAL) supernatant and lung tissue of mice that were exposed to air or CS for 4 or 24 weeks, complemented with data on inflammation in BAL and lungs. Moreover, using a translational approach, we checked for overlapping miRNAs in lung tissue between CS-exposed mice and patients with COPD. This information will highlight relevant miRNAs in the CS-induced inflammation.

MATERIALS AND METHODS

Mice

Male C57BL/6 wild-type (WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were kept under a 12h light-dark cycle in autoclaved cages and bedding, with unlimited access to water and food. The ethics committee for animal experimentation of the faculty of Medicine and Health Sciences (Ghent University) approved all in vivo manipulations.

Smoke exposure

Mice (n=8 per group) were exposed to air or CS, as described previously [360]. Briefly, ~~all~~ the mice were exposed whole body to the mainstream tobacco smoke of 5 simultaneously lit 3R4F reference cigarettes (without filter, University of Kentucky, Lexington, KY), 4 times a day with a 30 minutes smoke-free interval. Therefore, the mice were placed in a plexiglass chamber of 7500 cm^3, connected to a smoking chamber. The mice were exposed for 5 days per week, for 4 weeks (subacute exposure) or 24 weeks (chronic exposure). An optimal smoke-to-air ratio of 1:6 was maintained. The control groups were exposed to air.

Bronchoalveolar lavage (BAL)

Via a tracheal cannula, lungs were first lavaged using 3 times 300 µl HBSS (free of Ca2+ and Mg2+ and supplemented with 1% BSA). Supernatant of this fraction was used for ELISA and collected for miRNA profiling. Then, lungs were lavaged using 3 times 1 ml HBSS supplemented with 0.6 mM EDTA. The six lavage fractions were pooled, centrifuged, and the cell pellet was resuspended in 200 µl FACS buffer (PBS supplemented with 1 % BSA, 5mM EDTA and 0.1 % sodium azide). Subsequently, total cell counts were obtained using a Bürker chamber and differential cell counts (on at least 400 cells) were performed on cytocentrifuged preparations after May-Grünwald-Giemsa staining. Furthermore, BAL cells were used for flow cytometric analysis.

Preparation of single cell suspension of lung tissue

Following BAL, the pulmonary and systemic circulation was rinsed with saline, supplemented with 5mM EDTA. The left lung was used for histology, as described previously [360]. The major lobe of the right lung was taken and thoroughly minced, enzymatically digested and subjected to red blood cell lysis. After passage through a 50µm cell strainer, cells were counted with a Z2 particle counter (Beckman-Coulter, USA) and left on ice until labeling for further flow cytometric analysis. Another lobe of the right lung was stored for RNA extraction which was later used for miRNA profiling.

Quantification of inflammation

Flow cytometry was used to enumerate inflammatory cells in BAL fluid and in lung tissue. The analysis was performed on a FACS Calibur (4 weeks exposure experiment) or an LSR Fortessa (24 weeks exposure experiment) (BD Biosciences, San Diego, USA) and data were analyzed with FlowJo software (Tree Star Inc., Ashland, USA). The flow cytometry data in BAL were supplemented with cytospin

counts. Chemokines (CCL2 and CXCL1) were measured in BAL via commercially available ELISA kits (R&D systems).

RNA extraction

Total RNA from lung tissue and 100µl of cell-free BAL supernatant was extracted using the miRNeasy mini kit (Qiagen) according to the manufacturer's instructions. Afterwards, RNA was collected and measured using the Nanodrop 2000 (Thermo Fischer scientific).

miRNA profiling on lung tissue of mice that were exposed to air or CS for 4 or 24 weeks

Total RNA, including the small RNA fraction, was reverse transcribed with the miRNA reverse transcription kit (Applied Biosystems, Life Technologies) in combination with a stem-loop Megaplex miRNA primer pool (Applied Biosystems, Life Technologies) consisting of primers for 523 miRNAs and 15 endogenous controls as described previously [374]. After this RT reaction the cDNA was pre-amplified using the TaqMan PreAmp Master Mix and Primer Mix (Applied Biosystems, Life Technologies) . This pre-amplification increases the detection sensitivity. The pre-amplified cDNA was diluted 1,600 times. qPCR amplification of 523 mature miRNAs was performed using miRNA TaqMan assays (Applied Biosystems, Life Technologies). The qPCR mixture contained 4 µL of Universal qPCR mastermix, 3 µL of a 1/15 dilution of miRNA TaqMan assay, and 1 µL of diluted preamplified cDNA. All reactions were run on a 7900HT qPCR cycler (Applied Biosystems, Life Technologies) under the following cycling conditions: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. If the Cq-value was below 32, the miRNAs were considered expressed. The miRNA expression data were normalized using the global mean [389,390].

Only miRNAs that could be detected (had a Cq-value < 32) in at least 80% of the samples per group were included in our study, resulting in 225 miRNAs out of 523 in mouse lung.

miRNA profiling on bronchoalveolar lavage supernatant of mice that were exposed to air or CS for 4 or 24 weeks

The same workflow was followed for miRNA profiling on cell-free murine BAL supernatant.

Annotation of miRNA

Annotation of all differentially expressed miRNAs was updated using miRBase tracker [379] in Table 1 and 2, and in Figure S2 and S3.

Statistical analysis

Continuous variables were analyzed using non-parametric tests i.e. Mann-Whitney U test when comparing unrelated data using SPSS 24.0 software (SPSS Inc, Chicago, IL, USA). Heatmaps were generated using the heatmap.2 function from the gplots package [429], where samples were clustered using manhattan distances and the Ward's method (R statistical programming language, version 3.3.1) [430]. Spearman rank correlation tests between expressed miRNAs from lung and BAL, and a) flow cytometry data in lung and BAL, respectively; b) cytospin data in BAL; c) cytokine/chemokine levels in

BAL, were carried out using the cor.test function (R software, version 3.3.1). For the correlation analysis, all miRNAs were included when expressed in at least half of the murine samples. Data were kept when Rs ≥ 0.5 and adjusted p-value ≤ 0.05. The Benjamini-Hochberg procedure was used for multiple testing correction and p-values < 0.05 were considered statistically significant.

RESULTS

miRNA expression profiling in lung tissue of air- and CS-exposed mice

miRNA profiling was performed on lung tissue of C57BL/6 mice (8 per group), that were exposed to either air or CS for 4 or 24 weeks. Of the 523 miRNAs tested by stem-loop RT-qPCR, 255 miRNAs could be detected. After 4 weeks of CS exposure, 9 miRNAs exhibited differential pulmonary expression compared to the air-exposed mice (4 down-regulated and 5 up-regulated). After 24 weeks of CS exposure, 31 miRNAs showed a significant differential expression of which 16 were down-regulated and 15 were up-regulated. The results are represented in **Figure 1**. A list of all differentially expressed miRNAs in lung tissue can be found in **Table 1**.

In lung tissue, all 5 significantly up-regulated miRNAs after 4 weeks of CS exposure, were even more increased following chronic CS exposure, indicating a robust and progressive miRNA signature. On top of the list, miR-135b displayed the highest fold change in lung tissue (Fold change = 13.59, **Table 1**). From the significantly down-regulated miRNAs following 4 weeks of CS exposure in lung, only miR-322* was still significantly decreased after 24 weeks of CS exposure (**Figure 1e**).

Figure 1. miRNA expression profiling in lung tissue of air- and CS-exposed mice. Volcano plots showing the differential miRNA expression (in fold change on the x-axis) and significance level (-log10-adjusted p-value on y-axis). The detected miRNAs are plotted as black dots. The horizontal line indicates the 0.05 significance level. In the heatmaps, only the significantly differentially expressed miRNAs are represented. These were hierarchically clustered across the air- and smoke-exposed groups. Each row represents a miRNA and each column represents a murine lung sample. The color code indicates the expression level: red= higher expression following CS exposure, blue= lower expression, grey= miRNA was not detected ("NA" values). (a) Heatmap showing the differentially expressed miRNAs in murine lung tissue following subacute (4 weeks) air or CS exposure. (b) Volcano plot representing the miRNA profiling data following subacute CS exposure compared to air exposure. (c) Heatmap showing the differentially expressed miRNAs in murine lung tissue following chronic (24 weeks) air or CS exposure. (d) Volcano plot representing the miRNA profiling data following chronic CS exposure versus air exposure. (e) Overlap in differentially expressed miRNAs in murine lung tissue between subacute (4 weeks) and chronic (24 weeks) CS exposure compared to air exposure. (b,d) Both volcano plots with annotated miRNAs can be found in supplemental Figure S2.

Figure 2. miRNA expression profiling in cell-free BAL supernatant of air- and CS-exposed mice. Volcano plots showing the differential miRNA expression (in fold change on the x-axis) and significance level (-log10-adjusted p-value on y-axis). The detected miRNAs are plotted as black dots. The horizontal line indicates the 0.05 significance level. In the heatmaps, only the significantly differentially expressed miRNAs are represented. These were hierarchically clustered across the air- and smoke-exposed groups. Each row represents a miRNA and each column represents a murine BAL supernatant sample. The color code indicates the expression level: red= higher expression following CS exposure, blue= lower expression, grey= miRNA was not detected ("NA" values). (a) Heatmap showing the differentially expressed miRNAs in murine BAL supernatant following subacute (4 weeks) air or CS exposure. (b) Volcano plot representing the miRNA profiling data in BAL supernatant following subacute CS exposure compared to air exposure. (c) Heatmap showing the differentially expressed miRNAs in murine BAL supernatant following chronic (24 weeks) air or CS exposure. An enlargement of the heatmap can be found in Figure S1 of the supplemental data. (d) Volcano plot representing the miRNA profiling data in BAL supernatant following chronic CS exposure versus air exposure. (e) Overlap in differentially expressed miRNAs in murine BAL supernatant between subacute (4 weeks) and chronic (24 weeks) CS exposure compared to air exposure. (b,d) Both volcano plots with annotated miRNAs can be found in supplemental Figure S3.

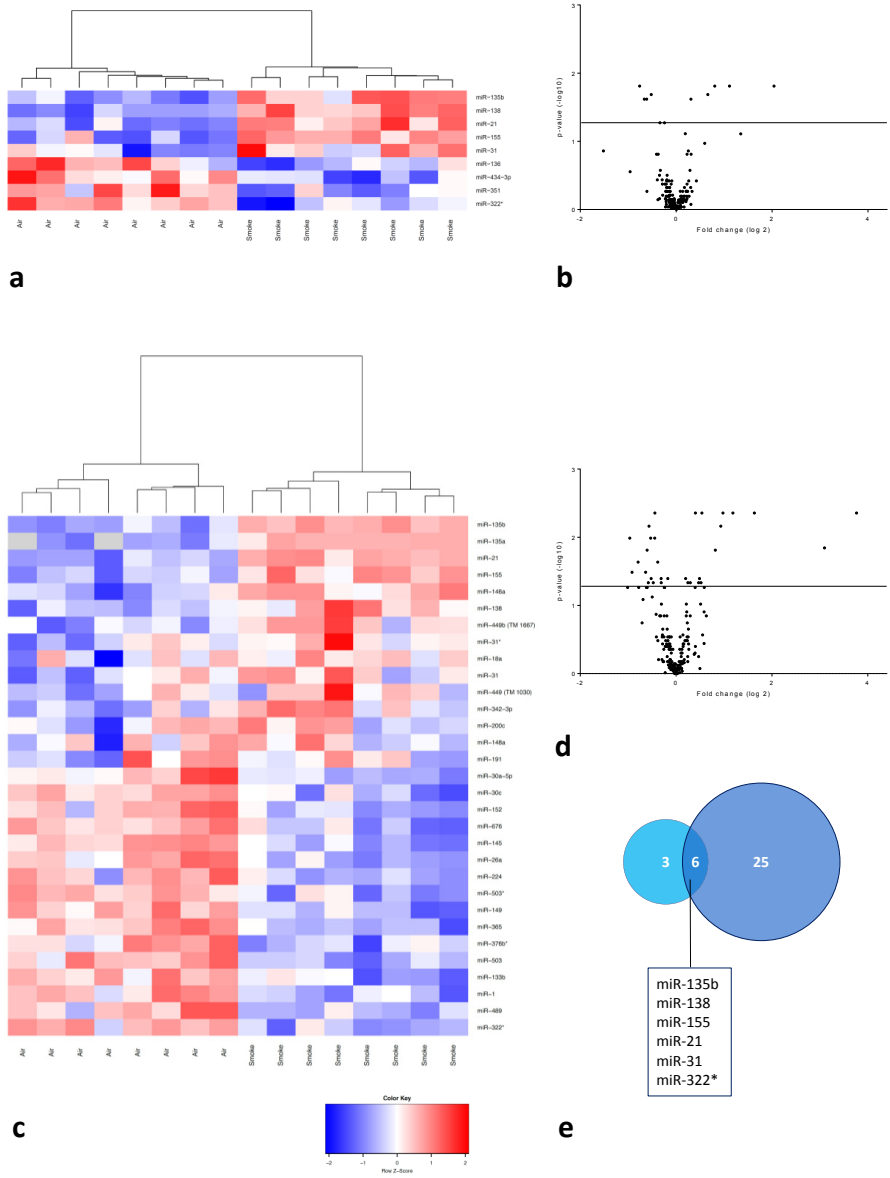


Figure 1. miRNA expression profiling in lung tissue of air- and CS-exposed mice.

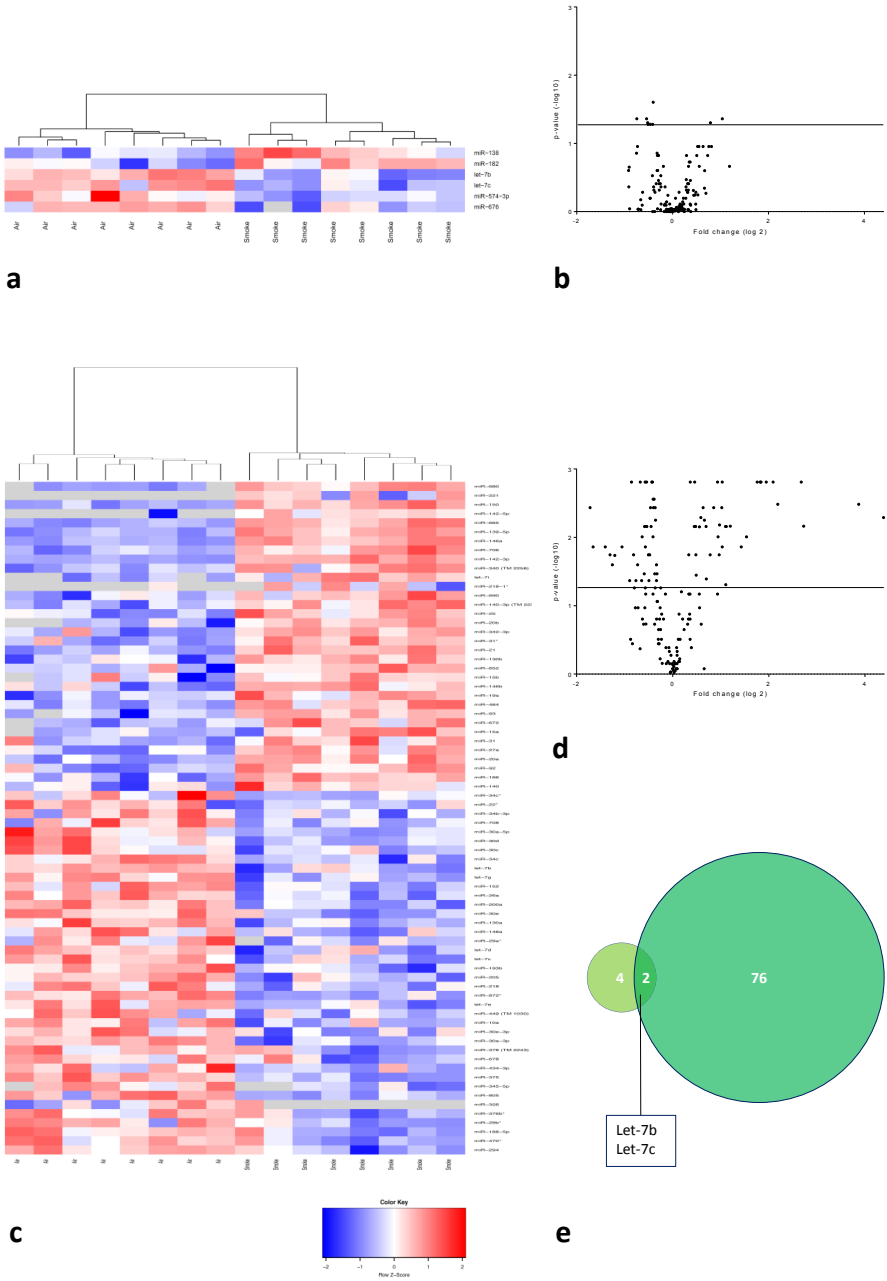


Figure 2. miRNA expression profiling in cell-free BAL supernatant of air- and CS-exposed mice.

miRNA expression profiling in supernatant of BAL of air- and CS-exposed mice

miRNA expression profiling was performed on cell-free BAL supernatant of mice exposed to air or CS for 4 or 24 weeks. Of the 523 miRNAs evaluated, 160 miRNAs could be detected in BAL supernatant. After 4 weeks of CS exposure, only 6 miRNAs were significantly differentially expressed (4 down-regulated and 2 up-regulated). However, after 24 weeks of CS smoke exposure, 78 miRNAs exhibited significant differential expression in BAL supernatant of which 40 were down-regulated and 38 were up-regulated. The results are represented in **Figure 2**. A list of all differentially expressed miRNAs in BAL supernatant can be found in **Table 2**.

In BAL supernatant, let-7b and let-7c were significantly reduced after 4 and 24 weeks of CS exposure (**Figure 2e**). Moreover, miR-680 showed the highest fold change (Fold change = 20.92, **Table 2**) following 24 weeks of CS exposure compared to air exposure.

Differentially expressed miRNAs following CS exposure in both lung tissue and BAL supernatant

Following subacute CS exposure, only miR-138 overlaps as being differentially expressed in both lung and BAL supernatant (**Figure 3a**). Following chronic CS exposure, 12 miRNAs (miR-146a, miR-148a, miR-152, miR-21, miR-26a, miR-30a-5p, miR-30c, miR-31, miR-31*, miR-342-3p, miR-376b* and miR-449) were differentially expressed in both lung tissue and BAL supernatant of which 10 showed concordant up- or down-regulation. Only miR-449 and miR-148a displayed different expression patterns in the two compartments (**Figure 3b**).

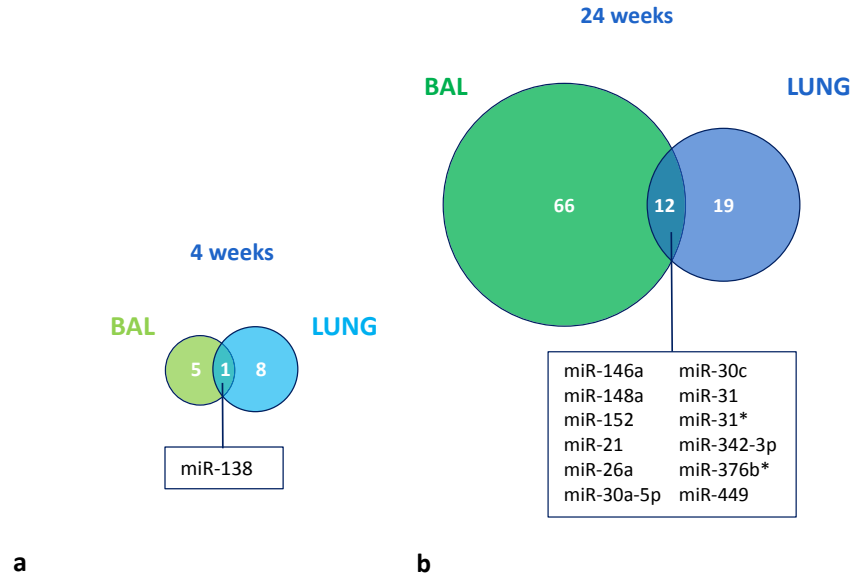


Figure 3. miRNAs that are differentially expressed in both lung tissue and BAL supernatant following (a) 4 weeks of CS exposure and following (b) 24 weeks of CS exposure.

Furthermore, a multidimensional scaling plot (**Figure 4**) showed a clear separation between normalized miRNA profiles (only containing miRNAs expressed in both fractions) from BAL supernatant and lung tissue, indicating two compartments with different cellular identity, anatomical structure and organization. miRNA profiles of lung tissue clustered more together than miRNA profiles of BAL supernatant. Moreover, following 24 weeks of CS exposure in BAL, a larger separation in miRNA profiles was noticed compared to lung, underlining the more relative diversity in cell types in BAL following long-term CS exposure. Also, due to this fact and a more activated state of immune cells, a global increase in miRNA abundance was found in BAL supernatant following 24 weeks of CS exposure (Mann-Whitney U test; p-value = 0.007, **Figure 5**).

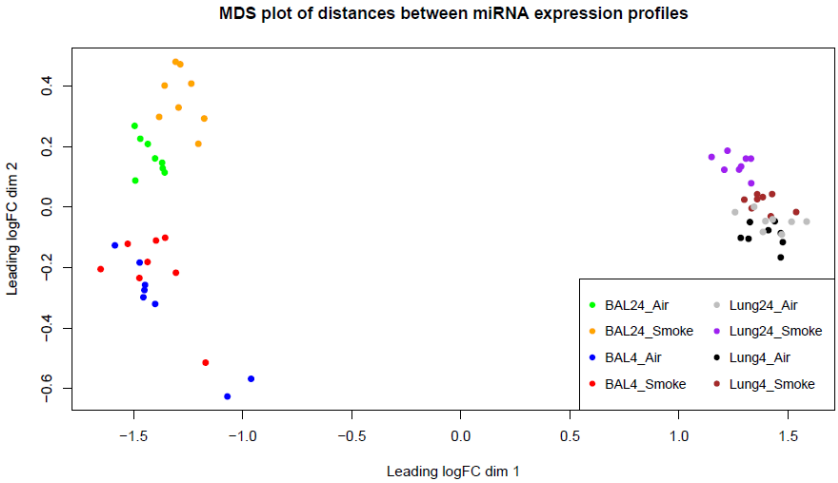


Figure 4. Multidimensional scaling (MDS) plot. This plot visualizes how miRNA profiling experiments cluster together.

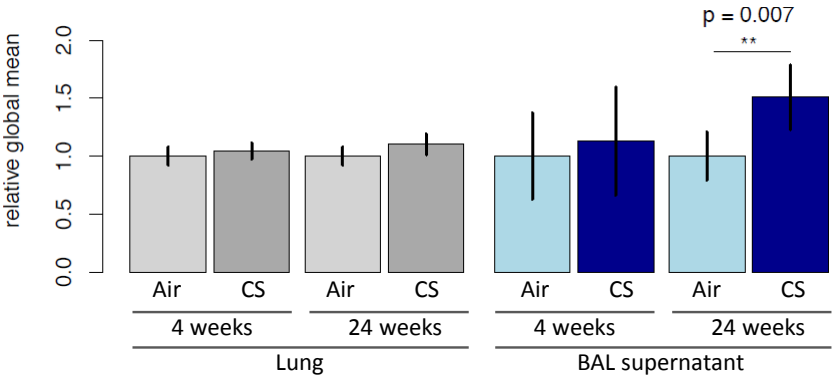


Figure 5. Relative global mean. This graph is obtained by analyzing the global difference in Cq-values between the 2 groups (air versus CS) per condition (relative to the air-exposed group) by performing a Mann-Whitney U test, enabling to detect a possible shift in miRNA expression.

Inflammation in lungs and BAL fluid of mice following CS exposure

Four weeks of CS exposure induced an inflammatory response in lung and BAL with recruitment of inflammatory cells and activation of immune signaling. In BAL fluid, there was a significant increase in total cell numbers (p-value = 0.007, **Figure 6a**) with more diversity in immune cell subsets such as a significant increase in macrophages (p-value = 0.0104), neutrophils (p-value = 0.00016), dendritic cells (DCs) (p-value = 0.00016) and $CD4^+$ (p-value = 0.007) and $CD8^+$T lymphocytes (p-value = 0.0104) compared with air-exposed mice (**Figure 6b-f**). After 24 weeks of CS exposure, the inflammatory response was severely augmented in BAL with a strong increase in total cell numbers as well as in macrophages, neutrophils, DCs and T lymphocytes (all p-values < 0.001, **Figure 6a-f**).

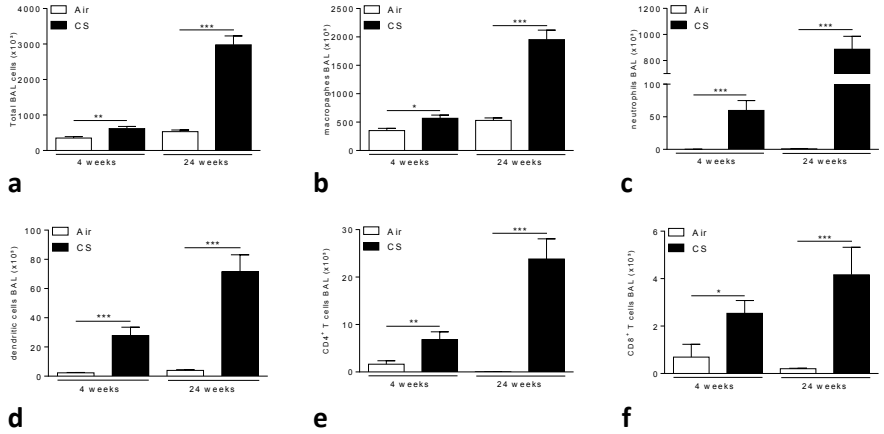


Figure 6. Inflammation in BAL following 4 and 24 weeks of air or CS exposure. (a) Total cell numbers in BAL, (b) Total macrophages in BAL, (c) Total neutrophils in BAL, (d) Total dendritic cells (DCs) in BAL, (e) Total $CD4^+$ T cells in BAL, (f) Total $CD8^+$ T cells in BAL. * p < 0.05, ** p < 0.01, *** p < 0.001

In addition, in lung tissue, the increase in dendritic cells is corresponding to the smoke-induced inflammation and proportionally to subacute or chronic CS exposure compared with air exposure (**Figure 7a**). Also, B cell numbers were augmented following chronic CS exposure (**Figure 7b**).

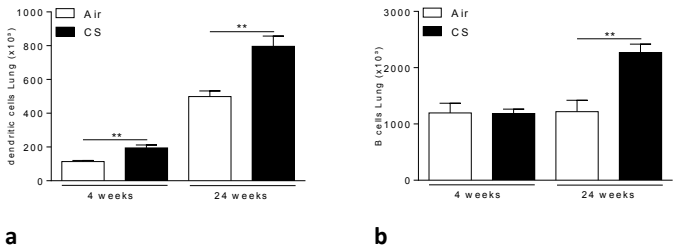


Figure 7. Inflammation in lung following 4 and 24 weeks of air or CS exposure. (a) Total dendritic cells in lung, (b) Total B cell numbers in lung following 4 or 24 weeks of air or CS exposure. ** p < 0.01

Correlation of miRNA expression with inflammatory cell subsets in lung tissue and BAL supernatant

To assess whether the change in cell types following CS exposure could be associated with the alteration in miRNA expression, we correlated the miRNA expression with populations of immune cells and levels of inflammatory chemokines. After subacute CS exposure, miR-135b correlated strongly with percentage DCs (adj. p-value = 0.017, **Figure 8a**). Following chronic CS exposure, miR-155 correlated significantly with percentage B cells (adj. p-value = 0.0067, **Figure 8b**) and miR-152, miR-30a-5p, miR-30c, miR-218 and miR-26a correlated with several immune cell types in lung tissue.

In BAL supernatant, miR-21 correlated significantly with macrophage numbers and CCL2 (adj. p-value = 0.0014, **Figure 8c, Figure S4a**), while miR-142-3p, miR-21, miR-146a as well as miR-218 and let-7 family members correlated with neutrophil numbers (**Figure 8d-e**). miR-26a and miR-146a correlated with several immune cell types whereas miR-31* correlated significantly with DC numbers (adj. p-value = 0.026).

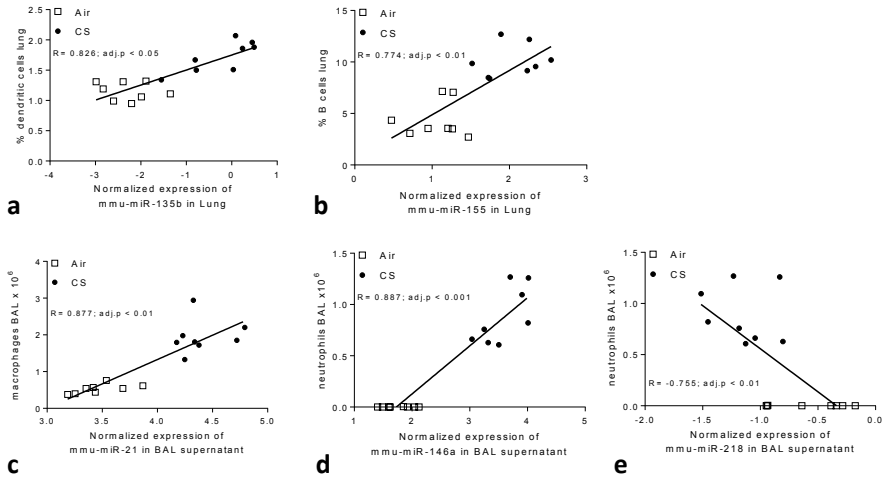


Figure 8. Spearman correlation analyses between the expression of (a) miR-135b and % dendritic cells (DCs) in lung following 4 weeks of air or CS exposure. Spearman correlation analysis between the expression of (b) miR-155 and % B cells in lung, (c) miR-21 and total macrophage numbers in BAL supernatant, (d) miR-146a and total neutrophil numbers in BAL supernatant and between (e) miR-218 and total neutrophils in BAL supernatant following 24 weeks of air or CS exposure.

Overlap in miRNA expression pattern between chronic CS-exposed mice and patients with COPD

Generally, miRNAs are highly conserved RNA molecules. Therefore, we evaluated a possible overlap in miRNA expression between mice following long-term CS exposure and patients with COPD [154].

First, we evaluated the overlap in lung tissue of mice exposed to CS for 24 weeks and our previously reported miRNA profiling in lung tissue of current smoking COPD patients compared to never-smokers without COPD, all assessed by stem-loop RT-qPCR. Interestingly, miR-135b and miR-155 were

significantly up-regulated, both in COPD patients (Adj. p-value (miR-135b) = 0.017; Adj. p-value (miR-155) = 0.0022) versus never-smoking controls and in mice following chronic CS-exposure (Adj. p-value (miR-135b and miR-155) = 0.004) (**Figure 9a**) (**Table S2**).

Second, by integrating data from patients participating in the GLUCOLD study where miRNA expression was newly profiled in bronchial biopsies between current-smoking and ex-smoking patients with moderate to severe COPD [175], we found an overlap for miR-31* with miRNA profiling in lung tissue of mice chronically exposed to CS compared to air-exposed mice, but also with lung tissue of current-smoking COPD patients compared to never-smokers (adj. p-value = 0.012 in GLUCOLD study; adj. p-value = 0.0012 in lung tissue of COPD patients and adj. p-value = 0.004 in mice) (**Figure 9b**)(**Table S3**). The clinical characteristics of the GLUCOLD study cohort can be found in the online **Table S1**.

Third, in previously published miRNA profiling data, assessed by stem-loop RT-qPCR, in induced sputum supernatant of current smoking patients with COPD compared to never-smokers, 8 miRNAs were significantly down-regulated in COPD [153]. Of these 8 miRNAs, 5 miRNAs overlapped with the miRNA profile obtained in BAL supernatant of mice chronically exposed to CS compared to air-exposed mice **(Figure 9c)**. Remarkably, let-7c and miR-218 were significantly reduced (**Table S4**).

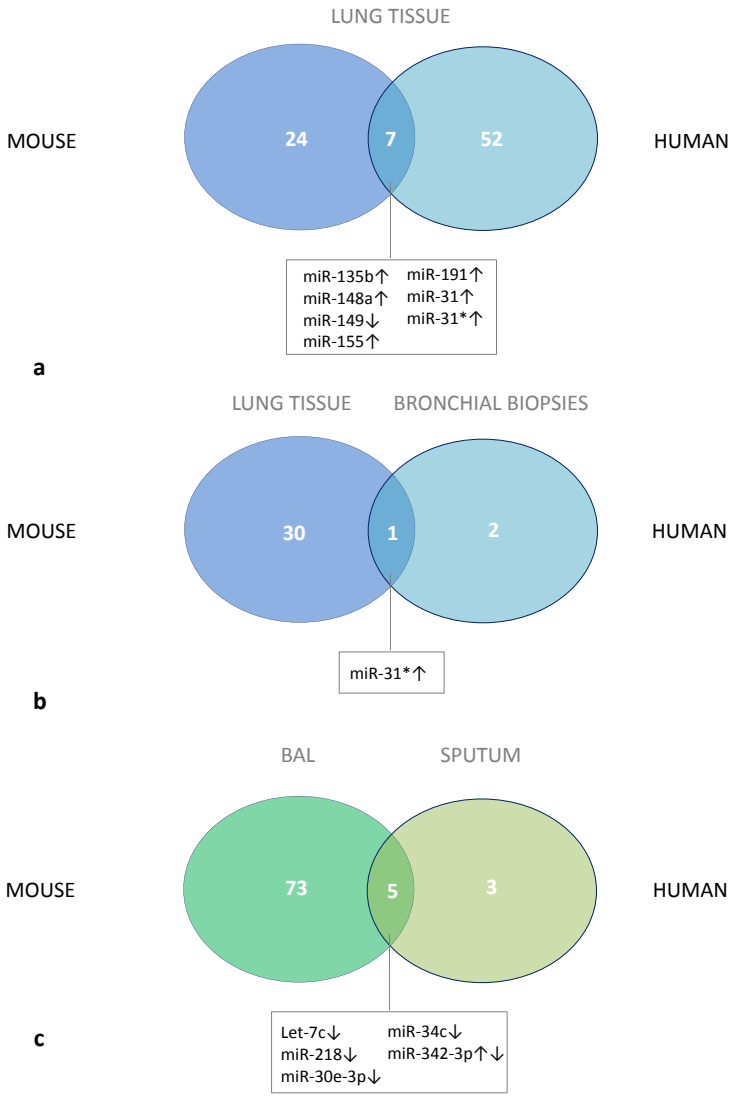


Figure 9. overlap in miRNA expression between chronic CS-exposed mice and patients with COPD. Differential miRNA expression was evaluated in (a) lung tissue of mice that were exposed to 24 weeks of air or CS, and in lung tissue of current smoking patients with COPD GOLD II (n=12) compared to never-smokers (n=8). (b) lung tissue of mice that were exposed to 24 weeks of air or CS, and in bronchial biopsies of current smoking patients with COPD GOLD II-III (n=42) compared to ex-smoking patients with COPD GOLD II-III (n=21). (c) in BAL supernatant of mice that were exposed to 24 weeks of air or CS, and in induced sputum supernatant of current smoking patients with COPD GOLD II (n=12) compared to never-smokers (n=10). Numbers of differentially expressed miRNAs are represented as well as the number of overlapping miRNAs. For the overlapping miRNAs, the direction of change in expression following chronic CS exposure /current smoking and having COPD is indicated with arrows. The level of the change in miRNA expression can be found in the supplemental data (Table S2-4). COPD: chronic obstructive pulmonary disease, GOLD: Global Initiative for Chronic Obstructive Lung Disease

DISCUSSION

We have developed a murine model in which CS inhalation – the main etiologic agent in most COPD cases – initiates COPD-like manifestations, enabling us to investigate the CS-induced pathogenesis of COPD [360]. Using these mice, we observed smoke-induced inflammation in BAL and lungs which coincided with changes in miRNA expression in those two compartments. To our knowledge, this is the first time that both lung and BAL supernatant have been thoroughly investigated regarding the miRNA profile.

Several miRNAs were strongly affected by smoke in lung tissue and BAL supernatant. Remarkably, we did not observe an overall down-regulation upon CS exposure in lung as is frequently noted in other miRNA profiling studies [149,150,154,248].

By focusing on the overlap between subacute and chronic CS exposure within the same compartment, or the overlap between miRNAs with altered expression levels in BAL and lung, we narrowed the pool of interesting miRNAs down to 18: let7b, let-7c, miR-135b, miR-138, miR-146a, miR-148a, miR-152, miR-155, miR-21, miR-26a, miR-30a-5p, miR-30c, miR-31, miR-31*, miR-322*, miR-342-3p, miR-376b* and miR-449.

We also assessed whether miRNA profiles clustered together between BAL and lung samples and whether the effect of smoke on the miRNA profile could be distinguished from air-exposed mice (Figure 4). The clear separation in miRNA expression between BAL fluid and lung mirrors its different cellular content and structural organization. Moreover, lung tissue was obtained from lavaged mice, meaning that all BAL cells were removed. Also, smoking obviously affected miRNA profiles, both in lung as in BAL supernatant. As expected, a higher diversity in immune cells – often in a more activated state – and a greater amount of cells populate the airways and alveolar spaces following CS exposure, which could lead to a global increase in miRNA abundance in BAL supernatant, favoring certain immune cell-specific miRNAs. To correct for this, we normalized with the mean expression of all miRNAs, rendering a profile relative to this global shift.

CS exposure altered immune cell subsets both in lung and in BAL, as well as it affected miRNA expression levels. We correlated miRNA expression with numbers of inflammatory cells in BAL and lung and found that many of our initially indicated interesting miRNAs were highly correlated. This could mean that altered miRNAs could be implicated in recruitment of these immune cells to the lung or airways, or that they are highly immune cell-specific.

Interestingly, an overlap was observed for miR-135b, miR-148a, miR-149, miR-155, miR-191, miR-31 and miR-31* between lungs of mice chronically exposed to CS and smoking patients with COPD compared to non-smoking controls, suggesting a potential role for these miRNAs in the pathogenesis of COPD [154]. In addition, there was also an overlap for miR-31* between these aforementioned two groups with patients with moderate to severe COPD participating in the GLUCOLD study where the

differential miRNA expression was assessed in bronchial biopsies between current- and ex-smoking patients [175]. By surveying the overlap between miRNA profiling data in human sputum supernatant of current smokers with or without airflow limitation compared to never-smokers, and our results in murine BAL supernatant, we found several miRNAs that were concordantly reduced including let-7c, miR-218, miR-26a and miR-449. Although detected in both human sputum and murine BAL supernatant, some miRNAs were expressed in the opposite direction such as miR-146a, miR-342-3p and miR-150 [153].

Regarding miRNA alterations in lung, gradual elevation of miR-155 is generally expected due to its inherent involvement in inflammation since miR-155 modulates both the innate and adaptive immune system [431-434] and is induced following TLR activation. In agreement with our data, an increase in miR-135b-5p expression was already demonstrated in lungs of mice that were exposed to CS for 4 days and for 18 months, besides the observation that miR-135b-5p is also highly inducible upon challenge of the airways with other noxious particles [435-437]. It is also not surprising that miR-21 is up-regulated in BAL supernatant and in lung tissue. First, miR-21 is suggested to be concordantly expressed between tumor tissue and matched plasma or serum [438,439]. Second, miR-21 is up-regulated in activated immune cell subsets accumulating in the lung upon an inflammatory stimulus, explaining its gradual increase following prolonged smoke exposure. Third, miR-21 is primarily expressed in cells of the macrophage lineage that are already present in the airways without prior CS trigger. Macrophages increase in number following CS exposure but not to the same extent as the newly arrived other immune cells present in BAL, explaining the only moderate increase in miR-21 expression in BAL supernatant.

As expected, miR-21 correlated with macrophage numbers in BAL. Additionally, miR-21 also correlated with CCL2 (MCP-1) protein expression in BAL. It has been shown in a mouse model of abdominal aortic aneurysm that mice exposed to nicotine displayed higher miR-21 levels, which was associated with a reduction in tumor suppressor genes, as well as with an augmentation of inflammatory genes such as IL-6 and MCP-1. Moreover, administering a pre-miR-21 to these mice augmented MCP-1 levels [440]. miR-155, although highly correlated with most immune cell types, showed high association (Rs > 0.750) with B cell, $CD11b^{+}$DC and monocyte-derived $CD11b^{+}$DC numbers, highlighting its intricate role in B cell and DC functionality [306,309]. Interestingly, reduced miR-218 expression was negatively correlated (Rs < -0.750) with both neutrophils, B cells, T cells and DC numbers, suggesting that a reduced miR-218 expression could be implicated in directional migration of these cell types towards the inflamed lung [154]. In addition, our results highlight a strong association of miR-31* with active smoking in mice and both cohorts of patients with COPD, as well as with DC subsets. An association of miR-31* with DC numbers is in agreement with a robust increase in both miR-31 transcripts in myeloid dendritic cells upon TLR stimulation in hypoxic conditions [441].

These results put forward some interesting miRNAs considerably altered following CS exposure both in lung tissue and BAL supernatant of matched murine samples. Correlation of altered miRNA expression with the change in inflammatory profile, suggests a possible implication of these miRNAs in CS-induced inflammation. Evaluating the miRNA expression profile in two different respiratory compartments augments the relevance of our findings, although mechanistic data are lacking. Interestingly, we translated some of our findings to the human situation by discussing the overlap between our murine data and miRNA profiling data in human sputum and lung [153,154].

In conclusion, we highlight some interesting miRNAs in CS-induced inflammation in the lung by integrating *in vivo* miRNA profiling data in both BAL supernatant and lung tissue following subacute and chronic CS exposure and *in silico* correlations with inflammatory parameters. Future research will be needed to investigate the exact impact of these miRNAs in CS-induced inflammation and the pathogenesis of COPD.

Table 1A. Differentially expressed microRNAs in murine lung tissue following 4 weeks of cigarette smoke exposure

Up-regulated in CS-exposed mice	miRBase release 21	Fold change	Adjusted p-value
mmu-miR-135b	mmu-miR-135b-5p	4.110	0.015
mmu-miR-138	mmu-miR-138-5p	2.160	0.015
mmu-miR-21	mmu-miR-21a-5p	1.747	0.015
mmu-miR-155	mmu-miR-155-5p	1.580	0.021
mmu-miR-31	mmu-miR-31-5p	1.238	0.024

Down-regulated in CS-exposed mice	miRBase release 21	Fold change	Adjusted p-value
mmu-miR-322x	mmu-miR-322-3p	1.695	0.015
mmu-miR-351	mmu-miR-351-5p	1.585	0.024
mmu-miR-434-3p	mmu-miR-434-3p	1.531	0.024
mmu-miR-136	mmu-miR-136-5p	1.531	0.021

First column: annotation of miRNA during microRNA profiling; Second column: annotation of miRNA according to miRBase release 21

Table 1B. Differentially expressed microRNAs in murine lung tissue following 24 weeks of cigarette smoke exposure

Up-regulated in CS-exposed mice	miRBase release 21	Fold change	Adjusted p-value
mmu-miR-135b	mmu-miR-135b-5p	13.593	0.004
mmu-miR-135a	mmu-miR-135a-5p	8.557	0.014
mmu-miR-21	mmu-miR-21a-5p	3.103	0.004
mmu-miR-155	mmu-miR-155-5p	2.274	0.004
mmu-miR-146a	mmu-miR-146a-5p	1.969	0.004
mmu-miR-138	mmu-miR-138-5p	1.908	0.007
mmu-miR-449b	mmu-miR-449c-5p	1.764	0.015
mmu-miR-31x	mmu-miR-31-3p	1.452	0.004
mmu-miR-18a	mmu-miR-18a-5p	1.435	0.046
mmu-miR-31	mmu-miR-31-5p	1.403	0.040
mmu-miR-449	mmu-miR-449a-5p	1.386	0.046
mmu-miR-342-3p	mmu-miR-342-3p	1.322	0.004
mmu-miR-200c	mmu-miR-200c-3p	1.229	0.046
mmu-miR-148a	mmu-miR-148a-3p	1.186	0.046
mmu-miR-191	mmu-miR-191-5p	1.148	0.040

Down-regulated in CS-exposed mice	miRBase release 21	Fold change	Adjusted p-value
mmu-miR-322x	mmu-miR-322-3p	1.953	0.010
mmu-miR-489	mmu-miR-489-3p	1.890	0.033
mmu-miR-1	mmu-miR-1a-3p	1.733	0.023
mmu-miR-133b	mmu-miR-133b-3p	1.553	0.033
mmu-miR-503	mmu-miR-503-5p	1.520	0.015
mmu-miR-376bx	mmu-miR-376b-5p	1.493	0.046
mmu-miR-365	mmu-miR-365-3p	1.479	0.007
mmu-miR-149	mmu-miR-149-5p	1.443	0.010
mmu-miR-503x	mmu-miR-503-3p	1.433	0.040
mmu-miR-224	mmu-miR-224-5p	1.393	0.046
mmu-miR-26a	mmu-miR-26a-5p	1.362	0.010
mmu-miR-145	mmu-miR-145a-5p	1.359	0.004
mmu-miR-676	mmu-miR-676-3p	1.302	0.023
mmu-miR-152	mmu-miR-152-3p	1.242	0.040
mmu-miR-30c	mmu-miR-30c-5p	1.241	0.046
mmu-miR-30a-5p	mmu-miR-30a-5p	1.127	0.040

First column: annotation of miRNA during microRNA profiling; Second column: annotation of miRNA according to miRBase release 21

Table 2A. Differentially expressed microRNAs in bronchoalveolar lavage supernatant following 4 weeks of cigarette smoke exposure

Up-regulated in CS-exposed mice	miRBase release 21	Fold change	Adjusted p-value
mmu-miR-138	mmu-miR-138-5p	2.048	0.044
mmu-miR-182	mmu-miR-182-5p	1.727	0.050

Down-regulated in CS-exposed mice	miRBase release 21	Fold change	Adjusted p-value
mmu-miR-676	mmu-miR-676-3p	1.678	0.044
mmu-miR-574-3p	mmu-miR-574-3p	1.458	0.044
mmu-let-7c	mmu-let-7c-5p	1.433	0.050
mmu-let-7b	mmu-let-7b-5p	1.325	0.025

First column: annotation of miRNA during microRNA profiling; Second column: annotation of miRNA according to miRBase release 21

Table 2B. Up-regulated microRNAs in bronchoalveolar lavage supernatant following 24 weeks of cigarette smoke exposure

Up-regulated in CS-exposed mice	miRBase release 21	Fold change	Adjusted p-value
mmu-miR-680	mmu-miR-680	20.924	0.005
mmu-miR-221	mmu-miR-221-3p	14.606	0.003
mmu-miR-297ax	mmu-miR-297a-3p	6.614	0.007
mmu-miR-150	mmu-miR-150-5p	6.382	0.002
mmu-miR-142-5p	mmu-miR-142a-5p	4.554	0.003
mmu-miR-685		4.256	0.002
mmu-miR-139-5p	mmu-miR-139-5p	3.853	0.002
mmu-miR-146a	mmu-miR-146a-5p	3.573	0.002
mmu-miR-706	mmu-miR-706	3.531	0.002
mmu-miR-142-3p	mmu-miR-142a-3p	3.382	0.002
mmu-miR-340	mmu-miR-340-3p	2.900	0.010
mmu-miR-592	mmu-miR-592-5p	2.684	0.014
mmu-let-7ax	mmu-let-7a-1-3p	2.294	0.007
mmu-let-7i	mmu-let-7i-5p	2.165	0.007
mmu-miR-218-1x	mmu-miR-218-1-3p	2.149	0.049
mmu-miR-690	mmu-miR-690	2.143	0.007
mmu-miR-140-3p	mmu-miR-140-3p	2.043	0.002
mmu-miR-9x	mmu-miR-9-3p	2.042	0.014
mmu-miR-25	mmu-miR-25-3p	2.030	0.004
mmu-miR-20b	mmu-miR-20b-5p	1.964	0.007
mmu-miR-342-3p	mmu-miR-342-3p	1.954	0.018
mmu-miR-31x	mmu-miR-31-3p	1.923	0.010
mmu-miR-21	mmu-miR-21a-5p	1.909	0.002
mmu-miR-130b	mmu-miR-130b-3p	1.807	0.004
mmu-miR-652	mmu-miR-652-3p	1.717	0.018
mmu-miR-15b	mmu-miR-15b-5p	1.625	0.041
mmu-miR-146b	mmu-miR-146b-5p	1.619	0.007
mmu-miR-19a	mmu-miR-19a-3p	1.580	0.006
mmu-miR-484	mmu-miR-484	1.550	0.004
mmu-miR-93	mmu-miR-93-5p	1.501	0.005
mmu-miR-672	mmu-miR-672-5p	1.471	0.007
mmu-miR-15a	mmu-miR-15a-5p	1.403	0.036
mmu-miR-31	mmu-miR-31-5p	1.397	0.018
mmu-miR-27a	mmu-miR-27a-3p	1.392	0.007
mmu-miR-20a	mmu-miR-20a-5p	1.373	0.002
mmu-miR-92	mmu-miR-92a-3p	1.365	0.007
mmu-miR-186	mmu-miR-186-5p	1.289	0.002
mmu-miR-140	mmu-miR-140-5p	1.261	0.018

First column: annotation of miRNA during microRNA profiling; Second column: annotation of miRNA according to miRBase release 21

Table 2C. Down-regulated microRNAs in bronchoalveolar lavage supernatant following 24 weeks of cigarette smoke exposure

Down-regulated in CS-exposed mice	miRBase release 21	Fold change	Adjusted p-value
mmu-miR-294	mmu-miR-294-3p	3.289	0.004
mmu-miR-470x	mmu-miR-470-3p	3.155	0.014
mmu-miR-188-5p	mmu-miR-188-5p	2.646	0.014
mmu-miR-29bx	mmu-miR-29b-1-5p	2.469	0.018
mmu-miR-376bx	mmu-miR-376b-5p	2.387	0.025
mmu-miR-326	mmu-miR-326-3p	2.304	0.018
mmu-miR-805		2.079	0.014
mmu-miR-345-5p	mmu-miR-345-5p	1.862	0.043
mmu-miR-375	mmu-miR-375-3p	1.812	0.002
mmu-miR-434-3p	mmu-miR-434-3p	1.757	0.018
mmu-miR-678	mmu-miR-678	1.701	0.043
mmu-miR-378	mmu-miR-378-5p	1.658	0.025
mmu-miR-30a-3p	mmu-miR-30a-3p	1.595	0.002
mmu-miR-30e-3p	mmu-miR-30e-3p	1.565	0.043
mmu-miR-10a	mmu-miR-10a-5p	1.553	0.010
mmu-miR-449	mmu-miR-449a-5p	1.506	0.034
mmu-let-7e	mmu-let-7e-5p	1.499	0.007
mmu-miR-872x	mmu-miR-872-3p	1.488	0.002
mmu-miR-218	mmu-miR-218-5p	1.481	0.014
mmu-miR-205	mmu-miR-205-5p	1.466	0.007
mmu-miR-193b	mmu-miR-193b-3p	1.458	0.002
mmu-let-7c	mmu-let-7c-5p	1.451	0.007
mmu-let-7d	mmu-let-7d-5p	1.420	0.018
mmu-miR-29ax	mmu-miR-29a-5p	1.408	0.043
mmu-miR-148a	mmu-miR-148a-3p	1.393	0.018
mmu-miR-130a	mmu-miR-130a-3p	1.377	0.004
mmu-miR-30e	mmu-miR-30e-5p	1.364	0.007
mmu-miR-200a	mmu-miR-200a-3p	1.353	0.002
mmu-miR-26a	mmu-miR-26a-5p	1.339	0.002
mmu-miR-152	mmu-miR-152-3p	1.328	0.010
mmu-let-7g	mmu-let-7g-5p	1.328	0.003
mmu-let-7b	mmu-let-7b-5p	1.321	0.004
mmu-miR-34c	mmu-miR-34c-5p	1.307	0.003
mmu-miR-30c	mmu-miR-30c-5p	1.295	0.034
mmu-miR-30d	mmu-miR-30d-5p	1.287	0.004
mmu-miR-30a-5p	mmu-miR-30a-5p	1.280	0.006
mmu-miR-708	mmu-miR-708-3p	1.264	0.043
mmu-miR-34b-3p	mmu-miR-34b-3p	1.264	0.025
mmu-miR-22x	mmu-miR-22-5p	1.263	0.043
mmu-miR-34cx	mmu-miR-34c-3p	1.253	0.034

First column: annotation of miRNA during microRNA profiling; Second column: annotation of miRNA according to miRBase release 21

Online Tables and Figures

Table S1. Patient characteristics of the GLUCOLD study population

	ex-smoker with COPD	current-smoker with COPD
n	22	41
male n (%)	20 (90.9)	34 (82.9)
age (years)	63.36 ± 8.19	58.51 ± 7.97
FEV_1 % predicted	60.77 ± 9.96	63.25 ± 10.44

Table S2. Overlap in miRNA expression in murine and human lung tissue following chronic cigarette smoke exposure and in patients with COPD. This table provides extra numeric information regarding Figure 6a.

	MOUSE		HUMAN	
	Fold change	Adj. p-value	Fold change	Adj. p-value
miR-135b	13.593	0.004	1.442	0.017
miR-148a	1.186	0.046	1.285	0.029
miR-149	-1.443	0.010	-1.514	0.024
miR-155	2.274	0.004	1.695	0.002
miR-191	1.148	0.040	1.265	0.034
miR-31	1.403	0.040	2.273	0.049
miR-31*	1.452	0.004	2.743	0.013

Table S3. Overlap in miRNA expression in murine lung tissue following chronic cigarette smoke exposure compared to air exposure and in bronchial biopsies of current smoking patients with COPD compared to ex-smoking patients with COPD. This table provides extra numeric information regarding Figure 6b.

	MOUSE		HUMAN	
	Fold change	Adj. p-value	Fold change	Adj. p-value
miR-31*	1.452	0.004	2.315	0.012

Table S4. Overlap in miRNA expression in murine BAL supernatant following chronic cigarette smoke exposure and in sputum supernatant of current smoking patients with COPD compared to non-smoking controls. This table provides extra numeric information regarding Figure 6c.

	MOUSE		HUMAN	
	Fold change	Adj. p-value	Fold change	Adj. p-value
let-7c	-1.451	0.007	-2.59	<0.001
miR-218	-1.481	0.014	-3.61	<0.001
miR-30e-3p	-1.565	0.043	-2.02	0.001
miR-34c	-1.307	0.003	-3.77	<0.001
miR-342-3p	1.954	0.018	-2.33	<0.001

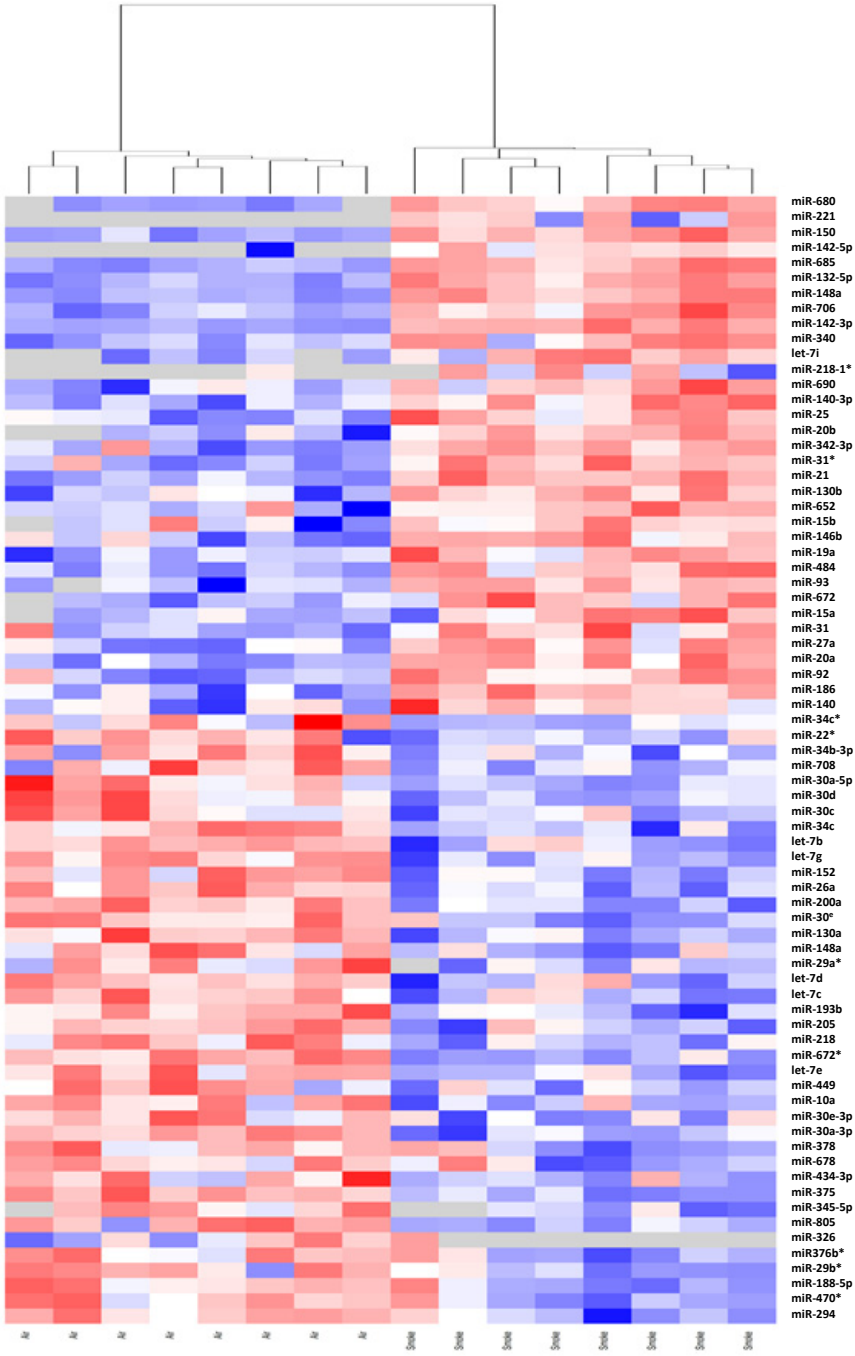


Figure S1. Enlargement of Figure 2c

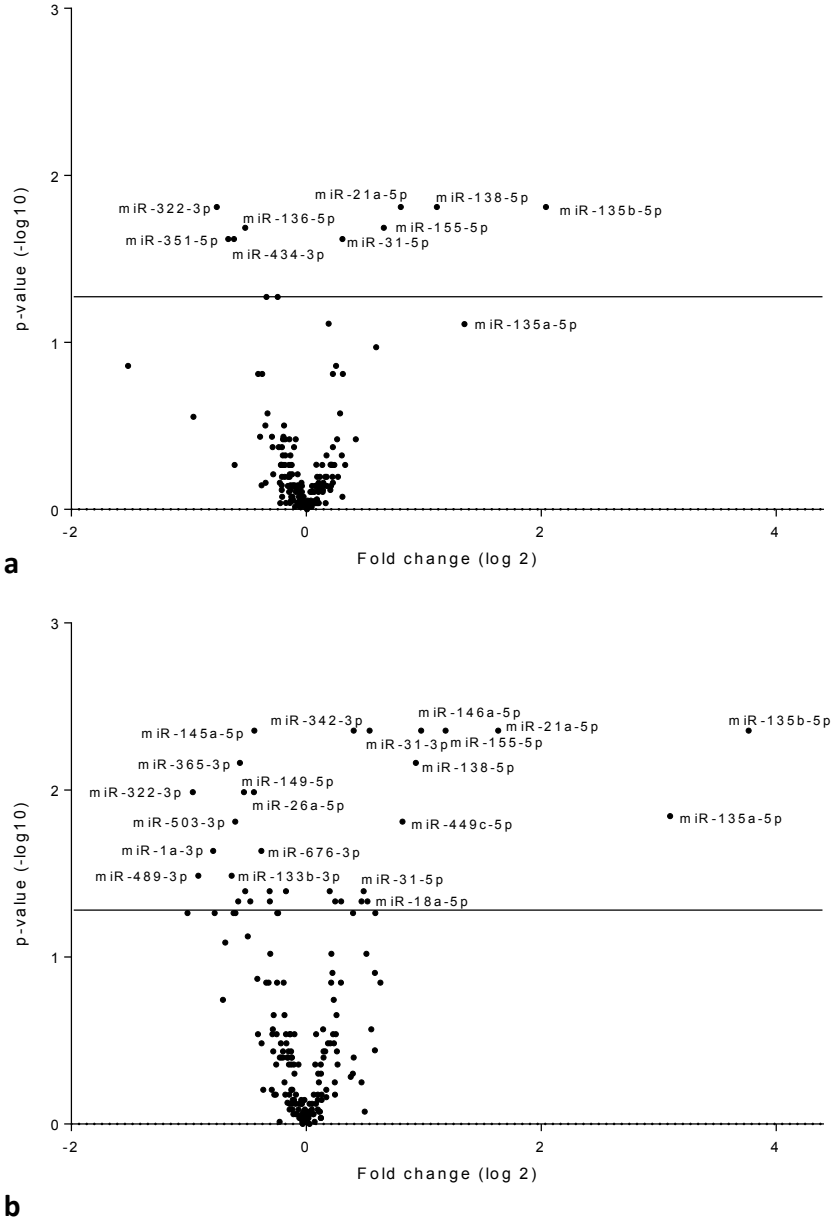
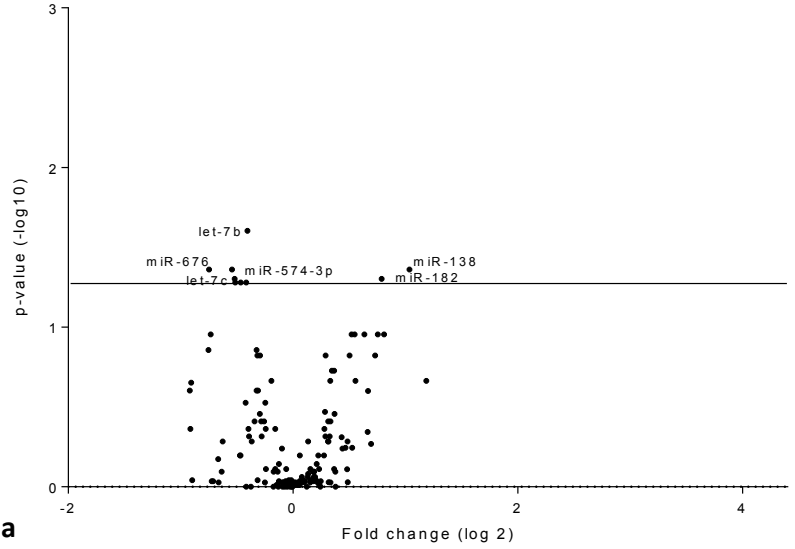


Figure S2. Volcano plots with significantly annotated miRNAs in lung tissue of cigarette smoke-exposed compared to air-exposed mice. (**a**) 4 weeks of CS/air exposure, (**b**) 24 weeks of CS/Air exposure. Volcano plots showing the differential miRNA expression (in fold change on the x-axis) and significance level (-log10-adjusted p-value on y-axis). The detected miRNAs are plotted as black dots. The horizontal line indicates the 0.05 significance level. This volcano plot is already presented in Figure 1 (without miRNA annotation).



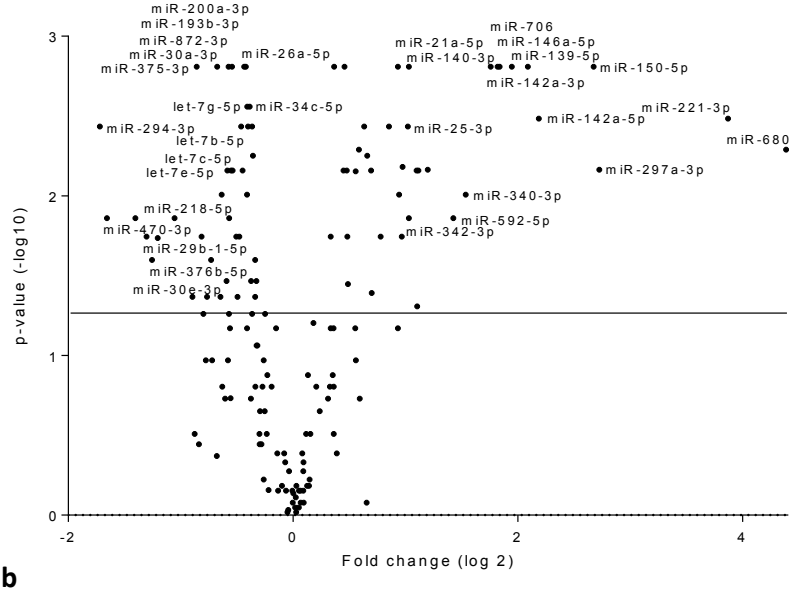


Figure S3. Volcano plots with significantly annotated miRNAs in bronchoalveolar lavage supernatant of cigarette smoke-exposed compared to air-exposed mice. (**a**) 4 weeks of CS/air exposure, (**b**) 24 weeks of CS/Air exposure. Volcano plots showing the differential miRNA expression (in fold change on the *x*-axis) and significance level (-$\log_{10}$-adjusted p-value on *y*-axis). The detected miRNAs are plotted as black dots. The horizontal line indicates the 0.05 significance level. This volcano plot is already presented in Figure 2 (without miRNA annotation).

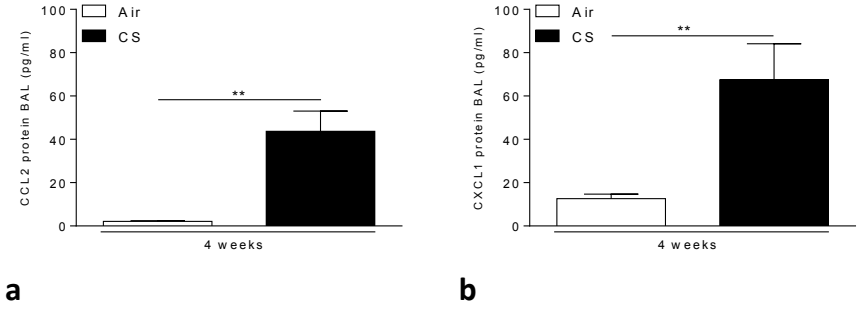


Figure S4. Chemokine levels in BAL supernatant of mice exposed to air or CS for 4 weeks. (a) Chemokine (C-C Motif) Ligand 2 (CCL2). (b) chemokine (C-X-C motif) ligand 1 (CXCL1). (n=8)

CHAPTER 7: Discussion and future perspectives

DISCUSSION AND FUTURE PERSPECTIVES

This research is concentrated on investigating the involvement of microRNAs (miRNAs), small regulatory non-protein coding RNA molecules, in the pathogenesis of COPD. Based on the knowledge that miRNAs play important regulatory roles in immune cell development and function, and that smoking affects miRNA expression levels, alterations in the expression pattern of miRNAs might be involved in the pathogenesis of COPD.

For this purpose, we accurately measured miRNA expression levels by stem-loop RT-qPCR in human and murine lung, and in murine cell-free bronchoalveolar lavage (BAL) supernatant. Some miRNAs were highlighted for their potential contribution to CS-induced inflammation and COPD pathogenesis. In particular, the functional role of miR-218-5p was assessed in detail by a translational research approach.

7.1 microRNA profiling in lung tissue of patients with COPD

As a first step, we performed a miRNA profiling on lung tissue of a screening cohort consisting of 12 patients with COPD, 10 smokers without airflow limitation and 8 never-smokers. Of the 740 miRNAs profiled, 377 miRNAs could be detected. Twenty-nine miRNAs were differentially expressed between smokers without airflow limitation and never-smokers, and 59 miRNAs were differentially expressed between smokers with COPD compared to never-smokers. Only 3 miRNAs were differentially expressed between smokers with or without airflow limitation. In another study, 70 miRNAs were differentially expressed in lung tissue between smokers with or without airflow limitation [152]. This could probably be caused by the fact that only patients with moderate COPD (GOLD II) were included in our study while in the other study a lot of patients had severe COPD (GOLD IV).

Across the 3 patient groups, 57 miRNAs were differentially expressed. After correcting for multiple testing, only 5 miRNAs remained significantly differentially expressed (**Figure 26**). Overall, most miRNAs were down-regulated in patients with COPD compared to never-smokers, indicating that the suppressive effect of these miRNAs on their target genes is abrogated. The observation that more miRNAs were down-regulated is in agreement with other miRNA profiling studies following CS exposure [149,150,248].

7.2 microRNA profiling in lung tissue and bronchoalveolar lavage supernatant of cigarette smoke-exposed mice

To perform basic research, our COPD mouse model is of great value. Although obvious species differences exist between mice and humans, the murine model has enabled us throughout the years

to answer specific questions of importance in the pathogenesis of COPD. Therefore, we used this CS-induced mouse model of COPD to identify alterations in miRNA expression levels in lung tissue and in BAL supernatant of mice that were exposed to air or CS for 4 (subacute) or 24 (chronic) weeks. Analogous to this, we investigated miRNA expression changes in lung tissue and in sputum supernatant of smokers with or without airflow limitation compared to never-smokers [153,154]. The advantage of investigating these miRNA expression changes in mice is that 1) we have matched murine samples both in lung and BAL supernatant, 2) we also acquired matched data on inflammatory cell subsets both in lung tissue and in BAL, 3) these mice have the same genotype, and 4) since most miRNAs are often highly conserved between humans and rodents, our data can be surveyed for overlapping miRNAs with human lung tissue and sputum supernatant data.

First, we started by performing a miRNA expression profiling in lung tissue and cell-free BAL supernatant of mice that were subacutely and chronically exposed to air or CS. miRNA profiling in lung tissue of rodents exposed to environmental CS was already performed, but not yet in combination with a miRNA profiling in BAL supernatant [150,151]. As expected, due to the different cellular content and tissue composition, a clear separation between miRNA profiles in BAL supernatant and lung was shown. Also, in BAL supernatant, the separation in miRNA profile between mice chronically exposed to air or CS was most obvious, corresponding to the relative increase in inflammatory cell numbers and cellular diversity following CS exposure.

In a next step, the overlap within each compartment as well as between compartments was assessed. Most differentially expressed miRNAs showed the same expression pattern in both compartments such as miR-146a-5p and miR-21a-5p. By correlating the miRNA expression profile with data on inflammatory cell subsets and chemokines, we could highlight some miRNAs, interesting for their contribution to CS-induced inflammation or because of their highly immune-cell specific expression or activity. Of note, miR-21a-5p, which is highly expressed in macrophages, correlated with macrophage numbers and CCL2 (MCP-1) expression in BAL. This is in agreement with data reporting that administration of a miR-21a-5p mimic induced MCP-1 levels [440]. In addition, miR-155-5p correlated with B cells, accentuating its role in B cell functionality [306]. We further searched for overlap with the previously assessed miRNA profiling data in human lung and sputum supernatant, all performed with the same stem-loop RT-qPCR technique and thus in that way, comparable [153,154,374]. This resulted in a selection of interesting differentially expressed miRNAs, putting forward these miRNAs (let-7b-5p, let-7c-5p, miR-135b-5p, miR-146a-5p, miR-149-5p, miR-155-5p, miR-21a-5p, miR-218-5p, miR-26a-5p, miR-31-3p, miR-31-5p, miR-332-3p, miR-342-3p, miR-376b-5p and miR-449a-5) as relevant in the CS-induced inflammation and in COPD (**Figure 26**).

7.3 miR-218-5p in cigarette smoke-induced inflammation and COPD

7.3.1 Expression of miR-218-5p

We showed that miR-218-5p was significantly down-regulated in patients with COPD. Both in our screening group of 30 patients as in our validation group of 71 patients, miR-218-5p levels were reduced in actively smoking individuals with or without airflow limitation compared to never-smokers. Remarkably, in ex-smokers without airflow limitation that quitted smoking for more than 1 year, miR-218-5p expression displayed normal levels, indicating that expression of miR-218-5p could be restored following smoking cessation. However, this was not the case in ex-smoking patients with COPD, especially not in those patients with severe COPD. Another research group classified miR-218-5p as a 'smoking cessation persistent miRNA' since after 3 months of smoking cessation, the expression of miR-218-5p remained significantly reduced compared to controls [34]. Apparently, more than 3 months of smoking cessation is needed for miR-218-5p levels to normalize in healthy smokers. Also, in our data, expression of miR-218-5p correlated with lung function parameters, meaning that low miRNA levels corresponded with worse lung function. Additionally, severe COPD was associated with reduced miR-218-5p expression, even when correcting for covariates including age and current smoking.

A decreased expression of miR-218-5p was also evaluated in human bronchial epithelial cells (HBECs) that were exposed to CS and in bronchial biopsies of patients with COPD, indicating a robust expression signature. Our findings in human airway epithelium confirm data of Schembri and coworkers where the expression of miR-218-5p was down-regulated in airway epithelium of smokers as well as in HBECs exposed to CSC compared to normal airway epithelium [149].

Besides the reported association with smoking, miR-218-5p was also found associated with COPD. However, miR-218-5p was not yet in-depth investigated in the context of COPD. In literature, miRNA profiling and consecutive cluster analysis was performed on the cellular fraction of BAL samples of patients with COPD and adenocarcinoma. Intriguingly, a significantly down-regulated miR-218-5p clustered to the COPD group [321]. In sputum supernatant, miR-218-5p was significantly lower expressed in smokers without airflow limitation and in smokers with COPD compared to never-smokers [153].

Interestingly, miRNA expression analysis in specific immune cell subsets revealed particular differential expression profiles following diverse stimuli. In human T cells, miR-218-5p was highly up-regulated following CD3/CD28 co-stimulation after 48h [442]. In human monocyte-derived macrophages challenged with different doses and durations of LPS, miR-218-5p was robustly down-regulated [443]. The longer the exposure, the more its expression was reduced.

7.3.2 Functional role of miR-218-5p

The two precursors of miR-218-5p, mir-218-1 and mir-218-2, are encoded on 4p15.31 and 5q35.1 within their respective host genes SLIT2 and SLIT3. Co-transcription of the intronic miR-218-5p with its host genes has been suggested, implying that both miR-218-5p and (one of) its host genes could be involved in the same processes [403,444]. SLIT2 is reported to inhibit directional migration of inflammatory cells (neutrophils, dendritic cells and T cells), which express the Roundabout (ROBO) 1 receptor, to the site of inflammation [445-449]. The pulmonary expression of SLIT2 is reduced in smokers with COPD versus smokers without airflow limitation, thus a lower expression of both miR-218-5p and SLIT2 might be involved in stimulating migration of inflammatory cells [152,168]. In this way, we hypothesized that miR-218-5p could contribute to the persistent inflammation in patients with COPD.

Furthermore, miR-218-5p has been described in many tumor types to function as a tumor suppressor thereby being able to influence cancer metastasis and proliferation but also to serve as a suitable biomarker to predict prognosis, staging of the tumor, response to therapy and survival [450-456]. As miR-218-5p is often down-regulated in tumors, reduced expression mostly implies a more invasive tumor and worse prognosis [457]. Reduced expression of the tumor suppressor miR-218-5p and its host genes has been found in the majority (80%) of NSCLCs [402]. Importantly, we assessed the expression of miR-218-5p in lung tissue of patients without lung cancer, or in tissue taken by the pathologist as far as possible from the tumor lesion. It has been shown that the expression of miR-218-5p varies in lung squamous cell carcinoma with tumor stage and according to location ranging from the lowest expression within the tumor to the highest expression at a distance from the tumor [416]. Intriguingly, we measured lowest expression values for miR-218-5p in lung tissue of severe ex-smoking COPD patients, none of them diagnosed with lung cancer.

In COPD, an inflammation/immune response-driven disease, we demonstrated by *in silico*, *in vitro* and *in vivo* analyses that miR-218-5p may have potential as a therapeutic. *In silico* analyses in human lung tissue and in normal HBECs revealed that a reduced expression of miR-218-5p was inversely associated with an enrichment for genes involved in immune, defense and inflammatory responses. *In vitro* transfection of normal HBECs with a miR-218-5p mimic showed that miR-218-5p has anti-inflammatory properties in CS-exposed cells compared to controls. In an *in vivo* murine experiment, we chose to intranasally administer a miR-218-5p inhibitor to air- or CS-exposed mice, in accordance with the reduced expression of miR-218-5p in lungs of patients with COPD. Since we observed more inflammatory cells in airways of mice receiving the miR-218-5p inhibitor compared to mice receiving the scrambled control sequence, we concluded that a lower expression of miR-218-5p is likely involved in the active recruitment of inflammatory cells towards the airways (**Figure 26**).

7.3.3 Future perspectives

Investigating miRNA expression in tissue leaves open questions about the impact of a given miRNA on the entire organism or on specific cell-types, as well as their interplay. In this context, we focused on normal HBECs and demonstrated the anti-inflammatory properties of miR-218-5p. In the future, it would be interesting to also include primary epithelial cells from COPD patients or smokers which can provide useful information regarding expression alterations linked to smoking history or disease.

Our preliminary results in a pre-clinical mouse model of COPD suggest that miR-218-5p is suited as a therapeutic, but further research is warranted. First, efficient delivery and uptake of a miR-218-5p mimic needs to be accomplished, preferably through the intranasal route in order to stimulate uptake through the bronchial epithelium, which is the cell type with a high expression of miR-218-5p in the lung. This should also improve the local distribution, avoiding extra-pulmonary effects. In a next step, the therapeutic potential should be assessed by administering miR-218-5p to mice that have been exposed to long-term CS with improvement on inflammation and characteristics of COPD pathology as primary end-points.

7.4 General future perspectives

Building on our miRNA profiling data in mice and humans, it would be interesting to investigate miR-135b-5p, miR-146a-5p, miR-155-5p, miR-21a-5p and miR-31-3p/-5p for their involvement in the pathogenesis of COPD. miR-146a-5p and miR-21a-5p are sensitive to TLR signaling and are crucial for their contribution to the resolution of inflammation [295,299]. CS induced expression of miR-31 in human bronchial epithelium and TLR stimulation in hypoxic conditions resulted in a robust increase of both miR-31 transcripts in myeloid DCs [441,458]. miR-155-5p is up-regulated following TLR activation and is intricately connected with the innate and adaptive immune response, especially with B cell and DC development and function [459]. miR-155-5p is regarded as a pro-inflammatory actor, of which the expression gradually increases with higher inflammatory state.

We started investigating miR-135b-5p and miR-21a-5p more in-depth for their contribution to the pathogenesis of COPD in collaboration with the lab of Prof. Hansbro.

For miR-21a-5p, we report a general increase in miR-21-5p expression in lungs of patients with COPD, which was associated with a reduction in lung function. Also, in both BALB/c mice with nose-only exposure and in C57BL/6 mice with whole body CS exposure, miR-21a-5p levels were up-regulated from 4 weeks of CS exposure onwards. Furthermore, a novel mode of action was identified for miR-21a-5p, mediating the NFκB pathway via SATB1 and S100A9 in CS-induced inflammation and the pathogenesis of COPD. *In vivo* inhibition of miR-21a-5p resulted in a reduction in pulmonary inflammation, remodeling and improvement in lung function, suggesting that inhibiting miR-21-5p might be a therapeutic option (manuscript in preparation).

A vital role for miR-21-5p in immune cell development and function has been frequently reported, as well as its contribution in repair and tissue regeneration [460-467]. In addition, the expression of miR-21-5p is dynamically regulated at several key points in its biogenesis [468]. For example, TGF-β signaling members regulate the processing of miR-21-5p [469]. miR-21-5p is a key miRNA in balancing between a pro- and an anti-inflammatory state. Thus, it is not surprising that a dysregulated miR-21-5p will have a major impact on the overall immune response, manifested ultimately as disease. Several arguments indicate potential relevance of miR-21-5p in COPD. First, there is a reported link with smoking history and COPD [470-472]. Although miR-21-5p was significantly elevated and miR-181a significantly lower expressed in serum samples of both asymptomatic heavy smokers and patients with COPD compared to healthy controls, an increased miR-21-5p to miR-181a ratio in serum was suggested as a valuable tool in predicting the occurrence of COPD in asymptomatic heavy smokers [470]. Further, miR-21-5p was significantly lower expressed in exhaled breath of COPD and asthma patients versus healthy controls [326]. Second, miR-21-5p is generally considered a biomarker of inflammation-associated diseases. Third,

since miR-21-5p is involved in both regeneration/repair and fibrosis, processes that partly rely on common mediators, it might also contribute to airway remodeling in patients with COPD. With the effect on remodeling in mind, it would also be interesting to investigate the functional role of miR-21-5p in cultured primary fibroblasts from patients with COPD compared with fibroblasts from controls.

Although miR-135b-5p is associated with CS-induced inflammation, its role in COPD remained to be elucidated. Therefore, we investigated the expression, localization and functional role of miR-135b-5p in the pathogenesis of COPD. First, the expression of miR-135b-5p was elevated in patients with COPD compared to never-smokers. Second, miR-135b-5p was highly expressed in bronchial epithelium, although its induction following CS was suggested to be mainly localized to alveolar type II cells. Further, by inhibiting miR-135b-5p in a COPD-mouse model (nose-only, 8 weeks air- or CS-exposure), we demonstrated that the CS-induced pulmonary inflammation is partially dependent on miR-135b-5p, as well as that miR-135b-5p is involved in emphysema development or progression, since mice receiving the miR-135b-5p inhibitor showed less parenchymal destruction and a better work of breathing (manuscript in preparation).
miR-135b-5p is strongly inducible upon challenge of the airways with different kinds of noxious particles (CS, nanotitanium dioxide, carbon black nanoparticles) [435,436,473]. Likely, the up-regulation of miR-135b-5p in particle- and CS-induced inflammation is IL-1RI-mediated. Once the inflammatory process is propagated, miR-135b-5p targets the IL-1RI in a negative feed-back loop in an attempt to resolve the inflammation. Target prediction and pathway analyses point towards a role for miR-135b-5p in WNT, TGF-β and BMP signaling by targeting TGFβR2, SMAD5, TGFβR1, ACVR1B and BMPR2 which are all involved in inflammatory responses [474-477].

miR-21-5p and miR-135b-5p have already been shown to be shed from exosomes to modulate the transcriptome in exosome-incorporating cells, thereby expanding their regulatory function beyond the cell of origin [478,479]. It has been reported that CS enhances the secretion of extracellular vesicles from lung epithelial cells, endothelial cells and alveolar macrophages. Also, the composition of these extracellular vesicles can be modified through this stressful condition [480]. In the future, it would be interesting to investigate whether miR-21-5p and miR-135b-5p play a role in this intercellular communication upon CS exposure. For example, by causing CS-induced injury to epithelial cells and using this supernatant for culturing fibroblasts.

Intriguingly, up-regulation of miR-135b-5p and miR-21-5p following acute inflammatory stimuli primarily led to resolution of the inflammation. However, in manifested disease, both contributed to the hallmarks of COPD.

Both miR-21-5p and miR-135b-5p have been identified as an oncomiR in several cancers and are often correlated with clinical stage and poor outcome [481,482]. This suggests a close and dangerous liaison between chronic inflammation and oncogenic transformation. However, the acquisition of a neoplastic state may require the succession of multiple cancer hallmarks. As a part of this multistep process, inflammation is regarded as an emerging hallmark for its tumor-promoting properties [483].

A complex disease such as COPD is never the result of one single perturbed gene or miRNA. Whether individual patients express different sets of disease-driving miRNAs remains to be tested. By performing research, our understanding of miRNA biology and functions within the lung will increase which will augment the opportunities to safely pursue miRNAs as therapeutic modalities. In light of therapeutic development of miRNA inhibitors or mimics, inhalation-based delivery should be aimed for in order to optimize a bio-distribution favoring the lung compartment and/or airways.

For the miRNA profiling in this research, we relied on the accurate and sensitive stem-loop RT-qPCR method. One disadvantage is that we could not detect isomiRs or discover new small RNA molecules that could have been present. Building further on this basis, performing RNA sequencing in lung tissue, sputum and blood – preferably matched – of patients with COPD compared to controls would add extra layers of information to unravel the pathogenesis of COPD. In that way, we obtain knowledge on small RNA, mRNA, lncRNA, and preferably also exosomal RNA, content.

More broadly, investigating non-coding RNA can represent a new step in understanding disease mechanisms. miRNAs and lncRNAs are highly dynamic regulatory molecules whose expression can vary spatially, temporally, or in response to stimuli. Their involvement in many diseases indicates that these non-coding RNAs may represent a gold mine of future biomarkers and drug targets. However, a large portion of lncRNAs has not yet been characterized. Some challenges in regard to sequencing analysis, annotation, the low level of cross-species conservation, the heterogeneity in structure and function, their predominant nuclear localization and often low expression levels hamper lncRNA research. Interestingly, these lncRNA molecules can serve as a source of miRNAs or can negatively regulate miRNA expression levels (ceRNA) [484].

The relevance of this work lays in the presentation of valuable miRNAs in COPD research. Although lower expressed, miRNAs can be detected in various body fluids. In line with identification of non-invasive miRNA biomarkers in diseases, we first highlight the miRNA profile in murine BAL fluid following subacute and chronic CS exposure in combination with the lung miRNA profile of the same mice, providing insights in miRNA alterations in both lung tissue and BAL supernatant. Investigating overlap or directional changes in differentially expressed miRNAs between both compartments can give information about their **potential as biomarkers** of CS-induced lung disease. Second, we show that 5 miRNAs out of the 8 differentially expressed miRNAs in sputum supernatant of patients with COPD overlap with the differentially expressed

miRNAs in BAL supernatant following chronic CS exposure, indicating the robustness of these 5 miRNAs as indicators of chronic CS exposure.

In addition, we pinpoint overlapping differentially expressed murine miRNAs within and between two lung compartments, as well as miRNAs that overlap with aberrantly expressed miRNAs in current smoking patients with COPD compared to never-smokers. The set of miRNA molecules that are transcribed in a certain condition reflects the current state of these cells or tissue, which can reveal pathological mechanisms underlying disease. Moreover, individual miRNAs can be drivers of disease. In this thesis, we highlight the anti-inflammatory properties of miR-218-5p, the influence of miR-21-5p on pulmonary inflammation and remodeling, and the impact of miR-135b-5p on development or progression of emphysema in COPD. However, their precise mode of action within this disease is still unknown and needs to be investigated in the future. In my opinion, restoring these miRNA levels, alongside others, bears **therapeutic potential** in COPD.

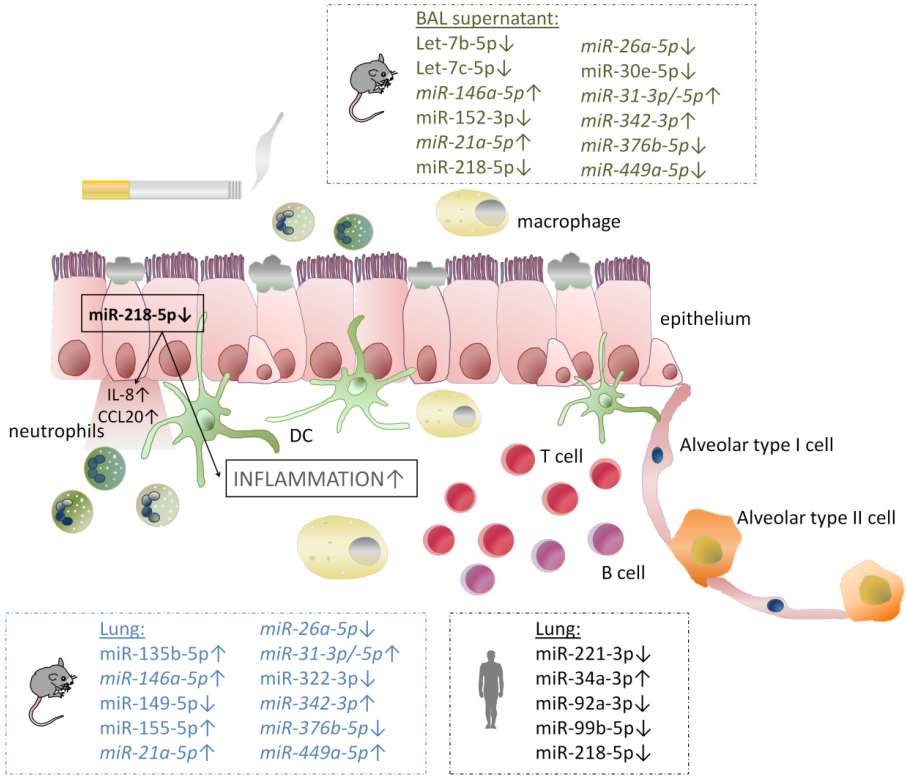


Figure 26. Schematic overview of the research work presented in this thesis

A reduced expression of miR-218-5p, predominantly localized in the bronchial epithelium, contributes to the sustained inflammatory response in patients with COPD by recruiting inflammatory cells.

miRNA profiling in both murine lung tissue and BAL supernatant revealed several interesting miRNAs. A selection was made based on 1) consistent overlap in the same compartment between subacute and chronic CS exposure, 2) overlap between murine BAL supernatant and lung following chronic CS exposure, 3) overlap between human samples (sputum supernatant, bronchial biopsies, lung tissue) of patients with COPD compared to never-smokers and murine samples (BAL supernatant, lung tissue) following chronic CS exposure and 4) fold change above 1.3. miRNAs that were as thus detected in both compartments are represented in italic. The direction of the arrow indicates the miRNA expression following CS exposure or in patients with COPD compared to non-smoking controls.

IL-8: interleukin-8; CCL20: Chemokine (C-C motif) ligand 20; DC: dendritic cell

CHAPTER 8: Summary/ Samenvatting

SUMMARY

Chronic obstructive pulmonary disease (COPD) is a respiratory condition that affects more than 200 million people worldwide and which is characterized by a progressive airflow limitation. The pathological changes are defined by underlying disease processes such as chronic bronchitis, obstructive bronchiolitis and emphysema. In addition, its pathology is hallmarked by an exaggerated inflammatory reaction in the lungs and airways following long-term inhalation of cigarette smoke. Remarkably, although smoking cessation slows down the accelerated decline in lung function, the inflammation in the lungs persists. Development of COPD in only a subgroup of smokers can be due to variability in genetic or epigenetic susceptibility.

The main objective of this thesis was to evaluate whether microRNAs (miRNAs) could contribute to the pathogenesis of COPD, as these small regulatory molecules are involved in inflammatory and immune responses, with as ultimate goal to identify promising miRNAs as therapeutic target.

First, we identified differentially expressed miRNAs in lung tissue of patients with COPD compared to never-smoking controls. The vast majority of miRNAs was down-regulated in COPD. After correction for multiple testing, 5 miRNAs remained significantly altered (**Figure 26**).

In a next step, we identified differentially expressed miRNAs in lung tissue and matched cell-free bronchoalveolar lavage (BAL) fluid of mice that were exposed to air or CS for 4 and 24 weeks. Importantly, miRNAs are generally highly conserved throughout evolution, making mouse models appropriate models to study miRNA expression and function. By digging into the miRNA profile of lung and matched BAL supernatant, combined with data on inflammation in both compartments, we were able to put forward some interesting miRNAs. Moreover, we showed concordant overlap with the differential miRNA expression in human lung, bronchial biopsies and sputum supernatant of patients with COPD (**Figure 26**).

Second, we investigated the functional role of miR-218-5p in COPD. miR-218-5p was one of the lower expressed miRNAs in patients with COPD compared to never-smokers. This reduced expression was validated in a large patient cohort, in human bronchial biopsies, in lung tissue of CS-exposed mice and in HBECs. *In situ* hybridization in human and murine lung revealed highest expression of miR-218-5p in the bronchial airway epithelium. Moreover, miR-218-5p correlated strongly with airway obstruction and GSEA indicated an inverse relationship of miR-218-5p with inflammatory responses. By performing *in vitro* and *in vivo* perturbation experiments, we demonstrated that a reduced expression of miR-218-5p is involved in the recruitment of inflammatory cells towards the airways, thereby assisting in the sustained chronic inflammation in patients with COPD (**Figure 26**).

SAMENVATTING

Chronisch obstructief longlijden (COPD) is een respiratoire aandoening die wereldwijd meer dan 200 miljoen mensen treft en die gekenmerkt wordt door een progressief bemoeilijkte ademhaling. De pathologische veranderingen worden bepaald door onderliggende ziekteprocessen zoals chronische bronchitis, obstructieve bronchiolitis en emfyseem. De pathologie wordt ook gekenmerkt door een overdreven ontstekingsreactie in de longen en luchtwegen als gevolg van jarenlange inhalatie van sigarettenrook. Treffend is dat zelfs wanneer er wordt gestopt met roken de ontstekingsreactie in de longen blijft voortbestaan, ondanks een verminderde achteruitgang van de longfunctie. Slechts een bepaalde subgroep van rokers ontwikkelt deze ziekte. Waarschijnlijk is een zekere variatie in genetische of epigenetische susceptibiliteit hiervoor verantwoordelijk.
We wilden tijdens dit onderzoek te weten komen of microRNAs (miRNAs), kleine RNA moleculen die inflammatoire en immunologische processen reguleren, betrokken zijn in de pathogenese van COPD, met als belangrijkste doel het identificeren van beloftevolle miRNAs als therapeutisch target.

Ten eerste hebben we miRNAs geïdentificeerd die differentieel tot expressie komen in longweefsel van COPD patiënten in vergelijking met longweefsel van mensen die nooit rookten, waarvan de meeste miRNAs waren gedaald in COPD patiënten. Na correctie voor meervoudig toetsen kwamen 5 miRNAs significant differentieel uit deze analyse (**Figuur 26**).
Nadien identificeerden we differentieel geëxpresseerde miRNAs in longweefsel en broncho-alveolair (BAL) lavage vocht van aan lucht of sigarettenrook blootgestelde muizen. Het is ook belangrijk om weten dat de sequentie van miRNAs goed bewaard is gebleven tijdens de evolutie zodat muismodellen geschikt zijn voor het bestuderen van de expressie en functie van miRNAs. Door het miRNA profiel in muizenlong en gepaarde BAL supernatans stalen te onderzoeken, samen met gegevens over het inflammatoir profiel in deze muizen, konden we enkele interessante miRNAs naar voor schuiven. Ook toonden we een overlap aan met differentieel geëxpresseerde miRNAs in humaan longweefsel, bronchiale biopsieën en sputum supernatans van COPD patiënten (**Figuur 26**).
Ten tweede hebben we de functionele rol van miR-218-5p bestudeerd in COPD. miR-218-5p was één van de miRNAs die gedaald was in COPD. Deze gedaalde expressie werd gevalideerd in een grote patiënten cohorte, in humane bronchiale biopsieën, in longen van aan rook blootgestelde muizen en in humane bronchiale epitheelcellen. *In situ* hybridisatie in humane- en muizenlong toonde aan dat miR-218-5p zeer hoog tot expressie komt in het bronchiale epitheel. De expressie van miR-218-5p correleerde sterk met luchtwegobstructie en GSEA duidde een omgekeerde associatie aan met de inflammatoire respons. *In vitro* en *in vivo* perturbatie experimenten geven aan dat een gedaalde expressie van miR-218-5p betrokken is bij het rekruteren van inflammatoire cellen naar de luchtwegen waardoor dit miRNA bijdraagt aan de chronische inflammatie in COPD patiënten (**Figuur 26**).

III. *ADDENDUM*

REFERENCES

CURRICULUM VITAE

LIST OF PUBLICATIONS

REFERENCES

1 Vogelmeier, C. F. *et al.* Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Lung Disease 2017 Report: GOLD Executive Summary. *Am J Respir Crit Care Med*, doi:10.1164/rccm.201701-0218PP (2017).

2 Decramer, M., Janssens, W. & Miravitlles, M. Chronic obstructive pulmonary disease. *Lancet* **379**, 1341-1351, doi:10.1016/S0140-6736(11)60968-9 (2012).

3 Buist, A. S. *et al.* International variation in the prevalence of COPD (the BOLD Study): a population-based prevalence study. *Lancet* **370**, 741-750, doi:10.1016/S0140-6736(07)61377-4 (2007).

4 Lamprecht, B. *et al.* Determinants of underdiagnosis of COPD in national and international surveys. *Chest* **148**, 971-985, doi:10.1378/chest.14-2535 (2015).

5 Pauwels, R. A. & Rabe, K. F. Burden and clinical features of chronic obstructive pulmonary disease (COPD). *Lancet* **364**, 613-620, doi:10.1016/S0140-6736(04)16855-4 (2004).

6 Mannino, D. M. & Buist, A. S. Global burden of COPD: risk factors, prevalence, and future trends. *Lancet* **370**, 765-773, doi:10.1016/s0140-6736(07)61380-4 (2007).

7 Anthonisen, N. R. *et al.* Effects of smoking intervention and the use of an inhaled anticholinergic bronchodilator on the rate of decline of FEV1. The Lung Health Study. *JAMA* **272**, 1497-1505 (1994).

8 Barnes, P. J. New anti-inflammatory targets for chronic obstructive pulmonary disease. *Nature reviews. Drug discovery* **12**, 543-559 (2013).

9 Vestbo, J. *et al.* Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med* **187**, 347-365, doi:10.1164/rccm.201204-0596PP (2013).

10 Stockley, R. A., Mannino, D. & Barnes, P. J. Burden and pathogenesis of chronic obstructive pulmonary disease. *Proceedings of the American Thoracic Society* **6**, 524-526, doi:10.1513/pats.200904-016DS (2009).

11 Lozano, R. *et al.* Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* **380**, 2095-2128, doi:10.1016/S0140-6736(12)61728-0 (2012).

12 WHO. Burden of disease. (accessed August 2017). Available from http://www.who.int/topics/global_burden_of_disease/en/.

13 European Respiratory Society. The European Lung White Book - *Chapter 1: the burden of lung disease.* (2013).

14 Terzikhan, N. *et al.* Prevalence and incidence of COPD in smokers and non-smokers: the Rotterdam Study. *Eur J Epidemiol* **31**, 785-792, doi:10.1007/s10654-016-0132-z (2016).

15 Afonso, A. S., Verhamme, K. M., Sturkenboom, M. C. & Brusselle, G. G. COPD in the general population: prevalence, incidence and survival. *Respir Med* **105**, 1872-1884, doi:10.1016/j.rmed.2011.06.012 (2011).

16 Muka, T. *et al.* The global impact of non-communicable diseases on healthcare spending and national income: a systematic review. *Eur J Epidemiol* **30**, 251-277, doi:10.1007/s10654-014-9984-2 (2015).

17 Kessler, R. *et al.* Symptom variability in patients with severe COPD: a pan-European cross-sectional study. *Eur Respir J* **37**, 264-272, doi:10.1183/09031936.00051110 (2011).

18 Bestall, J. C. *et al.* Usefulness of the Medical Research Council (MRC) dyspnoea scale as a measure of disability in patients with chronic obstructive pulmonary disease. *Thorax* **54**, 581-586 (1999).

19 Jones, P. W. *et al.* Development and first validation of the COPD Assessment Test. *Eur Respir J* **34**, 648-654, doi:10.1183/09031936.00102509 (2009).

20 Karloh, M. *et al.* The COPD Assessment Test: What Do We Know So Far?: A Systematic Review and Meta-Analysis About Clinical Outcomes Prediction and Classification of Patients Into GOLD Stages. *Chest* **149**, 413-425, doi:10.1378/chest.15-1752 (2016).
21 Saetta, M. *et al.* Goblet cell hyperplasia and epithelial inflammation in peripheral airways of smokers with both symptoms of chronic bronchitis and chronic airflow limitation. *Am J Respir Crit Care Med* **161**, 1016-1021, doi:10.1164/ajrccm.161.3.9907080 (2000).
22 Wedzicha, J. A. & Seemungal, T. A. COPD exacerbations: defining their cause and prevention. *Lancet* **370**, 786-796, doi:10.1016/S0140-6736(07)61382-8 (2007).
23 Barnes, P. J. & Celli, B. R. Systemic manifestations and comorbidities of COPD. *Eur Respir J* **33**, 1165-1185, doi:10.1183/09031936.00128008 (2009).
24 Agusti, A. *et al.* Characterisation of COPD heterogeneity in the ECLIPSE cohort. *Respir Res* **11**, 122, doi:10.1186/1465-9921-11-122 (2010).
25 Mannino, D. M., Thorn, D., Swensen, A. & Holguin, F. Prevalence and outcomes of diabetes, hypertension and cardiovascular disease in COPD. *Eur Respir J* **32**, 962-969, doi:10.1183/09031936.00012408 (2008).
26 Rennard, S. I. & Vestbo, J. COPD: the dangerous underestimate of 15%. *Lancet* **367**, 1216-1219, doi:10.1016/S0140-6736(06)68516-4 (2006).
27 Sandford, A. J. & Silverman, E. K. Chronic obstructive pulmonary disease. 1: Susceptibility factors for COPD the genotype-environment interaction. *Thorax* **57**, 736-741 (2002).
28 Stoller, J. K. & Aboussouan, L. S. alpha 1-antitrypsin deficiency. *Lancet* **365**, 2225-2236, doi:Doi 10.1016/S0140-6736(05)66781-5 (2005).
29 Wu, X., Yuan, B., Lopez, E., Bai, C. & Wang, X. Gene polymorphisms and chronic obstructive pulmonary disease. *J Cell Mol Med* **18**, 15-26, doi:10.1111/jcmm.12159 (2014).
30 Guida, F. *et al.* Dynamics of smoking-induced genome-wide methylation changes with time since smoking cessation. *Human molecular genetics* **24**, 2349-2359, doi:10.1093/hmg/ddu751 (2015).
31 Zeilinger, S. *et al.* Tobacco smoking leads to extensive genome-wide changes in DNA methylation. *Plos One* **8**, e63812, doi:10.1371/journal.pone.0063812 (2013).
32 Zhang, Y., Yang, R., Burwinkel, B., Breitling, L. P. & Brenner, H. F2RL3 methylation as a biomarker of current and lifetime smoking exposures. *Environmental health perspectives* **122**, 131-137, doi:10.1289/ehp.1306937 (2014).
33 Ito, K. *et al.* Decreased histone deacetylase activity in chronic obstructive pulmonary disease. *The New England journal of medicine* **352**, 1967-1976, doi:10.1056/NEJMoa041892 (2005).
34 Wang, G. *et al.* Persistence of Smoking-Induced Dysregulation of MiRNA Expression in the Small Airway Epithelium Despite Smoking Cessation. *Plos One* **10**, e0120824, doi:10.1371/journal.pone.0120824 (2015).
35 Salvi, S. S. & Barnes, P. J. Chronic obstructive pulmonary disease in non-smokers. *Lancet* **374**, 733-743, doi:10.1016/S0140-6736(09)61303-9 (2009).
36 MacNee, W. & Tuder, R. M. New paradigms in the pathogenesis of chronic obstructive pulmonary disease I. *Proceedings of the American Thoracic Society* **6**, 527-531, doi:10.1513/pats.200905-027DS (2009).
37 Silva, G. E., Sherrill, D. L., Guerra, S. & Barbee, R. A. Asthma as a risk factor for COPD in a longitudinal study. *Chest* **126**, 59-65, doi:10.1378/chest.126.1.59 (2004).
38 Lange, P. *et al.* Lung-Function Trajectories Leading to Chronic Obstructive Pulmonary Disease. *The New England journal of medicine* **373**, 111-122, doi:10.1056/NEJMoa1411532 (2015).
39 Barker, D. J. *et al.* Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease. *BMJ* **303**, 671-675 (1991).
40 Hayden, L. P. *et al.* Childhood pneumonia increases risk for chronic obstructive pulmonary disease: the COPDGene study. *Respir Res* **16**, 115, doi:10.1186/s12931-015-0273-8 (2015).
41 Bush, A. Lung Development and Aging. *Annals of the American Thoracic Society* **13**, S438-S446, doi:10.1513/AnnalsATS.201602-112AW (2016).

42 Martinez, F. D. Early-Life Origins of Chronic Obstructive Pulmonary Disease. *The New England journal of medicine* **375**, 871-878, doi:10.1056/NEJMra1603287 (2016).
43 Tager, I. B., Ngo, L. & Hanrahan, J. P. Maternal smoking during pregnancy. Effects on lung function during the first 18 months of life. *Am J Respir Crit Care Med* **152**, 977-983, doi:10.1164/ajrccm.152.3.7663813 (1995).
44 Guerra, S. *et al.* Combined effects of parental and active smoking on early lung function deficits: a prospective study from birth to age 26 years. *Thorax* **68**, 1021-1028, doi:10.1136/thoraxjnl-2013-203538 (2013).
45 Hylkema, M. N. & Blacquiere, M. J. Intrauterine effects of maternal smoking on sensitization, asthma, and chronic obstructive pulmonary disease. *Proceedings of the American Thoracic Society* **6**, 660-662, doi:10.1513/pats.200907-065DP (2009).
46 Fletcher, C. & Peto, R. The natural history of chronic airflow obstruction. *Br Med J* **1**, 1645-1648 (1977).
47 Brusselle, G. G. Matrix Metalloproteinase 12, Asthma, and COPD. *New England Journal of Medicine* **361**, 2664-2665, doi:10.1056/NEJMe0910626 (2009).
48 Rabe, K. F. *et al.* Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med* **176**, 532-555, doi:10.1164/rccm.200703-456SO (2007).
49 Han, M. K. *et al.* GOLD 2011 disease severity classification in COPDGene: a prospective cohort study. *Lancet Respir Med* **1**, 43-50, doi:10.1016/S2213-2600(12)70044-9 (2013).
50 Shapiro, S. D. End-stage chronic obstructive pulmonary disease: the cigarette is burned out but inflammation rages on. *Am J Respir Crit Care Med* **164**, 339-340, doi:10.1164/ajrccm.164.3.2105072c (2001).
51 Willemse, B. W. *et al.* Effect of 1-year smoking cessation on airway inflammation in COPD and asymptomatic smokers. *Eur Respir J* **26**, 835-845, doi:10.1183/09031936.05.00108904 (2005).
52 Hodge, S., Hodge, G., Holmes, M. & Reynolds, P. N. Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. *Eur Respir J* **25**, 447-454, doi:10.1183/09031936.05.00077604 (2005).
53 Fahy, J. V. & Dickey, B. F. Airway mucus function and dysfunction. *The New England journal of medicine* **363**, 2233-2247, doi:10.1056/NEJMra0910061 (2010).
54 Hogg, J. C. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. *Lancet* **364**, 709-721, doi:10.1016/s0140-6736(04)16900-6 (2004).
55 Hogg, J. C., Macklem, P. T. & Thurlbeck, W. M. Site and nature of airway obstruction in chronic obstructive lung disease. *The New England journal of medicine* **278**, 1355-1360, doi:10.1056/NEJM196806202782501 (1968).
56 Hogg, J. C., McDonough, J. E. & Suzuki, M. Small airway obstruction in COPD: new insights based on micro-CT imaging and MRI imaging. *Chest* **143**, 1436-1443, doi:10.1378/chest.12-1766 (2013).
57 Hogg, J. C. & Timens, W. The pathology of chronic obstructive pulmonary disease. *Annual review of pathology* **4**, 435-459, doi:10.1146/annurev.pathol.4.110807.092145 (2009).
58 Hogg, J. C. *et al.* The nature of small-airway obstruction in chronic obstructive pulmonary disease. *The New England journal of medicine* **350**, 2645-2653, doi:10.1056/NEJMoa032158 (2004).
59 McDonough, J. E. *et al.* Small-airway obstruction and emphysema in chronic obstructive pulmonary disease. *The New England journal of medicine* **365**, 1567-1575, doi:10.1056/NEJMoa1106955 (2011).
60 Magee, F., Wright, J. L., Wiggs, B. R., Pare, P. D. & Hogg, J. C. Pulmonary vascular structure and function in chronic obstructive pulmonary disease. *Thorax* **43**, 183-189 (1988).
61 Peinado, V. I. *et al.* Endothelial dysfunction in pulmonary arteries of patients with mild COPD. *The American journal of physiology* **274**, L908-913 (1998).

62 Munoz-Esquerre, M. *et al.* Systemic and Pulmonary Vascular Remodelling in Chronic Obstructive Pulmonary Disease. *Plos One* **11**, e0152987, doi:10.1371/journal.pone.0152987 (2016).
63 Chaouat, A., Naeije, R. & Weitzenblum, E. Pulmonary hypertension in COPD. *Eur Respir J* **32**, 1371-1385, doi:10.1183/09031936.00015608 (2008).
64 Singh, D. *et al.* Current Controversies in the Pharmacological Treatment of Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* **194**, 541-549, doi:10.1164/rccm.201606-1179PP (2016).
65 Celli, B. R. *et al.* The body-mass index, airflow obstruction, dyspnea, and exercise capacity index in chronic obstructive pulmonary disease. *The New England journal of medicine* **350**, 1005-1012, doi:10.1056/NEJMoa021322 (2004).
66 Puhan, M. A. *et al.* Expansion of the prognostic assessment of patients with chronic obstructive pulmonary disease: the updated BODE index and the ADO index. *Lancet* **374**, 704-711, doi:10.1016/S0140-6736(09)61301-5 (2009).
67 Jones, R. C. *et al.* Multi-component assessment of chronic obstructive pulmonary disease: an evaluation of the ADO and DOSE indices and the global obstructive lung disease categories in international primary care data sets. *NPJ Prim Care Respir Med* **26**, 16010, doi:10.1038/npjpcrm.2016.10 (2016).
68 Agusti, A. & Vestbo, J. Current controversies and future perspectives in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **184**, 507-513, doi:10.1164/rccm.201103-0405PP (2011).
69 Mesquita, R. *et al.* Activity levels and exercise motivation in COPD patients and their resident loved ones. *Chest*, doi:10.1016/j.chest.2016.12.021 (2017).
70 Ferreira, I. M., Brooks, D., White, J. & Goldstein, R. Nutritional supplementation for stable chronic obstructive pulmonary disease. *Cochrane Database Syst Rev* **12**, CD000998, doi:10.1002/14651858.CD000998.pub3 (2012).
71 Janssens, W. *et al.* Vitamin D deficiency is highly prevalent in COPD and correlates with variants in the vitamin D-binding gene. *Thorax* **65**, 215-220, doi:10.1136/thx.2009.120659 (2010).
72 Holick, M. F. Vitamin D deficiency. *The New England journal of medicine* **357**, 266-281, doi:10.1056/NEJMra070553 (2007).
73 Heulens, N. *et al.* Vitamin D deficiency exacerbates COPD-like characteristics in the lungs of cigarette smoke-exposed mice. *Respir Res* **16**, 110, doi:10.1186/s12931-015-0271-x (2015).
74 Young, R. P., Hopkins, R. J. & Marsland, B. The Gut-Liver-Lung Axis. Modulation of the Innate Immune Response and Its Possible Role in Chronic Obstructive Pulmonary Disease. *Am J Respir Cell Mol Biol* **54**, 161-169, doi:10.1165/rcmb.2015-0250PS (2016).
75 Sethi, S. & Murphy, T. F. Infection in the pathogenesis and course of chronic obstructive pulmonary disease. *The New England journal of medicine* **359**, 2355-2365, doi:10.1056/NEJMra0800353 (2008).
76 King, P. T., MacDonald, M. & Bardin, P. G. Bacteria in COPD; their potential role and treatment. *Transl Respir Med* **1**, 13, doi:10.1186/2213-0802-1-13 (2013).
77 Curtis, J. L., Freeman, C. M. & Hogg, J. C. The immunopathogenesis of chronic obstructive pulmonary disease: insights from recent research. *Proceedings of the American Thoracic Society* **4**, 512-521, doi:10.1513/pats.200701-002FM (2007).
78 Cosio, M. G., Saetta, M. & Agusti, A. Immunologic aspects of chronic obstructive pulmonary disease. *The New England journal of medicine* **360**, 2445-2454, doi:10.1056/NEJMra0804752 (2009).
79 MacNee, W., Wiggs, B., Belzberg, A. S. & Hogg, J. C. The effect of cigarette smoking on neutrophil kinetics in human lungs. *The New England journal of medicine* **321**, 924-928, doi:10.1056/NEJM198910053211402 (1989).
80 Medzhitov, R. Origin and physiological roles of inflammation. *Nature* **454**, 428-435, doi:10.1038/nature07201 (2008).

81 Hallstrand, T. S. *et al.* Airway epithelial regulation of pulmonary immune homeostasis and inflammation. *Clin Immunol* **151**, 1-15, doi:10.1016/j.clim.2013.12.003 (2014).
82 Brusselle, G. G., Joos, G. F. & Bracke, K. R. New insights into the immunology of chronic obstructive pulmonary disease. *Lancet* **378**, 1015-1026, doi:10.1016/s0140-6736(11)60988-4 (2011).
83 Schulz, C. *et al.* Expression and release of interleukin-8 by human bronchial epithelial cells from patients with chronic obstructive pulmonary disease, smokers, and never-smokers. *Respiration* **70**, 254-261, doi:72006 (2003).
84 Zhang, X. *et al.* Increased interleukin (IL)-8 and decreased IL-17 production in chronic obstructive pulmonary disease (COPD) provoked by cigarette smoke. *Cytokine* **56**, 717-725, doi:10.1016/j.cyto.2011.09.010 (2011).
85 Nadigel, J., Audusseau, S., Baglole, C. J., Eidelman, D. H. & Hamid, Q. IL-8 production in response to cigarette smoke is decreased in epithelial cells from COPD patients. *Pulm Pharmacol Ther* **26**, 596-602, doi:10.1016/j.pupt.2013.03.002 (2013).
86 Bracke, K. R. *et al.* Cigarette smoke-induced pulmonary inflammation and emphysema are attenuated in CCR6-deficient mice. *J Immunol* **177**, 4350-4359 (2006).
87 Demedts, I. K. *et al.* Accumulation of dendritic cells and increased CCL20 levels in the airways of patients with chronic obstructive pulmonary disease. *Am J Resp Crit Care* **175**, 998-1005, doi:10.1164/rccm.200608-1113OC (2007).
88 Shaykhiev, R. *et al.* Smoking-dependent reprogramming of alveolar macrophage polarization: implication for pathogenesis of chronic obstructive pulmonary disease. *J Immunol* **183**, 2867-2883, doi:10.4049/jimmunol.0900473 (2009).
89 De Grove, K. C. *et al.* Characterization and Quantification of Innate Lymphoid Cell Subsets in Human Lung. *Plos One* **11**, e0145961, doi:10.1371/journal.pone.0145961 (2016).
90 Vermaelen, K. Y., Carro-Muino, I., Lambrecht, B. N. & Pauwels, R. A. Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. *The Journal of experimental medicine* **193**, 51-60 (2001).
91 Shan, M. *et al.* Lung myeloid dendritic cells coordinately induce TH1 and TH17 responses in human emphysema. *Sci Transl Med* **1**, 4ra10, doi:10.1126/scitranlsmed.3000154 (2009).
92 Freeman, C. M. *et al.* Cytotoxic potential of lung CD8(+) T cells increases with chronic obstructive pulmonary disease severity and with in vitro stimulation by IL-18 or IL-15. *J Immunol* **184**, 6504-6513, doi:10.4049/jimmunol.1000006 (2010).
93 Knobloch, J. *et al.* The T-helper cell type 1 immune response to gram-negative bacterial infections is impaired in COPD. *Am J Respir Crit Care Med* **183**, 204-214, doi:10.1164/rccm.201002-0199OC (2011).
94 Roos, A. B. *et al.* IL-17A and the Promotion of Neutrophilia in Acute Exacerbation of Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* **192**, 428-437, doi:10.1164/rccm.201409-1689OC (2015).
95 Bozinovski, S. & Vlahos, R. Multifaceted Role for IL-17A in the Pathogenesis of Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* **191**, 1213-1214, doi:10.1164/rccm.201504-0714ED (2015).
96 Roos, A. B. *et al.* IL-17A Is Elevated in End-Stage Chronic Obstructive Pulmonary Disease and Contributes to Cigarette Smoke-induced Lymphoid Neogenesis. *Am J Respir Crit Care Med* **191**, 1232-1241, doi:10.1164/rccm.201410-1861OC (2015).
97 Miossec, P., Korn, T. & Kuchroo, V. K. Interleukin-17 and type 17 helper T cells. *The New England journal of medicine* **361**, 888-898, doi:10.1056/NEJMra0707449 (2009).
98 Alcorn, J. F., Crowe, C. R. & Kolls, J. K. TH17 cells in asthma and COPD. *Annual review of physiology* **72**, 495-516, doi:10.1146/annurev-physiol-021909-135926 (2010).
99 Di Stefano, A. *et al.* T helper type 17-related cytokine expression is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients. *Clin Exp Immunol* **157**, 316-324, doi:10.1111/j.1365-2249.2009.03965.x (2009).

100 Burzyn, D., Benoist, C. & Mathis, D. Regulatory T cells in nonlymphoid tissues. *Nature immunology* **14**, 1007-1013, doi:10.1038/ni.2683 (2013).
101 Polverino, F., Seys, L. J., Bracke, K. R. & Owen, C. A. B cells in chronic obstructive pulmonary disease: moving to center stage. *American journal of physiology. Lung cellular and molecular physiology* **311**, L687-L695, doi:10.1152/ajplung.00304.2016 (2016).
102 MacNee, W. Pulmonary and systemic oxidant/antioxidant imbalance in chronic obstructive pulmonary disease. *Proceedings of the American Thoracic Society* **2**, 50-60, doi:10.1513/pats.200411-056SF (2005).
103 McGuinness, A. J. & Sapey, E. Oxidative Stress in COPD: Sources, Markers, and Potential Mechanisms. *J Clin Med* **6**, doi:10.3390/jcm6020021 (2017).
104 Malhotra, D. *et al.* Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis. *Nucleic Acids Res* **38**, 5718-5734, doi:10.1093/nar/gkq212 (2010).
105 Malhotra, D. *et al.* Decline in NRF2-regulated antioxidants in chronic obstructive pulmonary disease lungs due to loss of its positive regulator, DJ-1. *Am J Respir Crit Care Med* **178**, 592-604, doi:10.1164/rccm.200803-380OC (2008).
106 Rangasamy, T. *et al.* Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. *The Journal of clinical investigation* **114**, 1248-1259, doi:10.1172/JCI21146 (2004).
107 Mercado, N. *et al.* Decreased histone deacetylase 2 impairs Nrf2 activation by oxidative stress. *Biochem Biophys Res Commun* **406**, 292-298, doi:10.1016/j.bbrc.2011.02.035 (2011).
108 Boutten, A., Goven, D., Artaud-Macari, E., Boczkowski, J. & Bonay, M. NRF2 targeting: a promising therapeutic strategy in chronic obstructive pulmonary disease. *Trends in molecular medicine* **17**, 363-371, doi:10.1016/j.molmed.2011.02.006 (2011).
109 MacNee, W. Accelerated lung aging: a novel pathogenic mechanism of chronic obstructive pulmonary disease (COPD). *Biochemical Society transactions* **37**, 819-823, doi:10.1042/BST0370819 (2009).
110 Kirkwood, T. B. Understanding the odd science of aging. *Cell* **120**, 437-447, doi:10.1016/j.cell.2005.01.027 (2005).
111 Tsuji, T., Aoshiba, K. & Nagai, A. Cigarette smoke induces senescence in alveolar epithelial cells. *Am J Respir Cell Mol Biol* **31**, 643-649, doi:10.1165/rcmb.2003-0290OC (2004).
112 Suga, T. *et al.* Disruption of the klotho gene causes pulmonary emphysema in mice. Defect in maintenance of pulmonary integrity during postnatal life. *Am J Respir Cell Mol Biol* **22**, 26-33, doi:10.1165/ajrcmb.22.1.3554 (2000).
113 Maruyama, N. *et al.* Senescence marker protein-30 knockout mouse as an aging model. *Annals of the New York Academy of Sciences* **1019**, 383-387, doi:10.1196/annals.1297.068 (2004).
114 Agusti, A. G. *et al.* Skeletal muscle apoptosis and weight loss in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **166**, 485-489, doi:10.1164/rccm.2108013 (2002).
115 Langen, R. C. *et al.* Muscle wasting and impaired muscle regeneration in a murine model of chronic pulmonary inflammation. *Am J Respir Cell Mol Biol* **35**, 689-696, doi:10.1165/rcmb.2006-0103OC (2006).
116 Berenson, C. S., Garlipp, M. A., Grove, L. J., Maloney, J. & Sethi, S. Impaired phagocytosis of nontypeable Haemophilus influenzae by human alveolar macrophages in chronic obstructive pulmonary disease. *The Journal of infectious diseases* **194**, 1375-1384, doi:10.1086/508428 (2006).
117 Taylor, A. E. *et al.* Defective macrophage phagocytosis of bacteria in COPD. *Eur Respir J* **35**, 1039-1047, doi:10.1183/09031936.00036709 (2010).
118 Sethi, S., Maloney, J., Grove, L., Wrona, C. & Berenson, C. S. Airway inflammation and bronchial bacterial colonization in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **173**, 991-998, doi:10.1164/rccm.200509-1525OC (2006).

119 Eltzschig, H. K. & Carmeliet, P. Hypoxia and inflammation. *The New England journal of medicine* **364**, 656-665, doi:10.1056/NEJMra0910283 (2011).
120 Yadava, K. *et al.* Microbiota Promotes Chronic Pulmonary Inflammation by Enhancing IL-17A and Autoantibodies. *Am J Respir Crit Care Med* **193**, 975-987, doi:10.1164/rccm.201504-0779OC (2016).
121 Sze, M. A. *et al.* Host Response to the Lung Microbiome in Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* **192**, 438-445, doi:10.1164/rccm.201502-0223OC (2015).
122 Wang, Z. *et al.* Lung microbiome dynamics in COPD exacerbations. *Eur Respir J* **47**, 1082-1092, doi:10.1183/13993003.01406-2015 (2016).
123 Tuder, R. M. & Petrache, I. Pathogenesis of chronic obstructive pulmonary disease. *The Journal of clinical investigation* **122**, 2749-2755, doi:10.1172/JCI60324 (2012).
124 Sandford, A. J., Joos, L. & Pare, P. D. Genetic risk factors for chronic obstructive pulmonary disease. *Current opinion in pulmonary medicine* **8**, 87-94 (2002).
125 Aierken, H. *et al.* Polymorphisms of the ADAM33 gene and chronic obstructive pulmonary disease risk: a meta-analysis. *Clin Respir J* **8**, 108-115, doi:10.1111/crj.12046 (2014).
126 Lomas, D. A. & Silverman, E. K. The genetics of chronic obstructive pulmonary disease. *Respir Res* **2**, 20-26 (2001).
127 Hofman, A. *et al.* The Rotterdam Study: 2014 objectives and design update. *Eur J Epidemiol* **28**, 889-926, doi:10.1007/s10654-013-9866-z (2013).
128 Manolio, T. A. Genomewide association studies and assessment of the risk of disease. *The New England journal of medicine* **363**, 166-176, doi:10.1056/NEJMra0905980 (2010).
129 Hindorff, L. A. *et al.* Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. *Proc Natl Acad Sci U S A* **106**, 9362-9367, doi:10.1073/pnas.0903103106 (2009).
130 Altshuler, D., Daly, M. J. & Lander, E. S. Genetic mapping in human disease. *Science (New York, N.Y.)* **322**, 881-888, doi:10.1126/science.1156409 (2008).
131 Hancock, D. B. *et al.* Meta-analyses of genome-wide association studies identify multiple loci associated with pulmonary function. *Nature genetics* **42**, 45-52, doi:10.1038/ng.500 (2010).
132 Loth, D. W. *et al.* Genome-wide association analysis identifies six new loci associated with forced vital capacity. *Nature genetics*, doi:10.1038/ng.3011 (2014).
133 Soler Artigas, M. *et al.* Genome-wide association and large-scale follow up identifies 16 new loci influencing lung function. *Nature genetics* **43**, 1082-1090, doi:10.1038/ng.941 (2011).
134 Boueiz, A. *et al.* Genome-Wide Association Study of the Genetic Determinants of Emphysema Distribution. *Am J Respir Crit Care Med*, doi:10.1164/rccm.201605-0997OC (2016).
135 Hancock, D. B. *et al.* Genome-wide joint meta-analysis of SNP and SNP-by-smoking interaction identifies novel loci for pulmonary function. *PLoS genetics* **8**, e1003098, doi:10.1371/journal.pgen.1003098 (2012).
136 Repapi, E. *et al.* Genome-wide association study identifies five loci associated with lung function. *Nature genetics* **42**, 36-44, doi:10.1038/ng.501 (2010).
137 Wain, L. V. *et al.* Genome-wide association analyses for lung function and chronic obstructive pulmonary disease identify new loci and potential druggable targets. *Nature genetics*, doi:10.1038/ng.3787 (2017).
138 Hobbs, B. D. *et al.* Genetic loci associated with chronic obstructive pulmonary disease overlap with loci for lung function and pulmonary fibrosis. *Nature genetics*, doi:10.1038/ng.3752 (2017).
139 Brusselle, G. G. & Bracke, K. R. Elucidating COPD pathogenesis by large-scale genetic analyses. *Lancet Respir Med* **3**, 737-739, doi:10.1016/S2213-2600(15)00360-4 (2015).
140 Wilk, J. B. *et al.* Genome-Wide Association Studies Identify CHRNA5/3 and HTR4 in the Development of Airflow Obstruction. *Am J Resp Crit Care* **186**, 622-632, doi:10.1164/rccm.201202-0366OC (2012).

141 Dupont, L. L. *et al.* Investigation of 5-HT receptors in bronchial hyperresponsiveness in cigarette smoke-exposed mice. *Pulm Pharmacol Ther*, doi:10.1016/j.pupt.2013.10.003 (2013).
142 Pillai, S. G. *et al.* Loci identified by genome-wide association studies influence different disease-related phenotypes in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **182**, 1498-1505, doi:10.1164/rccm.201002-0151OC (2010).
143 Obeidat, M. *et al.* Molecular mechanisms underlying variations in lung function: a systems genetics analysis. *Lancet Respir Med* **3**, 782-795, doi:10.1016/S2213-2600(15)00380-X (2015).
144 Rakyan, V. K., Down, T. A., Balding, D. J. & Beck, S. Epigenome-wide association studies for common human diseases. *Nat Rev Genet* **12**, 529-541, doi:10.1038/nrg3000 (2011).
145 Fraga, M. F. *et al.* Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci U S A* **102**, 10604-10609, doi:10.1073/pnas.0500398102 (2005).
146 Breton, C. V. *et al.* Prenatal tobacco smoke exposure affects global and gene-specific DNA methylation. *Am J Respir Crit Care Med* **180**, 462-467, doi:10.1164/rccm.200901-0135OC (2009).
147 Liu, F. *et al.* Epigenomic alterations and gene expression profiles in respiratory epithelia exposed to cigarette smoke condensate. *Oncogene* **29**, 3650-3664, doi:10.1038/onc.2010.129 (2010).
148 Szulakowski, P. *et al.* The effect of smoking on the transcriptional regulation of lung inflammation in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **174**, 41-50, doi:10.1164/rccm.200505-725OC (2006).
149 Schembri, F. *et al.* MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium. *Proc Natl Acad Sci U S A* **106**, 2319-2324, doi:10.1073/pnas.0806383106 (2009).
150 Izzotti, A. *et al.* Downregulation of microRNA expression in the lungs of rats exposed to cigarette smoke. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **23**, 806-812, doi:10.1096/fj.08-121384 (2009).
151 Izzotti, A., Calin, G. A., Steele, V. E., Croce, C. M. & De Flora, S. Relationships of microRNA expression in mouse lung with age and exposure to cigarette smoke and light. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **23**, 3243-3250, doi:10.1096/fj.09-135251 (2009).
152 Ezzie, M. E. *et al.* Gene expression networks in COPD: microRNA and mRNA regulation. *Thorax* **67**, 122-131, doi:10.1136/thoraxjnl-2011-200089 (2012).
153 Van Pottelberge, G. R. *et al.* MicroRNA Expression in Induced Sputum of Smokers and Patients with Chronic Obstructive Pulmonary Disease. *Am J Resp Crit Care* **183**, 898-906, doi:DOI 10.1164/rccm.201002-0304OC (2011).
154 Conickx, G. *et al.* MicroRNA Profiling Reveals a Role for MicroRNA-218-5p in the Pathogenesis of Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med* **195**, 43-56, doi:10.1164/rccm.201506-1182OC (2017).
155 De Smet, E. G., Mestdagh, P., Vandesompele, J., Brusselle, G. G. & Bracke, K. R. Non-coding RNAs in the pathogenesis of COPD. *Thorax* **70**, 782-791, doi:10.1136/thoraxjnl-2014-206560 (2015).
156 Thai, P. *et al.* Characterization of a Novel Long Non-Coding RNA, SCAL1, Induced by Cigarette Smoke and Elevated in Lung Cancer Cell Lines. *Am J Respir Cell Mol Biol*, doi:10.1165/rcmb.2013-0159RC (2013).
157 Bi, H. *et al.* Microarray analysis of long non-coding RNAs in COPD lung tissue. *Inflammation research : official journal of the European Histamine Research Society ... [et al.]* **64**, 119-126, doi:10.1007/s00011-014-0790-9 (2015).
158 Bosse, Y. *et al.* Molecular signature of smoking in human lung tissues. *Cancer Res* **72**, 3753-3763, doi:10.1158/0008-5472.CAN-12-1160 (2012).

159 Landi, M. T. *et al.* Gene expression signature of cigarette smoking and its role in lung adenocarcinoma development and survival. *Plos One* **3**, e1651, doi:10.1371/journal.pone.0001651 (2008).
160 Miura, K. *et al.* Laser capture microdissection and microarray expression analysis of lung adenocarcinoma reveals tobacco smoking- and prognosis-related molecular profiles. *Cancer Res* **62**, 3244-3250 (2002).
161 Powell, C. A. *et al.* Gene expression in lung adenocarcinomas of smokers and nonsmokers. *Am J Respir Cell Mol Biol* **29**, 157-162, doi:10.1165/rcmb.2002-0183RC (2003).
162 Ning, W. *et al.* Comprehensive gene expression profiles reveal pathways related to the pathogenesis of chronic obstructive pulmonary disease. *Proc Natl Acad Sci U S A* **101**, 14895-14900, doi:10.1073/pnas.0401168101 (2004).
163 Wang, I. M. *et al.* Gene expression profiling in patients with chronic obstructive pulmonary disease and lung cancer. *Am J Respir Crit Care Med* **177**, 402-411, doi:10.1164/rccm.200703-390OC (2008).
164 Faner, R. *et al.* Network Analysis of Lung Transcriptomics Reveals a Distinct B-Cell Signature in Emphysema. *Am J Respir Crit Care Med* **193**, 1242-1253, doi:10.1164/rccm.201507-1311OC (2016).
165 Steiling, K., Lenburg, M. E. & Spira, A. Airway gene expression in chronic obstructive pulmonary disease. *Proceedings of the American Thoracic Society* **6**, 697-700, doi:10.1513/pats.200907-076DP (2009).
166 Brody, J. S. & Steiling, K. Interaction of cigarette exposure and airway epithelial cell gene expression. *Annual review of physiology* **73**, 437-456, doi:10.1146/annurev-physiol-012110-142219 (2011).
167 Gower, A. C., Steiling, K., Brothers, J. F., 2nd, Lenburg, M. E. & Spira, A. Transcriptomic studies of the airway field of injury associated with smoking-related lung disease. *Proceedings of the American Thoracic Society* **8**, 173-179, doi:10.1513/pats.201011-066MS (2011).
168 Steiling, K. *et al.* A dynamic bronchial airway gene expression signature of chronic obstructive pulmonary disease and lung function impairment. *Am J Respir Crit Care Med* **187**, 933-942, doi:10.1164/rccm.201208-1449OC (2013).
169 Spira, A. *et al.* Effects of cigarette smoke on the human airway epithelial cell transcriptome. *Proc Natl Acad Sci U S A* **101**, 10143-10148, doi:10.1073/pnas.0401422101 (2004).
170 Harvey, B. G. *et al.* Modification of gene expression of the small airway epithelium in response to cigarette smoking. *Journal of molecular medicine (Berlin, Germany)* **85**, 39-53, doi:10.1007/s00109-006-0103-z (2007).
171 Beane, J. *et al.* Characterizing the impact of smoking and lung cancer on the airway transcriptome using RNA-Seq. *Cancer prevention research (Philadelphia, Pa.)* **4**, 803-817, doi:10.1158/1940-6207.capr-11-0212 (2011).
172 Beane, J. *et al.* Reversible and permanent effects of tobacco smoke exposure on airway epithelial gene expression. *Genome biology* **8**, R201, doi:10.1186/gb-2007-8-9-r201 (2007).
173 Sridhar, S. *et al.* Smoking-induced gene expression changes in the bronchial airway are reflected in nasal and buccal epithelium. *BMC genomics* **9**, 259, doi:10.1186/1471-2164-9-259 (2008).
174 Zhang, X. *et al.* Similarities and differences between smoking-related gene expression in nasal and bronchial epithelium. *Physiological genomics* **41**, 1-8, doi:10.1152/physiolgenomics.00167.2009 (2010).
175 van den Berge, M. *et al.* Airway gene expression in COPD is dynamic with inhaled corticosteroid treatment and reflects biological pathways associated with disease activity. *Thorax*, doi:10.1136/thoraxjnl-2012-202878 (2013).
176 Heguy, A. *et al.* Gene expression profiling of human alveolar macrophages of phenotypically normal smokers and nonsmokers reveals a previously unrecognized subset of genes

modulated by cigarette smoking. *Journal of molecular medicine (Berlin, Germany)* **84**, 318-328, doi:10.1007/s00109-005-0008-2 (2006).
177 Charlesworth, J. C. *et al.* Transcriptomic epidemiology of smoking: the effect of smoking on gene expression in lymphocytes. *BMC medical genomics* **3**, 29, doi:10.1186/1755-8794-3-29 (2010).
178 Barabasi, A. L., Gulbahce, N. & Loscalzo, J. Network medicine: a network-based approach to human disease. *Nat Rev Genet* **12**, 56-68, doi:10.1038/nrg2918 (2011).
179 Vidal, M., Cusick, M. E. & Barabasi, A. L. Interactome networks and human disease. *Cell* **144**, 986-998, doi:10.1016/j.cell.2011.02.016 (2011).
180 McDonald, M. L. *et al.* Beyond GWAS in COPD: probing the landscape between gene-set associations, genome-wide associations and protein-protein interaction networks. *Hum Hered* **78**, 131-139, doi:10.1159/000365589 (2014).
181 Sarkar, M., Bhardwaj, R., Madabhavi, I. & Khatana, J. Osteoporosis in chronic obstructive pulmonary disease. *Clin Med Insights Circ Respir Pulm Med* **9**, 5-21, doi:10.4137/CCRPM.S22803 (2015).
182 Maclay, J. D. & MacNee, W. Cardiovascular disease in COPD: mechanisms. *Chest* **143**, 798-807, doi:10.1378/chest.12-0938 (2013).
183 Durham, A. L. & Adcock, I. M. The relationship between COPD and lung cancer. *Lung cancer (Amsterdam, Netherlands)* **90**, 121-127, doi:10.1016/j.lungcan.2015.08.017 (2015).
184 Yohannes, A. M. & Ershler, W. B. Anemia in COPD: a systematic review of the prevalence, quality of life, and mortality. *Respiratory care* **56**, 644-652, doi:10.4187/respcare.01002 (2011).
185 Burgel, P. R. *et al.* Clinical COPD phenotypes: a novel approach using principal component and cluster analyses. *Eur Respir J* **36**, 531-539, doi:10.1183/09031936.00175109 (2010).
186 Han, M. K. *et al.* Chronic obstructive pulmonary disease phenotypes: the future of COPD. *Am J Respir Crit Care Med* **182**, 598-604, doi:10.1164/rccm.200912-1843CC (2010).
187 Rennard, S. I. *et al.* Identification of five chronic obstructive pulmonary disease subgroups with different prognoses in the ECLIPSE cohort using cluster analysis. *Annals of the American Thoracic Society* **12**, 303-312, doi:10.1513/AnnalsATS.201403-125OC (2015).
188 Celli, B. R. *et al.* An Official American Thoracic Society/European Respiratory Society Statement: Research questions in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **191**, e4-e27, doi:10.1164/rccm.201501-0044ST (2015).
189 Grana, R., Benowitz, N. & Glantz, S. A. E-cigarettes: a scientific review. *Circulation* **129**, 1972-1986, doi:10.1161/CIRCULATIONAHA.114.007667 (2014).
190 Ernst, P., Saad, N. & Suissa, S. Inhaled corticosteroids in COPD: the clinical evidence. *Eur Respir J* **45**, 525-537, doi:10.1183/09031936.00128914 (2015).
191 Pauwels, R. A. *et al.* Long-term treatment with inhaled budesonide in persons with mild chronic obstructive pulmonary disease who continue smoking. European Respiratory Society Study on Chronic Obstructive Pulmonary Disease. *The New England journal of medicine* **340**, 1948-1953, doi:10.1056/NEJM199906243402503 (1999).
192 Loke, Y. K., Cavallazzi, R. & Singh, S. Risk of fractures with inhaled corticosteroids in COPD: systematic review and meta-analysis of randomised controlled trials and observational studies. *Thorax* **66**, 699-708, doi:10.1136/thx.2011.160028 (2011).
193 Wedzicha, J. A. *et al.* Indacaterol-Glycopyrronium versus Salmeterol-Fluticasone for COPD. *The New England journal of medicine* **374**, 2222-2234, doi:10.1056/NEJMoa1516385 (2016).
194 Brusselle, G. G., Bracke, K. & Lahousse, L. Targeted therapy with inhaled corticosteroids in COPD according to blood eosinophil counts. *Lancet Respir Med* **3**, 416-417, doi:10.1016/S2213-2600(15)00145-9 (2015).
195 Brightling, C. E. *et al.* Sputum eosinophilia and the short term response to inhaled mometasone in chronic obstructive pulmonary disease. *Thorax* **60**, 193-198, doi:10.1136/thx.2004.032516 (2005).

196 Singh, D. *et al.* Eosinophilic inflammation in COPD: prevalence and clinical characteristics. *Eur Respir J* **44**, 1697-1700, doi:10.1183/09031936.00162414 (2014).
197 Cazzola, M. *et al.* Influence of N-acetylcysteine on chronic bronchitis or COPD exacerbations: a meta-analysis. *Eur Respir Rev* **24**, 451-461, doi:10.1183/16000617.00002215 (2015).
198 Calverley, P. M. *et al.* Roflumilast in symptomatic chronic obstructive pulmonary disease: two randomised clinical trials. *Lancet* **374**, 685-694, doi:10.1016/S0140-6736(09)61255-1 (2009).
199 Sanz, M. J., Cortijo, J. & Morcillo, E. J. PDE4 inhibitors as new anti-inflammatory drugs: Effects on cell trafficking and cell adhesion molecules expression. *Pharmacology & therapeutics* **106**, 269-297, doi:10.1016/j.pharmthera.2004.12.001 (2005).
200 Hatzelmann, A. *et al.* The preclinical pharmacology of roflumilast--a selective, oral phosphodiesterase 4 inhibitor in development for chronic obstructive pulmonary disease. *Pulm Pharmacol Ther* **23**, 235-256, doi:10.1016/j.pupt.2010.03.011 (2010).
201 Wedzicha, J. A., Calverley, P. M. & Rabe, K. F. Roflumilast: a review of its use in the treatment of COPD. *International journal of chronic obstructive pulmonary disease* **11**, 81-90, doi:10.2147/COPD.S89849 (2016).
202 Boswell-Smith, V. *et al.* The pharmacology of two novel long-acting phosphodiesterase 3/4 inhibitors, RPL554 [9,10-dimethoxy-2(2,4,6-trimethylphenylimino)-3-(n-carbamoyl-2-aminoethyl)-3,4,6, 7-tetrahydro-2H-pyrimido[6,1-a]isoquinolin-4-one] and RPL565 [6,7-dihydro-2-(2,6-diisopropylphenoxy)-9,10-dimethoxy-4H-pyrimido[6,1-a]isoquino lin-4-one]. *J Pharmacol Exp Ther* **318**, 840-848, doi:10.1124/jpet.105.099192 (2006).
203 Franciosi, L. G. *et al.* Efficacy and safety of RPL554, a dual PDE3 and PDE4 inhibitor, in healthy volunteers and in patients with asthma or chronic obstructive pulmonary disease: findings from four clinical trials. *Lancet Respir Med* **1**, 714-727, doi:10.1016/S2213-2600(13)70187-5 (2013).
204 Hodge, S. *et al.* Azithromycin improves macrophage phagocytic function and expression of mannose receptor in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **178**, 139-148, doi:10.1164/rccm.200711-1666OC (2008).
205 Rennard, S. I. *et al.* The safety and efficacy of infliximab in moderate to severe chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **175**, 926-934, doi:10.1164/rccm.200607-995OC (2007).
206 Janda, S., Park, K., FitzGerald, J. M., Etminan, M. & Swiston, J. Statins in COPD: a systematic review. *Chest* **136**, 734-743, doi:10.1378/chest.09-0194 (2009).
207 Lahousse, L. *et al.* Statins, systemic inflammation and risk of death in COPD: the Rotterdam study. *Pulm Pharmacol Ther* **26**, 212-217, doi:10.1016/j.pupt.2012.10.008 (2013).
208 Lipworth, B., Wedzicha, J., Devereux, G., Vestbo, J. & Dransfield, M. T. Beta-blockers in COPD: time for reappraisal. *Eur Respir J* **48**, 880-888, doi:10.1183/13993003.01847-2015 (2016).
209 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* **6**, e28457, doi:10.1371/journal.pone.0028457 (2011).
210 Eltom, S. *et al.* Role of the inflammasome-caspase1/11-IL-1/18 axis in cigarette smoke driven airway inflammation: an insight into the pathogenesis of COPD. *Plos One* **9**, e112829, doi:10.1371/journal.pone.0112829 (2014).
211 Pinkerton, J. W. *et al.* Inflammasomes in the lung. *Mol Immunol*, doi:10.1016/j.molimm.2017.01.014 (2017).
212 Babu, K. S. & Morjaria, J. B. Emerging therapeutic strategies in COPD. *Drug Discov Today* **20**, 371-379, doi:10.1016/j.drudis.2014.11.003 (2015).
213 Chapman, K. R., Stockley, R. A., Dawkins, C., Wilkes, M. M. & Navickis, R. J. Augmentation therapy for alpha1 antitrypsin deficiency: a meta-analysis. *Copd* **6**, 177-184 (2009).
214 Dirksen, A. *et al.* A randomized clinical trial of alpha(1)-antitrypsin augmentation therapy. *Am J Respir Crit Care Med* **160**, 1468-1472, doi:10.1164/ajrccm.160.5.9901055 (1999).

215 Brusselle, G. & Bracke, K. Targeting immune pathways for therapy in asthma and chronic obstructive pulmonary disease. *Annals of the American Thoracic Society* **11 Suppl 5**, S322-328, doi:10.1513/AnnalsATS.201403-118AW (2014).
216 Tripple, J. W., McCracken, J. L. & Calhoun, W. J. Biologic Therapy in Chronic Obstructive Pulmonary Disease. *Immunol Allergy Clin North Am* **37**, 345-355, doi:10.1016/j.iac.2017.01.009 (2017).
217 Wise, R. A. *et al.* Lack of Effect of Oral Sulforaphane Administration on Nrf2 Expression in COPD: A Randomized, Double-Blind, Placebo Controlled Trial. *Plos One* **11**, e0163716, doi:10.1371/journal.pone.0163716 (2016).
218 Woodruff, P. G. *et al.* Safety and efficacy of an inhaled epidermal growth factor receptor inhibitor (BIBW 2948 BS) in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* **181**, 438-445, doi:10.1164/rccm.200909-1415OC (2010).
219 Sugawara, A. *et al.* Novel 12-membered non-antibiotic macrolides from erythromycin A; EM900 series as novel leads for anti-inflammatory and/or immunomodulatory agents. *Bioorg Med Chem Lett* **21**, 3373-3376, doi:10.1016/j.bmcl.2011.04.004 (2011).
220 Mushtaq, Y. The COPD pipeline. *Nature reviews. Drug discovery* **13**, 253, doi:10.1038/nrd4254 (2014).
221 Hughes, A. D., McNamara, A. & Steinfeld, T. Multivalent dual pharmacology muscarinic antagonist and beta(2) agonist (MABA) molecules for the treatment of COPD. *Prog Med Chem* **51**, 71-95, doi:10.1016/B978-0-12-396493-9.00003-0 (2012).
222 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* **63**, 822-825 (2014).
223 Guell, M. R. *et al.* Benefits of Long-Term Pulmonary Rehabilitation Maintenance Program in Patients with Severe Chronic Obstructive Pulmonary Disease. Three-Year Follow-up. *Am J Respir Crit Care Med* **195**, 622-629, doi:10.1164/rccm.201603-0602OC (2017).
224 Cantone, I. & Fisher, A. G. Epigenetic programming and reprogramming during development. *Nature structural & molecular biology* **20**, 282-289, doi:10.1038/nsmb.2489 (2013).
225 Smith, Z. D. & Meissner, A. DNA methylation: roles in mammalian development. *Nat Rev Genet* **14**, 204-220, doi:10.1038/nrg3354 (2013).
226 Wright, M. W. & Bruford, E. A. Naming 'junk': human non-protein coding RNA (ncRNA) gene nomenclature. *Hum Genomics* **5**, 90-98 (2011).
227 Ecker, J. R. *et al.* Genomics: ENCODE explained. *Nature* **489**, 52-55, doi:10.1038/489052a (2012).
228 Taft, R. J., Pheasant, M. & Mattick, J. S. The relationship between non-protein-coding DNA and eukaryotic complexity. *BioEssays : news and reviews in molecular, cellular and developmental biology* **29**, 288-299, doi:10.1002/bies.20544 (2007).
229 Mestdagh, P., Vandesompele, J., Brusselle, G. & Vermaelen, K. Non-coding RNAs and respiratory disease. *Thorax* **70**, 388-390, doi:10.1136/thoraxjnl-2014-206404 (2015).
230 Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281-297 (2004).
231 Brown, D., Rahman, M. & Nana-Sinkam, S. P. MicroRNAs in respiratory disease. A clinician's overview. *Annals of the American Thoracic Society* **11**, 1277-1285, doi:10.1513/AnnalsATS.201404-179FR (2014).
232 Saini, H. K., Griffiths-Jones, S. & Enright, A. J. Genomic analysis of human microRNA transcripts. *Proc Natl Acad Sci U S A* **104**, 17719-17724, doi:10.1073/pnas.0703890104 (2007).
233 Ha, M. & Kim, V. N. Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol* **15**, 509-524, doi:10.1038/nrm3838 (2014).
234 Graves, P. & Zeng, Y. Biogenesis of mammalian microRNAs: a global view. *Genomics Proteomics Bioinformatics* **10**, 239-245, doi:10.1016/j.gpb.2012.06.004 (2012).

235 Ghildiyal, M. & Zamore, P. D. Small silencing RNAs: an expanding universe. *Nature Reviews Genetics* **10**, 94-108, doi:10.1038/nrg2504 (2009).
236 Altuvia, Y. *et al.* Clustering and conservation patterns of human microRNAs. *Nucleic Acids Res* **33**, 2697-2706, doi:10.1093/nar/gki567 (2005).
237 Denli, A. M., Tops, B. B., Plasterk, R. H., Ketting, R. F. & Hannon, G. J. Processing of primary microRNAs by the Microprocessor complex. *Nature* **432**, 231-235, doi:10.1038/nature03049 (2004).
238 Khvorova, A., Reynolds, A. & Jayasena, S. D. Functional siRNAs and miRNAs exhibit strand bias (vol 115, pg 209, 2003). *Cell* **115**, 505-505, doi:Doi 10.1016/S0092-8674(03)00893-6 (2003).
239 Ro, S., Park, C., Young, D., Sanders, K. M. & Yan, W. Tissue-dependent paired expression of miRNAs. *Nucleic Acids Res* **35**, 5944-5953, doi:10.1093/nar/gkm641 (2007).
240 Li, S. C. *et al.* miRNA arm selection and isomiR distribution in gastric cancer. *BMC genomics* **13 Suppl 1**, S13, doi:10.1186/1471-2164-13-S1-S13 (2012).
241 Winter, J., Jung, S., Keller, S., Gregory, R. I. & Diederichs, S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nature cell biology* **11**, 228-234, doi:10.1038/ncb0309-228 (2009).
242 Eulalio, A., Behm-Ansmant, I. & Izaurralde, E. P bodies: at the crossroads of post-transcriptional pathways. *Nat Rev Mol Cell Biol* **8**, 9-22, doi:10.1038/nrm2080 (2007).
243 Filipowicz, W., Bhattacharyya, S. N. & Sonenberg, N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* **9**, 102-114, doi:10.1038/nrg2290 (2008).
244 Xia, J. & Zhang, W. A meta-analysis revealed insights into the sources, conservation and impact of microRNA 5'-isoforms in four model species. *Nucleic Acids Res* **42**, 1427-1441, doi:10.1093/nar/gkt967 (2014).
245 Chugh, P. & Dittmer, D. P. Potential pitfalls in microRNA profiling. *Wiley Interdiscip Rev RNA* **3**, 601-616, doi:10.1002/wrna.1120 (2012).
246 Tuna, M., Machado, A. S. & Calin, G. A. Genetic and epigenetic alterations of microRNAs and implications for human cancers and other diseases. *Genes Chromosomes Cancer* **55**, 193-214, doi:10.1002/gcc.22332 (2016).
247 Feng, Z., Zhang, C., Wu, R. & Hu, W. Tumor suppressor p53 meets microRNAs. *J Mol Cell Biol* **3**, 44-50, doi:10.1093/jmcb/mjq040 (2011).
248 Gross, T. J. *et al.* A microRNA processing defect in smokers' macrophages is linked to SUMOylation of the endonuclease DICER. *The Journal of biological chemistry*, doi:10.1074/jbc.M114.565473 (2014).
249 van den Beucken, T. *et al.* Hypoxia promotes stem cell phenotypes and poor prognosis through epigenetic regulation of DICER. *Nature communications* **5**, 5203, doi:10.1038/ncomms6203 (2014).
250 Forman, J. J., Legesse-Miller, A. & Coller, H. A. A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence. *Proc Natl Acad Sci U S A* **105**, 14879-14884, doi:10.1073/pnas.0803230105 (2008).
251 Martello, G. *et al.* A MicroRNA targeting dicer for metastasis control. *Cell* **141**, 1195-1207, doi:10.1016/j.cell.2010.05.017 (2010).
252 Rupaimoole, R. *et al.* Hypoxia-upregulated microRNA-630 targets Dicer, leading to increased tumor progression. *Oncogene* **35**, 4312-4320, doi:10.1038/onc.2015.492 (2016).
253 Obernosterer, G., Leuschner, P. J., Alenius, M. & Martinez, J. Post-transcriptional regulation of microRNA expression. *RNA (New York, N.Y.)* **12**, 1161-1167, doi:10.1261/rna.2322506 (2006).
254 Roberts, T. C. The MicroRNA Biology of the Mammalian Nucleus. *Molecular therapy. Nucleic acids* **3**, e188, doi:10.1038/mtna.2014.40 (2014).

255 Ciafre, S. A. & Galardi, S. microRNAs and RNA-binding proteins: a complex network of interactions and reciprocal regulations in cancer. *RNA biology* **10**, 935-942, doi:10.4161/rna.24641 (2013).
256 Tay, Y., Rinn, J. & Pandolfi, P. P. The multilayered complexity of ceRNA crosstalk and competition. *Nature* **505**, 344-352, doi:10.1038/nature12986 (2014).
257 Thomson, D. W. & Dinger, M. E. Endogenous microRNA sponges: evidence and controversy. *Nat Rev Genet* **17**, 272-283, doi:10.1038/nrg.2016.20 (2016).
258 Thery, C., Ostrowski, M. & Segura, E. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* **9**, 581-593, doi:10.1038/nri2567 (2009).
259 Chen, X., Liang, H., Zhang, J., Zen, K. & Zhang, C. Y. Secreted microRNAs: a new form of intercellular communication. *Trends Cell Biol* **22**, 125-132, doi:10.1016/j.tcb.2011.12.001 (2012).
260 EL Andaloussi, S., Maeger, I., Breakefield, X. O. & Wood, M. J. A. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nature Reviews Drug Discovery* **12**, 348-358, doi:10.1038/nrd3978 (2013).
261 Mitchell, P. S. *et al.* Circulating microRNAs as stable blood-based markers for cancer detection. *P Natl Acad Sci USA* **105**, 10513-10518, doi:10.1073/pnas.0804549105 (2008).
262 Friedman, R. C., Farh, K. K., Burge, C. B. & Bartel, D. P. Most mammalian mRNAs are conserved targets of microRNAs. *Genome research* **19**, 92-105, doi:10.1101/gr.082701.108 (2009).
263 Liu, J. D. *et al.* Argonaute2 is the catalytic engine of mammalian RNAi. *Science (New York, N.Y.)* **305**, 1437-1441, doi:10.1126/science.1102513 (2004).
264 Salomon, W. E., Jolly, S. M., Moore, M. J., Zamore, P. D. & Serebrov, V. Single-Molecule Imaging Reveals that Argonaute Reshapes the Binding Properties of Its Nucleic Acid Guides. *Cell* **162**, 84-95, doi:10.1016/j.cell.2015.06.029 (2015).
265 Bartel, D. P. MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215-233, doi:10.1016/j.cell.2009.01.002 (2009).
266 Helwak, A., Kudla, G., Dudnakova, T. & Tollervey, D. Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. *Cell* **153**, 654-665, doi:10.1016/j.cell.2013.03.043 (2013).
267 Brodersen, P. & Voinnet, O. Revisiting the principles of microRNA target recognition and mode of action. *Nat Rev Mol Cell Biol* **10**, 141-148, doi:10.1038/nrm2619 (2009).
268 Shin, C. *et al.* Expanding the microRNA targeting code: functional sites with centered pairing. *Molecular cell* **38**, 789-802, doi:10.1016/j.molcel.2010.06.005 (2010).
269 Grimson, A. *et al.* MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Molecular cell* **27**, 91-105, doi:10.1016/j.molcel.2007.06.017 (2007).
270 Xiao, C. & Rajewsky, K. MicroRNA control in the immune system: basic principles. *Cell* **136**, 26-36, doi:10.1016/j.cell.2008.12.027 (2009).
271 Orom, U. A., Nielsen, F. C. & Lund, A. H. MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. *Molecular cell* **30**, 460-471, doi:10.1016/j.molcel.2008.05.001 (2008).
272 Place, R. F., Li, L. C., Pookot, D., Noonan, E. J. & Dahiya, R. MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci U S A* **105**, 1608-1613, doi:10.1073/pnas.0707594105 (2008).
273 Vasudevan, S., Tong, Y. & Steitz, J. A. Switching from repression to activation: microRNAs can up-regulate translation. *Science (New York, N.Y.)* **318**, 1931-1934, doi:10.1126/science.1149460 (2007).
274 Pasquinelli, A. E. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. *Nat Rev Genet* **13**, 271-282, doi:10.1038/nrg3162 (2012).
275 Lu, L. *et al.* Epigenetic silencing of miR-218 by the lncRNA CCAT1, acting via BMI1, promotes an altered cell cycle transition in the malignant transformation of HBE cells induced by

cigarette smoke extract. *Toxicology and applied pharmacology* **304**, 30-41, doi:10.1016/j.taap.2016.05.012 (2016).
276 Lu, L. *et al.* Feedback circuitry via let-7c between lncRNA CCAT1 and c-Myc is involved in cigarette smoke extract-induced malignant transformation of HBE cells. *Oncotarget*, doi:10.18632/oncotarget.15195 (2017).
277 Lu, L. *et al.* Posttranscriptional silencing of the lncRNA MALAT1 by miR-217 inhibits the epithelial-mesenchymal transition via enhancer of zeste homolog 2 in the malignant transformation of HBE cells induced by cigarette smoke extract. *Toxicology and applied pharmacology* **289**, 276-285, doi:10.1016/j.taap.2015.09.016 (2015).
278 Fabbri, M. *et al.* MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc Natl Acad Sci U S A* **104**, 15805-15810, doi:10.1073/pnas.0707628104 (2007).
279 Leuenberger, C. *et al.* MicroRNA-223 controls the expression of histone deacetylase 2: a novel axis in COPD. *Journal of molecular medicine (Berlin, Germany)* **94**, 725-734, doi:10.1007/s00109-016-1388-1 (2016).
280 Kim, D. H., Saetrom, P., Snove, O., Jr. & Rossi, J. J. MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci U S A* **105**, 16230-16235, doi:10.1073/pnas.0808830105 (2008).
281 Hansen, T. B. *et al.* miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA. *Embo J* **30**, 4414-4422, doi:10.1038/emboj.2011.359 (2011).
282 Tang, R. *et al.* Mouse miRNA-709 directly regulates miRNA-15a/16-1 biogenesis at the posttranscriptional level in the nucleus: evidence for a microRNA hierarchy system. *Cell research* **22**, 504-515, doi:10.1038/cr.2011.137 (2012).
283 Rasko, J. E. & Wong, J. J. Nuclear microRNAs in normal hemopoiesis and cancer. *J Hematol Oncol* **10**, 8, doi:10.1186/s13045-016-0375-x (2017).
284 Wong, J. J. *et al.* Identification of nuclear-enriched miRNAs during mouse granulopoiesis. *J Hematol Oncol* **7**, 42, doi:10.1186/1756-8722-7-42 (2014).
285 Bourc'his, D. & Voinnet, O. A small-RNA perspective on gametogenesis, fertilization, and early zygotic development. *Science (New York, N.Y.)* **330**, 617-622, doi:10.1126/science.1194776 (2010).
286 Baccarini, A. *et al.* Kinetic analysis reveals the fate of a microRNA following target regulation in mammalian cells. *Current biology : CB* **21**, 369-376, doi:10.1016/j.cub.2011.01.067 (2011).
287 Landgraf, P. *et al.* A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell* **129**, 1401-1414, doi:10.1016/j.cell.2007.04.040 (2007).
288 Bernstein, E. *et al.* Dicer is essential for mouse development. *Nature genetics* **35**, 215-217, doi:10.1038/ng1253 (2003).
289 Hatfield, S. D. *et al.* Stem cell division is regulated by the microRNA pathway. *Nature* **435**, 974-978, doi:10.1038/nature03816 (2005).
290 Mudhasani, R. *et al.* Loss of miRNA biogenesis induces p19Arf-p53 signaling and senescence in primary cells. *The Journal of cell biology* **181**, 1055-1063, doi:10.1083/jcb.200802105 (2008).
291 de Yebenes, V. G., Bartolome-Izquierdo, N. & Ramiro, A. R. Regulation of B-cell development and function by microRNAs. *Immunological reviews* **253**, 25-39, doi:10.1111/imr.12046 (2013).
292 Koralov, S. B. *et al.* Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell* **132**, 860-874, doi:10.1016/j.cell.2008.02.020 (2008).
293 Harris, K. S., Zhang, Z., McManus, M. T., Harfe, B. D. & Sun, X. Dicer function is essential for lung epithelium morphogenesis. *Proc Natl Acad Sci U S A* **103**, 2208-2213, doi:10.1073/pnas.0510839103 (2006).
294 Cohen, T. S. Role of MicroRNA in the Lung's Innate Immune Response. *Journal of innate immunity*, doi:10.1159/000452669 (2016).

295 O'Neill, L. A., Sheedy, F. J. & McCoy, C. E. MicroRNAs: the fine-tuners of Toll-like receptor signalling. *Nat Rev Immunol* **11**, 163-175, doi:10.1038/nri2957 (2011).
296 Montagner, S., Orlandi, E. M., Merante, S. & Monticelli, S. The role of miRNAs in mast cells and other innate immune cells. *Immunological reviews* **253**, 12-24, doi:10.1111/imr.12042 (2013).
297 Taganov, K. D., Boldin, M. P., Chang, K. J. & Baltimore, D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A* **103**, 12481-12486, doi:10.1073/pnas.0605298103 (2006).
298 O'Connell, R. M., Rao, D. S., Chaudhuri, A. A. & Baltimore, D. Physiological and pathological roles for microRNAs in the immune system. *Nat Rev Immunol* **10**, 111-122, doi:10.1038/nri2708 (2010).
299 Quinn, S. R. & O'Neill, L. A. A trio of microRNAs that control Toll-like receptor signalling. *International immunology* **23**, 421-425, doi:10.1093/intimm/dxr034 (2011).
300 Gantier, M. P. The not-so-neutral role of microRNAs in neutrophil biology. *J Leukoc Biol* **94**, 575-583, doi:10.1189/jlb.1012539 (2013).
301 Johnnidis, J. B. *et al.* Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature* **451**, 1125-1129, doi:10.1038/nature06607 (2008).
302 Baek, D. *et al.* The impact of microRNAs on protein output. *Nature* **455**, 64-71, doi:10.1038/nature07242 (2008).
303 Mehta, A. & Baltimore, D. MicroRNAs as regulatory elements in immune system logic. *Nat Rev Immunol* **16**, 279-294, doi:10.1038/nri.2016.40 (2016).
304 Baltimore, D., Boldin, M. P., O'Connell, R. M., Rao, D. S. & Taganov, K. D. MicroRNAs: new regulators of immune cell development and function. *Nature immunology* **9**, 839-845, doi:10.1038/ni.f.209 (2008).
305 Tsitsiou, E. & Lindsay, M. A. microRNAs and the immune response. *Curr Opin Pharmacol* **9**, 514-520, doi:10.1016/j.coph.2009.05.003 (2009).
306 Vigorito, E. *et al.* microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity* **27**, 847-859, doi:10.1016/j.immuni.2007.10.009 (2007).
307 Baumjohann, D. & Ansel, K. M. MicroRNA-mediated regulation of T helper cell differentiation and plasticity. *Nat Rev Immunol* **13**, 666-678, doi:10.1038/nri3494 (2013).
308 Chatila, W. M. *et al.* Blunted expression of miR-199a-5p in regulatory T cells of patients with chronic obstructive pulmonary disease compared to unaffected smokers. *Clin Exp Immunol* **177**, 341-352, doi:10.1111/cei.12325 (2014).
309 Rodriguez, A. *et al.* Requirement of bic/microRNA-155 for normal immune function. *Science (New York, N.Y.)* **316**, 608-611, doi:10.1126/science.1139253 (2007).
310 Wei, B. & Pei, G. microRNAs: critical regulators in Th17 cells and players in diseases. *Cellular & molecular immunology* **7**, 175-181, doi:10.1038/cmi.2010.19 (2010).
311 Weldon, S. *et al.* miR-31 dysregulation in cystic fibrosis airways contributes to increased pulmonary cathepsin S production. *Am J Respir Crit Care Med* **190**, 165-174, doi:10.1164/rccm.201311-1986OC (2014).
312 Oglesby, I. K., McElvaney, N. G. & Greene, C. M. MicroRNAs in inflammatory lung disease--master regulators or target practice? *Respir Res* **11**, 148, doi:10.1186/1465-9921-11-148 (2010).
313 Graff, J. W. *et al.* Cigarette smoking decreases global microRNA expression in human alveolar macrophages. *Plos One* **7**, e44066, doi:10.1371/journal.pone.0044066 (2012).
314 Russ, R. & Slack, F. J. Cigarette-Smoke-Induced Dysregulation of MicroRNA Expression and Its Role in Lung Carcinogenesis. *Pulmonary medicine* **2012**, 791234, doi:10.1155/2012/791234 (2012).
315 Takahashi, K. *et al.* Cigarette smoking substantially alters plasma microRNA profiles in healthy subjects. *Toxicology and applied pharmacology* **272**, 154-160, doi:10.1016/j.taap.2013.05.018 (2013).

316 Rogers, S. *et al.* Aryl hydrocarbon receptor (AhR)-dependent regulation of pulmonary miRNA by chronic cigarette smoke exposure. *Scientific reports* **7**, 40539, doi:10.1038/srep40539 (2017).
317 Hassan, F. *et al.* MiR-101 and miR-144 regulate the expression of the CFTR chloride channel in the lung. *Plos One* **7**, e50837, doi:10.1371/journal.pone.0050837 (2012).
318 Perdomo, C., Spira, A. & Schembri, F. MiRNAs as regulators of the response to inhaled environmental toxins and airway carcinogenesis. *Mutation research* **717**, 32-37, doi:10.1016/j.mrfmmm.2011.04.005 (2011).
319 Huang, W. & Li, M. D. Differential allelic expression of dopamine D1 receptor gene (DRD1) is modulated by microRNA miR-504. *Biol Psychiatry* **65**, 702-705, doi:10.1016/j.biopsych.2008.11.024 (2009).
320 Shi, L., Xin, Q., Chai, R., Liu, L. & Ma, Z. Ectopic expressed miR-203 contributes to chronic obstructive pulmonary disease via targeting TAK1 and PIK3CA. *International journal of clinical and experimental pathology* **8**, 10662-10670 (2015).
321 Molina-Pinelo, S. *et al.* MicroRNA clusters: dysregulation in lung adenocarcinoma and COPD. *Eur Respir J*, doi:10.1183/09031936.00091513 (2014).
322 Shen, W. *et al.* Repression of Toll-like receptor-4 by microRNA-149-3p is associated with smoking-related COPD. *International journal of chronic obstructive pulmonary disease* **12**, 705-715, doi:10.2147/COPD.S128031 (2017).
323 Akbas, F., Coskunpinar, E., Aynaci, E., Oltulu, Y. M. & Yildiz, P. Analysis of serum micro-RNAs as potential biomarker in chronic obstructive pulmonary disease. *Exp Lung Res* **38**, 286-294, doi:10.3109/01902148.2012.689088 (2012).
324 Soeda, S. *et al.* Clinical relevance of plasma miR-106b levels in patients with chronic obstructive pulmonary disease. *International journal of molecular medicine* **31**, 533-539, doi:10.3892/ijmm.2013.1251 (2013).
325 Dang, X. *et al.* Bioinformatic analysis of microRNA and mRNA Regulation in peripheral blood mononuclear cells of patients with chronic obstructive pulmonary disease. *Respir Res* **18**, 4, doi:10.1186/s12931-016-0486-5 (2017).
326 Pinkerton, M. *et al.* Differential expression of microRNAs in exhaled breath condensates of patients with asthma, patients with chronic obstructive pulmonary disease, and healthy adults. *The Journal of allergy and clinical immunology* **132**, 217-219, doi:10.1016/j.jaci.2013.03.006 (2013).
327 Christenson, S. A. *et al.* miR-638 regulates gene expression networks associated with emphysematous lung destruction. *Genome Med* **5**, 114, doi:10.1186/gm519 (2013).
328 Savarimuthu Francis, S. M. *et al.* MicroRNA-34c is associated with emphysema severity and modulates SERPINE1 expression. *BMC genomics* **15**, 88, doi:10.1186/1471-2164-15-88 (2014).
329 Mizuno, S. *et al.* MicroRNA-199a-5p is associated with hypoxia-inducible factor-1alpha expression in lungs from patients with COPD. *Chest* **142**, 663-672, doi:10.1378/chest.11-2746 (2012).
330 Kasahara, Y. *et al.* Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *The Journal of clinical investigation* **106**, 1311-1319, doi:10.1172/JCI10259 (2000).
331 O'Leary, L. *et al.* Airway smooth muscle inflammation is regulated by microRNA-145 in COPD. *Febs Lett* **590**, 1324-1334, doi:10.1002/1873-3468.12168 (2016).
332 Donaldson, A. *et al.* Increased skeletal muscle-specific microRNA in the blood of patients with COPD. *Thorax* **68**, 1140-1149, doi:10.1136/thoraxjnl-2012-203129 (2013).
333 Puig-Vilanova, E. *et al.* Epigenetic mechanisms in respiratory muscle dysfunction of patients with chronic obstructive pulmonary disease. *Plos One* **9**, e111514, doi:10.1371/journal.pone.0111514 (2014).
334 Booton, R. & Lindsay, M. A. Emerging role of MicroRNAs and long noncoding RNAs in respiratory disease. *Chest* **146**, 193-204, doi:10.1378/chest.13-2736 (2014).

335 Rupani, H., Sanchez-Elsner, T. & Howarth, P. MicroRNAs and respiratory diseases. *Eur Respir J*, doi:10.1183/09031936.00212011 (2012).
336 Osei, E. T. *et al.* Unravelling the complexity of COPD by microRNAs: it's a small world after all. *Eur Respir J*, doi:10.1183/13993003.02139-2014 (2015).
337 Nana-Sinkam, S. P. *et al.* Integrating the MicroRNome into the study of lung disease. *Am J Respir Crit Care Med* **179**, 4-10, doi:10.1164/rccm.200807-1042PP (2009).
338 Angulo, M., Lecuona, E. & Sznajder, J. I. Role of MicroRNAs in lung disease. *Archivos de bronconeumologia* **48**, 325-330, doi:10.1016/j.arbres.2012.04.011 (2012).
339 Esau, C. C. Inhibition of microRNA with antisense oligonucleotides. *Methods (San Diego, Calif.)* **44**, 55-60, doi:10.1016/j.ymeth.2007.11.001 (2008).
340 Selby, L. I., Cortez-Jugo, C. M., Such, G. K. & Johnston, A. P. Nanoescapology: progress toward understanding the endosomal escape of polymeric nanoparticles. *Wiley Interdiscip Rev Nanomed Nanobiotechnol*, doi:10.1002/wnan.1452 (2017).
341 Mook, O. *et al.* In vivo efficacy and off-target effects of locked nucleic acid (LNA) and unlocked nucleic acid (UNA) modified siRNA and small internally segmented interfering RNA (sisiRNA) in mice bearing human tumor xenografts. *Artificial DNA, PNA & XNA* **1**, 36-44, doi:10.4161/adna.1.1.12204 (2010).
342 Montgomery, R. L. *et al.* MicroRNA mimicry blocks pulmonary fibrosis. *Embo Mol Med* **6**, 1347-1356, doi:10.15252/emmm.201303604 (2014).
343 Rupaimoole, R. & Slack, F. J. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nature reviews. Drug discovery*, doi:10.1038/nrd.2016.246 (2017).
344 Dahlman, J. E. *et al.* In vivo endothelial siRNA delivery using polymeric nanoparticles with low molecular weight. *Nature nanotechnology*, doi:10.1038/nnano.2014.84 (2014).
345 McKiernan, P. J., Cunningham, O., Greene, C. M. & Cryan, S. A. Targeting miRNA-based medicines to cystic fibrosis airway epithelial cells using nanotechnology. *International journal of nanomedicine* **8**, 3907-3915, doi:10.2147/IJN.S47551 (2013).
346 De Backer, L. *et al.* Bio-inspired pulmonary surfactant-modified nanogels: A promising siRNA delivery system. *Journal of controlled release : official journal of the Controlled Release Society* **206**, 177-186, doi:10.1016/j.jconrel.2015.03.015 (2015).
347 Stenvang, J., Petri, A., Lindow, M., Obad, S. & Kauppinen, S. Inhibition of microRNA function by antimiR oligonucleotides. *Silence* **3**, 1, doi:10.1186/1758-907x-3-1 (2012).
348 Jeker, L. T. & Marone, R. Targeting microRNAs for immunomodulation. *Curr Opin Pharmacol* **23**, 25-31, doi:10.1016/j.coph.2015.05.004 (2015).
349 Gambari, R. *et al.* Targeting microRNAs involved in human diseases: a novel approach for modification of gene expression and drug development. *Biochemical pharmacology* **82**, 1416-1429, doi:10.1016/j.bcp.2011.08.007 (2011).
350 van der Ree, M. H. *et al.* Long-term safety and efficacy of microRNA-targeted therapy in chronic hepatitis C patients. *Antiviral Res* **111**, 53-59, doi:10.1016/j.antiviral.2014.08.015 (2014).
351 Thibault, P. A. *et al.* Regulation of Hepatitis C Virus Genome Replication by Xrn1 and MicroRNA-122 Binding to Individual Sites in the 5' Untranslated Region. *J Virol* **89**, 6294-6311, doi:10.1128/JVI.03631-14 (2015).
352 Lewis, A. P. & Jopling, C. L. Regulation and biological function of the liver-specific miR-122. *Biochemical Society transactions* **38**, 1553-1557, doi:10.1042/BST0381553 (2010).
353 van der Ree, M. H. *et al.* Safety, tolerability, and antiviral effect of RG-101 in patients with chronic hepatitis C: a phase 1B, double-blind, randomised controlled trial. *Lancet* **389**, 709-717, doi:10.1016/S0140-6736(16)31715-9 (2017).
354 Elmen, J. *et al.* LNA-mediated microRNA silencing in non-human primates. *Nature* **452**, 896-899, doi:10.1038/nature06783 (2008).

355 Lanford, R. E. *et al.* Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science (New York, N.Y.)* **327**, 198-201, doi:10.1126/science.1178178 (2010).
356 Tsai, W. C. *et al.* MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. *The Journal of clinical investigation* **122**, 2884-2897, doi:10.1172/JCI63455 (2012).
357 Stahlhut, C. & Slack, F. J. Combinatorial Action of MicroRNAs let-7 and miR-34 Effectively Synergizes with Erlotinib to Suppress Non-small Cell Lung Cancer Cell Proliferation. *Cell cycle (Georgetown, Tex.)* **14**, 2171-2180, doi:10.1080/15384101.2014.1003008 (2015).
358 Trang, P. *et al.* Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. *Molecular therapy : the journal of the American Society of Gene Therapy* **19**, 1116-1122, doi:10.1038/mt.2011.48 (2011).
359 Lu, Y. *et al.* A single anti-microRNA antisense oligodeoxyribonucleotide (AMO) targeting multiple microRNAs offers an improved approach for microRNA interference. *Nucleic Acids Res* **37**, e24, doi:10.1093/nar/gkn1053 (2009).
360 D'Hulst A, I., Vermaelen, K. Y., Brusselle, G. G., Joos, G. F. & Pauwels, R. A. Time course of cigarette smoke-induced pulmonary inflammation in mice. *Eur Respir J* **26**, 204-213, doi:10.1183/09031936.05.00095204 (2005).
361 Brusselle, G. G. *et al.* Murine models of COPD. *Pulmonary Pharmacology & Therapeutics* **19**, 155-165, doi:10.1016/j.pupt.2005.06.001 (2006).
362 Guerassimov, A. *et al.* The development of emphysema in cigarette smoke-exposed mice is strain dependent. *Am J Respir Crit Care Med* **170**, 974-980, doi:10.1164/rccm.200309-1270OC (2004).
363 Yao, H. *et al.* Cigarette smoke-mediated inflammatory and oxidative responses are strain-dependent in mice. *American journal of physiology. Lung cellular and molecular physiology* **294**, L1174-1186, doi:10.1152/ajplung.00439.2007 (2008).
364 Churg, A., Cosio, M. & Wright, J. L. Mechanisms of cigarette smoke-induced COPD: insights from animal models. *American journal of physiology. Lung cellular and molecular physiology* **294**, L612-631, doi:10.1152/ajplung.00390.2007 (2008).
365 Oglesby, I. K., Agrawal, R., Mall, M. A., McElvaney, N. G. & Greene, C. M. miRNA-221 is elevated in cystic fibrosis airway epithelial cells and regulates expression of ATF6. *Mol Cell Pediatr* **2**, 1, doi:10.1186/s40348-014-0012-0 (2015).
366 van Rooij, E. The art of microRNA research. *Circulation research* **108**, 219-234, doi:10.1161/circresaha.110.227496 (2011).
367 Shi, R. & Chiang, V. L. Facile means for quantifying microRNA expression by real-time PCR. *BioTechniques* **39**, 519-525 (2005).
368 Yin, J. Q., Zhao, R. C. & Morris, K. V. Profiling microRNA expression with microarrays. *Trends Biotechnol* **26**, 70-76, doi:10.1016/j.tibtech.2007.11.007 (2008).
369 Fox, S., Filichkin, S. & Mockler, T. C. Applications of ultra-high-throughput sequencing. *Methods in molecular biology (Clifton, N.J.)* **553**, 79-108, doi:10.1007/978-1-60327-563-7_5 (2009).
370 Baker, M. MicroRNA profiling: separating signal from noise. *Nature methods* **7**, 687-692, doi:10.1038/nmeth0910-687 (2010).
371 Mestdagh, P. *et al.* Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study. *Nature methods* **11**, 809-815, doi:10.1038/nmeth.3014 (2014).
372 Leshkowitz, D., Horn-Saban, S., Parmet, Y. & Feldmesser, E. Differences in microRNA detection levels are technology and sequence dependent. *RNA (New York, N.Y.)*, doi:10.1261/rna.036475.112 (2013).
373 Git, A. *et al.* Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. *RNA (New York, N.Y.)* **16**, 991-1006, doi:10.1261/rna.1947110 (2010).

374 Mestdagh, P. *et al.* High-throughput stem-loop RT-qPCR miRNA expression profiling using minute amounts of input RNA. *Nucleic Acids Res* **36**, e143, doi:10.1093/nar/gkn725 (2008).
375 Finotello, F. & Di Camillo, B. Measuring differential gene expression with RNA-seq: challenges and strategies for data analysis. *Brief Funct Genomics* **14**, 130-142, doi:10.1093/bfgp/elu035 (2015).
376 Soneson, C. & Delorenzi, M. A comparison of methods for differential expression analysis of RNA-seq data. *BMC bioinformatics* **14**, 91, doi:10.1186/1471-2105-14-91 (2013).
377 Pritchard, C. C., Cheng, H. H. & Tewari, M. MicroRNA profiling: approaches and considerations. *Nat Rev Genet* **13**, 358-369, doi:10.1038/nrg3198 (2012).
378 Takada, S. & Asahara, H. Current strategies for microRNA research. *Mod Rheumatol* **22**, 645-653, doi:10.1007/s10165-011-0583-8 (2012).
379 Van Peer, G. *et al.* miRBase Tracker: keeping track of microRNA annotation changes. *Database : the journal of biological databases and curation* **2014**, doi:10.1093/database/bau080 (2014).
380 Chou, C. H. *et al.* miRTarBase 2016: updates to the experimentally validated miRNA-target interactions database. *Nucleic Acids Res* **44**, D239-247, doi:10.1093/nar/gkv1258 (2016).
381 Sablok, G. *et al.* isomiRex: Web-based identification of microRNAs, isomiR variations and differential expression using next-generation sequencing datasets. *Febs Lett*, doi:10.1016/j.febslet.2013.06.047 (2013).
382 Chi, S. W., Zang, J. B., Mele, A. & Darnell, R. B. Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. *Nature* **460**, 479-486, doi:10.1038/nature08170 (2009).
383 Imig, J. *et al.* miR-CLIP capture of a miRNA targetome uncovers a lincRNA H19-miR-106a interaction. *Nat Chem Biol* **11**, 107-114, doi:10.1038/nchembio.1713 (2015).
384 Li, J. H., Liu, S., Zhou, H., Qu, L. H. & Yang, J. H. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. *Nucleic Acids Res* **42**, D92-97, doi:10.1093/nar/gkt1248 (2014).
385 Hassan, T. *et al.* Isolation and identification of cell-specific microRNAs targeting a messenger RNA using a biotinylated anti-sense oligonucleotide capture affinity technique. *Nucleic Acids Res* **41**, e71, doi:10.1093/nar/gks1466 (2013).
386 Bassett, A. R. *et al.* Understanding functional miRNA-target interactions in vivo by site-specific genome engineering. *Nature communications* **5**, 4640, doi:10.1038/ncomms5640 (2014).
387 Eulalio, A. *et al.* Functional screening identifies miRNAs inducing cardiac regeneration. *Nature* **492**, 376-381, doi:10.1038/nature11739 (2012).
388 Vandesompele, J. *et al.* Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* **3**, Research0034 (2002).
389 Mestdagh, P. *et al.* A novel and universal method for microRNA RT-qPCR data normalization. *Genome biology* **10**, R64, doi:10.1186/gb-2009-10-6-r64 (2009).
390 D'Haene, B., Mestdagh, P., Hellemans, J. & Vandesompele, J. miRNA expression profiling: from reference genes to global mean normalization. *Methods in molecular biology (Clifton, N.J.)* **822**, 261-272, doi:10.1007/978-1-61779-427-8_18 (2012).
391 Bustin, S. A. *et al.* The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry* **55**, 611-622, doi:10.1373/clinchem.2008.112797 (2009).
392 Chen, C. *et al.* Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* **33**, e179, doi:10.1093/nar/gni178 (2005).
393 Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* **102**, 15545-15550, doi:10.1073/pnas.0506580102 (2005).

394 Nuovo, G. J. In situ detection of microRNAs in paraffin embedded, formalin fixed tissues and the co-localization of their putative targets. *Methods (San Diego, Calif.)* **52**, 307-315, doi:10.1016/j.ymeth.2010.08.009 (2010).
395 Yamamichi, N. *et al.* Locked nucleic acid in situ hybridization analysis of miR-21 expression during colorectal cancer development. *Clinical cancer research : an official journal of the American Association for Cancer Research* **15**, 4009-4016, doi:10.1158/1078-0432.ccr-08-3257 (2009).
396 Hwang, H. W., Wentzel, E. A. & Mendell, J. T. Cell-cell contact globally activates microRNA biogenesis. *Proc Natl Acad Sci U S A* **106**, 7016-7021, doi:10.1073/pnas.0811523106 (2009).
397 Conickx, G., Mestdagh, P., Vandesompele, J., Brusselle, G. G. & Bracke, K. R. Reply: Direct Detection of Circulating MicroRNAs Unveiled the Absence of MicroRNA-218-5p in Smoker Subjects. *Am J Respir Crit Care Med* **196**, 533, doi:10.1164/rccm.201701-0224LE (2017).
398 Sin, D. D., Anthonisen, N. R., Soriano, J. B. & Agusti, A. G. Mortality in COPD: Role of comorbidities. *Eur Respir J* **28**, 1245-1257, doi:10.1183/09031936.00133805 (2006).
399 Verhamme, F. M. *et al.* Role of activin-A in cigarette smoke-induced inflammation and COPD. *Eur Respir J* **43**, 1028-1041, doi:10.1183/09031936.00082413 (2014).
400 Mestdagh, P. *et al.* The microRNA body map: dissecting microRNA function through integrative genomics. *Nucleic Acids Res* **39**, e136, doi:10.1093/nar/gkr646 (2011).
401 Traves, S. L., Culpitt, S. V., Russell, R. E., Barnes, P. J. & Donnelly, L. E. Increased levels of the chemokines GROalpha and MCP-1 in sputum samples from patients with COPD. *Thorax* **57**, 590-595 (2002).
402 Davidson, M. R. *et al.* MicroRNA-218 is deleted and downregulated in lung squamous cell carcinoma. *Plos One* **5**, e12560, doi:10.1371/journal.pone.0012560 (2010).
403 Baskerville, S. & Bartel, D. P. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. *RNA (New York, N.Y.)* **11**, 241-247, doi:10.1261/rna.7240905 (2005).
404 Barnes, P. J. The cytokine network in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* **41**, 631-638, doi:10.1165/rcmb.2009-0220TR (2009).
405 Wang, H. & Morse, H. C., 3rd. IRF8 regulates myeloid and B lymphoid lineage diversification. *Immunologic research* **43**, 109-117, doi:10.1007/s12026-008-8055-8 (2009).
406 Tamura, T., Kurotaki, D. & Koizumi, S. Regulation of myelopoiesis by the transcription factor IRF8. *International journal of hematology* **101**, 342-351, doi:10.1007/s12185-015-1761-9 (2015).
407 Hausser, J. & Zavolan, M. Identification and consequences of miRNA-target interactions - beyond repression of gene expression. *Nat Rev Genet* **15**, 599-612, doi:10.1038/nrg3765 (2014).
408 Osella, M., Bosia, C., Cora, D. & Caselle, M. The role of incoherent microRNA-mediated feedforward loops in noise buffering. *PLoS computational biology* **7**, e1001101, doi:10.1371/journal.pcbi.1001101 (2011).
409 Pilewski, J. M., Liu, L., Henry, A. C., Knauer, A. V. & Feghali-Bostwick, C. A. Insulin-like growth factor binding proteins 3 and 5 are overexpressed in idiopathic pulmonary fibrosis and contribute to extracellular matrix deposition. *Am J Pathol* **166**, 399-407, doi:10.1016/S0002-9440(10)62263-8 (2005).
410 Veraldi, K. L. *et al.* Role of insulin-like growth factor binding protein-3 in allergic airway remodeling. *Am J Respir Crit Care Med* **180**, 611-617, doi:10.1164/rccm.200810-1555OC (2009).
411 Schnapp, L. M. *et al.* Mining the acute respiratory distress syndrome proteome: identification of the insulin-like growth factor (IGF)/IGF-binding protein-3 pathway in acute lung injury. *Am J Pathol* **169**, 86-95, doi:10.2353/ajpath.2006.050612 (2006).
412 Halappanavar, S., Russell, M., Stampfli, M. R., Williams, A. & Yauk, C. L. Induction of the interleukin 6/ signal transducer and activator of transcription pathway in the lungs of mice

sub-chronically exposed to mainstream tobacco smoke. *BMC medical genomics* **2**, 56, doi:10.1186/1755-8794-2-56 (2009).
413 Kang, C. B., Hong, Y., Dhe-Paganon, S. & Yoon, H. S. FKBP family proteins: immunophilins with versatile biological functions. *Neurosignals* **16**, 318-325, doi:10.1159/000123041 (2008).
414 Nath, P. R. & Isakov, N. Insights into peptidyl-prolyl cis-trans isomerase structure and function in immunocytes. *Immunol Lett* **163**, 120-131, doi:10.1016/j.imlet.2014.11.002 (2015).
415 Kucharska, A., Rushworth, L. K., Staples, C., Morrice, N. A. & Keyse, S. M. Regulation of the inducible nuclear dual-specificity phosphatase DUSP5 by ERK MAPK. *Cellular signalling* **21**, 1794-1805, doi:10.1016/j.cellsig.2009.07.015 (2009).
416 Wang, J. *et al.* Characterization of microRNA transcriptome in tumor, adjacent, and normal tissues of lung squamous cell carcinoma. *The Journal of thoracic and cardiovascular surgery*, doi:10.1016/j.jtcvs.2015.02.012 (2015).
417 Arora, A. MicroRNA targets: potential candidates for indirect regulation by drugs. *Pharmacogenet Genomics* **25**, 107-125, doi:10.1097/FPC.0000000000000111 (2015).
418 Stolzenburg, L. R., Wachtel, S., Dang, H. & Harris, A. miR-1343 attenuates pathways of fibrosis by targeting the TGF-beta receptors. *The Biochemical journal* **473**, 245-256, doi:10.1042/BJ20150821 (2016).
419 Geiss, G. K. *et al.* Direct multiplexed measurement of gene expression with color-coded probe pairs. *Nature biotechnology* **26**, 317-325, doi:10.1038/nbt1385 (2008).
420 Malkov, V. A. *et al.* Multiplexed measurements of gene signatures in different analytes using the Nanostring nCounter Assay System. *BMC Res Notes* **2**, 80, doi:10.1186/1756-0500-2-80 (2009).
421 Szymczak, I., Wieczfinska, J. & Pawliczak, R. Molecular Background of miRNA Role in Asthma and COPD: An Updated Insight. *BioMed research international* **2016**, 7802521, doi:10.1155/2016/7802521 (2016).
422 Yang, L., Xu, Q., Xie, H., Gu, G. & Jiang, J. Expression of serum miR-218 in hepatocellular carcinoma and its prognostic significance. *Clin Transl Oncol* **18**, 841-847, doi:10.1007/s12094-015-1447-z (2016).
423 Yu, H. *et al.* Decreased expression of miR-218 is associated with poor prognosis in patients with colorectal cancer. *International journal of clinical and experimental pathology* **6**, 2904-2911 (2013).
424 Wozniak, M. B. *et al.* Circulating MicroRNAs as Non-Invasive Biomarkers for Early Detection of Non-Small-Cell Lung Cancer. *Plos One* **10**, e0125026, doi:10.1371/journal.pone.0125026 (2015).
425 Vogelmeier, C. F. *et al.* Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Lung Disease 2017 Report: GOLD Executive Summary. *Eur Respir J* **49**, doi:10.1183/13993003.00214-2017 (2017).
426 Holloway, R. A. & Donnelly, L. E. Immunopathogenesis of chronic obstructive pulmonary disease. *Current opinion in pulmonary medicine* **19**, 95-102, doi:10.1097/MCP.0b013e32835cfff5 (2013).
427 Mendell, J. T. & Olson, E. N. MicroRNAs in stress signaling and human disease. *Cell* **148**, 1172-1187, doi:10.1016/j.cell.2012.02.005 (2012).
428 Izzotti, A. *et al.* Dose-responsiveness and persistence of microRNA expression alterations induced by cigarette smoke in mouse lung. *Mutation research* **717**, 9-16, doi:10.1016/j.mrfmmm.2010.12.008 (2011).
429 Gregory R. Warnes, B. B., Lodewijk Bonebakker, Robert Gentleman, Wolfgang Huber Andy Liaw, Thomas Lumley, Martin Maechler, Arni Magnusson, Steffen Moeller, Marc Schwartz, Bill Venables. gplots: Various R Programming Tools for Plotting Data. R package version 3.0.1. https://CRAN.R-project.org/package=gplots. (2016).
430 The R project for Statistical Computing. Available from: https://www.r-project.org/

431 O'Connell, R. M. *et al.* Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *The Journal of experimental medicine* **205**, 585-594, doi:10.1084/jem.20072108 (2008).
432 O'Connell, R. M. *et al.* MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. *Immunity* **33**, 607-619, doi:10.1016/j.immuni.2010.09.009 (2010).
433 Yao, R. *et al.* MicroRNA-155 Modulates Treg and Th17 Cells Differentiation and Th17 Cell Function by Targeting SOCS1. *Plos One* **7**, e46082, doi:10.1371/journal.pone.0046082 (2012).
434 Dueck, A., Eichner, A., Sixt, M. & Meister, G. A miR-155-dependent microRNA hierarchy in dendritic cell maturation and macrophage activation. *Febs Lett* **588**, 632-640, doi:10.1016/j.febslet.2014.01.009 (2014).
435 Halappanavar, S. *et al.* Pulmonary response to surface-coated nanotitanium dioxide particles includes induction of acute phase response genes, inflammatory cascades, and changes in microRNAs: a toxicogenomic study. *Environ Mol Mutagen* **52**, 425-439, doi:10.1002/em.20639 (2011).
436 Halappanavar, S. *et al.* IL-1 Receptor Regulates microRNA-135b Expression in a Negative Feedback Mechanism during Cigarette Smoke-Induced Inflammation. *J Immunol*, doi:10.4049/jimmunol.1202456 (2013).
437 Luettich, K. *et al.* Systems toxicology approaches enable mechanistic comparison of spontaneous and cigarette smoke-related lung tumor development in the A/J mouse model. *Interdiscip Toxicol* **7**, 73-84, doi:10.2478/intox-2014-0010 (2014).
438 Shen, J. *et al.* Plasma microRNAs as potential biomarkers for non-small-cell lung cancer. *Lab Invest* **91**, 579-587, doi:10.1038/labinvest.2010.194 (2011).
439 Liu, X. G. *et al.* High expression of serum miR-21 and tumor miR-200c associated with poor prognosis in patients with lung cancer. *Medical oncology* **29**, 618-626, doi:10.1007/s12032-011-9923-y (2012).
440 Maegdefessel, L. *et al.* MicroRNA-21 blocks abdominal aortic aneurysm development and nicotine-augmented expansion. *Sci Transl Med* **4**, 122ra122, doi:10.1126/scitranslmed.3003441 (2012).
441 Pyfferoen, L. *et al.* The transcriptome of lung tumor-infiltrating dendritic cells reveals a tumor-supporting phenotype and a microRNA signature with negative impact on clinical outcome. *Oncoimmunology* **6**, e1253655, doi:10.1080/2162402X.2016.1253655 (2017).
442 Grigoryev, Y. A. *et al.* MicroRNA regulation of molecular networks mapped by global microRNA, mRNA, and protein expression in activated T lymphocytes. *J Immunol* **187**, 2233-2243, doi:10.4049/jimmunol.1101233 (2011).
443 Naqvi, A. R. *et al.* Expression Profiling of LPS Responsive miRNA in Primary Human Macrophages. *J Microb Biochem Technol* **8**, 136-143, doi:10.4172/1948-5948.1000276 (2016).
444 Rodriguez, A., Griffiths-Jones, S., Ashurst, J. L. & Bradley, A. Identification of mammalian microRNA host genes and transcription units. *Genome research* **14**, 1902-1910, doi:10.1101/gr.2722704 (2004).
445 Guan, H. *et al.* Neuronal repellent Slit2 inhibits dendritic cell migration and the development of immune responses. *J Immunol* **171**, 6519-6526 (2003).
446 Kanellis, J. *et al.* Modulation of inflammation by slit protein in vivo in experimental crescentic glomerulonephritis. *Am J Pathol* **165**, 341-352, doi:10.1016/S0002-9440(10)63301-9 (2004).
447 Prasad, A., Qamri, Z., Wu, J. & Ganju, R. K. Slit-2/Robo-1 modulates the CXCL12/CXCR4-induced chemotaxis of T cells. *J Leukoc Biol* **82**, 465-476, doi:10.1189/jlb.1106678 (2007).
448 Tole, S. *et al.* The axonal repellent, Slit2, inhibits directional migration of circulating neutrophils. *J Leukoc Biol* **86**, 1403-1415, doi:10.1189/jlb.0609391 (2009).
449 Ye, B. Q., Geng, Z. H., Ma, L. & Geng, J. G. Slit2 regulates attractive eosinophil and repulsive neutrophil chemotaxis through differential srGAP1 expression during lung inflammation. *J Immunol* **185**, 6294-6305, doi:10.4049/jimmunol.1001648 (2010).

450 Zhang, C., Ge, S., Hu, C., Yang, N. & Zhang, J. MiRNA-218, a new regulator of HMGB1, suppresses cell migration and invasion in non-small cell lung cancer. *Acta biochimica et biophysica Sinica* **45**, 1055-1061, doi:10.1093/abbs/gmt109 (2013).
451 Fan, R. *et al.* microRNA-218 increase the sensitivity of gastrointestinal stromal tumor to imatinib through PI3K/AKT pathway. *Clinical and experimental medicine*, doi:10.1007/s10238-014-0280-y (2014).
452 Yuan, W. *et al.* MicroRNA-218 enhances the radiosensitivity of human cervical cancer via promoting radiation induced apoptosis. *Int J Med Sci* **11**, 691-696, doi:10.7150/ijms.8880 (2014).
453 Chiu, K. L. *et al.* ADAM9 enhances CDCP1 protein expression by suppressing miR-218 for lung tumor metastasis. *Scientific reports* **5**, 16426, doi:10.1038/srep16426 (2015).
454 Lu, Y. F., Zhang, L., Waye, M. M., Fu, W. M. & Zhang, J. F. MiR-218 mediates tumorigenesis and metastasis: Perspectives and implications. *Experimental cell research* **334**, 173-182, doi:10.1016/j.yexcr.2015.03.027 (2015).
455 Tian, H. *et al.* miR-218 suppresses tumor growth and enhances the chemosensitivity of esophageal squamous cell carcinoma to cisplatin. *Oncol Rep* **33**, 981-989, doi:10.3892/or.2014.3657 (2015).
456 Xie, J. *et al.* MicroRNA-218 regulates cisplatin (DPP) chemosensitivity in non-small cell lung cancer by targeting RUNX2. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*, doi:10.1007/s13277-015-3831-2 (2015).
457 Xin, S. Y. *et al.* Reduced expression of circulating microRNA-218 in gastric cancer and correlation with tumor invasion and prognosis. *World J Gastroenterol* **20**, 6906-6911, doi:10.3748/wjg.v20.i22.6906 (2014).
458 Xi, S. *et al.* Cigarette smoke induces C/EBP-beta-mediated activation of miR-31 in normal human respiratory epithelia and lung cancer cells. *Plos One* **5**, e13764, doi:10.1371/journal.pone.0013764 (2010).
459 Faraoni, I., Antonetti, F. R., Cardone, J. & Bonmassar, E. miR-155 gene: A typical multifunctional microRNA. *Bba-Mol Basis Dis* **1792**, 497-505, doi:DOI 10.1016/j.bbadis.2009.02.013 (2009).
460 Cheng, Y. *et al.* MicroRNA-21 protects against the H(2)O(2)-induced injury on cardiac myocytes via its target gene PDCD4. *J Mol Cell Cardiol* **47**, 5-14, doi:10.1016/j.yjmcc.2009.01.008 (2009).
461 Hashimi, S. T. *et al.* MicroRNA profiling identifies miR-34a and miR-21 and their target genes JAG1 and WNT1 in the coordinate regulation of dendritic cell differentiation. *Blood* **114**, 404-414, doi:10.1182/blood-2008-09-179150 (2009).
462 Lu, T. X., Munitz, A. & Rothenberg, M. E. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. *J Immunol* **182**, 4994-5002, doi:10.4049/jimmunol.0803560 (2009).
463 Velu, C. S., Baktula, A. M. & Grimes, H. L. Gfi1 regulates miR-21 and miR-196b to control myelopoiesis. *Blood* **113**, 4720-4728, doi:10.1182/blood-2008-11-190215 (2009).
464 Liu, G. *et al.* miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. *The Journal of experimental medicine* **207**, 1589-1597, doi:10.1084/jem.20100035 (2010).
465 Barnes, N. A., Stephenson, S., Cocco, M., Tooze, R. M. & Doody, G. M. BLIMP-1 and STAT3 counterregulate microRNA-21 during plasma cell differentiation. *J Immunol* **189**, 253-260, doi:10.4049/jimmunol.1101563 (2012).
466 Tan, K. S. *et al.* Micro-RNAs in regenerating lungs: an integrative systems biology analysis of murine influenza pneumonia. *BMC genomics* **15**, 587, doi:10.1186/1471-2164-15-587 (2014).
467 Cavarretta, E. & Condorelli, G. miR-21 and cardiac fibrosis: another brick in the wall? *Eur Heart J* **36**, 2139-2141, doi:10.1093/eurheartj/ehv184 (2015).
468 Krichevsky, A. M. & Gabriely, G. miR-21: a small multi-faceted RNA. *J Cell Mol Med* **13**, 39-53, doi:10.1111/j.1582-4934.2008.00556.x (2009).

469 Davis, B. N., Hilyard, A. C., Lagna, G. & Hata, A. SMAD proteins control DROSHA-mediated microRNA maturation. *Nature* **454**, 56-61, doi:10.1038/nature07086 (2008).
470 Xie, L. *et al.* An increased ratio of serum miR-21 to miR-181a levels is associated with the early pathogenic process of chronic obstructive pulmonary disease in asymptomatic heavy smokers. *Mol Biosyst* **10**, 1072-1081, doi:10.1039/c3mb70564a (2014).
471 Zhang, Y., Pan, T., Zhong, X. & Cheng, C. Nicotine upregulates microRNA-21 and promotes TGF-beta-dependent epithelial-mesenchymal transition of esophageal cancer cells. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* **35**, 7063-7072, doi:10.1007/s13277-014-1968-z (2014).
472 Stanitz, E. *et al.* Evaluation of MicroRNA expression pattern of gastric adenocarcinoma associated with socioeconomic, environmental and lifestyle factors in northwestern Hungary. *Anticancer Res* **33**, 3195-3200 (2013).
473 Bourdon, J. A. *et al.* Carbon black nanoparticle intratracheal installation results in large and sustained changes in the expression of miR-135b in mouse lung. *Environ Mol Mutagen* **53**, 462-468, doi:10.1002/em.21706 (2012).
474 Nagel, R. *et al.* Regulation of the adenomatous polyposis coli gene by the miR-135 family in colorectal cancer. *Cancer Res* **68**, 5795-5802, doi:10.1158/0008-5472.can-08-0951 (2008).
475 Necela, B. M., Carr, J. M., Asmann, Y. W. & Thompson, E. A. Differential expression of microRNAs in tumors from chronically inflamed or genetic (APC(Min/+)) models of colon cancer. *Plos One* **6**, e18501, doi:10.1371/journal.pone.0018501 (2011).
476 Bhinge, A. *et al.* MiR-135b is a direct PAX6 target and specifies human neuroectoderm by inhibiting TGF-beta/BMP signaling. *Embo J* **33**, 1271-1283, doi:10.1002/embj.201387215 (2014).
477 Li, J. *et al.* miR-135b Promotes Cancer Progression by Targeting Transforming Growth Factor Beta Receptor II (TGFBR2) in Colorectal Cancer. *Plos One* **10**, e0130194, doi:10.1371/journal.pone.0130194 (2015).
478 Liao, J., Liu, R., Shi, Y. J., Yin, L. H. & Pu, Y. P. Exosome-shuttling microRNA-21 promotes cell migration and invasion-targeting PDCD4 in esophageal cancer. *Int J Oncol* **48**, 2567-2579, doi:10.3892/ijo.2016.3453 (2016).
479 Umezu, T. *et al.* Exosomal miR-135b shed from hypoxic multiple myeloma cells enhances angiogenesis by targeting factor-inhibiting HIF-1. *Blood*, doi:10.1182/blood-2014-05-576116 (2014).
480 Fujita, Y., Kosaka, N., Araya, J., Kuwano, K. & Ochiya, T. Extracellular vesicles in lung microenvironment and pathogenesis. *Trends in molecular medicine* **21**, 533-542, doi:10.1016/j.molmed.2015.07.004 (2015).
481 Valeri, N. *et al.* MicroRNA-135b Promotes Cancer Progression by Acting as a Downstream Effector of Oncogenic Pathways in Colon Cancer. *Cancer cell* **25**, 469-483, doi:10.1016/j.ccr.2014.03.006 (2014).
482 Melnik, B. C. MiR-21: an environmental driver of malignant melanoma? *J Transl Med* **13**, 202, doi:10.1186/s12967-015-0570-5 (2015).
483 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:10.1016/j.cell.2011.02.013 (2011).
484 Dykes, I. M. & Emanueli, C. Transcriptional and Post-transcriptional Gene Regulation by Long Non-coding RNA. *Genomics Proteomics Bioinformatics* **15**, 177-186, doi:10.1016/j.gpb.2016.12.005 (2017).

CURRICULUM VITAE

Personal details

Name	Griet Conickx
Home address	Ottergemsesteenweg 143, 9000 Ghent
Date of birth	09-06-1980
Nationality	Belgian
Email address	griet_conickx@hotmail.com / griet.conickx@ugent.be / griet.conickx@uzgent.be

Work experience

2011-present	PhD candidate in Health Sciences – Department of Respiratory Medicine – Faculty of Medical and Health Sciences – Ghent University – Belgium
2010-2011	Basic research on identification of biomarkers for hepatocellular carcinoma – Department of Pathology – Faculty of Medical and Health Sciences – Ghent University – Belgium
2003-2010	Pharmacist

Education

1998-2003	Master in Pharmaceutical Sciences – KULeuven - Belgium
1992-1998	Latin and Sciences – Sint-Vincentiusinstituut – Dendermonde – Belgium

Additional courses

2016	Spirometry course – Ghent University Hospital – Belgium
2015	Applying for a postdoctoral job – Ghent University – Belgium
2014	Advanced Academic English: presentation skills – Ghent University – Belgium
	Advanced Academic English: writing skills – Ghent University – Belgium
	Clinical studies: study design, implementation and reporting – Ghent University – Belgium
2013	Statistical analysis with SPSS (advanced course) – Ghent University – Belgium
2012	Statistical course in R – Ghent University – Belgium
2011	Laboratory animal science (FELASA cat. C) – Ghent University – Belgium

Awards/grants

2017	Belgian Thoracic Society - GSK Basic Science Award in Pneumology 2017 – Brussels – Belgium
2016	Poster award at the 14th ERS Lung Science Conference – Estoril – Portugal
	Bursary to attend the 14th ERS Lung Science Conference – Estoril – Portugal
	Travel grant to attend the ATS conference – San Francisco – USA

Attended conferences

2017	Belgian Thoracic Society, GSK awards in Pneumology – Brussels – Belgium
	U4 meeting – Ghent – Belgium
2016	workshop 'light on the dark side of the genome' (f-TALES) – Ghent – Belgium
	14th ERS Lung Science Conference – Estoril – Portugal
	4th annual IUAP meeting, AIREWAY II – Ghent – Belgium
2015	The non-coding genome – EMBL – Heidelberg – Germany
	Mini-symposium 'non-coding RNAs in cancer' – Ghent – Belgium
	3rd annual IUAP meeting, AIREWAY II – Leuven – Belgium
	IRC Mini-symosium 'Inflammation Research in Ghent: IRC meets the University Hospital Ghent – VIB – Ghent – Belgium
	Belgian Thoracic Society, GSK awards in Pneumology – Brussels – Belgium
	Symposium "From big data to bedside: translational bioinformatics in cancer research" – Ghent – Belgium
2014	2nd annual IUAP meeting, AIREWAY II – Liège – Belgium
	Belgian Thoracic Society, GSK awards in Pneumology – Brussels – Belgium
	American Thoracic Society – San Diego – CA – USA
2013	19th DMBR workshop: 'disease models and cancer' – VIB – Ghent – Belgium
	Belgian Thoracic Society, GSK awards in Pneumology – Brussels – Belgium
	1st annual IUAP meeting, AIREWAY II – Ghent – Belgium
2012	18th DMBR workshop: 'adaptive immunity' – VIB – Ghent – Belgium

Abstracts and presentations

2017	Belgian Thoracic Society, GSK awards in Pneumology (Brussels) – Identification and functional characterization of microRNAs in the pathogenesis of COPD (oral presentation)

2016	4th annual IUAP meeting (Ghent) – microRNA profiling reveals a role for miR-218-5p in the pathogenesis of COPD (oral presentation)
	ATS (San Francisco) – microRNA-218-5p is implicated in the pathogenesis of chronic obstructive pulmonary disease (poster presentation)
	Belgian Thoracic Society, GSK awards in Pneumology (Brussels) – microRNA profiling reveals a role for miR-218-5p in the pathogenesis of COPD (oral presentation)
	14th ERS Lung Science Conference (Estoril) – microRNA profiling reveals a role for miR-218-5p in the pathogenesis of COPD (poster presentation)
	ERS (London) – microRNA profiling reveals a role for miR-218-5p in the pathogenesis of COPD (poster presentation)
2014	Belgian Thoracic Society, GSK awards in Pneumology (Brussels) – Expression of microRNA-218 is reduced in cigarette smoke-exposed mice and patients with COPD (oral presentation)
	ATS (San Diego) – Expression of microRNA-218 is reduced in cigarette smoke-exposed mice and patients with COPD (poster discussion)
2013	1st annual IUAP meeting, AIREWAY II (Ghent) – MicroRNAs in COPD: a research project (oral presentation)

LIST OF PUBLICATIONS

Allais L, Kumar S, Debusschere K, Verschuere S, Maes T, De Smet R, **Conickx G**, De Vos M, Laukens D, Joos GF, Brusselle GG, Elewaut D, Cuvelier CA, Bracke KR.
The Effect of Cigarette Smoke Exposure on the Development of Inflammation in Lungs, Gut and Joints of TNFDeltaARE Mice.
Plos One 2015; 10: e0141570.
IF: 3.057 / ranking in Multidisciplinary sciences: 11/63

Seys LJM, Verhamme FM, Dupont LL, Desauter E, Duerr J, Agircan AS, **Conickx G**, Joos GF, Brusselle GG, Mall MA, Bracke KR.
Airway Surface Dehydration Aggravates Cigarette Smoke-Induced Hallmarks of COPD in Mice.
Plos One 2015; 10:e0129897
IF: 3.057 / ranking in Multidisciplinary sciences: 11/63

Maes T, Cobos FA, Schleich F, Sorbello V, Henket M, De Preter K, Bracke KR, **Conickx G**, Mesnil C, Vandesompele J, Lahousse L, Bureau F, Mestdagh P, Joos GF, Ricciardolo FL, Brusselle GG, Louis R.
Asthma inflammatory phenotypes show differential microRNA expression in sputum.
The Journal of allergy and clinical immunology 2016; 137: 1433-1446.
Impact factor: 13.081 / ranking Allergy: 1/26

Conickx G, Mestdagh P, Avila Cobos F, Verhamme FM, Maes T, Vanaudenaerde BM, Seys LJ, Lahousse L, Kim RY, Hsu AC, Wark PA, Hansbro PM, Joos GF, Vandesompele J, Bracke KR, Brusselle GG.
MicroRNA Profiling Reveals a Role for MicroRNA-218-5p in the Pathogenesis of Chronic Obstructive Pulmonary Disease.
Am J Respir Crit Care Med 2017; 195: 43-56.
Impact factor: 13.204 / ranking in Respiratory system: 2/59

Conickx G, Mestdagh P, Vandesompele J, Brusselle GG, Bracke KR.
Reply to Direct Detection of Circulating microRNAs Unveiled the Absence of miR-218-5p in Smoker Subjects.
Am J Respir Crit Care Med 2017; 196: 533.
Impact factor: 13.204 / ranking in Respiratory system: 2/59

Conickx G[#], Avila Cobos F[#], van den Berge M, Faiz A, Timens W, Hiemstra PS, Joos GF, Brusselle GG, Mestdagh P*, Bracke KR*.
MicroRNA profiling in lung tissue and bronchoalveolar lavage supernatant of cigarette smoke-exposed mice and in COPD patients: a translational approach
revision submitted to Scientific Reports
Impact factor: 4.259 / ranking in Multidisciplinary sciences: 10/64

Sunkara KP†, Jarnicki AG†, Kim RY†, **Conickx G**†, Haw TJ, Wark PA, Brusselle GG, Bracke KR, Foster PS, Horvat JC, Hansbro PM
Identification of a novel MicroRNA-21-mediated SATB1/S100A9/NF-κB axis in the pathogenesis of chronic obstructive pulmonary disease
Manuscript in preparation.

Sunkara KP†, Jarnicki AG†, Kim RY†, **Conickx G**†, Brusselle GG, Wark PA, Foster PS, Horvat JC, Bracke KR†, Hansbro PM†
Role for microRNA-135b in the pathogenesis of chronic obstructive pulmonary disease
Manuscript in preparation.

DANKWOORD

Als ik terugblik op de voorbije jaren op de dienst longziekten, voel ik vooral dankbaarheid. Dankbaarheid omdat ik de kans kreeg dit doctoraat te mogen doen, dat ik zoveel heb geleerd en zoveel fijne mensen heb ontmoet. Dit was een enorme verrijking op velerlei vlakken. Dankjewel!

Aan het tevoorschijn 'toveren' van prachtige onderzoeksresultaten en een doctoraatsboekje gaat heel wat werk vooraf. Dit kon niet zonder jullie hulp.

Allereerst wil ik mijn promotor professor Brusselle bedanken. Bedankt omdat u me de kans gaf dit doctoraat te mogen doen. U besliste dat microRNAs het middelpunt van mijn onderzoek zouden uitmaken en begeleidde me doorheen de microRNA pieken en dalen. Het was een onvoorstelbaar leerzame ervaring! Bedankt voor je bemoedigingen en eeuwig optimisme ('daar zit muziek in'). Uw passie en gedrevenheid voor onderzoek zullen me bijblijven. Veel succes in uw verdere carrière!

Ken, mijn co-promotor, jij volgde mijn onderzoek van zeer nabij en diende als eerste aanspreekpunt. Ik heb veel voor je deur gestaan om onderzoeksresultaten te bespreken en altijd maakte je direct tijd vrij. Merci, dat waardeer ik echt. Bedankt ook om mijn schrijfsels en presentaties na te kijken. Ik vermoed dat ik er doorheen de jaren op vooruit ben gegaan, want je veranderde steeds minder ☺. Een spraakwaterval ben je niet, maar ik ken niemand die nog flauwere moppen kan vertellen. Bedankt voor je rustige en kundige begeleiding en je aangename gezelschap.

I would also like to thank the members of the jury: Prof. Dr. Elfride De Baere, Prof. Dr. Fabrice Bureau, Prof. Dr. Katleen De Preter, Prof. Dr. Catherine Greene, Prof. Dr. Koen Raemdonck, Dr. Mireille Van Gele and Prof. Dr. Karim Vermaelen. Thank you for thoroughly reading my dissertation and for your valuable suggestions. These certainly improved my thesis manuscript. I am pleased to have you here as experts from complementary disciplines. A special thanks to Prof. Dr. Catherine Greene and Prof. Dr. Fabrice Bureau who had to travel from Ireland and Luik, respectively, to attend my thesis defense.

Professor Joos, in de voorbijgaande jaren zag ik je carrière allerlei wendingen aannemen, diensthoofd longziekten, vakgroep voorzitter en zelfs ERS president. Toch bleef je heel bereikbaar en toegankelijk. Bedankt voor je vriendelijke bemoedigingen en het aansturen van ons labo!

Tania, jij bent samen met Ken de steunpilaar van ons labo. Waar PhD studenten komen en gaan is het zo belangrijk dat er enkele vaste waarden zorgen voor het voortbestaan en de erkenning van ons labo. Het was fijn om onze eerste ervaringen met een primaire celcultuur en transfecties te kunnen delen. Bedankt voor je directheid. Het was altijd eerlijk en recht uit het hart.

Pieter (Mestdagh), bedankt voor je begeleiding en hulp bij alle data-gerelateerde analyses. Bedankt ook voor je hulp bij het sturen van mijn onderzoek.

Pieter en Jo, het deed altijd zo'n deugd dat als ik mijn resultaten op een GOA meeting toonde, jullie 'open-minded' de resultaten interpreteerden en mee naar mogelijke oplossingen zochten. Ik heb hier veel uit geleerd. Merci!

Fien en Sharen, ondertussen allebei al goed 'gerodeerde' post-docs in ons labo, ik wens jullie veel succes in jullie verdere (academische) loopbaan. Fien, jij was mijn bureaugenootje in Blok B en kamergenootje in San Diego. We hebben veel gelachen, gebabbeld (tijdens de lunch ☺) en lief en leed gedeeld. Het ga je (jullie) goed, geniet van het onderzoek en ga ervoor! Sharen, je bent vaak in alle

stilte een berg werk aan het verzetten, ze zullen je hier missen als je vertrekt naar je nieuwe job. Katrien, jij deed je masterthesis in ons labo toen ik arriveerde en bent nu ook je doctoraatsonderzoek aan het afronden. Veel succes met het schrijven en afleggen van je doctoraatsthesis! Elise, mijn mede microRNA-genootje, het microRNA onderzoek is niet gemakkelijk. Daar kan je al van meespreken. Ik wens je nog veel succes met de muisproeven en daarna goede moed bij het schrijven van je boekje. Jaja, na al die afzwaaiende PhD studenten ligt de toekomst nu in Evy haar handen. Evy, je bent ondertussen al een vol jaar in ons labo en al goed op weg. Ook jou wens ik een boeiend PhD traject! Ik zal jullie missen!

En dan zijn er natuurlijk ook mijn voorgangers: Leen, Smitha, Lisa en Ellen. Jullie hebben ons labo ondertussen ingeruild voor andere oorden. Bedankt voor de fijne gesprekken tijdens de lunch of het fruitmomentje. Het ga jullie goed!

Constantinos, jij vervoegde ons labo voor een jaar. Thanks for your visits, the long hikes and your drawing!

Tine, jij werd een collega op de dienst pathologie nadat je onderzoek er op de dienst longziekten op zat. Ik herinner me nog je doctoraatsverdediging en weet ook dat ik al heel snel niet meer kon volgen. Wat wou dat BAL toch altijd zeggen? Ondertussen weet ik wel beter. Nadien kreeg ik ook de kriebels te pakken en ben ik in je voetsporen getreden.

Francisco, thanks for your kind help with the bio-informatics analyses and your friendship! I remember you giving us seeds of a Spanish pepper plant to grow in our garden. These became 6 beautiful plants with lots of peppers. We could not eat one, they were too spicy ☺. I wish you a happy future and good luck with your doctoral thesis!

En dan last but not least, de drijvende kracht achter ons labo: de laboranten. Ann, Anouck, Christelle, Eliane, Evelyn, Greet, Indra, Katleen, Lien en Marie-Rose, jullie zorgen voor de muisjes, het roken ('ik ga gaan roken' betekent geen tussentijdse pauze maar echte arbeid!), kleuringen, het opnemen van beelden, en nog zoveel meer. Jullie maken dat een eindpunt zo vlot verloopt als dat je ooit al hebt meegemaakt. Ook tijdens de middagpauzes zorgen jullie voor soms hilarische gespreksonderwerpen. Bedankt voor jullie onvermoeibare inzet, jullie enthousiasme en ongelooflijke hulp bij alles!

Ook bedankt aan de mensen van het animalarium om de muizen te verzorgen.

Nu wij in MRBII gehuisvest zijn lijkt de K12 een hele eind ver weg, maar ook daar zitten fijne collega's. Lies, Kevin, Lotte en Bibi, bedankt voor de fijne maar misschien iets schaarsere babbels! Lies, ondertussen verdedigde je je PhD, publiceerde je ongelooflijk veel wetenschappelijke artikels en onderwijs je studenten. Ik wens je veel succes in je verdere carrière! Kevin, jij verdedigde onlangs je doctoraat en vertoeft nu in Antwerpse oorden. Het is fijn om je af en toe eens weer te zien. Doe dat goed daar! Bibi, ook jij mocht na lang zwoegen deze 3 lettertjes achter je naam schrijven. Veel plezier in je verdere werk!

Bart, Annie en Chantal, om alles op wieltjes te laten lopen qua administratie en financiën zijn jullie echt van onmisbaar belang. Bedankt voor de korte maar fijne telefoontjes of leuke babbels als ik eens even binnen sprong of we samen toezicht hielden.

Nathalie en Emily, jullie zie ik maar heel af en toe. Ik wens jullie veel succes met jullie doctoraat in het kader van de Rotterdam studie!

Bedankt aan de datamanagers Frauke, Tasja en Stefanie en alle anderen om ons te helpen bij het afnemen van het 'informed consent'.

Kelly, Nancy, Veronique, Elisabeth, Melissa en Lotte, bedankt voor de fijne babbels. Het was altijd fijn om even bij jullie binnen te springen en raad te kunnen vragen. Bedankt voor de leuke samenwerking!

En nu het onderzoek verder evolueert richting toediening van (met siRNA of microRNA geladen) partikels voor inhalatie, lijkt het me fijn om Pieterjan van de farmacie nog even wegwijs te maken in ons labo en enkele knepen van het vak te leren. Pieterjan, hopelijk wordt dit veelbelovend onderzoek en het begin van een mooie samenwerking met ons labo!

Isabelle en Evelien, vroeger waren we collega's en nu is het zalig om af en toe af te spreken en bij te babbelen. Bedankt!

Rita, jij hebt alles van dichtbij mogen meemaken. Jij hebt me weten geboren worden, me weten studeren voor apotheker en daarna werden we collega's. Je heb ons huwelijk meegemaakt, kwam op babybezoek en nu kom je naar mijn doctoraatsverdediging. Bedankt voor je vriendschap, enthousiasme, de lekkere pannenkoeken en de fijne bezoeken!

Dat een doctoraat niet altijd van een leien dakje verloopt, zullen mijn vele voorgangers/PhD studenten wel beamen. Gelukkig werd ik ook ondersteund door familie en vrienden die me voldoende afleiding bezorgden.

Aan al mijn/onze vrienden, ongelooflijk bedankt voor jullie vriendschap en steun!!

Een speciale attentie gaat naar mijn ouders die me onvoorwaardelijk steunen en me eigenlijk niet vaak genoeg zien. Veel sprak ik niet over mijn onderzoek maar jullie wisten wel hoeveel het voor me betekende. Heel erg bedankt om er voor me te zijn, een kaarsje te branden als het nodig was en ik ben er zeker van dat jullie supertrots gaan zijn als jullie dit boekje in jullie handen hebben.

Een dikke merci aan mijn zus Tine. Bedankt voor je fijne wensen, leuke attenties, kaartjes en een luisterend oor. Ook voor jou en je gezin waren de voorbije jaren niet altijd even gemakkelijk. Goede moed aan jullie allen, veel musiceergenot en veel liefs!

Ook een dikke merci aan mijn schoonouders, schoonzus en familie! Bedankt voor de fijne bezoeken, het lekkere eten en de rustige zondagmiddagen in Poperinge.

En dan zijn er natuurlijk mijn twee grootste supporters, Pieter en Simon. Pieter, heel erg bedankt voor je liefde, steun en begrip. Het voorbije jaar was voor ons allebei zeer intens maar super waardevol. Simon, jouw komst is ons grootste geschenk. Bedankt voor je onvoorwaardelijke liefde en vele knuffels. Ik zie jullie graag!

Hartelijk dank aan iedereen!

Griet, september 2017