Identification of novel therapeutics for chronic obstructive pulmonary disease/emphysema

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

September 2016

Statement of Originality

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Krishna Priya Sunkara

September 2016

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my parents, my mother in-law and late father in-law. I thank them for coming to Australia to extend their continuous support to me. Without whom my PhD would not be possible.

A great thanks to my primary supervisor Prof. Phil Hansbro, for his guidance, support and supervision to make this thesis possible and also to my co-supervisors, Laureate Prof. Paul Foster and A/Prof. Peter Wark. A special thanks to my co-supervisor Dr. Jay Horvat for his encouragement and support throughout my PhD. I'd like to extend my sense of gratitude to Dr. Richard Kim for his ongoing guidance, immense support throughout my doctoral studies and also for reviewing my thesis. My thanks goes to Dr. Andrew Jarnicki for his supervision and guidance all throughout my PhD studies.

I thank Mr. Tatt Jhong Haw and all other Hansbro group members for their help throughout my PhD. I would also like to thank my colleagues and friends, Dr. Kamal Dua, Ms. Prema Nair, Dr. Alan Hsu, Ms. Fiona Eyers and Mr. Stuart Upward for their encouragement and moral support.

Finally, I thank my brothers and sister in-laws for all their encouragement. Last but not least, words cannot express my love and thanks towards my husband, Suresh for always being there for me and to my lovely children, Manasa and Advait.

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Synopsis

Chronic obstructive pulmonary disease (COPD) is characterised by progressive decline in lung function that is caused by aberrant inflammatory responses, small airway remodelling and emphysema. The key risk factor of COPD is cigarette smoking. Current mainstay therapies of COPD only provide symptomatic relief and fail to limit the disease progression. Thus there is an urgent requirement for the development of new therapies. However this is hampered by the lack of understanding of the mechanisms that drive COPD pathogenesis. Therefore there is a need for the elucidation of the mechanisms that underpin the development of COPD.

MicroRNAs (miRs) are evolutionarily conserved small noncoding RNAs that regulate the expression of their target genes at the post-transcriptional level. More than 1,000 human miRNAs have been identified and are known to regulate numerous biological processes such as cell differentiation and proliferation, apoptosis, and immune responses. Importantly, altered expression of miRs are implicated in the development of several cancers and inflammatory diseases including asthma. However, their role in the pathogenesis of COPD is limited. Thus, our studies were aimed to understand the roles of CS-induced dysregulated miRs and interrogate their potential for therapeutic targeting in experimental COPD

Using microarray-based miR profiling technique, we identified a range of dysregulated miRs in CS-induced experimental mouse model of COPD. Acute and chronic CS-exposure chronically upregulated the expression of four miRs (miR-9, -21, -135b and-146b) in the lungs. Using miR-specific antagomirs we inhibited the CS-induced miRs and demonstrated that targeting CS-induced miRs may be an effective therapy in COPD treatment. We showed that CS-induced miR-9 and miR-21 promote airway inflammation and small airway remodelling and worsened lung function in experimental COPD. Treatment with miR-9- and miR-21-specific antagomirs, Ant-9 and Ant-21 lead to reduced airway inflammation, suppressed small airway remodelling and improvement

in impaired lung function. Thus indicating a potential pathogenic role for the miRs in the development of COPD. Our studies identified increased levels of oxidative stress responsive transcription factor NRF2 and restored levels of cytokine signalling suppressor protein, SOCS5 to play important roles reducing the COPD pathologies. We also identified a novel miR-21-dependent pro-inflammatory pathway in COPD pathogenesis. We demonstrated that CS-exposure induces a miR-21/SATB1/S100A9/NF-κB axis in the lungs and thus advances our understanding of the pro-inflammatory role of miR-21 in COPD pathogenesis. Our studies also demonstrated that CS-miR-135b promotes neutrophilic airway inflammation and showed reduced BMPR2 expression, a potential mediator of macrophage recruitment and may play a role in small airway remodelling in experimental COPD. We also demonstrated that treatment with Ant-135b and Ant-146b suppresses airway remodelling and emphysema-like alveolar enlargement. This indicates that miR-135b and miR-146b may play potentially overlapping roles in mediating COPD pathogenesis. We also showed that miR-135b and miR-146b may promote emphysema through VEGF and IRAK1 and TRAF6-dependent mechanisms. Furthermore our studies demonstrated for the first time that inhibition of combinations of CS-induced miRs, may have beneficial effects in reducing some features of COPD. Further we also showed that combined inhibition of CS-induced miR-21 and miR-146b may be more effective in suppressing key features of COPD.

Collectively, these studies further extend our understanding of the pathogenesis of COPD and identifies CS-induced miRs as potential novel therapeutic strategies in the treatment of COPD. Therapeutic targeting of a CS-induced miR or in combination may be more beneficial as miRs regulate multiple pathogenic pathways. Further, exploration of the CS-induced miR-dependent mechanisms identified in our studies may assist in the development of miR-based therapeutic strategies for the treatment of COPD.

Publications arising from this thesis

KP Sunkara, AG Jarnicki, RY Kim, TJ Haw, PA Wark, JC Horvat, PS Foster, PM Hansbro Role of miR-9 in the pathogenesis of chronic obstructive pulmonary disease. Prepared for submission to *European Respiratory Journal*.

Krishna P. Sunkara, Andrew G. Jarnicki, Ken R. Bracke, Richard Y. Kim, Tatt Jhong Haw, Peter A. Wark, Griet Conickx, Guy G. Brusselle, Jay C. Horvat, Paul S. Foster and Philip M. Hansbro. miR-21 suppression of SATB1 induces S100A9/NF-κB and COPD pathogenesis. Prepared for submission to *Journal of Clinical Immunology.*

Future publications

Role of miR-135b and miR-146b in the pathogenesis of experimental chronic obstructive pulmonary disease

Combined targeting of cigarette smoke-induced miRs in experimental COPD

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Abbreviations

Akt: Protein kinase B

AP-1: Activator protein

ATP: Adenosine triphosphate

BALF: Bronchoalveolar lavage fluid

BM: Basement membrane

BMPR: Bone morphogenetic protein receptor

BSA: Bovine serum albumin

CCL: Chemokine (C-C motif) ligand

CR: C-C chemokine receptor

CS: Cigarette smoke

COPD: Chronic obstructive

COX-2: Cyclooxygenase-2

CXCL: Chemokine (C-X-C motif)

CXCR: C-X-C chemokine receptor type

DAMP: Damage-associated molecular

DC: Dendritic cell

DMSO: Dimethyl sulfoxide

ECM: Extracellular matrix

EGFR: Pro-epidermal growth factor

ELISA: Enzyme linked immunosorbent assay protein

FEV1: Forced expiratory volume in

FOXO: Forkhead box protein

FVC: Functional vital capacity

GCLC: Glutamate--cysteine ligase catalytic subunit

GM-CSF: Granulocyte macrophage colony-stimulating factor

GPX: Glutathione peroxidase

GST: Glutathione S-transferase

GSTP: Glutathione S transferase

H&E: Hematoxylin and eosin

HDAC: Histone deacetylase

HO: Heme oxygenase

HPRT: Hypoxanthine-guanine

HRP: Horseradish peroxidase

i.n.: Intranasally

ICAM: Intercellular adhesion molecule

IFN: Interferon

IL: Interleukin

IL-1R: IL-1 receptor

IRAK: Interleukin-1 receptor associated kinase

JAK: Tyrosine-protein kinase

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein

miR: MicroRNA

MMP: molecular pattern monophosphate

MUC: Mucin

MyD88: Myeloid differentiation

NE: Neutrophil elastase

NF-κB: Nuclear factor κB

NOX: NADPH oxidase

NRF: Nuclear factor erythroid

nt: nucleotide

PAIS: Protein inhibitor of activated STAT

pAkt: Phosphorylated Akt

PAMP: Pathogen-associated pattern

PBS: Phosphate-buffered saline

PBS-T: PBS and Tween 20

PDCD: Programmed cell death protein

PDGFRB: Platelet-derived growth factor receptor beta

PI3K: Phosphoinositide-3-kinase

pol II: Polymerase II

Pre-miRNA: Precursor-miRNA

primary response gene 88

Pri-miRNA: Primary miRNA

PRR: Pattern recognition receptors

PTEN: Phosphatase and tensin

pulmonary disease

PVDF: Polyvinylidene difluoride

qPCR: Quantitative PCR

RAGE: Advanced glycosylation end product-specific receptor

RECK: Reversion-inducing cysteine-rich protein with Kazal motifs region gene 8

RIN: RNA integrity number

RISC:RNA-induced-silencingcomplex

RNA: ribonucleic acid

RNAi: RNA interference

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RT: Room temperature

S100:S100 calcium-binding protein

SATB:Special AT-rich sequence-binding protein

Scram: Scrambled antagomir

SHIP: SH2 domain containing inositol signalling

siRNA: Short-interfering RNA

SIRT: Sirtuin

SMAD: Mothers against decapentaplegic homolog

snoRNA: Small nucleolar RNA

snRNA: Small nuclear RNA

SOCS: Suppressor of cytokine

SPRY: Protein sprouty homolog

STAT: Signal transducer and activator

TBP: TATA binding protein

TBS: Tris-buffered saline

TBS-T: TBS and Tween 20

TGF: Transforming growth factor

TH: T helper lymphocyte

TIMP: Metalloproteinase inhibitor

TLR: Toll-like receptor

TNF: Tumour necrosis factor

TRAF: TNF receptor-associated factor

UTR: Untranslated region

VEGF: Vascular endothelial growth factor

WOB: Work of breathing

WT: Wild-type

Chapter 1: INTRODUCTION

1.1 CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic obstructive pulmonary disease (COPD) is a progressive and disabling chronic airway condition that is characterised by irreversible, or incompletely reversible, airflow obstruction (1). Cigarette smoke (CS) is the key risk factor COPD (2). Previously it was estimated that at least 25% of continuous smokers would develop COPD (3), but in a recent report it is shown that nearly 50% of elderly smokers (> 45 years of age) developed COPD (4). However, there are several other causes of COPD such as inhalation of particulate matter and noxious gases present in environmental pollution which are generated by biomass combustion and occupational exposure, and genetic predisposition that are other important causes of COPD (5-7). Non-smokers account for between 17-39% of COPD cases worldwide with the percentage differing from developed to developing countries (8). In a recent study it was reported that in non-smokers COPD development is associated with genetic variants (9). It was also revealed that smokers with a set of genetic signatures are at lower risk of developing COPD than others (9).

COPD encompasses the conditions of chronic bronchitis, small airway disease and emphysema which together result in airflow limitation (10-12). Importantly, these pathophysiological conditions persist despite smoking cessation (13, 14) and are present in 80-90% of all cases of COPD (15), suggesting that once COPD is induced, patient's condition deteriorates. In affected individuals, lung function typically declines over time and results in dyspnoea (shortness of breath) and cough (16). Spirometry is used to measure the severity of airflow limitation and is considered as the gold standard for COPD diagnosis (17). However, some smokers may be symptomatic but do not have airflow limitation (18) and are more susceptible to exacerbations and death.

1.1.1 Epidemiology of COPD

COPD is one of the World's leading causes of morbidity and mortality. The World Health Organisation (WHO) currently estimates that ~65 million people have moderate-to-severe COPD globally (19). Thus, it causes significant socioeconomic burden on the individual, society and healthcare facilities. In 2005, more than 3 million deaths were attributed to COPD (19, 20). In Australia, COPD is one of the leading causes of death and accounted for more than 4% of all deaths in the year 2003 (21). In the years 2008-2009 the healthcare cost for COPD in Australia was estimated to be around $929 million, where the highest portion of expenditure was due to hospitalisation (22) .

1.1.2 Pathology of COPD

In COPD, the major sites of chronic bronchitis are trachea and bronchi (15). CS causes epithelial irritation and injury that initiates inflammatory process. Repeated cycles of injury exaggerates inflammatory responses which lead to tissue repair resulting in remodelling and thickening of the airway walls (23). Chronic bronchitis is associated with mucous hypersecretion, difficulty in breathing and chronic productive cough (15). In COPD, small airway disease (i.e. in airways <2mm in diameter) plays a significant role in airflow obstruction due to the structural remodelling induced by degradation of airway elastin by proteolytic enzymes (13, 15, 24). Emphysematous lesions mainly affect the alveolar parenchyma and are induced by excessive inflammation and tissue destruction (25-27).

1.1.3 Diagnosis and disease severity

Depending on the severity of airflow limitation, COPD is categorised into four stages based on the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (17). GOLD classification is based on forced expiratory volume in one second (FEV1) and FEV/FVC (functional vital capacity) values. Decreased forced expiratory volume (FEV1) in one second is a characteristic feature of COPD (17, 28). Patients with FEV1 more than

80% predicted value are classified as GOLD A or mild in terms of severity. Reduced values of FEV1% indicates increase in COPD severity which declines with disease progression as shown in (Table 1.1) (28).

Table 1.1: Gold Stages of COPD

GOLD Stage	FEV_1 % predicted	Disease severity
A	$FEV_1 \geq 80\%$ predicted	Mild
B	$FEV_1 = 50\text{-}80\%$ predicted	Moderate
C	$FEV_1 = 30\text{-}50\%$ predicted	Severe
D	$FEV_1 < 30\%$ predicted	Very Severe

1.2 COPD pathogenesis

Several mechanisms are implicated in the pathogenesis of COPD, including chronic airway inflammation, oxidative stress, apoptosis, autophagy and autoimmunity that lead to damage of lung tissue, and results in self-perpetuating inflammation. A large and increasing number of factors have been associated with these processes and are likely to have overlapping functions. Whilst there have been significant advances in the elucidation of these mechanisms, the precise roles of the vast majority of individual factors in COPD pathogenesis remains unclear (29, 30).

1.2.1 Inflammation

Chronic inflammation of both the large and small conducting airways, and the alveoli, is strongly associated with the development of COPD and disease severity(31). Inhalation of noxious particles, such as cigarette smoke (CS) and environmental pollutants, causes the influx of inflammatory cells into the airways that then release

various pro-inflammatory cytokines and chemokines such as tumour necrosis factor (TNF)α and interleukin (IL)-8 (also known as C-X-C motif ligand [CXCL] 8) (32). The release of these mediators in turn attracts more inflammatory cells and drives a positive-feedback pro-inflammatory loop that results in chronic airway inflammation in COPD (33). Inflammatory cells also produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) and tissue degrading enzymes that cause oxidative stress and tissue destruction, respectively. These processes precede the onset of emphysematous changes in the lungs (34). Importantly, several studies have shown that the number of inflammatory cells in the bronchoalveolar lavage fluid (BALF) is markedly increased in patients with COPD and that these levels can increase up to six fold depending on disease severity (35-37). Macrophages, neutrophils, T and B -lymphocytes and dendritic cells (DCs) have all been implicated in COPD pathogenesis (38, 39). These inflammatory cells initiate pro-inflammatory responses which further lead to airway remodelling, emphysema and self-perpetuating inflammation.

1.2.1.1 Epithelial cells

The respiratory epithelium acts as a passive barrier to inhaled particles and can orchestrate immune responses to infection and tissue injury (40). Significantly, CS exposure can compromise epithelial barrier functionality and induce the production of pro-inflammatory mediators such as IL-8, TNFα, interferon (IFN)-γ and IL-1β from epithelial cells (33). These mediators can also trigger the release of ROS, and RNS. Collectively, these mediators are strongly associated with the pathogenesis of COPD and may promote and perpetuate the inflammation and mucous hypersecretion that is observed in patients with COPD (41).

Under normal conditions, the respiratory epithelium regulates the activity of alveolar macrophages through the activation of the immunosuppressive cytokine, transforming growth factor (TGF)-β. However, exposure to CS disrupts TGF-β responses and promotes the release of pro-inflammatory mediators from alveolar macrophages (40, 42). Thus, increased TGF-β signalling induces fibroblast proliferation and fibrosis in the airways **(Figure 1.1)** that result in narrowing of the airway lumen and reduced airflow in COPD (43). Tosi et al, has shown that epithelial cells also interact with activated neutrophils through the expression of the intercellular adhesion molecule (ICAM)-1 and increase the production of TNFα and IL-1β augmenting the airway inflammatory airway responses (44). Furthermore, serum ICAM-1 is elevated in COPD patients during active smoking compared to non-COPD patients (45). Thus, the interaction between airway epithelial cells and infiltrating immune cells release a number of mediators and further increase the pro-inflammatory responses. Collectively, these studies show that CS-induced injury of epithelium initiates a cascade of innate immune responses in COPD.

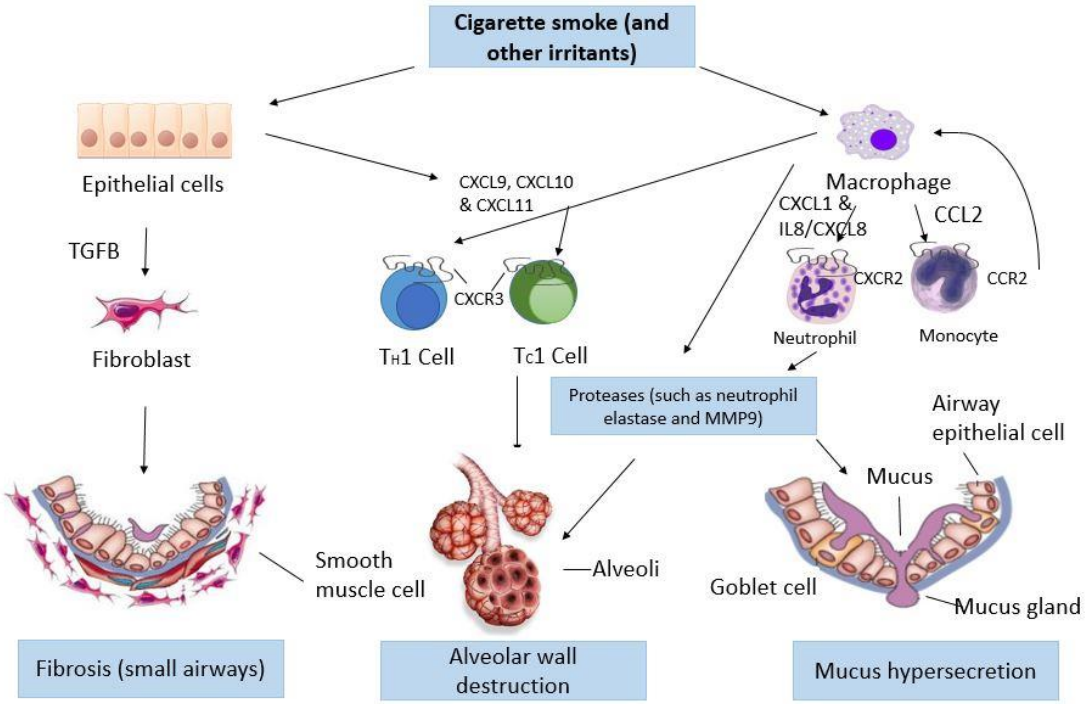


Figure 1.1: Inflammatory and immune cells involved in mucous hyper secretion, alveolar wall destruction and small airway remodelling in COPD pathogenesis. Cigarette smoke and particulate matter when inhaled, stimulates epithelial cells and macrophages to induce various pro-inflammatory mediators such as CC-chemokine ligand 2 (CCL2), CXC-chemokine ligand 1 (CXCL1) and CXCL8. CCL2 and CXCL1 and CXCL8 act on CC-chemokine receptor (CCR) 2 and CXC-chemokine receptor (CXCR) 2 receptors respectively and attract monocytes and neutrophils to lungs. Additionally, CXCL9, CXCL10 and CXCL11 act on the CXCR3 receptor to attract T-helper type (Th) 1 cells and type 1 cytotoxic T (TC1) cells into lungs. These inflammatory cells and epithelial cells release proteases such as matrix metalloproteinase 9 (MMP9) and neutrophil elastase which lead to tissue destruction and mucous hypersecretion. Epithelial cells also induce transforming growth factor β (TGF-β) which mediates fibroblast proliferation and result in small airway fibrosis. Adapted from (43).

1.2.1.2 Neutrophils

Several studies have shown that the number of neutrophils are increased in the airway lumen (33, 46) as well as in the interstitial tissue of the central and peripheral airways in patients with COPD (47, 48). Importantly, increased numbers of neutrophils in the sub-epithelial space is correlated with worsened airflow limitation, which highlights the potential importance of neutrophils in disease development, progression and severity (47, 49). Increased levels of neutrophils are also found in bronchial glands (50), BALF and sputum in COPD patients (33).

Neutrophils are recruited to the airways by chemotactic agents like IL-8, CXCL5 and leukotriene B4 (LTB4), which interact with CXCR2 receptors expressed on neutrophils. Importantly, IL-8 and LTB4 (49, 51) are secreted by epithelial cells, alveolar macrophages and neutrophils (33, 52, 53). TNFα, CXCL2/macrophage inflammatory protein (MIP)-2, and (C-C motif) ligand 7 (CCL7) are other examples of neutrophil chemoattractants that are increased in COPD. Thus, these neutrophil chemoattractants

produced from various cells induce neutrophil influx into the airways in COPD. TNFα and CXCL1 are increased in COPD patients (54) and CXCL2 levels are shown to be increased in CS-exposed mice (55), and recombinant CCL7 resulted in accumulation of neutrophils in the lungs of naïve mice (56). These data suggest that TNFα, CXCL1/2 and CCL7 produced by epithelial cells play important and potentially overlapping roles in the recruitment of neutrophils to the airways in COPD. Activated neutrophils also release a number of proteolytic enzymes, such as neutrophil elastase (NE), serine proteases and matrix metalloproteases (MMP), that cause extracellular matrix (ECM) destruction that results in emphysematous changes in COPD (13, 49). This suggests that, activated neutrophils and proteases together induce emphysema in COPD.

CS-exposure induces activation of neutrophils, macrophages and epithelial cells also result in the secretion of pro-inflammatory mediators called S100 molecules into the extracellular space (57, 58). S100A8 and S100A9 represent 45% of the cytosolic content of neutrophils and about 1% in monocytes (59, 60). They play key roles in maintaining cellular homeostasis like growth, differentiation and apoptosis (59, 61). S100A8 and S100A9, upon secretion from activated cells or on phagocytes, form S100A8/9 homodimers and S100A8/9 heterodimers and can function as damage associated molecular patterns (DAMPS) that are recognised by toll-like receptor (TLR) 4 and receptor for advanced glycation end products (RAGE) (62, 63). Receptor ligation results in NF-κB activation and induces the release of a suite of pro-inflammatory cytokines such as TNFα, IL-6 and IL-8 (62-64). Significantly, S100A8/9 levels are increased in the serum of patients with COPD and this is associated with decreased FEV_1 (65) and S100A/9 elevated levels were also found in sputum of COPD patients (65, 66). These data suggest that S100A8 and S100A9 play roles in mediating inflammatory responses and may be associated with impaired lung function in the pathogenesis of COPD.

1.2.1.3 Macrophages

In the lungs, macrophages play critical roles in initiating non-specific innate immunity and phagocytosing apoptotic, necrotic and infected cells (67). There are two major types of resident lung macrophage; alveolar macrophages and interstitial macrophages. Unlike alveolar macrophages that reside in alveoli, interstitial macrophages are derived from blood monocytes and mature following recruitment to the lungs (52). Significantly, macrophage numbers are increased 5-10 fold in the airways, lung parenchyma, BALF and sputum of patients with COPD compared to non-smokers, suggesting that macrophages are involved in the development of COPD (67). Several factors can promote the recruitment and activation of macrophages in the lungs including granulocyte-macrophage colony-stimulating factor (GM-CSF). It is a regulator of both macrophages and neutrophils (68), and is increased in the sputum of COPD patients (69). *In vivo* overexpression of GM-CSF induces increase in macrophage numbers (70) while neutralising CS-induced GM-CSF reduced inflammation by reducing TNFα and MMP12 mRNA (71). In our mouse model, airway macrophages were depleted with chlodronate-loaded liposomes that lead to depletion of macrophages and reduced CS-induced airway inflammation and remodelling and emphysema-like alveolar enlargement resulting in improved lung function in experimental COPD (72).

An important activator of macrophages is IFN-γ, which stimulates alveolar macrophages and epithelial cells by activating Janus kinases (JAK) 1 and 2 that are then phosphorylated and activate signal transducer and activator of transcription (STAT)1 (73). This results in the production of the chemokines CXCL9 (Mig), CXCL10 (IP-10) and CXCL11 (I-TAC) which interact with CXCR3 receptors expressed on Th 1 lymphocytes and CD8+ T cells, attracting lymphocytes cells to the lungs. On activation these T lymphocytes release more IFN-γ that augments the pro-inflammatory response and triggers chronic inflammatory responses (43, 50, 67, 74). Further on stimulation with CS, alveolar macrophages secrete the chemoattractants CCL2 (also known as monocyte chemo-attractant protein [MCP]1) that binds CC-chemokine receptor 2 (CCR2) and

attract monocytes to airways (67). CXCL1 and IL-8 binds to CXCR2 (51) and recruit circulating neutrophils to the airways further increasing the pro-inflammatory responses (43, 74). Additionally, alveolar macrophages can also produce the anti-inflammatory cytokine IL-10, however, IL-10 levels are known to be suppressed in COPD patients (75, 76). Thus, activated macrophages release a number of inflammatory mediators causing increased inflammatory cell influx into lungs that drive the disease development.

Macrophages can also generate ROS such as hydrogen peroxide and cysteine proteases and MMPs, which are known to contribute to tissue destruction and development of emphysema (74, 77). In addition macrophages are known to regulate the expression of transforming growth factor (TGF)α, which activates epidermal growth factor receptors (EGFRs) inducing mucous hypersecretion in COPD (67, 74).

Collectively, these data suggest that macrophages play key roles in perpetuating inflammatory responses, increase ROS and proteases production, and result in chronic inflammatory responses, mucous production and emphysema development in COPD.

1.2.1.4 T Lymphocytes

CD4+ and CD8+ T lymphocytes may play key roles in the pathogenesis of COPD as CD8+ T lymphocyte numbers (46) are strongly increased in central, as well as peripheral, airways and in the lung parenchyma (78, 79). These are also shown to be localised to airway smooth muscle bundles (80). Increased CD8+ T cell numbers in the small airways and alveoli has been shown to correlate with increased airflow limitation, suggesting a potential role in the disease development (13, 47, 50). While the predominant T lymphocytes in COPD are CD8+ cells, CD4+ T lymphocytes numbers are also markedly increased (approximately 3-fold) in the lungs of COPD patients (81).

CD4+ T cells produce the pro-inflammatory cytokines, IFN-γ and IL-12 contributing to the increase in IFN-γ levels which are also secreted by CD8+ T cells (43, 77, 81-83). CD4+ and CD8+ T cells are recruited to the lungs through the interaction of T cell surface receptor, CXCR3 with CXCL10 (43, 84). Following activation these cells

produce more IFN-γ, which results in a continuous cycle of T cell expression and their mobilisation to the airways enhancing the lung damage **(Figure1.1)** (43, 50, 84, 85). CD8+T cells also release proteases, granzymes and perforins and can induce apoptosis of alveolar epithelial and endothelial cells and contribute to parenchymal damage (33, 86). Increased concentrations of perforins have been detected in the sputum and BALF of COPD patients (10). Thus, the increased number of T lymphocytes in airway and parenchymal tissue play an important role in mediating COPD development.

Also another class of effector T cells, Th17 cells, have been associated with COPD development. Th17 cells produce the pro-inflammatory cytokines IL-17A and IL-17F (85, 87, 88), which have been shown to drive mucous hypersecretion through the induction of the mucin genes, mucin (MUC) 5AC and MUC5AB (88). A recent study showed that the increased expression of MUC5AC to be positively correlated with the severity of airflow limitation in patients with COPD (89). Moreover, IL-17 cytokines also stimulate airway epithelial cells to release IL-8 and MMPs further enhancing the inflammatory responses and mediating in tissue destruction in COPD.

1.2.1.5 B lymphocytes

B lymphocytes along with T lymphocytes play critical roles in adaptive immune responses. B lymphocytes are increased in small and large airways of COPD patients and the size of B cell-rich lymphoid follicles in COPD lungs are associated with disease severity (90, 91). The increase in B lymphocytes in the lungs of COPD patients are due to inflammatory responses and autoantibodies produced in reaction to cigarette smoke which further contribute to lung injury (90, 91). Studies with B lymphocyte deficient mice and COPD patients implicate the role of these cells in the pathogenesis of emphysema (92, 93).

1.2.1.5 Dendritic cells

In the lung, dendritic cells (DCs) normally exist as immature antigen presenting cells in the sub-epithelial, interstitial and pleural compartments (94). Some studies have shown that cigarette smoking markedly increases DC numbers in the airway epithelium and alveoli of COPD patients (43, 95). These cells are also shown to be activated in models of CS-exposure (96, 97), indicating their potential role in COPD. However, another study showed that DCs inhibit alveolar macrophage migration and prevent CS-induced pathogenesis in the early stages of disease development, suggesting that DCs are protective in COPD (98). Studies investigating the role of DCs in the pathogenesis of COPD are limited and show conflicting roles for DCs in COPD, therefore there is a need to further understand the role of DC in COPD pathogenesis.

1.2.2 Oxidative stress

Oxidative stress results from an imbalance between the amount of oxidants generated and the capacity of body to neutralise them. This imbalance results from increases in oxidants such as the ROS and RNS and decreased antioxidant capacity of the body (99, 100). Oxidant generation is essential for cell homeostasis and oxidants are endogenously produced by macrophages, neutrophils and other inflammatory cells (99, 100). CS contains approximately 6,000 chemical moieties (101, 102) and includes ROS and RNS such as peroxides, hydrogen peroxide, hydroxyl ions nitric oxide and nitrogen dioxide (29). In COPD, oxidative stress is purported to be induced by the combined effects of increased burden of oxidants present in CS and excessive oxidant production as a result of chronic inflammation (33, 102). ROS and RNS lead to damage of lung tissue, increasing cell death through the disruption of damage repair mechanisms (33, 102, 103). Importantly, ROS are known to activate the pro-inflammatory transcription factors NF-kB and activator protein (AP)-1 that induce the expression of TNFα, IL-8, IL-1, IL-6, MCP-1 and MMPs. ROS also activate signal transduction pathways, such as Mitogen-Activated Protein Kinase (MAPK) and Phosphoinositide 3-Kinase (PI3K), that

also induce the expression of pro-inflammatory cytokines (10, 34, 104-106). However, in the lungs of COPD patients, ROS also induces post-translational modification of histone deacetylase 2 (HDAC2) (107, 108) leading to its reduced expression (109). Moreover, HDAC2 is required in the glucocorticoid-mediated inactivation of the pro-inflammatory genes to suppress inflammatory processes. (34, 102, 106). Thus in COPD, ROS inactivates HDAC2 expression in the lungs, resulting in increased expression of pro-inflammatory mediators such as TNFα and IL-8 leading to perpetuating the inflammatory responses (106, 107, 110).

CS exposure also activates the oxidative stress responsive transcription factor, nuclear factor-erythroid 2 related factor 2 (NRF2). NRF2 induces the expression of protective antioxidant genes, including NAD (P)H: quinone oxidoreductase (NQO)-1 and glutathione S-transferase (GST), by binding to the promoter regions of antioxidant response elements (AREs) and attenuates the oxidative-stress responses (111, 112). Importantly, NRF2 is ubiquitously expressed in the lungs and is enriched in epithelial cells and macrophages (113). Significantly, NRF2 levels and activity are reduced in patients with COPD, and CS-exposed NRF2-deficient mice exhibit increased oxidative stress and develop severe emphysema (114-117). These studies suggest that NRF2 plays an important role in inducing antioxidant responses in the pathogenesis of CS-induced COPD

Oxidative stress can be assessed through the nitric oxide and hydroxyl derived compounds such as 3-nitrotyrosine and 8-isoprostanes which are protein and lipid peroxidation markers (118, 119). These are widely used as surrogate markers of oxidative stress and are increased in COPD patients. Importantly, increased levels of both of these markers are associated with disease severity as determined by FEV_1 (120, 121).

1.2.3 Protease: Anti-protease imbalance

Several studies have presented evidence for the role of protease: anti-protease imbalance in the development of COPD. This may result from persistent inflammation and oxidative stress that is induced by exposure to CS, which activates lung proteases and inactivates the anti-proteases (27, 122, 123). As discussed earlier, activated neutrophils secrete serine proteases such as NE, protease-3 and cathepsin G and MMPs such as MMP-8 and MMP-9, and activated macrophages express MMP-1, MMP-9 and MMP-12 which are capable of digesting elastin and causing ECM destruction (124-126). Consistent with this observation, numerous studies on animal models demonstrate the development of emphysema by the infusion of the elastolytic enzymes in the lungs (27, 102). NE degrades the ECM proteins, but NE is inhibited by α1-Anti trypsin (α1-AT). NE-mediated elastin degradation and emphysema has most strongly been associated with α1-AT deficiency (42). NE also activates MMP-9 which inactivating the endogenous MMP inhibitor tissue inhibitor of matrix metalloprotease (TIMP) and promotes epithelial cell death and mucous hypersecretion (27, 34, 127). Thus, MMP-9 causes elastolytic activity and remodelling of the tissue, increasing the inflammatory responses. It also degrades α1-AT, potentiating NE activity and thus enhances disease progression. Furthermore, both MMP-9 and MMP-12 are purported to play key roles in the early phase of COPD development, whereas MMP-1 predominates in severe forms of the disease (128). Thus, chronic inflammatory responses induce an imbalance in proteases and anti-proteases and potentially mediate increased tissue destruction.

1.2.4 Apoptosis

Apoptosis is a mechanism of programmed cell death in infected and damaged immune cells (129). In smokers with COPD, there is an increase in the apoptosis of interstitial and inflammatory alveolar epithelial and endothelial cells (130). These findings are supported by several mouse studies that have shown that alveolar epithelial and endothelial cell apoptosis contributes to the development of emphysema (27, 52).

Collectively, these studies report that apoptosis of type l alveolar cells (pneumocytes) leads to airspace enlargement and type II alveolar cells decreases the secretion of surfactant that results in a similar phenotype to human emphysema (100, 129). Importantly, apoptosis plays a critical role in many disease-causing processes that are associated with the pathogenesis of COPD, including inflammation, protease/anti-protease imbalance and oxidative stress (30) introducing additional layers of complexities that needs to be investigated.

1.2.4.1 Apoptosis and Inflammation

Apoptosis is induced as part of the normal resolution of inflammation, whereby damaged, inflammatory cells apoptose and are then removed by phagocytes such as macrophages, to contain the release of cytotoxic intracellular materials and limit tissue damage (123). However, in the presence of pro-inflammatory mediators activated neutrophils which are tolerant to apoptosis (131, 132) release NE, which cleaves the phosphatidylserine receptor on macrophages and results in impaired phagocytosis and removal of apoptotic cells (133). In COPD patients, DCs and CD8+ T cells can also promote apoptosis of alveolar epithelial cells through the release of perforins, granzyme-B and TNFα (131). These studies suggest that chronic inflammatory responses cause impaired apoptotic clearance as well as result in increased apoptotic cell death and mediate alveolar tissue destruction in the pathogenesis of COPD.

1.2.4.2 Apoptosis and Protease: Anti-protease imbalance

Apoptosis is an important mechanism which maintains cell numbers and tissue structure (134). Recent reports indicate the role of apoptosis and inadequate maintenance of alveolar tissue to promote emphysema development (30, 135). For example, vascular endothelial growth factor (VEGF) which is highly expressed in endothelial cells, if decreased leads to apoptosis of the structural cells and may result in emphysema (136). In COPD, the presence of elevated levels of the proteolytic enzymes

NE and MMPs results in increased degradation of the ECM that reduces the levels of growth factors from the basal membrane (134). This causes increased death of type I alveolar cells, resulting in increased alveolar tissue destruction and emphysema (29, 43). It has also been suggested that apoptosis of bronchial epithelium is modulated by cell:cell and cell:ECM interactions (137). In a mouse model of emphysema the authors have shown that apoptosis of epithelial cells causes increased elastolytic activity resulting in emphysematous changes in the lungs (138). This shows apoptosis-dependent alveolar destruction may play an important role in the development of emphysema in COPD.

1.2.4.3 Apoptosis and Oxidative Stress

Several studies using animal models of emphysema have shown a potential role for oxidative stress in inducing the apoptosis of alveolar cells, which suggests that this association plays an important role in the development of emphysema (27, 52, 139). Oxidative stress has been shown to reduce the expression of vascular endothelial growth factor (VEGF) (139, 140). VEGF is necessary for the growth and survival of endothelial cells and the apoptosis of epithelial cells decreases VEGF expression (130). As a consequence alveolar endothelial cell death takes place, compromising the microcirculation and further promoting the cycle of apoptosis **(Figure 1.2)** (27, 30, 52). In human studies, VEGF is present at low levels in the sputum of COPD patients, suggesting that reduced VEGF and VEGF-induced responses are important in the development of emphysema (52). However, there are other studies which reported elevated levels of VEFG in the distal airways and alveoli of COPD patients (141, 142). These reports indicate a protective response of VEGF against emphysema development in COPD, thus the role of VEGF in COPD is controversial and needs to be further explored. Further, in a model of emphysema, oxidative stress was inhibited through the administration of antioxidant N-acetyl-l-cysteine which suppressed the apoptosis of

alveolar cells and emphysema (140). This indicates that emphysema in COPD is regulated by apoptosis through an oxidative stress-dependent mechanism.

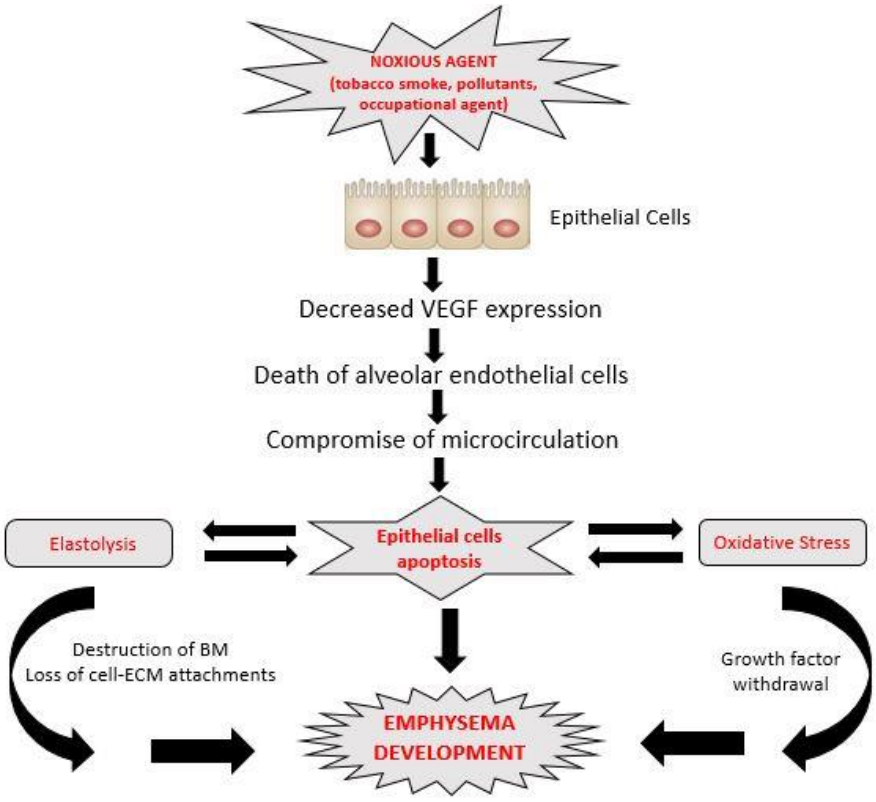


Figure 1.2: Lung epithelial cell apoptosis and development of emphysema mediated by decreased VEGF production. CS exposure increases epithelial cell apoptosis which may lead to reduced vascular endothelial growth factor (VEGF) expression, increased alveolar endothelial cell death and result in compromised microcirculation. This further promotes apoptosis of epithelial cells leading to feedback loop of increased digestion of elastin (elastolysis) and oxidative stress which results in alveolar damage. Destruction of alveolar tissue damages the basement membrane (BM) and extracellular matrix (ECM) attachments and further reduces growth factors. All these processes are implicated in emphysema development. Adapted from (129).

1.2.5 Autophagy

Autophagy plays important roles in development, homeostasis and in stress environments such as infection, hypoxia and oxidative stress (143, 144). It is a controlled cellular mechanism through which damaged proteins and organelles are degraded through a lysosome-dependent pathway (143, 145). Increasing evidence suggests that autophagy is both protective and deleterious (143, 146). Several studies have shown roles for autophagy in COPD (147, 148), and in recent work on COPD patients, Chen *et al*., showed increased expression of autophagic markers such as microtubule-associated protein-1 light chain (LC)3B-II, autophagy-associated genes (Atg) Atg4, Atg5–Atg12 and Atg7 and increased autophagosome formation (149). Consistent with human COPD studies, various *in-vivo* and *in-vitro* models of CS-exposure also showed increased autophagy (150-153). In a model of emphysema, mice exposed to chronic CS-exposure showed increases in autophagosome numbers and enhanced autophagy proteins levels. Conversely LC3B null mice or autophagy deficient mice exposed to chronic CS were shown to be resistant to emphysematous changes (149). The authors also showed that autophagy deficient mice were resistant to the disruption of airway mucocilicary clearance on exposure to acute CS-exposure (149). Further in a recent study by Zhu *et al*., the authors showed negative regulation of CSE-induced LC3B protein levels and autophagosome formation by the NRF2 anti-oxidant responsive system (154). This indicates that NRF2 may play an important role in regulating autophagy in CS-exposed systems. However, the exact mechanism through which autophagy mediates COPD pathologies is unclear and needs to be elucidated with further studies.

1.2.6 Autoimmunity

Autoimmune diseases occur when the adaptive immune system losses immunological tolerance and is pitched against self-antigens (155). In COPD, autoimmunity likely plays a role in pathogenesis. It arises as a result of persistent inflammatory responses that continue even after smoking cessation (156). Several

studies have shown the involvement of T and B cells in COPD development. In one study, lung CD4+T cells of COPD patients had Th1 phenotype and expressed cytokines MIG and IP-10 that mediated the upregulation of MMP-12 elastase associated with emphysema (157). In COPD patients, CD8+T cells are increased and induce the production of a range of pro-inflammatory cytokines, which indicates their involvement in COPD development (158, 159). However, these studies were unable to show the involvement of an auto-immune component of these cells in COPD pathogenesis. Nevertheless, recent studies of COPD patients have shown the increased presence of antibodies reactive to self-antigens of airway epithelial and endothelial cells, carbonyl-modified and citrullinated proteins induced from oxidative stress and elastin (160, 161). Thus increasing evidence implicates roles for autoimmunity in COPD pathogenesis.

1.3 COPD EXACERBATIONS

Exacerbations of COPD are most frequently caused by bacterial and viral respiratory infections (162). Patients with repeated exacerbations are grouped as a frequent exacerbator phenotype and have severe airflow obstruction, low quality life and poor prognosis (163). COPD exacerbations worsen disease severity increasing the lung damage (164). This was demonstrated in an *in vitro* study that primary bronchial epithelial cells (pBECs) from COPD show increased susceptibility to influenza through the exaggerated PI3 kinase activity which promotes increased viral entry resulting in increased inflammatory responses (164). Thus during an exacerbation, patients with COPD exhibit further increases in neutrophil, T lymphocyte, and macrophage numbers in the airways. These effects are associated with increases in the levels of TNFα, GM-CSF, IL-8 and other pro-inflammatory mediators in the bronchial mucosa and sputum (165-167). Likewise, oxidative stress is increased during COPD exacerbations and COPD patients with acute exacerbations show higher levels of hydrogen peroxide and nitric oxide levels in exhaled breath condensates and also show further decline in FEV_1 values (168, 169).

1.4 SYSTEMIC INFLAMMATION IN COPD

COPD also causes many extra-pulmonary effects such as generalised weakness, hypoxia, and systemic oxidative stress that can predispose to cardiovascular complications, which are the main cause of death in patients with COPD (50). In a study assessing systemic levels of pro-inflammatory mediators in COPD patients, the authors showed inverse correlation between partial pressure of oxygen and the circulating TNFα, which suggests that airflow obstruction in COPD may drive systemic hypoxia that further increases circulating TNFα levels (170). In COPD patients with emphysematous changes increased TNFα levels are linked with weight loss of the patients (42). Therefore, high levels of leukocytes, CRP, IL-6, TNFα are considered as markers of systemic inflammation in COPD (171). This shows that inflammation, oxidative stress and emphysema changes in COPD, together mediate exaggerated inflammatory responses resulting in systemic inflammation.

1.5 PATHOPHYSIOLOGY OF COPD

1.5.1 Airflow obstruction

A major pathophysiological outcome of COPD is expiratory airflow obstruction that results from increased airway resistance and loss of elastic recoil of the lung (172). In the large airways, chronic exposure to CS induces inflammatory cell influx and inflammatory responses in the epithelium and glands lining the large airways (173). This causes in increased mucous production resulting in airflow limitation (174). A recent study also indicate large airway remodelling and smooth muscle hypertrophy to contribute to airflow obstruction (175).

The major sites of airflow obstruction in COPD are the small airways (90). Small airways show persistent cell infiltration causing chronic inflammatory responses leading to repeated cycles of injury and repair which results in airway wall thickening (176, 177). Thus, small airways are the major contributors to airflow obstruction which results due to

the narrowing of the airway lumen due to fibrosis and smooth muscle hypertrophy (176, 177). Furthermore, elastolytic activity of proteases causes the disruption of alveolar attachments to the bronchioles, which predisposes the airways to early closure during expiration and obstructs airflow (14, 88, 178).

1.5.2 Emphysema

The cardinal feature of emphysema is the parenchymal destruction which is induced by excessive inflammation and tissue destruction (25-27). Parenchymal destruction also results in the loss of alveolar attachments to small airways and exaggerated inflammatory responses lead to narrowing of small airways (25-27). Thus the alveolar wall damage adversely affects the elastic recoil of the lung and also results in decreased surface area available for gas exchange (179). Emphysema is widely considered as an irreversible and abnormal enlargement of the air-spaces and is associated with breathlessness in patients with COPD (15, 29).

1.5.3 Mucous hypersecretion

Chronic cough and mucous hypersecretion in patients with COPD are potentially a result of epithelial disruption (25), decreased mucociliary clearance (13, 14), goblet cell hyperplasia, mucosal metaplasia and hypertrophy/hyperplasia of bronchial sub-mucosal glands. Most of the pathogenic mechanisms implicated in the development of COPD have deleterious effects on epithelial cells and goblet cells which induce excessive mucous secretion (102, 180). Increased mucous secretion is known to contribute to decline in lung function in COPD (181).

1.5.4 Fibrosis

Small airways in COPD have increased ECM and collagenous deposition around small airways resembling scar tissue which contract, accounting for the narrowing of the airway lumen and causing air flow obstruction. TGF-β plays a major role in fibrogenesis

by stimulating fibroblast recruitment, converting fibroblasts into myofibroblasts, and facilitating the production of ECM (25). TGF-β activates Mothers against decapentaplegic homolog (Smad) signalling pathways, particularly Smad 3 which plays a key role in wound healing and fibroblast activation (88, 102).

1.6 CURRENT THERAPIES

Current therapies for COPD only address the symptoms of disease and do not reduce the severity and progression of the disease (182, 183). Thus, cessation of smoking is considered to be the effective approach which improves patients overall health, but few patients are able to stop smoking (13, 14). However smoking cessation cannot reverse the lung pathologies and does not prevent decline in lung function. Currently, combinatorial treatment with anti-inflammatory glucocorticoids and long acting β2-agonists are the mainstay therapies for COPD, but have little efficacy in preventing chronic inflammation and fail to limit the progression of disease (184-186). Several drugs are now being developed to limit disease progression such as LTB4 antagonists, PDE4 inhibitors, and NE and MMP inhibitors (187, 188). However, combination therapies of this nature will be prohibitively expensive, a complicated course of therapy and may lead to enhanced side effects(189). Therefore, there is an urgent need for a new approach to drug therapy. Oligonucleotide therapy to suppress the activity of pivotal inflammatory molecules could potentially be used in the treatment of complex diseases such as COPD (190). One such potential oligonucleotide therapy is the manipulation of microRNAs (miRs) (190). A single miR can modulate numerous target genes, increasing the chances of effective treatment and miR-based therapy may be developed into a One-Drug, Multiple-Target therapy approach (191).

1.7 MICRORNAS (miRs)

miRs are small non-coding RNAs of approximately 22 nucleotides in length and are highly conserved between microorganisms, plants and animals. miRs are small

noncoding RNAs that regulate the expression of their target genes at the post-transcriptional level (192). miRs bind to 3' untranslated regions of mRNA and modulate their expression through RNA interference (RNAi) mechanisms(192). A single miR is known to regulate single, hundreds or thousands of downstream mRNAs, and a single mRNA can be simultaneously targeted by a number of miRs (193, 194). To date nearly 1000 miRs have been identified in humans and miRs constitute up to 3% of the human genome and about one third of human genes are regulated by miRs (194-196).

1.7.1 miR biogenesis and mechanism of action

miRs are primarily transcribed from intronic regions of coding and non-coding genes and are spliced out of the corresponding gene transcript as primary-miR (pri-miR). Pri-miRs are several hundred nucleotides long and are processed into mature miR (197). RNA polymerase type II (Pol-II) mediates the transcription of the long stem-loop pri-miR from the genome. The stem-loop structure of pri-miRs is sliced by an RNase III endonucleases known as Drosha/Pasha to produce a 70 nucleotide long hair pin loop precursor miR the pre-miR (198-200). Pre-miRs are transported out of the nucleus into the cytoplasm by Ran-GTP and the receptor Exportin-5 and then cleaved by another RNase III endonuclease, Dicer, into 18-25 nucleotide miR duplexes in the cytoplasm **(Figure 1.3)** (198-201). These mature miR duplexes are not complementary and are degraded into a non-functional (passenger strand) and a functional (guide) strand. The guide strand associates with Argonaute (Ago) proteins and is then incorporated into the RNA-induced silencing complex (RISC) (195, 197, 198, 202). With the formation of miR-ribonucleoprotein complex (RISC), post-transcriptional gene silencing is initiated (198-201). The miR-RISC ribonucleoprotein complex recognizes and promotes binding between the miR and the 3' untranslated region (UTR) of target mRNA. Depending on the degree of complementarity between the miR and the target mRNA, either degradation or translational inhibition of the mRNA occurs. Perfect miR and target mRNA

complementarity results in target mRNA degradation, whereas partial complementary results in translational repression (198-201).

In plants, miRs have been shown to bind perfectly to the mRNA targets and are cleaved by RNAi mechanisms through endonuclease activity (203). In contrast, mammalian miRs are mostly partially complementary to their target mRNA and result in inhibition of translation through deadenylation of target mRNAs (199, 200). However, in order to facilitate the development of miR-targeted therapies, it is important to further understand the mechanism through which miRs downregulate their target gene expression.

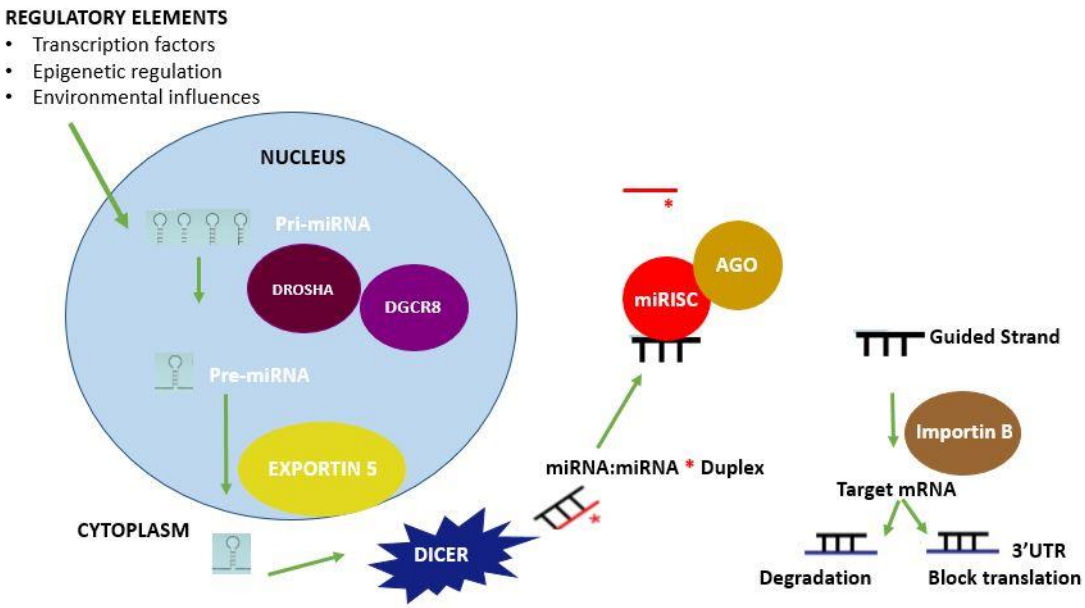


Figure 1.3: microRNA (miR) biogenesis. miR expression is induced by transcription factors and other regulatory proteins. In the nucleus primary miR (pri-miR) are processed by Drosha and dsRNA binding protein Pasha (DGCR8) into precursor miR (pre-miR) which is transported into cytoplasm by Exportin 5. The pre-miR is processed by Dicer into a miR duplex; the guide strand integrates with the RNA-induced silencing complex (RISC) forming miR-RISC (miRISC) and the miR*/antisense strand is degraded. Guide strand integration into miRISC is aided by Argonaute (Ago) proteins. Importin 8 facilitates

the movement of miR to the target mRNA and mediates binding to the 3'UTR region and induces target mRNA degradation or translational repression. Adapted from (204).

miRs regulate a wide variety of physiological processes and are essential in normal development, maintenance and functioning of individual cells and the organism as a whole. They are important in cell differentiation, inflammation and immune responses, apoptosis and many other normal physiological processes. Alterations in miR expression have been associated with the development of various diseases such as cancer and inflammatory, infectious, and autoimmune diseases. Thus, miRs are altered in chronic inflammatory diseases and as COPD has similar features of chronic inflammatory diseases, it is highly likely that miRs are potentially involved in COPD pathogenesis. (193, 205-207) .

1.7.2 miRs in immunity and COPD

Numerous clinical and experimental studies have implicated miRs and their altered expression in the pathogenesis of several chronic inflammatory diseases. A number of miRs ares involved in the regulation of immune responses and their aberrant expression may drive the pathogenesis of these diseases. Increasing evidence suggests that miRs play important roles in the development and function of the innate and adaptive immune system (193). TLRs are cell surface receptors that are expressed on macrophages, epithelial, endothelial cells and recognise microbial components called pathogen-associated molecular patterns (PAMPs) (208, 209). TLR ligation initiates downstream pro-inflammatory responses. TLR signalling is controlled by negative regulation in order to prevent abnormal inflammatory responses (210). Recent studies have shown that miRs modulate innate immune responses through negative regulation of the TLRs (210-212). Independent studies revealed that miR-146a, -146b, -155, -9 and -21 are TLR responsive miRs (211, 213-216).

miR-146a/b are induced from LPS/TNFα/IL-1β-stimulated human monocytes and NF-κB is activated increasing the pro-inflammatory responses(213). However, miR-146a

suppresses the signalling transducers, TNF receptor–associated factor (TRAF)-6 and IL-1 receptor associated kinase (IRAK1) of myeloid differentiation primary response protein (MyD)88-dependent TLR2, TLR4 and TLR5 pathways and thus negatively regulate TLR responses (216). Recent studies showed that miR-155 and miR-21 expression is induced in LPS-stimulated mouse macrophages (214, 215). TLR4 ligation induces miR-155 and miR-21 expression and increased miR-155 expression downregulates its target Src homology 2 (SH2) domain-containing inositol-5´-phosphatase (SHIP) 1, which is a negative regulator of TLR4 signalling (214). Whereas upregulated miR-21 suppresses its target programmed cell death (PDCD) 4 mRNA which in-turn decreases TLR4 signalling and NF-κB activation (214, 215, 217). Thus, miR-155 increases and miR-21 decreases TLR signalling, thus the miRs fine tune TLR signalling. Furthermore, stimulation of monocytes and neutrophils with LPS induces the expression of miR-9 through a TLR4-MyD88 and NF-κB dependent mechanism (213). This study demonstrated that miR-9 directly targets the p50 subunit of NF-κB and downregulates its expression (213), which suggests that miR-9 endogenously inhibits NF-κB-dependent pro-inflammatory responses.

miRs are also known to regulate adaptive immune responses. For example, it was shown that the dysregulation of miR biogenesis in lymphocyte stem cells resulted in impaired T and B cell development (193). Another example of miR-associated regulation of adaptive immune responses is, miR-155-dependent differentiation of naive T cells into Th17 cells (218) which are involved in the autoimmune diseases such as systemic lupus erythematosus (SLE) (219). Furthermore, a recent report also showed through an *in vivo* model, that miR-155 deficiency can decrease the inflammatory responses in SLE through the up-regulation of its target gene sphingosine-1-phosphate receptor 1 (*S1PR1*) (219). The authors also showed reduced IL-17a levels were associated with miR-155-deficiency. Furthermore, increased expression of miR-155 and miR-21 are also shown to inhibit the transcription factor special AT-rich binding protein 1 (SATB1), which under normal conditions, suppresses effector T cell differentiation and maintains regulatory T

(Treg) cell function (220). Importantly, SATB1 responses facilitate Th2 cell differentiation and induce the production of Th2 cytokines IL-4 and IL-5 (221, 222). Aberrant expression of SATB1 is also associated with several cancers, including breast and lung cancers, where increased SATB1 responses inhibit apoptosis (223, 224) and positively correlate with poor prognosis (225, 226). However, in colorectal cancer and squamous cell carcinoma of lung, reduced levels of SATB1 correlated with disease severity. Likewise in a recent study on clear cell renal carcinoma, increased expression of miR-21 is associated with decreased SATB1 levels and was a poor prognosis of disease (227-229). This indicates that role of miRs and their target genes are cell type and context dependent. Collectively, these reports show that miRs are strongly involved in regulating both innate and adaptive immune responses suggesting that miRs may mediate both innate and adaptive responses in COPD development.

To date several studies have strongly implicated miRs in the development of lung cancer, however, few have elucidated the role of miRs and their targets in the pathogenesis of COPD. A recent miR profiling study identified 57 upregulated, and 13 downregulated, miRs in the lungs of smokers with COPD compared to smokers without COPD (230). Interestingly, the expression of several miRs was altered at various stages of emphysema and in different regions of the lung (231, 232), suggesting that miRs are differential expressed in various tissues in COPD lungs and may be involved in the disease developmental processes. Further, in another study, the expression of miRs has also been shown to be altered in serum, sputum and exhaled breath condensate of patients with COPD (233-235). Importantly, most of the miR-based studies in COPD have only profiled the expression of miRs in different parts of lung tissue of COPD patients. Significantly, few studies have characterised the miR-mediated mechanisms that underpin the development of COPD. In a study on sputum of COPD patients, *let*-7c and miR-125b expression were reduced and were associated with increased levels of TNF receptor II (TNFR-II) (234). Importantly, the authors showed that with *let*-7c downregulation the levels of its predicted target TNFRII were increased which is

associated with inflammation in COPD (236) . Similarly in a study by Sato *et al.*, primary fibroblasts isolated from COPD patients stimulated with IL-1β and TNFα, showed reduced miR-146a expression compared with fibroblasts from smokers without COPD (237). Interestingly, reduced expression of miR-146a led to increased mRNA expression of its target cyclooxygenase (COX)-2 and increased levels of prostaglandin E2 (PGE2) (237). Significantly, PGE2 is a potent pro-inflammatory mediator and its levels are markedly elevated in the lungs of patients with COPD (237) suggesting that dysregulation of miR-146a in the lungs plays an important role in the chronic airway inflammation that is associated with COPD. Furthermore, a recent study showed that CS-exposed mice exhibit IL-1R1-dependent increases in the expression of miR-135b in the lungs (238). Increase in miR-135b expression led to the inhibition of IL-1α activation and, subsequently, suppressed airway inflammation (238). Thus, these studies indicate some of the potential roles of miRs in the pathogenesis of COPD.

1.7.3 Potential miR therapeutics

Research of miRs has gained prominence since their involvement in various diseases are identified. Now, less than 25 years following their discovery, research into miRs has reached the stage where abnormally expressed miRs are being interrogated as the next potential therapeutic targets for numerous diseases. Targeting miRs therapeutically has advantages, and may be beneficial as miRs can modulate multiple targets that are involved in numerous disease-causing pathways. Thus targeting a single miR potentially suppress several disease causing mechanisms.

In disease associated with decreased miR expression, miR mimics or viral vector-based approaches can be employed to boost miR expression (239, 240). Mimics are synthetic double-stranded miRs that can be injected intravenously or intra-tumorally (239, 240). The guide strand is usually synthesised to be identical to the target miR and is chemically modified to increase its stability (241, 242). The passenger strand is

cholesterol-conjugated at 3' end to aid in uptake across the plasma membrane (241, 242). Importantly, whilst miR mimics increase the expression of target miRs they can also induce TLR-dependent non-specific interferon responses at high doses (243). Thus, systemic delivery of miR mimics may also result in uptake by non-target tissues and, potentially, lead to off-target effects. Thus, an effective way of maximising the therapeutic potential of miR mimics would be to deliver them directly to target cells and/or tissues. Target miR expression can also be synthetically and stably increased by viral vector constructs of adeno-associated virus (AAV) or lentiviral origin (242). Viral vectors are more technically advantageous for exogenous overexpression of miRs as they can be chosen based on virus serotypes, tropism toward specific cell types and interaction with cellular receptors (242). In a study performed by Kota *et al.*, in a mouse model of hepatocellular carcinoma, AAV miR-26a overexpression construct administration suppressed tumour progression, indicating that the exogenous overexpression of miRs may be potentially used to increase the expression of miRs in disease (244).

A number of techniques have been developed to inhibit the expression of up-regulated miRs, such as miR gene knock out, miR 'sponges', and antagomir and locked nucleic acid (LNA). miR gene knock-out studies are practically challenging due to the difficulty in generation of genetic knockouts. miR sponges are miR inhibitors of cellular origin that contain multiple binding sites for a specific miR and work by sequestering endogenous miRs. Sponges are transgene can be delivered by a viral vector. However, their in *vivo* functions are yet to be evaluated (245, 246).

Antagomirs, or anti-miRs, are chemically engineered 2'-O-methyl group–modified oligonucleotides. LNA are modified anti-miR oligonucleotides where the 2'-O-oxygen is bridged to the 4' position through a methylene linker to form a stable duplex that is complementary to the miR (247, 248). Antagomirs are cholesterol-conjugated, single stranded RNA sequences oligonucleotides with partially, or completely, complementary sequences analogues to mature miR that are used to silence miRs and potentially upregulate the target genes involved (247, 249, 250). The first synthesised anti-miR,

developed Krutzfeldt and colleagues was miR-122-specific inhibitor antagomir (Ant)-122, for silencing the liver miR-122. As illustrated **(Figure 1.4)**, with intravenous administration of Ant-122, miR-122 expression was silenced in mice for more than a week in liver, heart, skin, intestine, lung and bone with the upregulation of the predicted target genes (250). However, similar to miR mimics, antagomirs may potentially lead to off-target effects.

At present there are two miR-targeted therapeutics in clinical trials. One of them is Miravirsen, a miR-122, an LNA-based anti-miR against chronic hepatitis C virus (HCV) infection and is in clinical phase IIA trial. The other one is MRX34, a miR-34 mimic against unresectable primary liver cancer and advanced or metastatic cancer with liver involvement which is in phase I clinical trials (251, 252), thus the miR-based therapeutics in clinical trials highlight the potential of targeting miRs in disease.

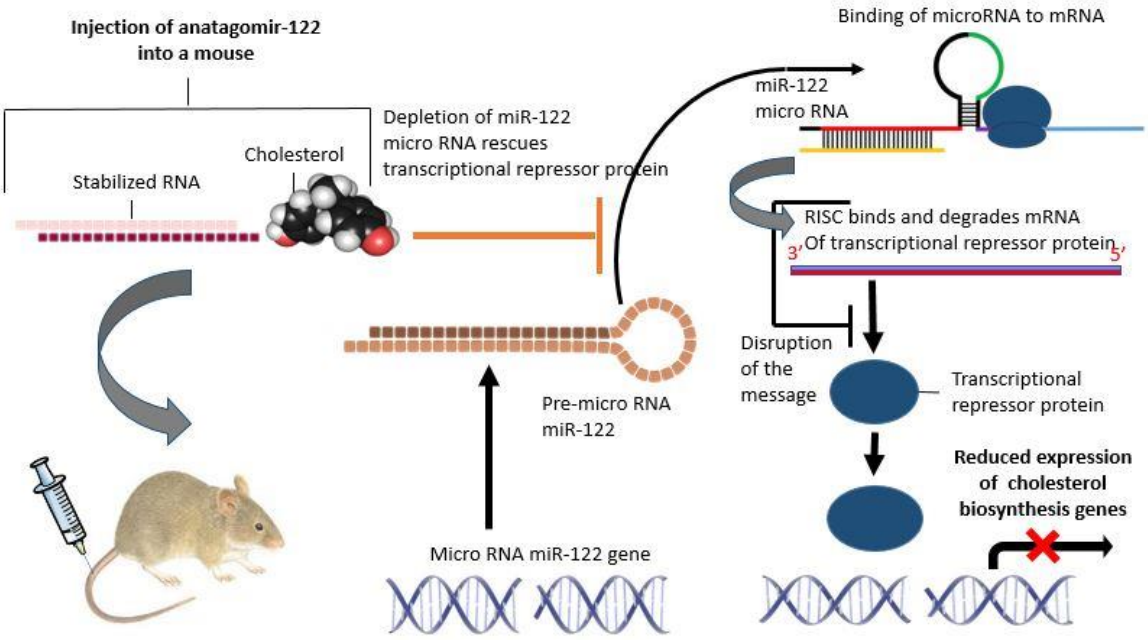


Figure 1.4: microRNA (miR) silencing *in vivo* with antagomirs. miR-122 is transcribed from DNA into a hairpin structure, pre-miR-122, which is processed into a 22 nucleotide long miR duplex. The guide strand of the miR duplex integrates with the RNA-induced silencing complex (RISC) complex to form miRSC enabling the antisense strand to link at the 3' UTR region of the target mRNA and induce translational repression. Thus, miR-122 may inhibit the repressor gene of the cholesterol synthesis pathway resulting in increased cholesterol production. Importantly, Krutzfeldt and colleagues (250), prepared a cholesterol-conjugated, single-stranded RNA, antagomir (Ant) with perfect complementary to mature miR-122. Intravenous administration of Ant-122 to mice, resulted in suppression of miR-122 and increased the expression of repressor gene of the cholesterol synthesis pathway and decreased cholesterol levels. Adapted from ***(253)*.**

1.8 STUDY RATIONALE

COPD is a chronic respiratory disease with aberrant inflammatory responses that drive structural damage of the lungs. Current treatments for COPD only suppress the symptoms of the disease and do not halt the disease progression. Thus, there is an urgent need to identify and elucidate the role of the pathogenic mediators that drive exaggerated inflammatory immune responses in COPD in order to develop effective therapeutic strategies. Increasing clinical and experimental evidence implicates the role of miRs in the pathogenesis of COPD. These studies show that CS exposure dysregulates the miR expression profile, which may concurrently alter the expression of miR-regulated genes and potentially induce the mechanisms that drive COPD pathogenesis. Targeting CS-induced miR expression may be more effective than current therapies as miRs regulate multiple target genes in several pathways associated with the development of COPD. Hence, targeting miRs represents a novel therapeutic approach for COPD. We thus hypothesised that cigarette smoke induces chronic inflammatory responses in the lungs that lead to pathogenesis of COPD. Smoking may

alter miRNA expression levels that regulate gene expression and induce the mechanisms that drive COPD pathogenesis. Modulating miRNA expression may be a viable and novel therapeutic approach for COPD. The studies described hereafter in this Thesis were designed to identify the miRs that are differentially expressed following exposure to cigarette smoke, their target genes, and the downstream immunological mechanisms that they regulate in the pathogenesis of COPD.

Our lab recently developed a novel, nose-only CS exposure-induced mouse model of experimental COPD that generates many of the key features of human COPD, including chronic airway inflammation and remodelling, emphysema and lung function impairment, within 8 weeks (72). In this model, mice are exposed to 12 cigarettes twice per day which is equivalent to one pack of cigarettes per day in humans. The development of characteristic features of COPD in a relatively short time period (i.e. 8wks) makes this model an ideal platform to elucidate the molecular mechanisms that underpin disease development. Although disease features are established by 8 weeks, further smoking for 4more weeks progressively worsens the disease (72). To identify the miRs potentially involved in the induction, development and progression phases of the disease, we used our 12 weeks smoke-exposure model to profile the CS-induced dysregulated miRs in experimental COPD.

Using this model, the following novel studies were conceived and performed during my PhD:

1. Characterise the lung miR expression profile at 4, 6, 8, and 12 weeks of CS-exposure.
2. To assess the functional impact of selected miRs with altered expression in COPD
3. Interrogation of the signalling axis of selected miRs involved in COPD pathophysiology.
4. To establish the therapeutic impact of specific miR antagomirs in COPD model.

Chapter 2: Methods

2.1 Ethics statement

This study was performed in accordance with the recommendations in the Australian code of practice for the care and use of animals for scientific purposes issued by the National Health and Medical Research Council of Australia. All protocols were approved by the Animal Ethics Committee of the University of Newcastle.

2.2 Induction of experimental COPD

We used an established model of CS-induced experimental COPD that was developed in our lab. Female wild-type BALB/c mice were exposed to the CS of twelve reference cigarettes (3R4F, University of Kentucky, Lexington, KY, USA) or normal air *via* the nose only to the CS for two times a day (total 75min exposure per day), 5 times a week, for either 4, 6, 8 and 12 weeks, as previously described previously (72, 162, 254-257).

2.3 RNA isolation and quantification

Total RNA was isolated from whole lung homogenates with TRIzol® and quantitated using a NanoDrop™, ND1000 spectrophotometer (NanoDrop ™ USA) (72). The RNA Integrity Number (RIN) of each sample was assessed using an Agilent 2100 Bioanalyser system (Agilent Technologies, USA) and Agilent RNA6000 Nano LabChip Kit (Agilent Technologies, USA) according to the manufacturer's instructions. In brief, 1μL of dye concentrate was added to 65μL of column-filtered gel matrix followed by sequential addition of 9μL of the gel-dye mix to the two wells on the chip to prime the chip. Sample and ladder wells were supplemented by 5μL of nano marker (buffer) and then 1μL each of heat-denatured RNA samples (70°C for 2 minutes) and ladder were added to the appropriate wells. Before starting the run, loaded chips were vortexed

(240rpm for 1 minute). Four samples from each group were selected based on their (RIN; >8) for microarray-based miRNA profiling.

2.4 Microarray-based miR expression profiling

To profile the miRs, Agilent unrestricted Mouse miRNA (8 x 15k arrays per slide, AMADID Number: 021828, Sanger Version 12) microarrays (Agilent Technologies, USA) was used.Total total RNA samples were fluorescently labelled using an Agilent miRNA Complete Labelling and Hyb Kit (Agilent Technologies, USA). Fluorescently labelled samples were purified, and hybridised onto Agilent unrestricted Mouse miRNA microarray slides. Briefly, 100ng of total RNA was incubated with 2µL of calf intestinal alkaline phosphatase (CIP) master mix2 and 2.8µL of dimethyl sulfoxide (DMSO) was added and denaturation was carried at 100°C for 5 minutes. These denatured samples were incubated with 4.5µL of ligation master mix to label them 3' end. These labelled samples were supplemented with nuclease-free water and purified from DMSO and free Cyanine3-pCp by elution through Micro Bio-Spin 6 columns (1000xg for 2 minutes) followed by drying with a vacuum concentrator for 50°C for 1 hour. Then reconstituted with 18µL of nuclease-free water and blocked with 4.5µL of kit-supplied 10X gene expression and supplied with 22.5µL of Hi-RPM hybridisation buffer. Samples were later heated and cooled for 5 mins at 100°C on ice and then hybridised to the microarray chips (55°C for 20 hours). Hybridised microarrays chips were washed with the supplied wash buffers and scanned with an Agilent microarray scanner. Raw microarray data was extracted using the scanner software's feature extraction function in a format compatible for analysis using GeneSpring GX 11.3 software (Agilent Technologies, USA).

2.5 Quantification of miR and gene expression through real-time quantitative PCR (qPCR)

Total RNA was extracted from snap frozen whole lung tissue and blunt-dissected airway and parenchyma using the TRIzol Reagent (Invitrogen, Life Technologies,

Australia) as described previously (72, 255). miR and mRNA expression levels were assessed by reverse transcribing the RNA to miR specific and gene cDNA respectively by Bioscript (Bioline, NSW, Australia), followed by real time quantitative polymerase chain reaction (qPCR) (257-259). Briefly qPCR assays were performed with SYBR Green Supermix (KAPA Biosystems) and ViiA7 thermo cycler (Life Technologies) using custom designed primers synthesised from (Integrated DNA technologies, Australia, Table 1). miR-9,-21,-135b and -146b expression were determined by normalising to the geometric mean of reference RNA's U6 small nuclear RNA (U6) and U49 small nucleolar RNA (U49). mRNA expression of NRF2, NQO-1, GSTP1, GCLC, HO-1, GPX2, CXCL2, CCL7, GM-CSF, MMP12, MARCO PDGFRβ, SPRY1, SPRY2, PDCD4, TIMP3, RECK, PTEN, SATB1, S100A9, S100A8, SIRT1, BMPR2, VEGF, IRAK1, TRAF6, and CCL2 were normalised to the house keeping gene hypoxanthineguanine phosphoribosyl transferase (HPRT). Custom designed primers synthesised from Integrated DNA technologies, Australia were used (Table 2.1) (260).

Table 2.1: Primers used in qPCR primers sequences

Primers	Sequences (5' →3')
NFR2 Forward	CTTTAGTCAGCGACAGAAGGAC
NRF2 Reverse	AGGCATCTTGTTTGGGAATGTG
CXCL2 Forward	TGCTGCTGGCCACCAACCAC
CXCL2 Reverse	AGTGTGACGCCCCCAGGACC
GM-CSF Forward	ATGCCTGTCACGTTGAATGAAGAG G
GM-CSF Reverse	AGGCGGGTCTGCACACATGTTA
MMP12 Forward	CCTCGATGTGGAGTGCCCGA
MMP12 Reverse	CCTCACGCTTCATGTCCGGAG
MARCO Forward	GCACTGCTGCTGATTCAAGTTC
MARCO Reverse	AGTTGCTCCTGGCTGGTATG
NQO-1 Forward	GTAGCGGCTCCATGTACTCTC
NQO-1 Reverse	AGGATGCCACTCTGAATCGG
GSTP1 Forward	GGCATGCCACCATACACCAT
GSTP1 Reverse	ATTCGCATGGCCTCACACC
GCLC Forward	CGACCAATGGAGGTGCAGTTA
GCLC Reverse	AACCTTGGACAGCGGAATGA
HO-1 Forward	GGTGCAAGATACTGCCCCTG
HO-1 Reverse	TGAGGACCCACTGGAGGAG
GPX2 Forward	ACCAGTTCGGACATCAGGAG
GPX2 Reverse	CCCAGGTCGGACATACTTGA

Primers	Sequences (5' →3')
SOCS5 Forward	ATCAGCATCGAGAAAGACAGTG
SOCS5 Reverse	CGCTTCGAGTTCTACCAAACTT
CCL7 Forward	GGGCCCAATGCATCCACATGC
CCL7 Reverse	TTCAGCGCAGACTTACATGCCC
SPRY1	CGAGGGCCTGTGTGCTGCAT
SPRY1 Reverse	ACTCTCGGCCAAATCCGGGC
SPRY2 Forward	ACGCGATGGTCAGCGATGGG
SPRY2 Reverse	AACCCGGCCTGTTCACTCGG
PDCD4 Forward	CCACTGACCCTGACAATTTAAGC
PDCD4 Reverse	TTTTCCGCAGTCGTCTTTTGG
TIMP3 Forward	AAAAGAGCGGCAGTCCCCGC
TIMP3 Reverse	TCCCACCACTTTGGCCCGGA
RECK Forward	TGTGCCGCAGTGTGACGTGT
RECK Reverse	AGGGCCGAGAGTGGGACGTG
PTEN Forward	TGGATTCGACTTAGACTTGACCT
PTEN Reverse	GCGGTGTCATAATGTCTCTCAG
SATB1 Forward	CATGTTACCAGTTTTCTGCGTG
SATB1 Reverse	GTGAATAGCCTAGAGACAGCAAC
S100A9 Forward	TGGGCTTACACTGCTCTTACC
S100A9 Reverse	GGTTATGCTGCGCTCCATCT

Primers	Sequences (5' →3')
S100A8 Forward	AAATCACCATGCCCTCTACAAG
S100A8 Reverse	CCCACTTTTATCACCATCGCAA
SIRT1 Forward	CAGCCGTCTCTGTGTCACAAA
SIRT1 Forward	CAGCCGTCTCTGTGTCACAAA
SIRT1 Forward	GCACCGAGGAACTACCTGAT
BMPR2 Forward	GTGTTATGGTCTGTGGGAGAAAT
BMPR2 Reverse	AAAGCGGTACGTTCCATTCTG
VEGF Forward	GGCTGCACCCACGACAGAAGG
VEGF Reverse	TCGGACGGCAGTAGCTTCGCT
IRAK1 Forward	CAGCTATCAAGGTTTCGTCACC
IRAK1 Reverse	AGGCAGTATAAGCCACTCTCTG
HPRT Forward	AGGCCAGACTTTGTTGGATTTGAA
HPRT Reverse	CAACTTGCGCTCATCTTAGGCTTT

2.6 Antagomir administration

CS-induced miR-9,-21,135b,-146b was inhibited with specific antagomirs (Ant): miR-9-specific- Ant-9, miR-21-specific-Ant-21, miR-135b-specific-Ant-135b, and miR-146b-specific-Ant-146b. The miR sequence for miR-9-21,-135b and-146b were obtained from miRBase (http://www.mirbase.org/) and complementary sequence/antagomirs for miR-9,-21-135b and-146b were designed. A scrambled (Scr) sequence RNA VIII matched against mouse genome was employed as a control to test the specificity of Ants. Ants were synthesised at Sigma Aldrich in lyophilised form and were resuspended with nuclease free water. BALB/c mice were anaesthetized with isoflurane and administered with specific Ant and scrambled intranasally (i.n.) once a week (2.5mg/Kg). Mice were sacrificed after 8 weeks of CS-exposure to assess the effects of the antagomir

on COPD pathogenesis.

2.7 Ant-9 and TMC administration

The specific NRF2 activator,2-trifluoromethyl-2'-methoxychalone (TMC), was initially solubilised in a co-solvent solution (4% DMSO, 4% ethanol) and diluted in PBS (vehicle). Groups of CS-exposed mice were treated intranasally (i.n.) with Ant-9 (2.5 mg/Kg in) or an equivalent amount of Scr antagomir once a week under isoflurane anaesthesia. In other groups, TMC (10mg/kg) or vehicle were administered i.n. three times a week with TMC. Mice were sacrificed after 8 weeks of smoking and the cardinal features of such as such as inflammation, small airway fibrosis, emphysematous air-space enlargement were COPD assessed.

2.8 *In situ* hybridisation (ISH)

miR-21 ISH was performed on formalin fixed paraffin embedded (FFPE) lung sections. All buffers were made up with nuclease free water (Invitrogen, Australia) and autoclaved. Lung sections slides were deparaffinised with xylene, rehydrated with graded ethanol washes and washed in PBS. Sections were treated with Proteinase K (15µg/mL) and slides washed and dehydrated with graded ethanol washes. Slides were pre-hybridised in a humidifying chamber with 1x ISH buffer (Exiqon) in a hybridiser (55ºC). Pre-hybridisation was followed by overnight hybridisation (55ºC) with double DIG (digoxigenins) labelled scrambled and miR-21 LNA Probes (Exiqon). Post hybridisation washes were carried out with varying concentrations of saline sodium citrate buffer and then blocked with 2% lamb serum in PBS-T, followed by incubation with 1:800 anti-DIG primary antibody conjugated to alkaline phosphatase (Roche) in 2% lamb serum. Sections were developed by incubating with BM purple (Roche), and 0.2mM Levimasol (Sigma) to inhibit endogenous alkaline phosphatase activity. On blue colour visualization colorimetric reactions were terminated with phosphatase stop solution and nuclei stained with Nuclear Fast Red counterstain (Vector Laboratories). Slides were then washed,

dehydrated and mounted with aqueous mounting medium (Vector Laboratories) with staining visualised using light microscopy (259).

2.9 Airway Inflammation

Airway inflammation was assessed by processing brochoalveolar lavage fluid (BALF) collected from left lobe of the lung tying the right lobes which are snap frozen for RNA and protein extraction. BALF was collected twice using 0.5ml of PBS each time. BALF was then centrifuged (300xg for 10 min at 4^{o}C) and supernatant was stored for further analysis. The pelleted cell mass was treated with red blood cell lysis buffer washed and spun down. Supernatant was discarded and the pellet was resuspended in 160 µl of HBSS. Trypan Blue was used to stain the cells and total cell numbers were determined by hemocytometer. The stored cell suspensions were cyto-centrifuged (300xg, 10 min; Thermo Fisher Scientific, Norwood, Australia) and stained with May Grunwald-Giemsa and airway inflammation was assessed by enumeration of different inflammatory cell types according to morphological features of leukocytes using light microscopy (40x magnification) as previously described (72, 162, 254, 255).

2.10 Protein Isolation

Proteins were extracted from snap frozen whole lung tissue and blunt dissected airways and parenchyma using radio-immunoprecipitation assay lysis buffer (Sigma Aldrich) and Dulbecco's phosphate-buffered saline (Life Technologies) respectively, as described previously (255). Total protein was quantified using BCA Protein assay Kit (Thermo, Scientific) according to the manufacturer's instructions.

2.11 Enzyme linked immunosorbent assay (ELISA)

Proteins were extracted from frozen lung tissue as described previously (255). CXCL1 and TNFα protein levels were measured using anti-mouse ELISA kits (R&D Systems, USA) according to the manufacturer's instructions and normalised to total

protein.

2.12 Airway remodelling

Small airway remodelling was assessed by staining longitudinal sections with Verhoff-Van Gieson stain as previously described (261). Image J software was used to quantify the area of fibrosis stained pink around small airways and at least four airways per section were measured. The area of fibrosis measured in μm^2 was normalised by dividing the area with the perimeter of the basement membrane measured in µm of the airway in consideration (Small airways were defined as having a basement membrane perimeter of ≤1,000 µm) (261).

2.13 Alveolar enlargement

Lungs were perfused, fixed with 10% buffered formalin, paraffin-embedded and 5µm longitudinal sections were cut and then stained with hematoxylin and eosin (H&E). Alveolar enlargement was assessed on 10 random images of the stained sections overlaid with an 11 lined counting grid. The average alveolar diameter was determined by counting the number of times the alveoli intercepts the lines on the grid known as the mean linear intercept method (72, 162, 254, 255). The percentage of destroyed space was also determined using the destructive index method, as described previously (262).

2.14 Lung function analysis

Mice were anaesthetised with Ketamine (100mg/kg) and Xylazine (10mg/kg) to determine the lung function parameters using flexiVent apparatus (SCIREQ, Canada) and the forced oscillatory technique (FOT)–Forced pulmonary maneuver system (Buxco, USA). This was carried out by tracheal cannulation of mice and trans-pulmonary resistance and dynamic compliance were determined through single frequency perturbation whereas elastance was measured by low frequency forced oscillatory technique using the flexiVent system. Forced vital capacity (FVC) was measured by

using fast flow maneuver and work of breathing (WoB) was determined through quasi-static pressure-volume curve values. For each maneuver, three perturbations were performed and average was calculated as described previously (72, 162, 254, 255).

2.15 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Sample proteins (20µg) and WesternC Standard (Bio-Rad) were loaded onto 4-15% gradient polyacrylamide gel (Bio-Rad) and the gels were electrophoresed. Briefly, a wet transfer system was used to transfer the proteins from the gel to a polyvinylidene difluoride (Bio-Rad). The blots were then blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline and Tween-20 (TBS-T). Blocking was followed by overnight incubation at 4ºC with primary antibodies against NRF2 (Cell Signalling Technology), SOCS5, β-actin, TATA binding protein (TBP) (Abcam, USA), anti-SATB1 and β-actin (Abcam, USA) and anti-pAKT (Ser 473) and anti-AKT (Cell Signalling USA) which were used according to manufacturer's instructions. Blots were washed and incubated with anti-Rabbit IgG HRP (R&D Systems, USA) and anti-mouse IgG peroxidase (Sigma-Aldrich, Australia) secondary antibodies in TBS-T. Blots were developed using West Femto substrate (Thermo, Scientific) and visualised by chemiluminescence (Bio-Rad, ChemiDoc MP System) and protein levels were assessed using Image J software.

2.16 Immunoflourescence (IF)

FFPE lung sections were deparaffinized in xylene and then rehydrated in graded ethanol washes, followed by a PBS wash. Antigen retrieval was performed in a steamer with citraconic anhydride buffer (0.05% citraconic anhydride in dH2O, pH 7.4). Slides were then washed in PBS-T and blocked with donkey serum (Sigma), and incubated in a humidifying chamber with rabbit-anti-SATB1 antibody (Abcam). The sections were washed in PBS-T and incubated with donkey anti-rabbit secondary antibody H&L Alexa Fluor 594 (abcam) for 1hr at RT. Slides were washed in PBS-T followed by incubation

with nuclear stain Hoechst (Thermo, Scientific), washed in PBS-T and mounted with Fluorsave. Fluorescence was detected with an Olympus fluorescent microscope.

2.17 NF-κB activity assays

Levels of DNA bound NF-κB (p65) subunit (active NF-κB) were measured by ELISA with the TransAM NF-kB family transcription factor assay (Active Motif, CA) as per manufacturer's instructions (255).

2.18 Combined antagomir administration

CS-induced miR-9, -21, -135b and -146b were inhibited with miR-specific antagomirs (Ant-9, -21, -135b, and -146b). The sequences for these miRs were obtained from miRBase (http://www.mirbase.org/) and complementary sequence/antagomir for miR-9 (Ant-9), miR-21 (Ant-21), miR-135b (Ant-135b) and miR-146b (Ant-146b) were designed. A scrambled (Scr) sequence RNA VIII matched against the mouse genome was employed as a control to test the specificity of miR-specific antagomirs. Antagomirs were synthesised by Sigma Aldrich and supplied in lyophilised form. They were resuspended with nuclease free water. BALB/c mice were anaesthetised with isoflurane and cominations of two miR-specific antagomirs were administered intranasally (i.n.) on alternate days, once a week and throughout CS-exposure (2.5mg/Kg; Table 2.2).

Table 2.2: Antagomir treatment regimen in experimental COPD

Day of Smoking	Group	Treatment with antagomir (Ant)
0	Smk+ Ant-(9+135b)	Ant-9
	Smk+ Ant-(9+146b)	Ant-9
	Smk+ Ant-(21+135b)	Ant-21
	Smk+ Ant-(21+146b)	Ant-21
	Smk+ Scr	Scr
1	Smk+ Ant-(9+135b)	Ant-135b
	Smk+ Ant-(9+146b)	Ant-146b
	Smk+ Ant-(21+135b)	Ant-135b
	Smk+ Ant-(21+146b)	Ant-146b
	Smk+ Scr	Scr

2.19 Statistics

Statistical analysis was performed using GraphPad Prism software version 6 (San Diego, California). Results are represented as ± standard error of the mean (SEM). Comparisons between two groups were made using Mann-Whitney test. With data involving three or more groups, Kolmogorov–Smirnov test was performed and as the data was normally distributed, data was analysed using One-way ANOVA with Uncorrected Fisher's LSD post-test.

CHAPTER 3: MICROARRAY-BASED MICRORNA (miR) EXPRESSION PROFILING IN EXPERIMENTAL COPD

3.1 INTRODUCTION

Current COPD therapies have limited efficacy in inhibiting chronic inflammation and do not reverse the tissue lesions such as airway remodelling and emphysema. These therapies fail to limit the progression of the disease and only target disease symptoms and reduce exacerbations (263, 264). Thus, there is a clear and demonstrated need for new therapies that can prevent the induction, or inhibit the progression of COPD. However, this is hampered by a lack of proper understanding of the mechanisms that underpin the disease process. Additionally, the identification of key biomarkers that accurately detect disease stage and predict the course of disease will facilitate the development of new therapeutic strategies that may be effective in COPD treatment.

MicroRNAs (miRs) play an integral role in the control of genes and associated networks through the post-transcriptional regulation of gene expression (192). A single miR targets multiple genes, which gives them the capacity to simultaneously regulate multiple physiological processes. (265). The importance of microarray platform for quantifying miRs expression has accelerated in recent years as microarray technologies simultaneously measure many miRs on a single microarray. A number of studies have used microarray profiling as a platform to identify the dysregulated miRs in COPD patients. Thus, several miRs were found to be altered at various stages of emphysema and in different regions of the lung (231, 232). This indicates that CS-induced dysregulated miRs exhibit spatiotemporal pattern in the pathogenesis of COPD, thus miRs may be altered in various lung tissues which may be involved in the disease developmental processes.

However, the development of new therapies has been severely hampered by the lack of animal models that recapitulate the hallmark features of human COPD in a reasonable time frame. To address this, we developed a nose-only smoke exposed

mouse model of COPD that develops the hallmark features of disease within 8 weeks (72). Further our model is also steroid-resistant like human COPD, as glucocorticoid treatment does not prevent lung inflammation or emphysema and does not restore lung function (72).

Here, we performed a microarray-based miR analysis to profile the altered miRs with CS-exposure and then to determine their role in COPD pathogenesis. We identified miRs-30c-1-3p, -122 and -133a-3p that were dysregulated in the induction phase of COPD and the miRs -9,-21,-132, -135b, -146b and -466g were altered from the induction phase and throughout the disease development. Hence these miRs may be potentially involved in the induction of various pathophysiological features of COPD, namely inflammation, airway remodelling, emphysema and reduced lung function. Thus, our study shows that CS-induces increases in miR expressions at various stages of COPD development, hence these miRs may be the potential regulators of induction and progression phases of COPD.

3.2 RESULTS

3.2.1 miR expression profile in lungs in response to CS exposure in experimental COPD

BALB/c mice were exposed to CS or normal air, for 4, 6 8, or 12 weeks and microarray was performed to determine the CS-induced dysregulated miRs in lungs. miRs that were differentially expressed between the CS-exposed and normal air-exposed groups by greater than 2 fold with a p-value <0.5 were considered to be significantly altered and pursued further. Of the 672 miRs assessed the expression of more than 60 miRs was differentially regulated (Table 3.1).

Table 3.1: microRNAs (miRs) with altered expression in the lung during CS exposure in experimental COPD

Week 4			
Name	Unpaired	Foldchange	Regulation
mmu-miR-135b	0.005718	250.52728	up
mmu-miR-764-5p	1.82E-07	124.02801	up
mmu-miR-721	0.010374	64.6149	up
mmu-miR-188-5p	0.01538	61.093224	up
mmu-miR-1906	0.027099	41.63484	up
mmu-miR-680	0.02133	35.016983	up
mmu-miR-877	0.033584	30.970171	up
mmu-miR-685	0.013848	15.975608	up
mmu-miR-705	0.021435	2.77686	up
mmu-miR-132	0.005101	2.5821474	up
mmu-miR-146b	7.11E-06	2.2941413	up
mmu-miR-193	0.038684	2.9623795	down
mmu-miR-142-5p	0.040775	2.224444	down

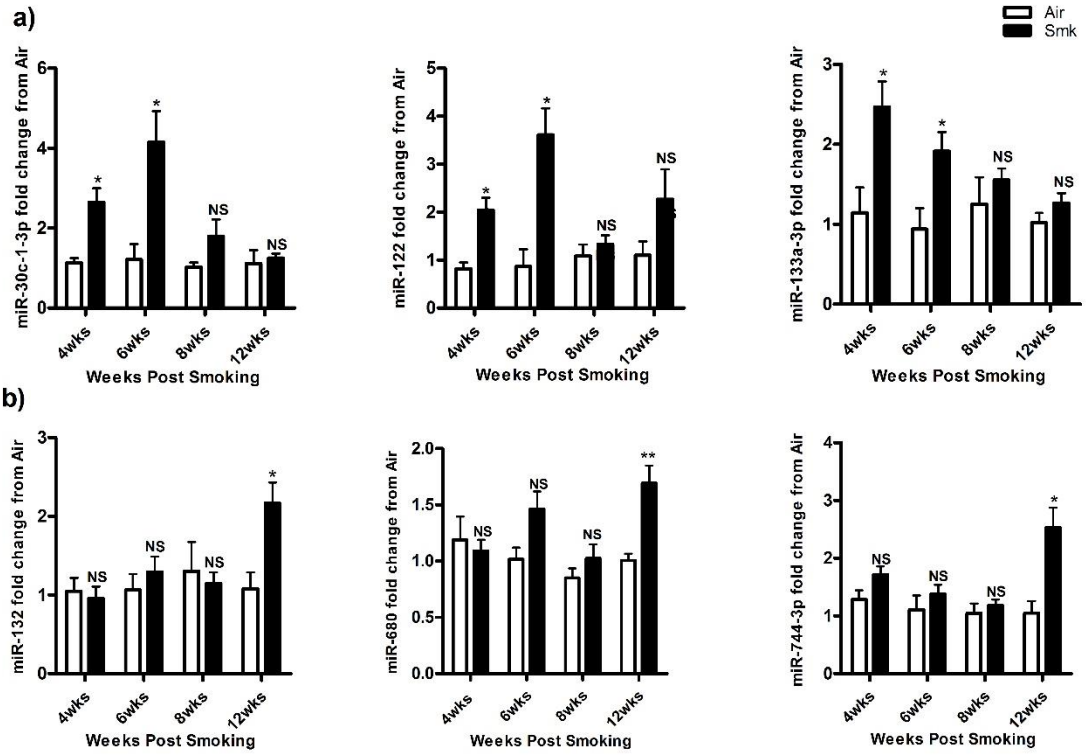
Week 6			
Name	Unpaired	Foldchange	Regulation
mmu-miR-135b	0.002487294	346.83612	up
mmu-miR-764-5p	6.43E-03	50.202267	up
mmu-miR-122	0.036430877	27.79126	up
mmu-miR-9	0.025990065	26.657883	up
mmu-miR-701	0.014825455	16.036392	up
mmu-miR-146b-3p	0.027516073	13.718124	up
mmu-miR-146b	1.8433E-08	2.402143	up
mmu-miR-705	0.014255607	2.3409803	up
mmu-miR-21	7.32083E-06	2.2245314	up
mmu-miR-32	0.024163112	2.01104	down

Week 8			
Name	Unpaired	Foldchange	Regulation
mmu-miR-135b	0.005881463	133.31866	up
mmu-miR-21	6.06534E-06	2.036141	up
mmu-miR-466h	0.040326253	11.838187	down
mmu-miR-207	0.03558116	30.228733	down
mmu-miR-669l	0.035304073	30.403603	down
mu-miR-1196	0.034805946	36.037186	down
mmu-miR-1894-5p	3.48E-02	36.661568	down
mmu-miR-467f	0.03209787	2.0443435	down
mmu-miR-214-3p	3.09E-02	13.534366	down
mmu-miR-466a-3p	0.025756592	2.3297324	down
mmu-miR-1906	0.019428063	31.907864	down
mmu-miR-154	0.01911295	32.314774	down
mmu-miR-30c-1-3p	0.015084208	19.250727	down
mmu-miR-18a-3p	0.013630453	21.617414	down
mmu-miR-200a-3p	0.01329037	2.3602736	down
mmu-miR-467e	0.011713621	2.1072156	down
mmu-miR-466f-3p	1.59E-03	3.6936483	down
mmu-miR-467a-3p	0.0005297	2.14742	down
mmu-miR-466g	5.06E-05	107.287056	down
mmu-miR-204	2.09238E-05	2.248159	down
mmu-miR-669n	1.79348E-05	3.4312658	down
mmu-miR-598	2.16164E-07	97.25102	down
mmu-miR-744-3p	1.42504E-07	40.19447	down
mmu-miR-467a	1.02665E-07	64.78448	down
mmu-miR-297a-3p	3.8248E-08	38.67033	down
mmu-miR-211	3.14658E-08	109.07946	down
mmu-miR-467c	5.93E-09	64.1924	down
mmu-miR-377	2.12385E-09	82.31992	down
mmu-miR-490	7.62E-10	124.861244	down

Week 12			
Name	Unpaired	Foldchange	Regulation
mmu-miR-135b	7.48E-10	4462.461	up
mmu-miR-764-5p	6.73E-08	78.52882	up
mmu-miR-680	0.003812	50.247734	up
mmu-miR-214-3p	3.2E-09	37.905373	up
mmu-miR-146b-3p	4.26E-08	31.914886	up
mmu-miR-188-5p	0.006754	3.0411222	up
mmu-miR-705	7.01E-04	2.8276794	up
mmu-miR-449a	3.94E-06	2.7704122	up
mmu-miR-21	8.29E-06	2.5910807	up
mmu-miR-146b	1.46E-06	2.577521	up

3.2.2 Validation of differentially regulated miR expression using qpCR

The altered expression of miRs identified by microarray analysis were further validated by qPCR using custom designed primers **(Table 3.2)**. Each miR selected for validation was analysed at individual time point of CS-exposure (4, 6, 8 and 12 weeks). We identified 3 miRs that exhibited increased expression prior to the onset of disease features (4 weeks) **(a).** 3 miRs that were significantly expressed only when features of COPD were evident **(b).** 6, CS-induced miRs expression were increased at all time points tested (4–12 weeks CS-exposure) **(c)** and 6 miRs were up-regulated at one or more time points of CS-exposure **(d)**



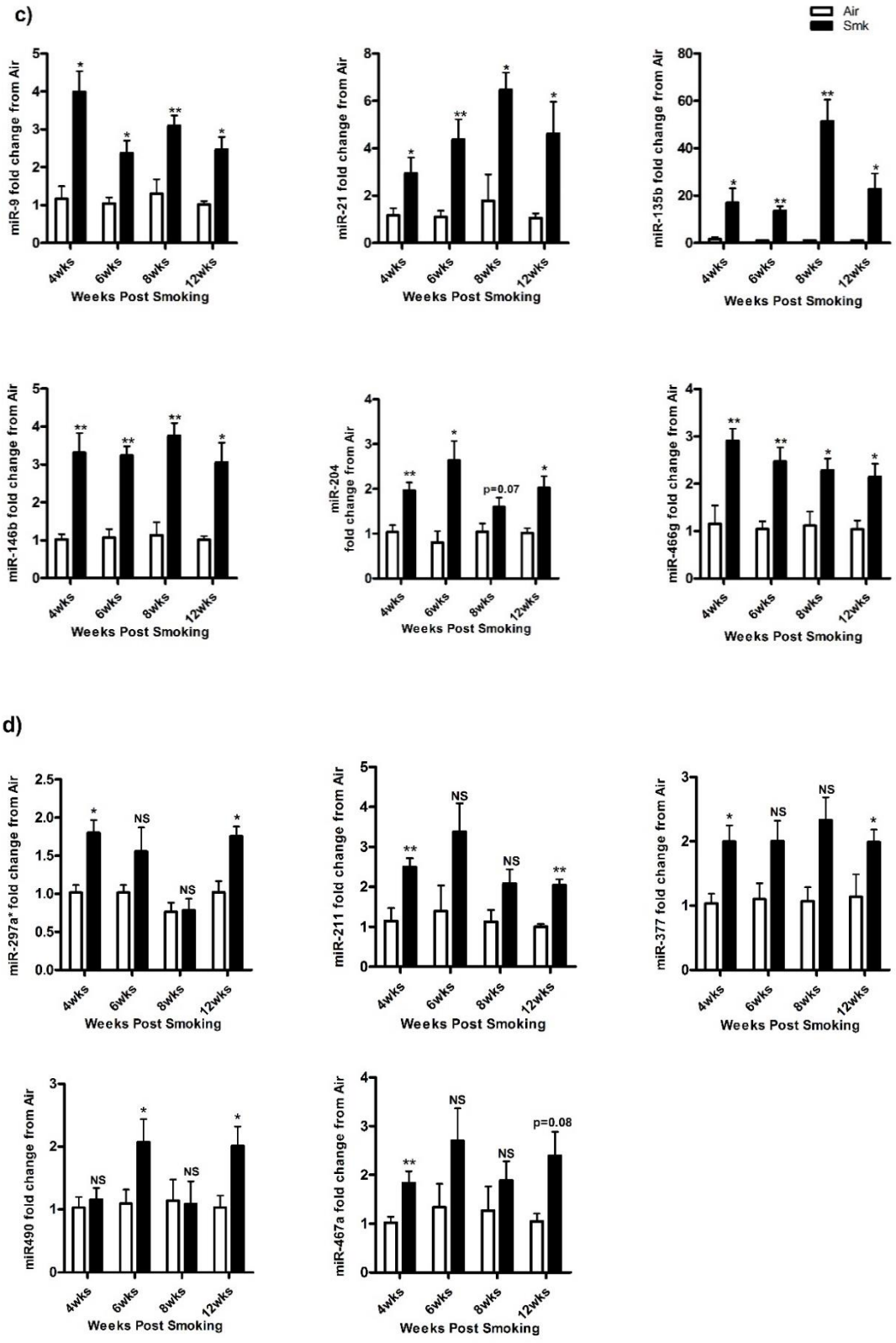

c)
Air
Smk
miR-9 fold change from Air
miR-21 fold change from Air
miR-135b fold change from Air
miR-146b fold change from Air
miR-204 fold change from Air
p=0.07
miR-466g fold change from Air
4wks
6wks
8wks
12wks
Weeks Post Smoking
d)
miR-297a* fold change from Air
miR-211 fold change from Air
miR-377 fold change from Air
miR490 fold change from Air
miR-467a fold change from Air
p=0.08
NS

Figure 3.1: Altered expression of microRNAs (miRs) during cigarette smoke (CS) exposure in experimental COPD. Microarray-based miR profiling was performed on total lung RNA from normal air and CS-exposed mice and identified miRs that were dysregulated with CS-exposure. miRs were validated by qPCR at each time point (4, 6, 8 and 12 weeks) and miR expression was normalised to the geometric mean of the housekeeping controls small nuclear RNA (snRNA) U6 and small nucleolar RNA (snoRNA) U49. miRs that were differentially expressed **(a)** only at 4-6 weeks, **(b)** only at 12 weeks, **(c)** throughout CS-exposure, and **(d)** at one or more time points of CS exposure. Data are presented as means ± s.e.m. NS; not significant, *P<0.05; **P<0.01. Data were analysed by two-tailed unpaired student's *t* test.

Table 3.2: Custom-designed microRNA (miR) primers used to validate microarray miR data by qPCR

Primers	Sequences (5' →3')	Target
U6 Forward	5'-CGGCAGCACATATACTAAAATTGG-3'	U6 SnRNA
U6 Reverse	5'-GCCATGCTAATCTTCTCTGTATC-3'	U6 SnRNA
U49 Forward	5'-ATCACTAATAGGAAGTGCCGTC-3'	U49 SnoRNA
U49 Reverse	5'-ACAGGAGTAGTCTTCGTCAGT-3'	U49 SnoRNA
miR-30c-1-3p Forward	5'-CTGGGAGAGGGTTG-3'	mmu-miR-30c-1-3p
miR-30c-1-3p Reverse	5'-GTAAAACGACGGCCAGTGGAGTAAA-3'	mmu-miR-30c-1-3p
miR-122 Forward	5'-TGGAGTGTGACAATG-3'	mmu-miR-122
miR-122 Reverse	5'-GTAAAACGACGGCCAGTCAAACAC-3'	mmu-miR-122
miR-133a-3p Forward[1]	5'-GCT+GGTAAAATGGA-3'	mmu-miR-133a-3p
miR-133a-3p Reverse	5'-GTAAAACGACGGCCAGTATTTGGT-3'	mmu-miR-133a-3p
miR-9 Forward[1]	5'-TCTTT+GGTTATCTAGC-3'	mmu-miR-9
miR-9 Reverse	5'-GTAAAACGACGGCCAGTTCATACA-3'	mmu-miR-9
miR-21 Forward[1]	5'-T+AGCTTATCAGACTG-3'	mmu-miR-21
miR-21 Reverse	5'-GTAAAACGACGGCCAGTTCAACAT-3'	mmu-miR-21
miR-132 Forward[1]	5'-TAAC+AGTCTACAGC-3'	mmu-miR-132
miR-132 Reverse	5'-GTAAAACGACGGCCAGTCGACCATG-3'	mmu-miR-132
miR-135b Forward	5'-TATGGCTTTTCATTCC-3'	mmu-miR-135b
miR-135b Reverse	5'-GTAAAACGACGGCCAGTTCACATA-3'	mmu-miR-135b
miR-146b Forward[1]	5'-TG+AGAACTGAATTCC-3'	mmu-miR-146b
miR-146b Reverse	5'-GTAAAACGACGGCCAGTAGCCTAT-3'	mmu-miR-146b
miR-466g Forward[1]	5'-ATA+CAGACACATGC-3'	mmu-miR-466g
miR-466g Reverse	5'-GTAAAACGACGGCCAGTTGTGTGT-3'	mmu-miR-466g
miR-490 Forward	5'-CAACCTGGAGGACT-3'	mmu-miR-490
miR-490 Reverse	5'-GTAAAACGACGGCCAGTCAGCATGG-3'	
miR-680 Forward	5'-GGGCATCTGCTGA-3'	mmu-miR-680
miR-680 Reverse	5'-GTAAAACGACGGCCAGTCCCCCATG-3'	mmu-miR-680
miR-744-3p Forward	5'-CTGTTGCCACTAAC-3'	mmu-miR-744-3p
miR-744-3p Reverse	5'-GTAAAACGACGGCCAGTAGGTTGAG-3'	mmu-miR-744-3p

[1] LNA modifies bases are preceded by (+) symbol

Table 3.3: Potential pathways, and associated diseases and functions linked with increased expression of microRNAs (miRs) and their targets (known and predicted) during the initiation phase of COPD and throughout CS-exposure in experimental COPD

Potential pathways of miRs dysregulated in the initiation phase of experimental COPD

miR-30c-1-3p	miR122	miR133a
Amboid Processing	HIFIαSignaling	Dolichyl-diphosphooligosaccharide Biosynthesis
Epithelial Adherens Junction Signaling	Ceramide Signaling	Isoleudne Degradation I
SonicHedgehog Signaling	Glycolysis I	CT LA4 Signaling in CytotoxicT Lymphocytes
Thrombin Signaling	GM-CSFSignaling	Actin CytoskeletonSignaling
G Protein Signaling Mediated by Tubby	CNTF Signaling	Clathrin-mediated Endocytosis Signaling
Signaling by Rho Family GTPases	VEGF Family Ligand-Receptor Interactions	Virus Entry via Endocytic Pathways
Mitotic Roles af Polo-Lice Kinase	FLT3 Signaling in Hematopoietic Progenitor Cells	Pentose Phosphate Pathway (Oxidative Branch)
RhoGDI Signaling	Sphintingosine-1-phosphate Signaling	Tetrahydrobiopterin Biosynthasis I
Mechanisms of Viral Exit from Host Cells	IL-17 Signaling	Tetrahyclrobiopterin Biosynthesis II
Axonal Guidance Signaling	NGFSignaling	Fcy Receptor-mediated Phagocytosis in Macrophages and Monocytes
Flavin Biosynthesis 1V (Mammalian)	Tight Junction Signaling	Inositol Pyrophosphates Biosynthesis
4-hydroxyproline Degradation I	IL-2 Signaling	Ketolysis
GDP-L-fucose Biosynthesis I (from GDP-D-mannose)	Xenobiotic Metabolism Signaling	Paxillin Signaling
Formaldehyde Oxidation II	T Cell Receptor Signaling	Epithelial Adherens Junction Signaling
Leptin Signaling in Obesity	Rac Signaling	Remodeling of Epithelial Adherens Junctions
IL-4 Signaling	Thrombin Signaling	Macropinocytosis Signaling
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	FAK Signaliry	Ardrogen Biosynthesis
Colorectal Cancer Metastasis Signaling	UVA-Induced MAPK Signaling	Pentose Phosphate Pathway
Pyrimidine Deoxyribonideotides De Novo Biosynthesis I	Signaling by Rho Family GTPases	Mevalonate Pathway I
HIPPO signaling	Antiproliferative Role of Somatostation Receptor 2	Leucine Degradation I

Diseases and functions of miRs dysregulated in the initiation phase of experimental COPD

miR-30c-1-3p		miR-122		miR-133a	
argiogenesis	1.65E-02	neutrophilia of lung	1.03E-02	autophagy of epithelial cells	2.39E-02
Asthma	2.04E-02	accumulation of alternatively activated macrophages	2.73E-02	bronchopulmonary sequestration	2.39E-02
tumorigenesis of lung	3.88E-02	accumulation of lung cells	2.73E-02	congenital cystic a denomatoid malformation	2.39E-02
production of mucus	4.79E-02	accumulation of monocyte-derived dendritic cells	2.73E-02	degradation of fibrin clot	2.39E-02
abnormal morphology of lurg pleura	2.26E-02	accumulation of monocytes	2.73E-02	fragmentation of DNA	2.39E-02
adhesion of Streptococcus pneumoniae	2.26E-02	chemotaxis of alveolar macrophages	2.73E-02	growth of tumor	3.20E-02
adhesion of nasopharyngeal epithelial cells	2.26E-02	disorganization of bronchial epithelial cells	2.73E-02	cellular homeostasis	4.28E-02
biogenesis of lateral plasma membrane	2.26E-02	neutrophilia of lung tissue	2.73E-02	tumorigenesis of lung	4.28E-02
length of apical membrane	2.26E-02	pleural effusion of lung	2.73E-02	atypical lung carcinoid tumor	4.72E-02
length of basal membrane	2.26E-02	proliferation of effector memory T lymphocytes	2.73E-02	expansion of bronchioalveolar stem cells	4.72E-02

Potential pathways of miRs dysregulated throughout the CS-exposure in experimental COPD

miR-	Potential Pathways	p-value	Ratio	miR-	Potential Pathways	p-value	Ratio
9	Actin Cytoskeleton signaling	1.58E-04	0.15	135	Realm signaling in Neurons	1.58E-03	0.11
	Regulation of Actin-based Motility by Rho	2.04E-04	0.21		Sonic Hedgehog Signaling	1.12E-02	0.14
	Tec Kinase signaling	1.07E-03	0.15		Amyloid Processing	1.35E-02	0.1
	Semaphorin signaling in Neurons	3.09E-03	0.22		Flavin Biosynthesis IV (Mammalian)	2.24E-02	1
	signaling by Rho Family GTPases	3.24E-03	0.12		GDP-L-fucose Biosynthesis I (from GDP-D-mannose)	2.24E-02	1
	RhoGDI signaling	5.75E-03	0.13		IL-4 Signaling	3.16E-02	0.08
	Axonal Guidance signaling	8.17E-03	0.14		Mitotic Roles of Polo-Like Kinase	4.37E-02	0.09
	Germ Cell-Sertoli Cell Junction signaling	8.78E-03	0.13		RhoGDI Signaling	4.79E-02	0.05
	Paxillin signaling	1.51E-02	0.14		Mouse Embryonic Stern Cell Pluripotency	5.37E-02	0.06
	Rac signaling	2.51E-02	0.13		Primary Immunodeficiency Signaling	5.50E-02	0.12
	PPARα/RXRα Activation	2.83E-02	0.11		G Protein Signaling Mediated by Tubby	6.17E-02	0.11
	Epithelial Adherens Junction signaling	2.75E-02	0.12		Thrombin Signaling	6.46E-02	0.05
	Ephrin Receptor signaling	2.75E-02	0.11		Zymosterol Biosynthesis	6.61E-02	0.33
	Hepatic Fibrosis / Hepatic Stellate Cell Activation	2.88E-02	0.11		Breast Cancer Regulation by Stathmin1	7.24E-02	0.05
	VDR/RXR Activation	3.02E-02	0.14		Gap Junction Signaling	7.94E-02	0.05
	Integrin signaling	3.24E-02	0.11		Epithelial Adherens Junction Signaling	8.32E-02	0.05
	PAK signaling	3.24E-02	0.13		Leptin signaling in Obesity	8.51E-02	0.07
	Androgen Biosynthesis	3.47E-02	0.4		Calcium Signaling	8.71E-02	0.05
	HMGB1 signaling	3.89E-02	0.12		Myo-inositol Biosynthesis	8.71E-02	0.25
	ILK signaling	4.47E-02	0.11		IL-15 Signaling	9.33E-02	0.06
21	TGF-β signaling	1.45E-02	0.08	146b	IL-15 Signaling	9.33E-03	0.14
	Coenzyme A Biosynthesis	3.02E-02	0.5		Isoleucine Degradation I	1.00E-02	0.38
	B Cell Receptor signaling	3.89E-02	0.04		Valine Degradation I	1.45E-02	0.33
	Neurotrophin/TRK signaling	3.98E-02	0.08		DNA double-strand break repair by non-homologous end joining	1.95E-02	0.3
	Aspartate Degradation II	5.89E-02	0.25		CMP-N-acetylneuraminate biosynthesis I (eukaryotes)	2.04E-02	0.5
	Toll-like Receptor signaling	8.48E-02	0.05		Dolichyldiphosphooligosaccharide biosynthesis	2.04E-02	0.5
	Guanosine Nucleotides Degradation III	7.41E-02	0.2		Wnt/Ca+ pathway	2.88E-02	0.17
	Adenosine Nucleotides Degradation II	7.41E-02	0.2		Mismatch Repair in Eukaryotes	3.31E-02	0.4
	Ephrin A signaling	7.94E-02	0.08		Leucine Degradation I	3.31E-02	0.4
	NAD Salvage Pathway II	8.71E-02	0.17		PPARα/RXRα Activation	4.27E-02	0.1
	Role of BRCA1 in DNA Damage Response	9.77E-02	0.08		RhoGDI signaling	4.90E-02	0.1
	CD27 signaling in Lymphocytes	9.77E-02	0.08		Role of BRCA1 in DNA Damage Response	5.89E-02	0.14
	Role of Lipids/Lipid Rafts in the Pathogenesis of Influenza	0.102	0.14		Protein Citrullination	6.03E-02	1
	Urate Biosynthesis/Inosine 5-phosphate Degradation	0.102	0.14		Flavin Biosynthesis IV Mammalian	6.03E-02	1
	Purine Nucleotides Degradation II (Aerobic)	2.00E-03	0.14		GDP-L- fucose Biosynthesis I ram GDP-D-mannose	6.03E-02	1
	Role of IL-17A in Arthritis	1.13E-01	0.05		Adenine and Adenosine Salvage VI	6.03E-02	1
	Cholesterol Biosynthesis I	1.15E-01	0.13		Glutamate Dependent Acid Resistance	6.03E-02	1
	Cholesterol Biosynthesis II (via 24,25-dhydrolanosterol)	1.15E-01	0.13		Estrogen Receptor signaling	6.92E-02	0.11
	Cholesterol Biosynthesis III (via Desmosterol)	1.15E-01	0.13		Androgen signaling	7.24E-02	0.11
	Role of IL-17A in Psoriasis	1.29E-01	0.11		Activation of IRF by Cytosolic Pattern Recognition Reciptors	7.94E-02	0.13

Diseases and functions of miRs dysregulated throughout the CS-exposure in experimental COPD

miR-	Diseases or Functional Annotation	p-Value
9	permeability of endothefal tissue	2.23E-03
	damage of endothelial tissue	2.37E-61
	degradation of DNA	3.93E-03
	transepithelial electrical resistance	7.10E-03
	fibrin Clot	1.13E-02
	formation of bronchiole	2.16E-02
	abnormal circulation in lung	3.46E-02
	migration of microvascufar endothelial cells	3.40E-02
	Phaagocytosis of phagocytes	3.46E-02
	transepthelial electrical resistance of bronchial epithelal cells	3.46E-02
	growth of organism	3.53E-02
	inflarnmation of respiratory system component	4.38E-02
	quantity of type 2 pneumocytes	4.38E-02
	chemotaxs of cells	4.98E-02
	morphogenesis of endoderm	4.98E-02
	stimulation of alveolar macrophages	4.98E-02
21	cell viabilty of fibroblasts	3.29E-03
	Apoptosis of fibroblasts	6.03E-03
	accumulation of adenosine	1.52E-02
	Agenesis of lung	1.52E-02
	biogenesis of lateral plasma membrane	1.52E-02
	chemoattraction	1.52E-02
	Chronic bronchitis	1.52E-02
	leakage Of lung	1.52E-02
	length of apical membrane	1.52E+00
	length of basal membrane	1.52E-02
	loss of lateral plasma membrane	1.52E-02
	priming of alveolar macrophages	1.52E-02
	tubulogenesis of endothelial cells	1.52E-02
	calcification of lung	3.01E-02
	generation of reactive oxygen Species	3.01E-02
	retention of leukocytes	3.01E-02
	seauestration of neutrophils	3.01E-02
	Cystic Fibrosis	4.48E-02
	Uptake of D-glucose	4.48E-02

miR-	Diseases or Functional Annotation	p-Value
135	angiogenesis	1.65E-02
	Asthma	2.04E-02
	abnormal morphology of lung pleura	2.26E-02
	adhesion of Streptococcus pneumoniae	2.26E-02
	adhesion of nasopharyngeal epithelial cells	2.26E-02
	biogenesis of lateral plasma membrane	2.26E-02
	length of apical membrane	2.26E-02
	length of basal membrane	2.26E-02
	loss of lateral plasma membrane	2.26E-02
	quantity of lung carcinoma	2.26E-02
	quantity of pulmonary adenoma	2.26E-02
	regeneration of lung	2.26E-02
	response of multipotent progenitor 2 cells	2.26E-02
	tumorigenesis of lung	3.88E-02
	apoptosis of adenocarcinoma cells	4.48E-02
	metastasis of benign tumor	4.48E-02
	onset of tumorigenesis of adenoma	4.48E-02
	permeability of vascular endothelial tissue	4.48E-02
	quantity of blood vessel	4.48E-02
	senescence of tumor cells	4.48E-02
	size of adenoma	4.48E-02
	transformation of bronchial epithelial cells	4.48E-02
	tubulation of endotheial cells	4.48E-02
	production of mucus	4.97E-02
146b	tubulation of cells	1.07E-02
	tubulation of epithelial tissue	1.07E-02
	congestion of lung	2.05E-02
	Pulmonary Hypertension	3.27E-02
	abnormal morphology of alveolar macrophages	4.72E-02

3.3 DISCUSSION AND CONCEPTION OF STUDIES

We have shown that CS exposure alters the expression of sets of miRs in the induction and progression phases, or throughout the time course, of experimental COPD. However, the mechanism by which CS exposure alters the expression of these miRs in the lungs is not clear. One possible explanation is that CS exposure induces epigenetic changes such as DNA methylation of miR encoding genes leading to miR dysregulation, such as the hypo-methylation of miR-9-3p encoding gene was observed in the oral epithelial cells of smokers (266). Thus, the altered miRs regulate the expression of their target genes post-transcriptionally and may potentially drive the pathogenesis of COPD.

The potential targets of altered miRs with CS-exposure were identified by open source algorithms such as Target Scan (267), and miRANDA (268), which are based on complementarity of seed sequence with the validated and predicted targets of the miRs. We employed these algorithms because of their advantages, such as Target Scan identifies the potential targets which exhibit perfect complementarity with the seed sequence and miRANDA provides tissue based miR-gene prediction information (269). These targets were then analysed by pathway predicting algorithms such as PANTHER pathway to assess the potential biological and pathological roles of these miRs. These associations were taken into consideration in selecting the miRs for qpCR validation. However only 18 miRs were validated at one or more time points of CS-exposure. Furthermore, at 8th week of CS-exposure most of the altered miRs identified by microarray analysis were not validated by qPCR. Several studies have reported poor association between miR expression identified by microarray analysis and qPCR-based validation (270, 271) as qPCR assays are more specific and sensitive than microarrays. (272). This is due to the increased non-specific hybridisation of the probes to the smaller and similar sequences of mature miRs and also due to the higher sequence similarities of the miRs among themselves. (273, 274)

The altered miRs with CS-exposure were qpCR validated, the miRs up-regulated at the initiation phase of the disease development, miR- (30c-1-3p, -122 and -133a-3p)

and miRs- (9, -21, -132, -135b, -146b and -466g) that were upregulated throughout the 12 weeks of CS exposure were selected for further investigations. However, with 8 weeks of CS exposure, the key features of COPD such as inflammation, airway remodelling, emphysema, are significantly increased which results in impaired lung function (72). This suggests that the dysregulated miRs expressed in the induction phase of disease development, and throughout CS exposure, may be involved in the pathogenesis of COPD and the miRs (upregulated at 12 weeks) may be a consequence of the disease process.

Additionally, to investigate the functional roles of these CS-induced miRs, we performed an initial pilot study with the miRs expressed during induction phase and all throughout the CS-exposure (data not shown), to assess the knock-down potential of the miR-specific inhibitors. We then selected four CS-induced miRs (miR-9, miR-21, miR-135b and miR-146b) to examine their functional roles in the pathogenesis of CS-induced experimental COPD. These miRs were selected based on the biological abundance at 8 weeks of CS-exposure, their association with various biological and pathogenic mechanisms **(Table 3.2 and 3.3)** and the efficacy of miR-specific inhibitors. These studies are described in Chapters 3-6 of this Thesis. In these chapters we have investigated the roles of the four selected miRs with 8weeks of CS-exposure which is the time point associated with the development of all the key features of experimental COPD (72). Thus, the outcomes of the microarray analysis were the foundation of my thesis based on which the novel PhD studies described hereafter were conceived, planned and implemented.

CHAPTER 4: ROLE OF miR-9 IN THE PATHOGENESIS OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE

4.1 INTRODUCTION

Cigarette smoke (CS) contains, and exposure induces the production of reactive oxygen (ROS) and nitrogen (RNS) species such as hydrogen peroxides, hydroxyl ions, nitric oxide, and peroxynitrite (275). These oxidants stimulate epithelial cells to secrete TNFα, IL-8 and IL-1β, which induces NF-kB signalling, increasing the expression of pro-inflammatory mediators (276). This leads to recruitment of neutrophils and monocytes which are major additional sources of ROS (277). ROS production perpetuates inflammation and leads to tissue injury (278, 279). Importantly, miR-9 is potently induced in neutrophils and macrophages following exposure to pro-inflammatory stimuli (213) and these cells are known to play important roles in COPD pathogenesis (72).

Nuclear factor-erythroid 2 related factor-2 (NRF2) is a transcription factor that is activated in response to oxidative stress and is a predicted target of miR-9. In normal conditions NRF2 is associated with its inhibitor Kelch-like ECH-associated protein 1(KEAP1) in the cytoplasm. Under oxidative stress, these factors dissociate and Nrf2 trans-locates to the nucleus where it binds to anti-oxidant responsive elements (AREs) to induce the expression of anti-oxidant genes such as NAD(P) H:quinone oxidoreductase-1 (NQO-1) (112, 280, 281). In COPD, NRF2 activity is decreased in the lungs of patients (282), and NRF2-deficient mice are more susceptible to CS-induced emphysema, which develops more rapidly and is more severe (117). Pharmacological activators have been developed to stimulate NRF2 activity and induce the expression of anti-oxidant genes. These include 4,2′,5′-trihydroxy-4′-methoxychalcone (TMC) that potently activates NRF2 responses in mice and cultured lung epithelial cells and induces the expression of NQO-1, heme oxygenase (HO)-1 and other anti-oxidant genes (283).

Inflammation is characterised by dysregulated cytokine signalling and suppressor of cytokine signalling proteins (SOCS) are a family of 7 proteins which are known as negative regulators of cytokine activation (284, 285). One of the SOCS gene, SOCS 5 is a known target of miR-9 (286). Knowledge of the role of SOCS5 in COPD is limited, but it is known to mediate T-helper (Th) cell differentiation, inhibit IL-4R/STAT6 mediated immunomodulatory responses in Th1 cells and promotes the differentiation of these cells (287). It also regulates epidermal growth factor receptor (EGFR) (288, 289), which is increased in bronchial biopsies of COPD patients (290). CS-induced EGFR positively regulates IL-8 secretion *in vitro* and its stimulation augmented phosphatidylinositol 3-kinase (PI3K) activity in COPD bronchial epithelial cells (291, 292).

In this study we investigated the role of CS-induced miR-9 and its predicted target NRF2 and its known target SOCS5 (286) in the pathogenesis of experimental of COPD. We used our well-established mouse model, which develops the hallmark features of the human disease after eight weeks of nose-only CS exposure (72, 162, 254-257), and replicates similar exposures to human smokers (293). Chronic CS exposure induced increases in miR-9 that was associated with the development of disease. miR-9 expression was depleted which lead to increases in NRF2 and SOSC5, resulting in the reduction of pathophysiological features of the disease such as inflammation, airway remodelling and impaired lung function.

4.2 RESULTS

4.2.1 CS exposure induces a persistent increase in miR-9 expression in experimental COPD

BALB/c mice were exposed to CS or normal air for 2, 4, 6 or 8 weeks. To identify miRs potentially involved in COPD pathogenesis microarray-based miR analysis was performed on total RNA from lungs. miR-9 was significantly upregulated at 4 and 8 weeks of CS exposure compared to normal air-exposed controls (manuscript under review). CS-induced increases in miR-9 expression were validated by real-time quantitative PCR

(qPCR) performed on lung RNA collected at 4, 6 and 8 weeks of CS exposure **(Figure 4.1a)**. To identify tissue specific changes in miR-9 expression, levels were assessed in blunt-dissected airways and parenchyma tissue at 8 weeks; the time point when all the hallmark features of CS-induced COPD are observed. miR-9 expression was significantly up-regulated in parenchyma but not in airways **(Figure 4.1 (b** and **c))**.

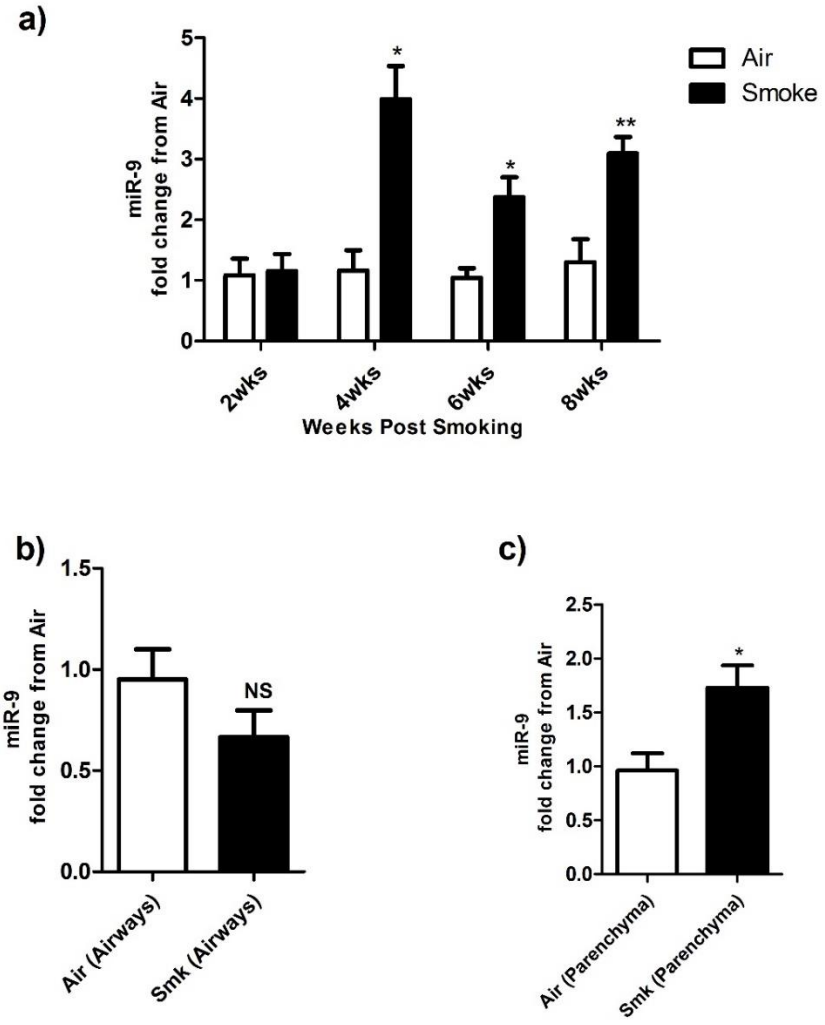


Figure 4.1: Acute and chronic cigarette smoke (CS) exposure induces increases in miR-9 expression in experimental chronic obstructive pulmonary disease (COPD). BALB/c mice were exposed to CS *via* the nose-only or normal air for 2, 4, 6 and 8 weeks**. a)** Whole lung miR-9 expression was assessed using qPCR. miR-9 expression was also assessed in blunt dissested lung tissue in **b)** airways and **c)** parenchyma after eight weeks of chronic CS-exposure. miR-9 expression is expressed as fold change

relative to normal air-exposed controls. n= 4-8 mice/group. Data are presented as means ± s.e.m, NS; not significant; *P<0.05; **P<0.01. Data were analysed by two-tailed Mann Whitney test

4.2.2 Inhibition of miR-9 suppresses pulmonary inflammation in CS-induced experimental COPD

To investigate the role(s) of miR-9 in the pathogenesis of COPD, CS- or normal air-exposed mice were administered i.n. with Ant-9 once per week during eight weeks of CS exposure **(Figure 4.2a)**, and key disease features assessed. Administration of Ant-9 completely inhibited CS-induced increases in miR-9 expression (Smk+Ant-9) in lung tissue compared to scrambled antagomir-treated controls (Smk+Scr) **(Figure 4.2b)**. The inhibition of miR-9, suppressed CS-induced cellular inflammation in airways **(Figure 4.2c)** and pro-inflammatory mediators expression and proteins levels in lungs **(Figure 4.2 (d** and **e)).**

These data demonstrate that inhibiting CS-induced miR-9 expression reduces pulmonary inflammation in experimental COPD.

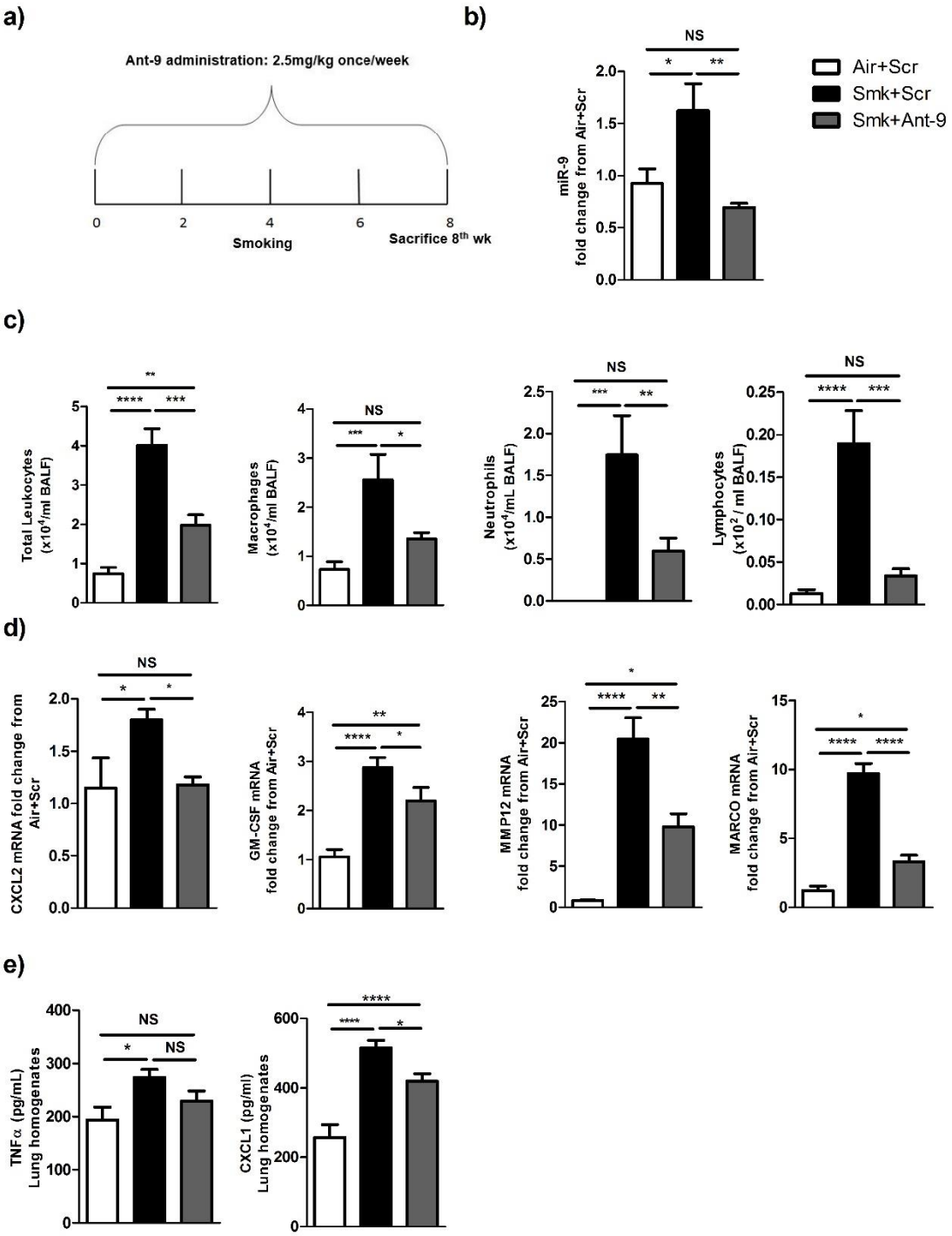


Figure 4.2: Cigarette smoke (CS)-induced increases in miR-9 expression are inhibited by antagomir-9 (Ant-9), which reduces pulmonary inflammation in experimental chronic obstructive pulmonary disease (COPD). a) BALB/c mice were exposed to CS *via* the nose-only, or normal air, for eight weeks and were treated with miR-9-specific antagomir Ant-9 or scrambled antagomir (Scr) intranasally once a week

during CS exposure. **b)** miR-9 expression was determined in whole lung tissue by qPCR, **c)** Total leukocyte, macrophage, neutrophil and lymphocyte numbers in bronchoalveolar lavage fluid. **d)** Pro-inflammatory chemokine (C-X-C motif) ligand 2 (CXCL2) and COPD related factors, granulocyte-macrophage colony-stimulating factor (GM-CSF), matrix metalloproteinase-12 (MMP-12) and macrophage receptor with collagenous structure (MARCO) expression in whole lung were assessed by qPCR. **e)** Tumour necrosis TNFα and CXCL1 levels in lung homogenates were quantified by ELISA. n=5-6 mice/group. Data are presented as means ± s.e.m. NS; not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 compared to normal air-exposed controls. Data were analysed with One-way ANOVA (uncorrected Fishers LSD test).

4.2.3 Depletion of CS-induced miR-9 expression inhibits airway fibrosis back to basal levels, causes a trend towards a decrease in emphysema and improves lung function in experimental COPD

We next assessed the role(s) of miR-9 in the induction of the pathophysiological features of experimental COPD. Treatment with Ant-9 completely inhibited CS-induced collagen deposition around the airways compared to sham treated CS-exposed controls with levels equivalent to those in normal air exposed controls **(Figure 4.3a**). Treatment also caused a close to statistically significant suppression of CS-inuced emphysema-like alveolar enlargement **(Figure 4.3b)**. Finally treatment also attenuated CS-induced impairement of lung function with reductions in dynamic compliance, forced vital capacity (FVC) and work of breathing **(Figure 4.3c)**.

These data demonstrate that inhibiting CS-induced miR-9 expression, prevents small airway remodelling and suppresses emphysema and impared lung function in experimental COPD

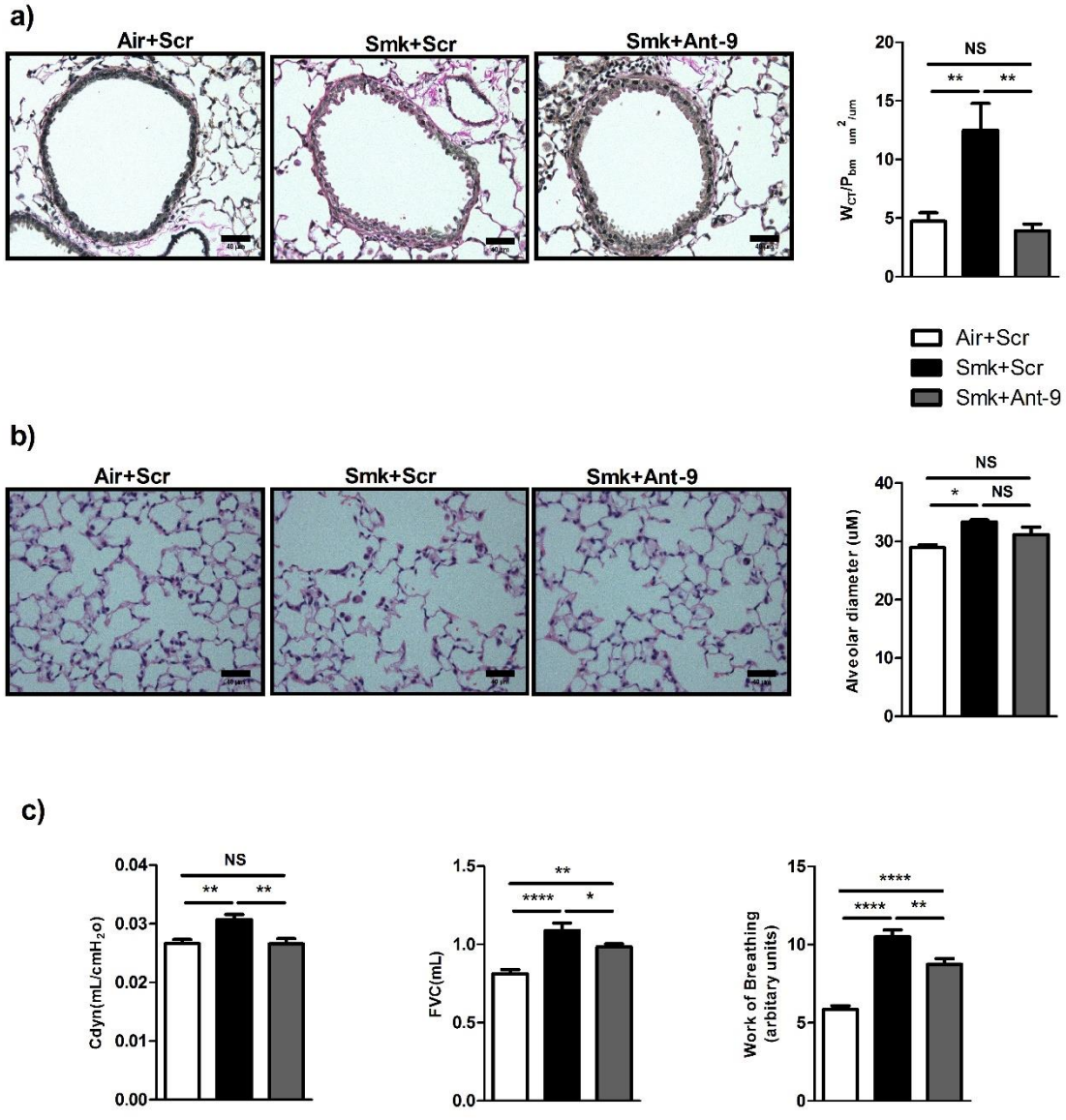


Figure 4.3: Cigarette smoke (CS)-induced increases in miR-9 expression are inhibited by antagomir-9 (Ant-9), suppressing small airway remodelling, decreasing emphysema and improving impaired lung function in experimental chronic obstructive pulmonary disease (COPD). BALB/c mice were exposed to CS *via* the nose-only, or normal air, for eight weeks and were treated with miR-9-specific antagomirAnt-9 or scrambled antagomir (Scr) intranasally once a week during CS exposure. **a)** Area of collagen deposition (μm^2) per perimeter of basement membrane (μm) in Verhoff-Van Gieson stained (pink) lung sections, scale bar = 40 μm. **b)** Mean alveolar diameter assessed in hematoxylin and eosin-stained lung sections using the

mean linear intercept technique. **c)** Lung function was assessed in terms of lung compliance (at 30cmH_2O), forced vital capacity and work of breathing. n=4-6 mice/group. Data are presented as means ± s.e.m. NS; not significant; *P<0.05; **P<0.01; ****P<0.0001 compared to normal air-exposed controls. Data were analysed with One-way ANOVA (uncorrected Fishers LSD test).

4.2.4 NRF2 expression and protein levels are increased with Ant-9 mediated inhibition of CS-induced miR-9 in experimental COPD

We then examined the mechanisms of how miR-9 promotes the pathogenesis of experimental COPD and the therapeutic benefit of its inhibition. We used the online target prediction algorithm MIRANDA to identify putative gene targets of miR-9. The seed sequence of miR-9 potentially binds to 3'UTR of NRF2 and may repress the expression of NRF2 mRNA **(Figure 4.4a)**. To investigate the role of NRF2 in experimental COPD, the expression of *NRF2* was assessed. CS exposure from eight weeks with sham treatment did not induce significant changes in expression but treatment with Ant-9 led to a two-fold increase in NRF2 mRNA levels in lung tissue **(Figure 4.4b)**. In contrast, CS-exposure led to a modest but significant increase in NRF2 protein and Ant-9 treatment NRF2 levels were further enhanced. To examine site-specific changes in NRF2 protein within the cellular compartment we assessed NRF2 levels in cytoplasmic and nuclear extracts of whole lung tissues, CS exposure induced marginal trends towards increased in levels in the nucleus and decreases in the cytoplasm giving some indication of nuclear accumulation **(Figure 4.4 (c** and **d))**.

These data show that inhibiting CS-induced miR-9 expression results in significant increase in NRF2 protein levels.

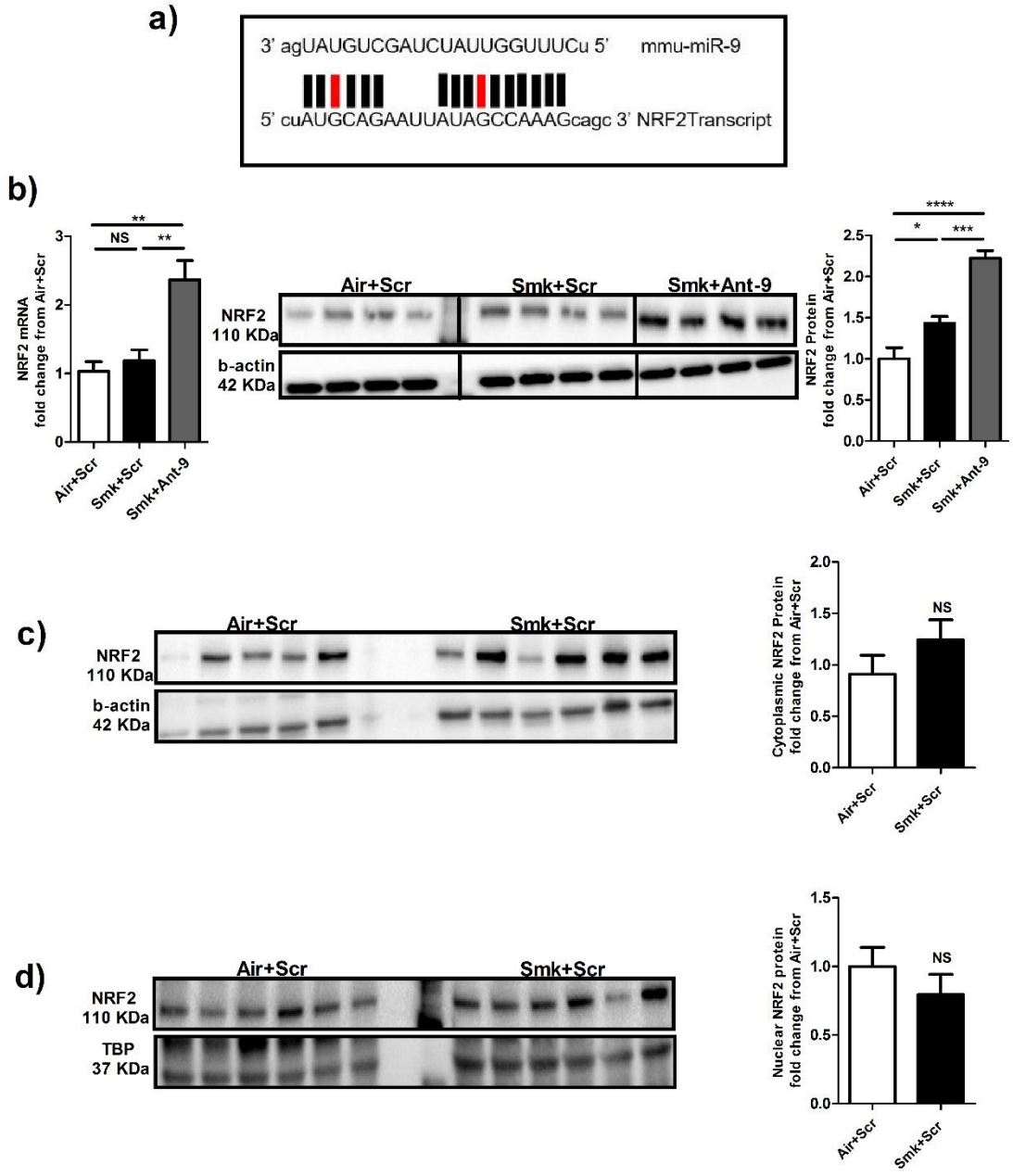


Figure 4.4: Inhibition of cigarette smoke (CS)-induced increases in miR-9 expression by antagomir-9 (Ant-9), increases nuclear factor-erythroid 2 related factor2 (NRF2) expression and protein levels in experimental chronic obstructive pulmonary disease (COPD). a) Seed sequence of miR-9 binding to the 3' UTR of NRF2 transcript. BALB/c mice were exposed to CS *via* the nose-only or normal air, for eight weeks and were treated with miR-9-specific anatgomir, Ant-9 or scrambled antagomir (Scr) intranasally once a week during CS-exposure. **b)** *NRF2* expression and protein

levels in whole lung were assessed by qPCR and immunoblot and densitometry. **c)** Cytoplsamic and **d)** nuclear NRF2 protein levels in whole lung tissues were assesed by immunoblot and densitometry. N=4-6 mice/group. Data are presented as means ± s.e.m. NS; not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 compared to normal air-exposed controls. Data in **(b)** and **(c and d)** were analysed with One-way ANOVA using uncorrected Fishers LSD test and two-tailed Mann Whitney test respectively.

4.2.5 NRF2 expression and NFR2 induced anti-oxidant gene expression are increased in CS-exposed airways but not parenchyma experimental COPD.

We next measured the expression of NRF2 and NFR2 induced anti-oxidants gene expression in blunt-dissected airways and parenchyma after either weeks of CS exposure. CS-induced increase in NRF2 expression in airways but not in parenchyma **(Figure 4.5a)**. In CS-exposed airways the expression of NQO-1, glutathione S-transferase Pi (GSTP1), glutamate-cysteine ligase catalytic subunit (GCLC) were increased but HO-1 and glutathione peroxidase 2 (GPX2) were not altered **(Figure 4.5b)**. In contrast, none of these anti-oxidant genes were altered in the parenchyma **(Figure 4.5c)**.

These data show that chronic CS exposure induces increases NRF2 and anti-oxidant gene expression specifically in the airways.

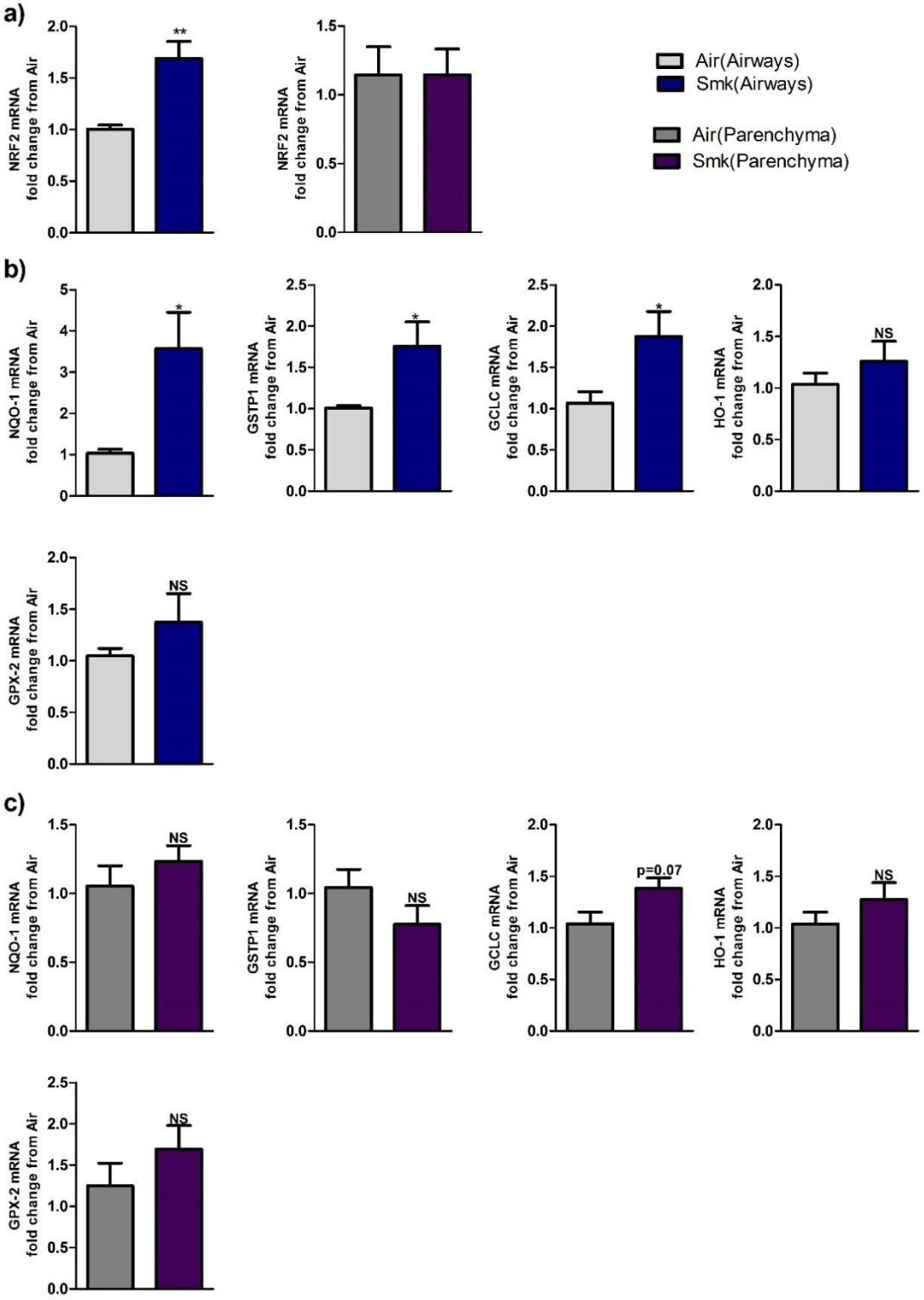

a)
NRF2 mRNA fold change from Air
**
NRF2 mRNA fold change from Air
Air(Airways)
Smk(Airways)
Air(Parenchyma)
Smk(Parenchyma)
b)
NQO-1 mRNA fold change from Air
*
GSTP1 mRNA fold change from Air
*
GCLC mRNA fold change from Air
*
HO-1 mRNA fold change from Air
NS
GPX-2 mRNA fold change from Air
NS
c)
NQO-1 mRNA fold change from Air
NS
GSTP1 mRNA fold change from Air
NS
GCLC mRNA fold change from Air
p=0.07
HO-1 mRNA fold change from Air
NS
GPX-2 mRNA fold change from Air
NS

Figure 4.5: Nuclear factor-erythroid-2 related factor2 (NRF2) and NFR2-induced anti-oxidant gene expression in airways and parenchymal tissue in cigarette smoke (CS)-induced experimental chronic obstructive pulmonary disease (COPD). BALB/c mice were exposed to CS *via* the nose-only or normal air, for eight weeks. **a)** NRF2 mRNA, **b)** and **c)** NAD(P)H: quinone oxidoreductase-1 (NQO1), glutathione S-transferase P1 (GSTP1), glutamate-cysteine ligase, catalytic subunit (GCLC), heme oxygenase-1 (HO-1) and glutathione peroxidase-2 (GPX2) expression in airways and parenchyma tissue sections of lung were assessed by qPCR. n=5-8 mice/group. Data are presented as means ± s.e.m. NS; not significant; *P<0.05; **P<0.01; compared to normal air-exposed controls. Data were analysed by two-tailed Mann Whitney test.

4.2.6 Treatment with 10mg/kg of 2-trifluoromethyl-2'-methoxychalone (TMC) suppresses CS-induced acute inflammation.

We next performed an optimisation experiment with the NRF2 activator TMC, to determine its optimal dose required to suppress CS-induced acute inflammation. Mice were exposed to CS or normal air for a week and were administered either 1mg/kg or 10mg/kg TMC or vehicle (Veh) and CS-induced inflammation and anti-oxidant genes expression was assessed.

Administration of 10mg/kg of TMC suppressed total number of inflammatory cells and macrophages in BALF and neutrophils and lymphocytes showed a trend towards reduction in (Smk+TMC) group compared with the (Smk+Veh) group. In contrast, neither total inflammatory cells nor individual inflammatory cell types were reduced with 1mg/kg of TMC treatment **(Figure 4.6a)**. Next we assessed the expression of anti-oxidant genes, treatment with both the doses of TMC increased the expression of NRF2-inducible anti-oxidant gene GpX2 compared with the (Smk+Veh) group. Higher dose of TMC was also associated with a trend towards increase in the expression of other anti-oxidant genes such as NQO-1 and GSTP1, whereas the expression of GCLC and HO-1 were not altered with both the doses of TMC **(Figure 4.6b)**.

These data show that the NRF2 activator, TMC suppresses CS-induced acute inflammation in the airways and also increases anti-oxidant gene expression in the lung tissue.

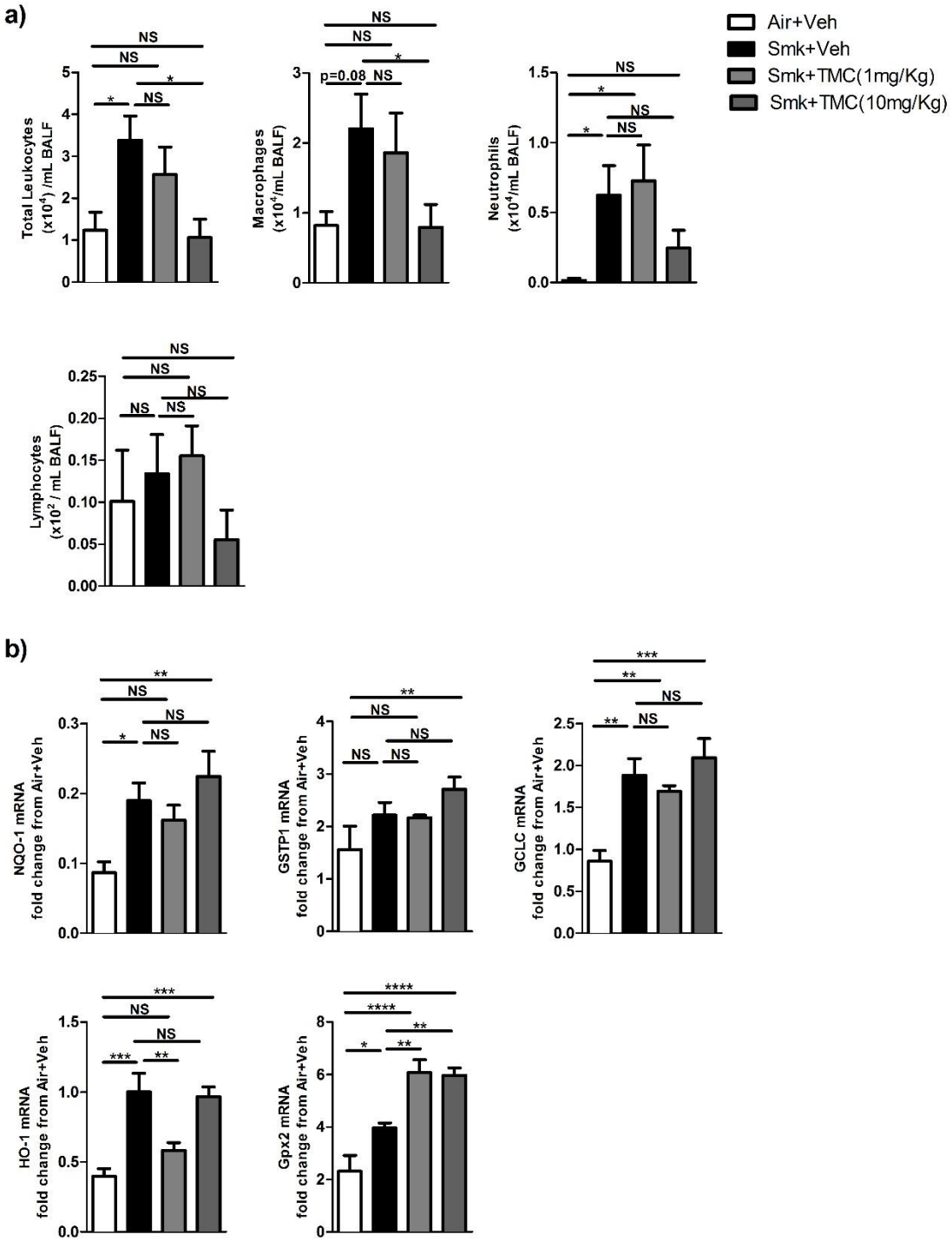


Figure 4.6: Optimsation of 2-trifluoromethyl-2'-methoxychalone (TMC) treatment doses in the suppression of inflammation induced by acute cigarette smoke (CS)-exposure. BALB/c mice were exposed to CS or normal air for a week and either 1mg/kg or 10mg/kg TMC or vehicle were administered intranasally on alternate days of CS-exposure. **a)** Total leukocyte, macrophage, neutrophil, and lymphocyte numbers in broncho-alveolar lavage fluid, and **b)** anti-oxidant gene expression of NAD(P)H: quinone oxidoreductase-1 (NQO1), glutathione S-transferase P1 (GSTP1), glutamate-cysteine ligase, catalytic subunit (GCLC), heme oxygenase-1 (HO-1), and glutathione peroxidase-2 (GPX2) were quantified in whole lung by qPCR. n=4-6 mice/group. Data are presented as means ± s.e.m. NS; not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 compared to normal air-exposed controls. Data were analysed with One-way ANOVA (uncorrected Fishers LSD test).

4.2.7 Ant-9 mediated inhibition of CS-induced miR-9 and TMC administration to activate NRF2 suppresses BALF inflammation in experimental COPD

To further investigate the roles and relationships, an experiment was performed where miR-9 was depleted and NRF2 activated, to determine whether the same effects were observed. This was achieved by depleting CS-induced miR-9 with Ant-9 and activating NRF2 with TMC administration to induce NRF2 activation. CS- or normal air-exposed mice were administered Ant-9 and TMC during eight weeks of CS exposure and key disease features were assessed **(Figure 4.7a).** Administration of Ant-9 again inhibited CS-induced miR-9 expression in the lungs in both Ant-9 treated groups (Smk+Ant-9+Veh and Smk+Ant-9+TMC) compared to the Scr-treated controls (Smk+Scr+Veh) **(Figure 4.7b).** The total number of inflammatory cells as well as macrophages and neutrophils in BALF were reduced with treatment with Ant-9 in both the Ant-9 treated groups. There were no major additional affects of TMC treatment. In contrast, neither total inflammatory cells nor individual inflammatory cells were reduced by treatment with only TMC (Smk+Scr+TMC) (**Figure 4.7c)**. We then assessed mRNA

expression of CS-induced lung pro-inflammatory mediators. Ant-9 treatment reduced the expression of CXCL2 in (Smk+Ant-9+Veh) group compared with the (Smk+Scr+Veh) group. Whereas the expression of other mediators such as GM-CSF, MMP12 and MARCO were not altered either with Ant-9 or TMC treatments **(Figure 4.7d)**. Next, we measured the effects of treatments on TNFα and CXCL1 protein levels in lung homogenates. Again these were generally reduced with Ant-9 treatment and TMC had no effect **(Figure 4.7e)**.

These data show that the major effects are driven through miR-9 and that in the continued presence of this miR, TMC treatment alone does not reduce pulmonary inflammation.

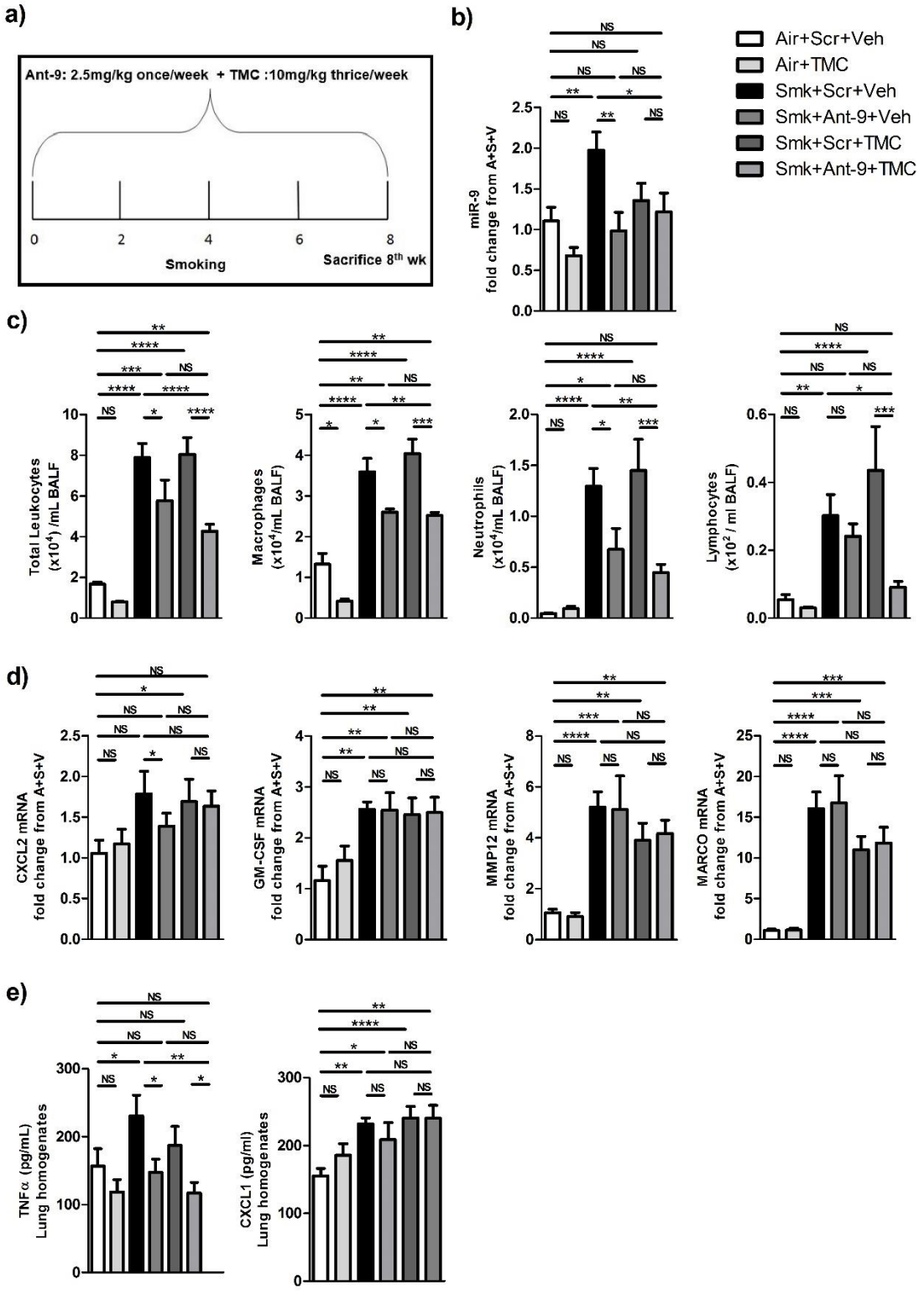


Figure 4.7: Inhibition of CS-induced miR-9 with antagomir-9 (Ant-9) and administration of 2-trifluoromethyl-2'-methoxychalone (TMC) reduces airway inflammation in experimental chronic obstructive pulmonary disease (COPD). a)

BALB/c mice were exposed to CS or normal air, for eight weeks and were treated with miR-9-specific antagomir Ant-9 or scrambled antagomir (Scr) intranasally (i.n.) once a week and TMC or vehicle of TMC were administered i.n. three times a week during CS-exposure. **b)** miR-9 expression in the lungs was assessed by qPCR. **c)** Total leukocyte, macrophage, neutrophil and lymphocyte numbers in bronchoalveolar lavage fluid. **d)** Pro-inflammatory mediators, chemokine (C-X-C motif) ligand 2 (CXCL2) and COPD related factors: granulocyte-macrophage colony-stimulating factor (GM-CSF), matrix metalloproteinase-12 (MMP-12) and macrophage receptor with collagenous structure (MARCO) expression in whole lung were assessed by qPCR. **e)** TNFα and CXCL1 levels in lung homogenates were quantified by ELISA. n=5-6 mice/group. Data are presented as means ± s.e.m. NS; not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 compared to normal air-exposed controls. Data were analysed with One-way ANOVA (uncorrected Fishers LSD test).

4.2.8 Ant-9 mediated inhibition of CS-induced miR-9 and TMC administration to activate NRF2 does not improve emphysema

We next assessed the effect of miR-9 inhibition and NFR2 activation on small airways fibrosis and emphysema,. There were no significant changes in collagen depostion between air-exposed controls (Air+Scr+Veh and Air+TMC) and CS-exposed scr-treated controls (Smk+Scr+Veh) **(Fig 4.8a).** However, CS-exposed scr treated controls showed increased emhysema like alveolar changes compared with air-exposed controls. Treatment with Ant-9 showed a trend towards decreae in alveolar enlargement in (Smk+9+veh) group but TMC treatmets had no effect on alveolar enlargement **(Fig 4.8b)**.

These data show that TMC administration does not affect emphysema-like alveolar enlargement.

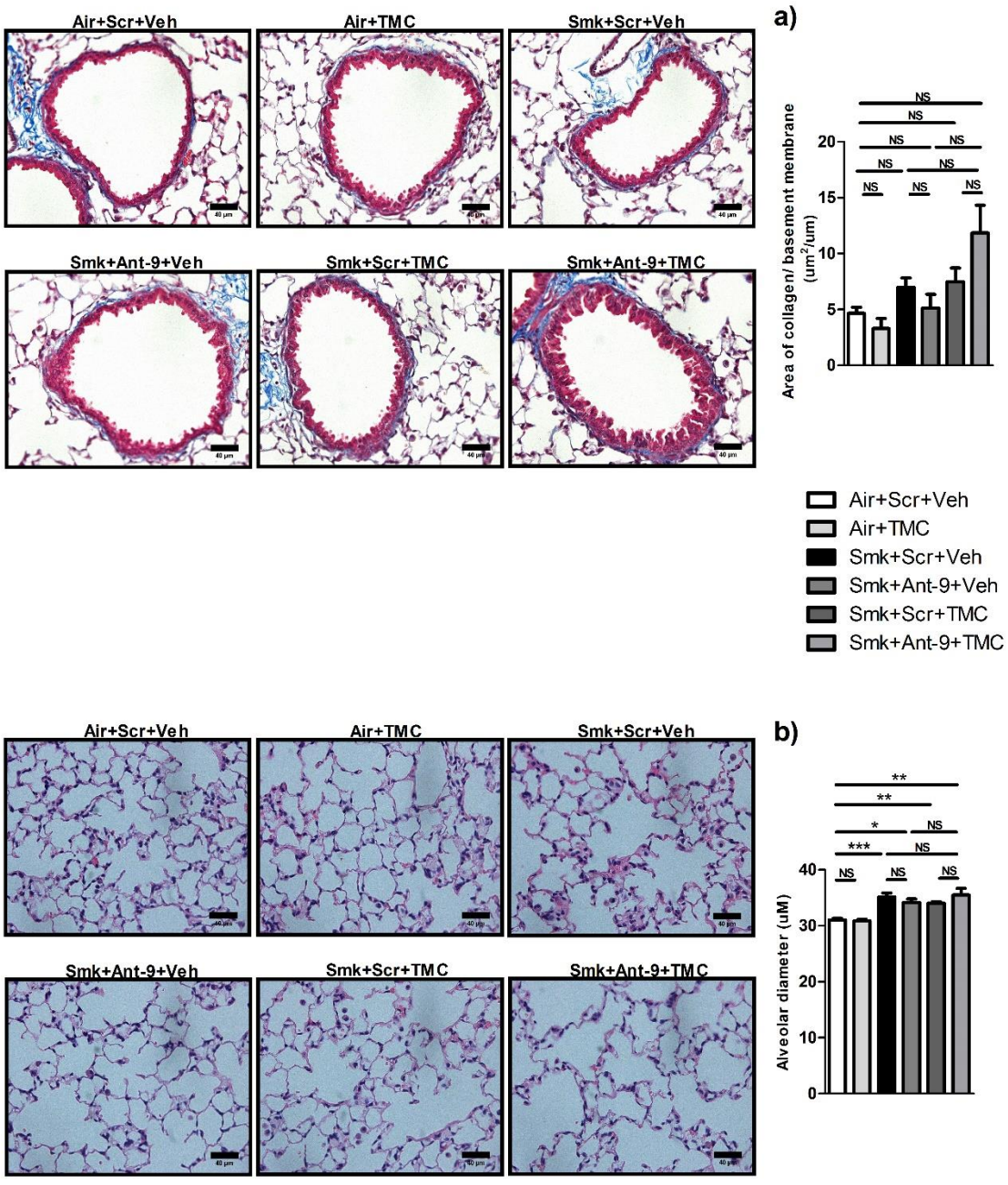


Figure 4.8: Effects of inhibition of CS-induced miR-9 and 2-trifluoromethyl-2'-methoxychalone (TMC) administration on airway remodelling and emphysema in experimental chronic obstructive pulmonary disease (COPD). BALB/c mice were exposed to CS or normal air for eight weeks and were treated with miR-9-specific antagomir Ant-9 or scrambled antagomir (Scr) intranasally (i.n.) once a week during CS-exposure. **a)** Area of collagen deposition (μm^2) per perimeter of basement membrane

(μm) in Masson-trichrome stained (blue) lung sections, scale bar = 40 μm. **b)** Mean alveolar diameter assessed in hematoxylin and eosin-stained lung sections using the mean linear intercept technique. n=6 mice/group. Data are presented as means ± s.e.m. NS; not significant; *P<0.05; **P<0.01; ***P<0.001 compared to normal air-exposed controls. Data were analysed with One-way ANOVA (uncorrected Fishers LSD test).

4.2.9 CS-induced increases in miR-9 expression inhibits SOCS5 levels which are restored by inhibition of miR-9 in experimental COPD

SOCS5 is a known target of miR-9 (286). The seed sequence of miR-9 binds to the 3'UTR of SOCS5 mRNA and represses its expression **(Figure 4.9a).** To investigate these potential interactions in experimental COPD, the expression of SOCS5 was assessed. CS-exposure did not induce significant changes but treatment with Ant-9 led to significant increases in *SOCS5* expression. In contrast CS-exposure led to a significant reduction in SOSC5 protein and with Ant-9 treatment SOSC5 levels were restored **(Figure 3.9b).** To determine tissue specific changes we analysed SOCS5 mRNA and protein levels in airways and parenchyma. In CS-exposed airways there was a trend to an increase in *SOCS5* mRNA, which was almost significant for protein levels **(**p=0.056, **Figure 4.9c).** In parenchyma *SOCS5* mRNA and protein levels were reduced **(Figure 4.9d).**

These data show that CS-induced miR-9 targets SOSC5 and with inhibition of miR-9 SOSC5 levels are restored.

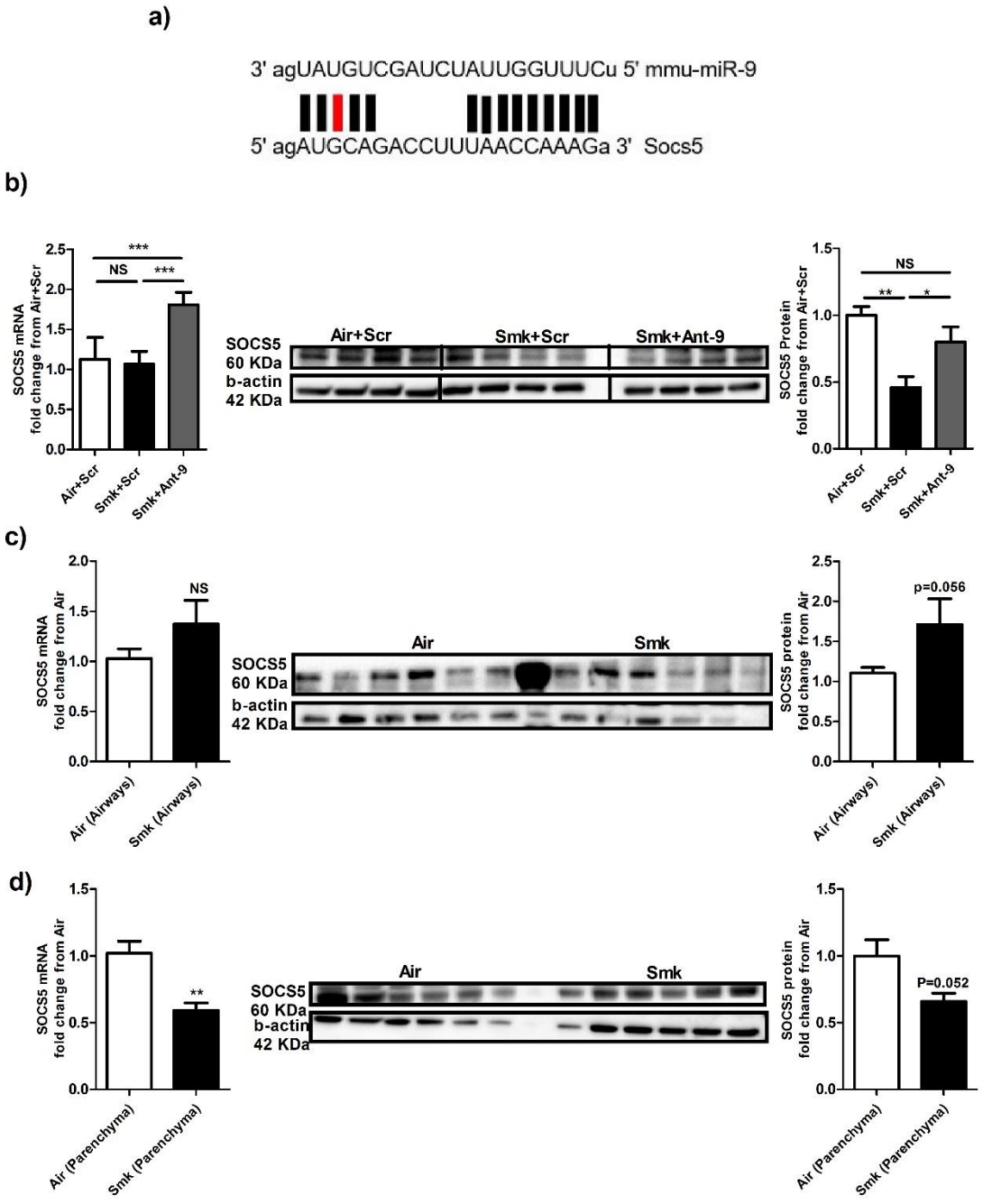


Figure 4.9: Cigarette smoke (CS)-induced increases in miR-9 expression inhibits SOCS5 levels and with the inhibition of miR-9 SOCS5 transcript and protein levels are increased in experimental chronic obstructive pulmonary disease (COPD). a) Seed sequence of miR-9 binding to the 3' UTR of SOCS5 transcript. BALB/c mice were exposed to CS *via* the nose-only or normal air, for eight weeks and were treated with miR-9-specific antagomir Ant-9 or scrambled antagomir (Scr) intranasally once a week

during CS-exposure. **b)** *SOCS5* expression and protein levels in whole lung and in **c)** airways and **d)** parenchyma tissues were assessed by qPCR and immunoblot and densitometry.. Data are presented as means ± s.e.m. NS; not significant; *P<0.05; **P<0.01; ***P<0.001 compared to normal air-exposed controls. Data in **(b)** and **(c-d)** were analysed with One-way ANOVA using uncorrected Fishers LSD test and two-tailed Mann Whitney test, respectively.

4.3 DISCUSSION

Here we examined the role(s) of CS-induced miR-9 in the pathogenesis of experimental COPD. Our model recapitulates the hallmark features of COPD in humans including chronic pulmonary inflammation, small airway remodelling, emphysema and impaired lung function (72, 162, 254-257). We showed that increased lung miR-9 expression was increased in association with pathogenesis in experimental COPD. Using an antagomir that specifically depletes miR-9 *in vivo* we define a previously unrecognized pathway that induces experimental COPD. With the increase in miR-9 expression the levels of its putative target NRF2 increased and the levels of its canonical target SOCS5 decreased. Inhibition of miR-9 increased NRF2 and restored SOSC5 levels resulting in a decrease in pulmonary inflammation, airway remodelling and impaired lung function in experimental COPD. This study identifies miR-9 as novel therapeutic target in pathogenesis of COPD.

Many disease mechanisms are implicated in the pathogenesis of COPD. This includes miRs which are known to regulate both innate and adaptive immune systems and are involved in the development of several inflammatory diseases. miRs are important because they regulate multiple downstream transcripts, which affect many pathological pathways involved in disease development. For example miR-218 has recently been shown to have a protective role and antagonism of miR-328 increases anti-microbial functions of macrophages in human and experimental COPD (257, 294). Our findings extend our understanding of the involvement of miRs in the pathogenesis

of COPD. miR-9 has also been shown to play an important role in promoting steroid resistance in a mouse model of IFN-ɣ/LPS-induced experimental asthma (295). Further as our COPD model is also steroid resistant and CS-induced increase in miR-9 expression in our model also indicates a potential role of the miR with steroid resistance in COPD.

miR-9 expression was increased in the lungs from four weeks of CS exposure. This coincides with the "induction phase" of experimental COPD suggesting that exaggerated miR-9 expression promotes the development of disease. Increased expression after eight weeks was observed in the lung parenchyma but not in the airway epithelium. To interrogate the roles in COPD pathogenesis, we depleted miR-9 *in vivo* throughout CS exposure which resulted in decreased inflammatory cell numbers in BALF after eight weeks. TNFα and CXCL1 protein levels were increased in experimental COPD but were significantly reduced with the inhibition of miR-9. Treatment with Ant-9 also reduced CS-induced increases in the mRNA expression of CXCL2, GM-CSF and COPD related factors MMP12 and MARCO TNFα, CXCL1, CXCL2, and MMP12 are known neutrophil chemo-attractants, whereas GM-CSF attracts both macrophages and neutrophils (68, 296-298). Thus, these results may explain the reduced macrophage and neutrophil infiltration in BALF. We demonstrate that miR-9 inhibition reduced neutrophils in the lungs and two other studies have shown that this miR promotes neutrophil influx (213, 295). Collectively, this provides strong evidence that CS-induced miR-9 may promote neutrophilic inflammation in experimental COPD. Further MARCO acts as a receptor for unopsonised particles, and is mainly expressed on macrophages and contributes to bacterial phagocytosis (299). It is shown that disruption of both NRF2 and MARCO results in reduced bacterial phagocytosis (300). With increases in CS-induced miR-9 expression, we demonstrated increases in both macrophage numbers and MARCO expression. Moreover, Ant-9 treatment reduced both macrophage numbers and MARCO expression indicating that CS-induced miR-9 may be associated with reduced anti-bacterial defence.

Increased miR-9 expression at eight weeks was accompanied by increased airway remodelling. Thus, miR-9 may promote collagen deposition in the lungs in CS-induced COPD, which was confirmed when the depletion of miR-9 completely inhibited small airway remodelling. This is in contrast with other recent studies showing that miR-9 is anti-fibrotic, as it attenuated the induction of mRNA transcripts of pro-fibrotic transforming growth factor-beta receptor-2 (TGFBR2) and NADPH oxidase-4 (NOX4) in human lung fibroblasts stimulated with ROS and platelet-derived growth factor receptorβ (PDGFRβ) in cardiac fibroblasts of rats, indicating a complex role of miR-9 in fibrosis (301, 302). In our model, treatment with Ant-9 had no effect on the anti-fibrotic gene PDGFRβ (data not shown), however the decrease in remodelling with inhibition of miR-9 may be due to reductions in pro-fibrotic MMP12 (303, 304).

Increased emphysema-like alveolar enlargement was also associated with increased miR-9 expression after eight weeks. With miR-9 suppression, a trending decrease in alveolar enlargement was observed, indicating that this miR may promote emphysema. This may be due to combined effect of reductions in the pro-emphysema factor MMP12, and pro-inflammatory mediators TNFα and CXCL1. These have all been associated with the development of emphysema in COPD (304-306).

In COPD the combination of chronic pulmonary inflammation, airway remodelling and emphysema combine to reduce lung function. In our study, reduced airway inflammation, airway remodelling and emphysema with Ant-9 treatment lead to improved lung function with the suppression of CS-induced increases in compliance and FVC, which in combination reduced the work of breathing.

Using bioinformatics approaches we identified that NRF2 mRNA possesses a putative binding site for miR-9 in its 3'-UTR. Since NRF2 regulates anti-oxidant enzymes such as NQO-1 and HO-1, we examined the association between miR-9 and NRF2 in experimental COPD. With increases in CS-induced miR-9, NRF2 mRNA was not altered but protein levels were modestly increased. However inhibition of miR-9 substantially

increased NRF2 mRNA and protein levels. Interestingly, most *in vitro* studies show that CS reduces NRF2 levels, and NRF2 levels are decreased in COPD patients (114, 282, 307). However, increased levels of NRF2 have also been observed in moderate smokers compared with heavy smokers (308). Nevertheless, our data provide convincing evidence that inhibiting miR-9 increases NRF2 activity that is associated with the suppression of disease features.

To address the possibility of NRF2 levels being masked by the presence of whole lung tissues, we measured tissue specific changes in our control groups. With the trending reduction of miR-9 in CS-exposed airways, NRF2 mRNA was significantly upregulated along with that of associated anti-oxidant genes including NQO-1, GSTP1, and GCLC. Others have also shown increased NRF2 and NQO-1 levels in airway epithelial cells exposed exposure to CS extract (154, 309). Notably, in our study the levels of NRF2 or anti-oxidants were not altered in the parenchyma. Significantly, inhibition of miR-9 increased the levels of both NRF2 mRNA and protein and suppressed key features of experimental COPD. Thus, NRF2 likely plays an important role in the airway epithelium regulating airway inflammation and the suppression of responses by miR-9 may contribute to COPD pathogenesis.

Previous studies have shown that NRF2-deficient mice develop more severe emphysema following CS exposure compared to $NRF2^{+/+}$ mice (116, 117). However, in our studies some emphysema-like changes were observed despite increased NRF2 levels following miR-9 inhibition. Thus although beneficial, other factors are also likely involved.

To further assess roles and interactions we inhibited miR-9 and activated NRF2 with TMC during CS exposure. TMC was used to activate NRF2 because it has been shown to induce the expression of anti-oxidant genes which attenuate oxidative stress and suppresses inflammation (283). We first performed a dose optimisation study Administration of TMC at 10mg/kg reduced inflammation with decreased numbers of macrophages and neutrophils in the BALF after one week of CS exposure. Importantly,

this was associated with increased expression of GpX2, a known NRF2-inducible anti-oxidant gene (310). Treatment also trended towards increasing the expression of NQO-1, a surrogate maker of NRF2 activation (283), and GSTP1. Again treatment with Ant-9 led to the suppression of miR-9 expression, which resulted in decrease in inflammation and disease features in both the Ant-9 treated groups. The suppressive effects of Ant-9 were reduced in this experiment compared to the initial inhibition study, which may be due to the effects of the vehicle for TMC. TMC had little additional benefit on top of Ant-9 suggesting that inhibition of miR-9 is sufficient to increase NFR2 activity to a maximum. TMC alone had no beneficial effects suggesting that miR-9-mediated suppression dominates over TMC-induced NRF2 activity. In another NRF2 activation study, mice administered with 50mg/kg of TMC by gavage, had increases in anti-oxidant genes NQO1 and GCLM expression after 24hrs in small intestine. This indicates that NRF2 activation with TMC is dependent on dose, time and route of administration, which needs to be further explored in experimental COPD.

We also assessed changes in 8-isoprostane levels with Ant-9 and TMC treatment, as this factor is a known biomarker of oxidative stress in COPD (120). Surprisingly, we did not observe significant changes in 8-isoprostane levels in BALF in any groups (data not shown). Few studies have shown increased 8-isoprostane levels in lung tissues of COPD patients (311, 312) but in a mouse model of CS-induced inflammation, 8-isoprostane levels were increased after 13 weeks of CS-exposure (313). This suggests that the elevation of 8-isoprostane is dependent on dose and time-period of smoke exposure. We also assessed NRF2 inducible anti-oxidants gene expression in lungs with Ant-9 and TMC treatment, which were also not altered with any treatment (data not shown). Taken together these data indicate that targeting miR-9 has greater effects than direct promotion of NRF2 and anti-oxidant activity in COPD.

Since SOCS5 is a known target of miR-9 (286) and a negative regulator of JAK/STAT signalling which are involved in the pathogenesis of COPD, we assessed the levels of SOCS5 mRNA and protein in CS-induced experimental COPD. Concomitant

with increases in miR-9, SOCS-5 mRNA was not altered but protein levels were significantly reduced. With inhibition of miR-9, both mRNA and protein levels were restored indicating that SOCS5 is negatively regulated by miR-9 and is involved in COPD pathogenesis. We also assessed tissue specific changes of SOCS5 mRNA and protein levels in the airways and parenchyma in our control groups. With trending reduction in miR-9 in CS-exposed airways, both SOCS5 mRNA and protein showed trending increase. In parenchyma, with increases in miR-9, both SOCS5 mRNA and protein levels were significantly reduced indicating that SOCS5 is regulated by miR-9 and may be involved in the pathogenesis of experimental COPD.

The mechanisms through which a reduction in SOCS5 mediates the pathogenesis of COPD is unclear but likely involves the control of cytokines/chemokines. A recent study showed increased phosphorylated STAT1 in lung parenchyma samples of COPD patients compared with smokers and non-smokers without COPD (314). Moreover, with CS-exposure we have shown decreased SOSC5 in lung parenchyma. Thus, a potential mechanism through which increases in CS-induced miR-9 and concurrent decreases in SOCS-5 mediates COPD pathogenesis may be through elevated STAT1 signalling that promotes cytokine and chemokine production that we observed. The inhibition of Ant-9 lead to increased SOCS5 and a suppression of cytokines/chemokines. However, the exact mechanisms downstream of SOCS-5 remains to be fully elucidated.

In summary, we demonstrate for the first time that miR-9 promotes inflammation, airway remodelling and emphysema that impairs lung function in experimental CS-induced COPD. This occurs through the suppression of NRF2 and dominates over therapeutic activation of NRF2 and anti-oxidant responses. We also identified SOCS5 to be negatively regulated by miR-9 that may play an important role in disease pathogenesis. Inhibition of CS-induced miR-9 increased NRF2 and restored SOCS5 levels, potentially mediating the decrease in pulmonary inflammation, small airway remodelling and improved lung function. This suggests that inhibiting CS-induced miR-9

may have beneficial effects in the treatment of COPD. However, there may be other mechanisms through which miR-9 promotes COPD pathogenesis and further studies are required.

CHAPTER 5: miR-21 SUPPRESSION OF SATB1 INDUCES S100A9/NF-KB AND COPD PATHOGENESIS

5.1 INTRODUCTION

miR-21-5p/miR-21 is a broadly conserved miR and is one the best characterised and is implicated in a variety of inflammatory diseases and cancers (315, 316). miR-21 expression is increased in serum of patients with mild to moderate COPD and asymptomatic smokers, compared to healthy controls, implicating its involvement in early pathogenesis (317). Increased expression of miR-21 has been associated with both increases and decreases in pro-inflammatory nuclear factor-kappa B (NF-κB) activity (215, 318). Thus, increased miR-21 expression can exert pro- or anti-inflammatory effects depending on the stimulus, cell type, tissue localisation, disease state, and target genes affected (215, 318). Notably, NF-κB activity is substantially elevated in sputum and bronchial biopsies of COPD patients (312, 319, 320) and is required for the development of cigarette smoke (CS)-induced experimental COPD in mice (255). Significantly, NF-κB can bind to promoter regions of miR-21 and induce its expression (321) suggesting that miR-21-dependent, NF-κB-mediated inflammatory responses have the capacity to self-perpetuate.

The potent neutrophil chemoattractants S100A8 and S100A9 responses induce NF-κB signalling (63) and are also NF-κB dependent (322). Besides, substantial clinical and experimental evidence demonstrates that S100A8 and S100A9 are negatively regulated by the transcriptional repressor (323), special AT rich sequence binding protein (SATB) 1 (324). Additionally SATB1 is a known target of miR-21 (220). Thus, we hypothesized that CS exposure may induce a miR-21-dependent decrease in SATB1 that results in S100A8 and S100A9 responses that drive the development of COPD. Here, we investigated the role, and potential for therapeutic targeting of miR-21 in CS-induced experimental COPD.

In this study we show that CS exposure substantially and chronically increases lung miR-21 expression which correlates with worsened lung function in experimental COPD. Levels of SATB1 were reduced and S100A9 increased, respectively. Treatment with a miR-21-specific inhibitor, antagomir (Ant)-21, during CS exposure restored SATB1 expression and suppressed S100A9 levels in the lung in experimental COPD. These effects were associated with reduced NF-κB activity resulting in decreased airway inflammation, and improved lung function. Thus, we define a novel miR-21/SATB1/S100A9/NF-κB axis in the pathogenesis of COPD and highlight miR-21 as a potential therapeutic target.

5.2 RESULTS

5.2.1 CS exposure increases miR-21 expression in experimental COPD

Microarray-based miR profiling was performed on total lung RNA isolated from BALB/c mice that were exposed to CS *via* the nose only, or normal air, for 2, 4, 6 or 8 weeks. miR-21 was the second-most highly up-regulated miR at 4, 6 and 8 weeks of CS exposure, compared to normal air-exposed controls (unpublished data). Increased miR-21 expression was validated by real-time quantitative PCR (qPCR) at 2, 4, 6 and 8 weeks of CS exposure **(Figure 5.1a)**. Since the hallmark features of COPD are observed at 8 weeks of CS exposure (72, 162, 254-257) we next assessed miR-21 expression in blunt-dissected lung airway and parenchymal tissue at this time point. CS exposure significantly increased miR-21 expression in both tissue compartments **(Figure 5.1 (a** and **b))**, compared to normal air-exposed controls.

Thus, acute and chronic CS exposure increases miR-21 expression in lungs in experimental COPD.

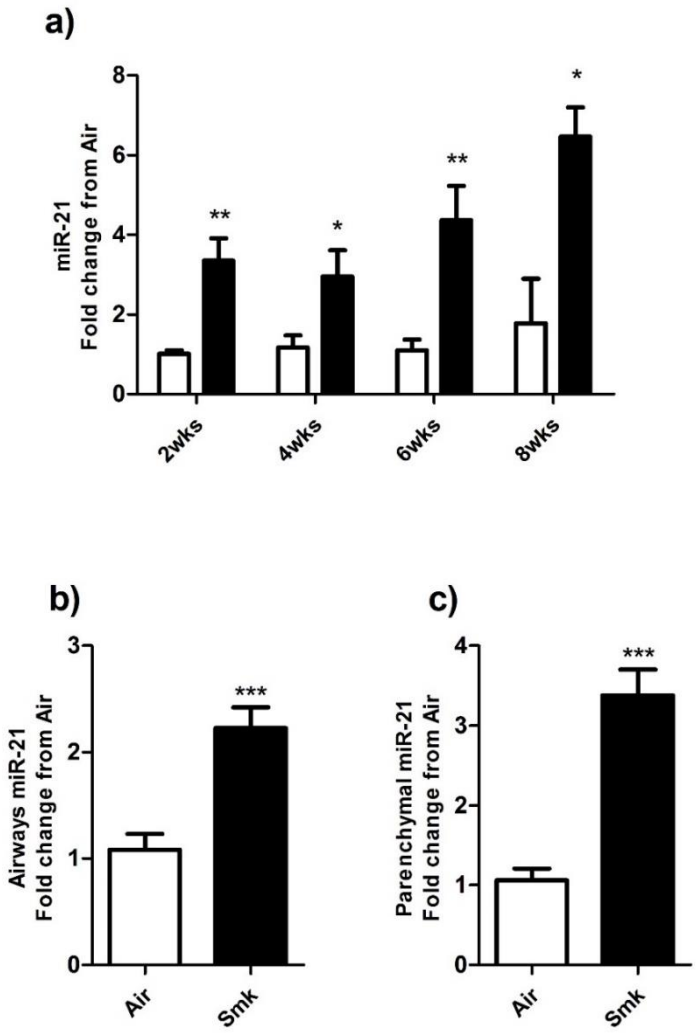
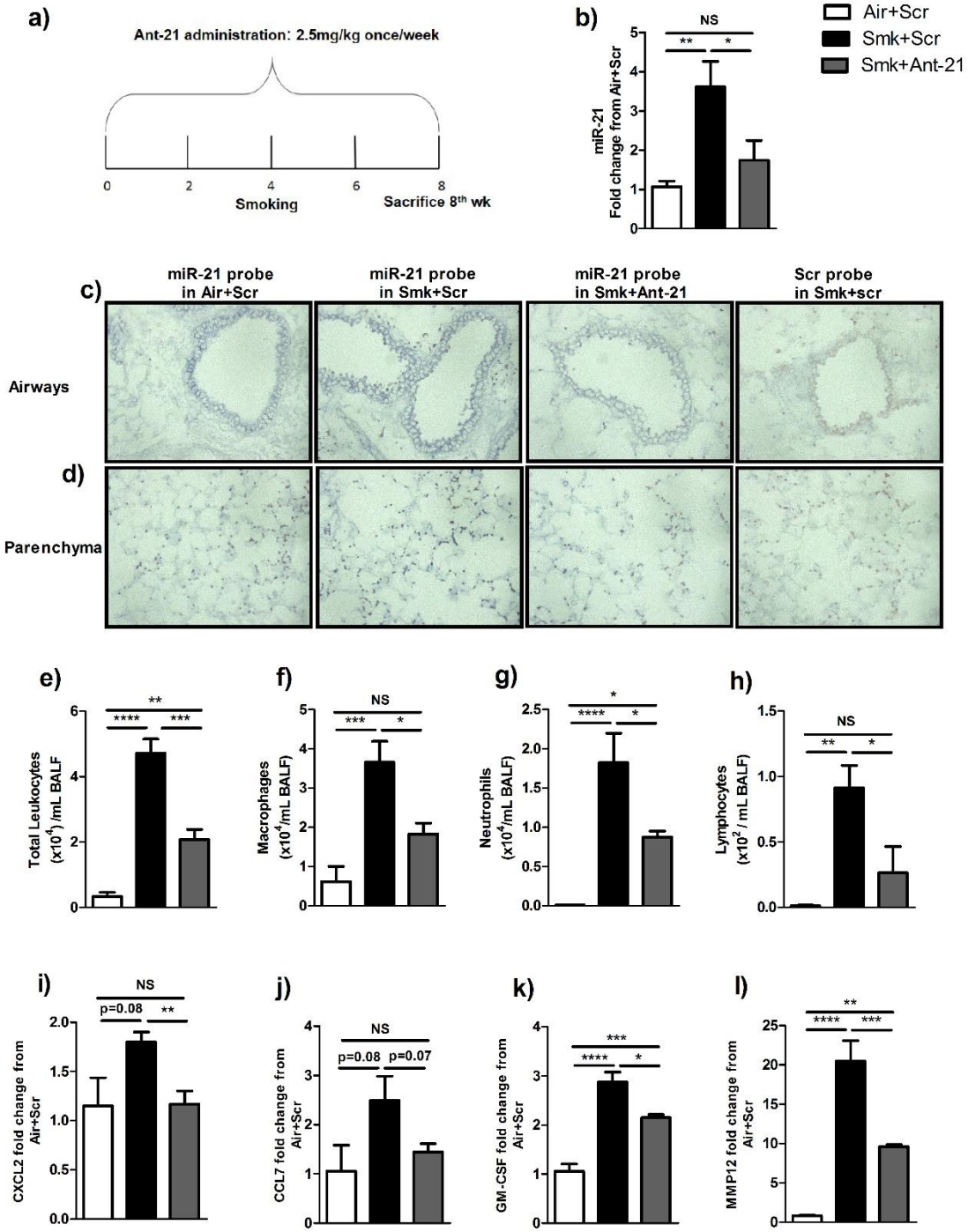


Figure 5.1: Cigarette smoke (CS) exposure increases lung miR-21 expression in experimental chronic obstructive pulmonary disease (COPD). a) BALB/c mice were exposed to CS *via* the nose only, or normal air, for 2, 4, 6 and 8 weeks. miR-21 expression was normalised to the geometric mean of the housekeeping controls small nuclear RNA U6 (SnRNA U6)and small nucleolar RNA U49 (SnoRNA U49). Lungs were blunt dissected to determine miR-21 expression in **b)** airways and **c)** parenchyema after 8 weeks of CS exposure. miR-21 expression is expressed as fold change from normal air-exposed controls. n= 4-8 mice. Data are presented as means ± s.e.m. *P<0.05; **P<0.01; ***P<0.001. Data were analysed by two-tailed Mann Whitney test.

5.2.2 Inhibition of CS-induced miR-21 suppresses airway inflammation in experimental COPD

We then assessed the role and tissue localisation of CS-induced miR-21 in experimental COPD. Mice were exposed to CS and treated with scrambled (Scr) control or miR-21-specific antagomir (Ant-21) for 8 weeks to induce experimental COPD. CS-exposed, Scr-treated (Smk+Scr) mice had increased lung miR-21 expression compared to air-exposed, Scr-treated controls **(Air+Scr; Figure 5.2 (a** and **b))** after 8 weeks. Treatment with Ant-21 suppressed CS-induced miR-21 expression at 8 weeks (Smk+Ant-21) back to baseline in air-exposed, Scr-treated controls. We then showed that miR-21 expression was widespread, occurring in airway epithelium and parenchyma of CS-exposed compared to normal air-exposed groups **(Figure 5.2 (c** and **d))**. Ant-21 treatment decreased miR-21 expression in both of these lung compartments. We then assessed the role and potential for therapeutic targeting of increased miR-21 expression in experimental COPD. Ant-21 treatment suppressed airway inflammation by reducing the numbers of total leukocytes, macrophages, neutrophils and lymphocytes in bronchoalveolar lavage fluid (BALF) compared to Scr-treated controls **(Figure 5.2 (e-h))**. Treatment with Ant-21 also reduced mRNA expression of pro-inflammatory mediators CXCL2, CCL7 and COPD related factors GM-CSF and MMP12 **(Figure 5.2 (i-l))**. Further Ant-21 treatment also inhibited the production of CS-induced TNFα and CXCL1 in the lungs back to baseline levels in normal air-exposed controls **(Figure 5.2 (m** and **n))**.

Collectively, these data show that CS-induced miR-21 expression in the airways and lungs plays an important role in promoting airway inflammation in experimental COPD.


a)
Ant-21 administration: 2.5mg/kg once/week
0
2
4
6
8
Smoking
Sacrifice 8th wk
b)
NS
**
*
miR-21
Fold change from Air+Scr
Air+Scr
Smk+Scr
Smk+Ant-21
c)
miR-21 probe in Air+Scr
miR-21 probe in Smk+Scr
miR-21 probe in Smk+Ant-21
Scr probe in Smk+scr
Airways
d)
Parenchyma
e)
Total Leukocytes (x10^4) /mL BALF
f)
Macrophages (x10^4/mL BALF)
g)
Neutrophils (x10^4/mL BALF)
h)
Lymphocytes (x10^2 / mL BALF)
i)
CXCL2 fold change from Air+Scr
p=0.08
j)
CCL7 fold change from Air+Scr
p=0.08
p=0.07
k)
GM-CSF fold change from Air+Scr
l)
MMP12 fold change from Air+Scr

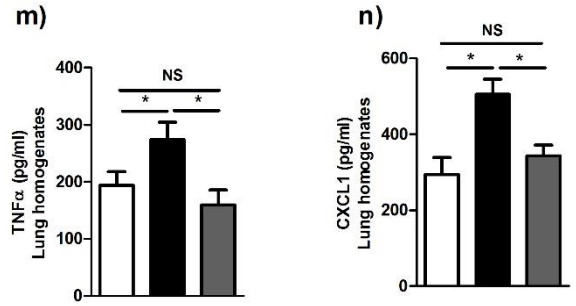
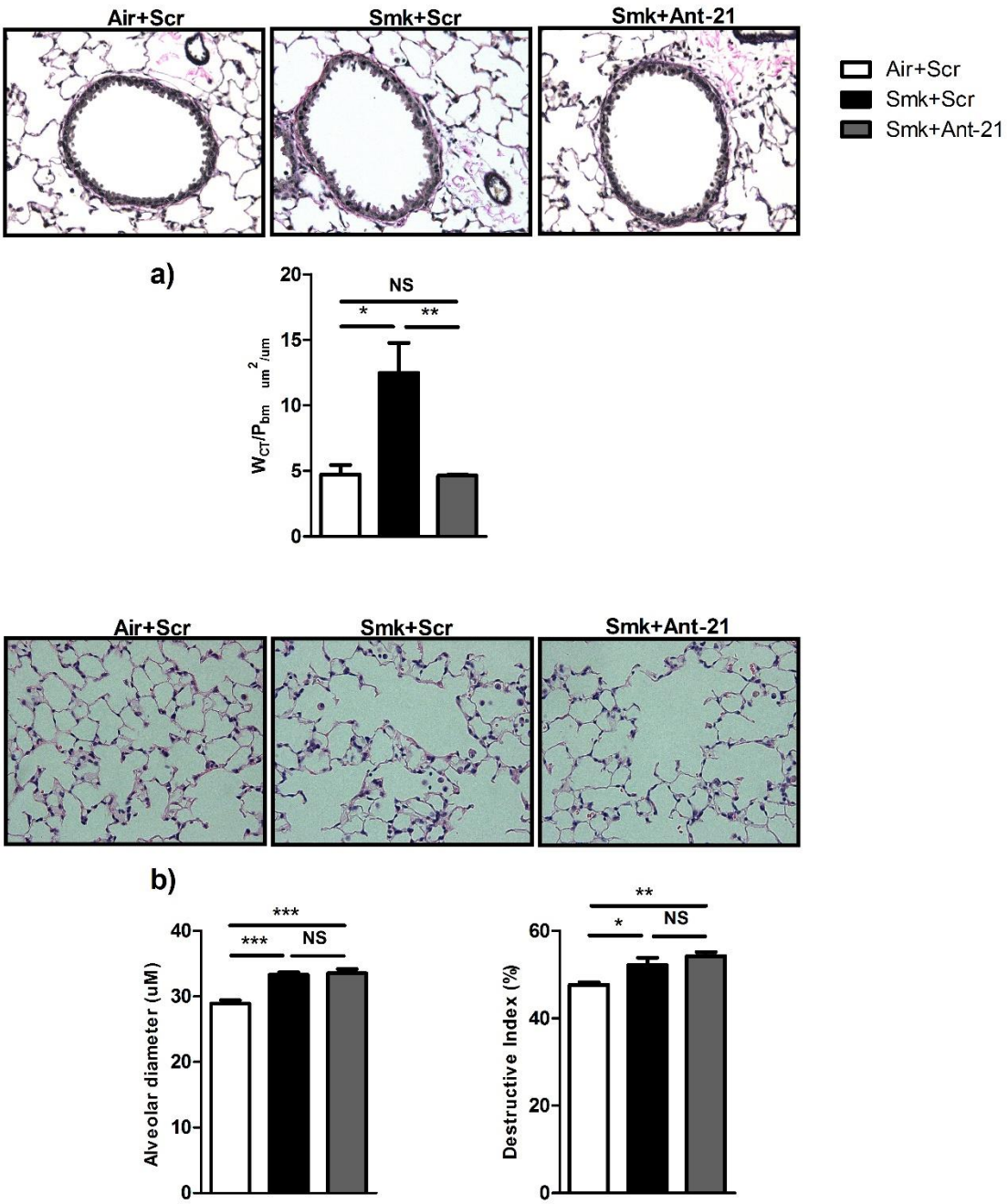


Figure 5.2: Cigarette smoke (CS)-induced miR-21 expression induces pulmonary inflammation in experimental chronic obstructive pulmonary disease (COPD). a) BALB/c mice were exposed to CS *via* the nose-only, or to normal air, and treated intranasally with miR-21-specific antagomir (Ant-21) or scrambled (Scr) antagomir once a week for 8 weeks. **b)** miR-21 expression was normalised to the geometric mean of the housekeeping controls small nuclear RNA U6 (snRNA U6) and small nucleolar RNA U49 (snoRNA U49). miR-21 and mRNA are presented as relative abundance compared to normal air-exposed controls. Representative photomicrographs (40x magnification) showing tissue and cellular localisation of miR-21 in histological sections of mouse lungs collected at 8 weeks. Localisation of miR-21 was characterised using *in situ* hybridisation analyses with a miR-21-specific locked nucleic acid (LNA™) probe. miR-21-positive signal (blue color) is visible in **c)** airways and **d)** parenchyma. miR-21-positive signal is not evident when Scr LNA™ miR probe was used. Nuclear Fast Red™ was used as a counterstain. **e)** Total leukocyte, **f)** macrophage **g)** neutrophil and **(h)** lymphocyte numbers stained with May-Grunwald Giemsa were enumerated in bronchoalveolar lavage fluid (BALF). Pro-inflammatory chemokines **i)** chemokine (C-X-C motif) ligand 2 (CXCL2), **j)** chemokine (C-C motif) ligand 7 (CCL7), and COPD related factors **k)** granulocyte-macrophage colony-stimulating factor (GM-CSF) and **l)** matrix metalloproteinase 12 (MMP12) mRNA expression in whole lung tissue normalised to hypoxanthine-guanine phosphoribosyltransferase (HPRT) **m)** Tumor necrosis factor-α (TNFα) and **n)** CXCL1 protein levels in whole lung homogenates. n=4 and 5-6

mice/group for **(c-d)** and **(b, e-n)** respectively. Data are presented as means ± s.e.m. NS; not significant *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 compared to normal air-exposed controls. Data were analysed with One-way ANOVA using Sidak post-test.

5.2.3 Inhibition of CS-induced miR-21 reduces small airway remodelling and improves lung function, but not emphysema, in experimental COPD

We next examined the role of miR-21 in the development of the key pathophysiological features of experimental COPD. We first assessed the effects of targeting miR-21 on small airway remodelling in experimental COPD **(Figure 5.3a)**. CS-exposed, Scr-treated (Smk+Scr) mice had markedly increased collagen deposition around the small airways compared to normal air-exposed controls (Air+Scr). Significantly, Ant-21 treatment (Smk+Ant-21) completely inhibited CS-induced small airway collagen deposition, which were returned to baseline levels observed in normal air-exposed controls. We next assessed the effects of Ant-21 treatment on CS-induced, emphysema-like alveolar enlargement **(Figure 5.3b)**. CS-exposed, Scr-treated mice had significantly increased mean alveolar diameter compared to normal air-exposed, Scr-treated controls. However, this was not suppressed by Ant-21 treatment. We next examined the effects of treatment on lung function. CS-exposed, Scr-treated mice had increased dynamic compliance and FVC, and decreased transpulmonary resistance and elastance compared to normal air-exposed, Scr-treated controls **(Figure 5.3c)**. The combined effect of these changes resulted in increased work of breathing. Significantly, treatment with Ant-21 completely inhibited these changes and restored all of the parameters of lung function back to baseline levels observed in normal air-exposed controls. Collectively these data demonstrate that CS exposure induces small airway emodelling and reduces lung function in experimental COPD through a miR-21-dependent mechanism, which can be targeted therapeutically.


Air+Scr
Smk+Scr
Smk+Ant-21
Air+Scr
Smk+Scr
Smk+Ant-21
a)
NS
*
**
W_{CT}/P_{bm} um^2/um
20
15
10
5
0
Air+Scr
Smk+Scr
Smk+Ant-21
b)
***
***
NS
Alveolar diameter (uM)
40
30
20
10
0
**
*
NS
Destructive Index (%)
60
40
20
0

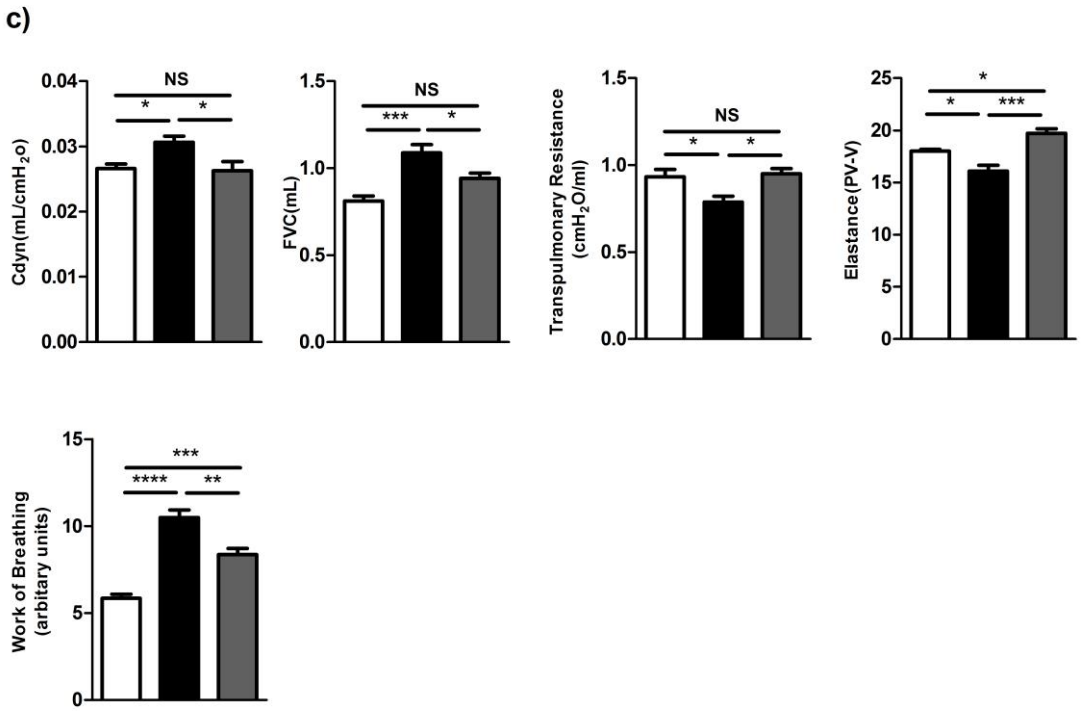


Figure 5.3: Cigarette smoke (CS)-induced miR-21 expression induces small airway remodelling and impairs lung function, but not emphysema-like alveolar enlargement, in experimental chronic obstructive pulmonary disease (COPD). BALB/c mice were exposed to CS *via* the nose-only, or to normal air, and treated intranasally with miR-21-specific antagomir (Ant-21) or scrambled (Scr) antagomir once a week for 8 weeks. **a)** Representative photomicrographs (40x magnification) of Verhoff-Van Gieson stained sections showing collagen deposition around the small airways (pink) and quantification of collagen deposition around the small airways per μm of basement membrane perimeter. **b)** Representative photomicrographs (40x magnification) of hematoxylin and eosin-stained lung sections and emphysema-like alveolar enlargement assessed using the mean linear intercept technique and destructive index. **c)** Lung function was assessed in terms of lung compliance (at 30cmH_2O), forced vital capacity (FVC), transpulmonary resistance, elastance, and work of breathing. n= 4-6 mice/group. Data are presented as means ± s.e.m. *P<0.05;

**P<0.01; ***P<0.001; ****P<0.0001 compared to normal air-exposed controls. Data were analysed with One-way ANOVA using Sidak post-test.

5.2.4 Inhibition of CS-induced miR-21 selectively inhibits canonical targets in experimental COPD

The expression of mRNA targets can be modulated by miRNAs through gene silencing. To determine the mechanistic role of CS-induced miR-21 in the pathogenesis of experimental COPD, changes in the mRNA expression of its targets were evaluated. The predicted targets of miR-21-5p were identified and their expression was assessed. With the increase in CS-induced miR-21-5p, the expression of SPRY1, SPRY2, PDCD4 and RECK **(Fig 5.4 a-d)** were decreased and the expression of TIMP3 and PTEN **(Fig 5.4 e** and **f)** were not affected compared with the CS-exposed scr treated controls (Smk+Scr). Ant-21 treatment did not increase expression of SPRY1, SPRY2 and PDCD4, whereas the expression TIMP3, RECK and PTEN was further decreased in (Smk+Ant-21) group compared with Scr treated controls.

Thus these data demonstrate that inhibition of CS-induced miR-21 does not affect the expression of its target genes

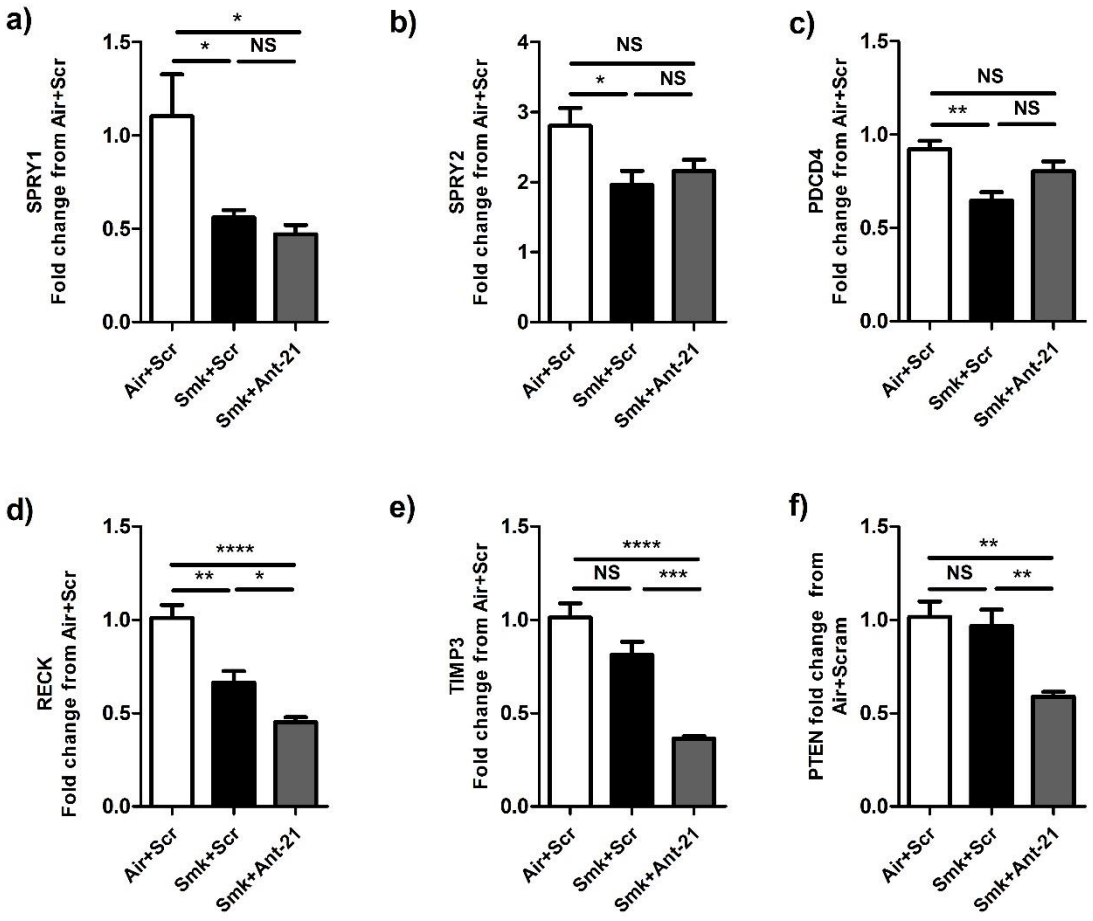


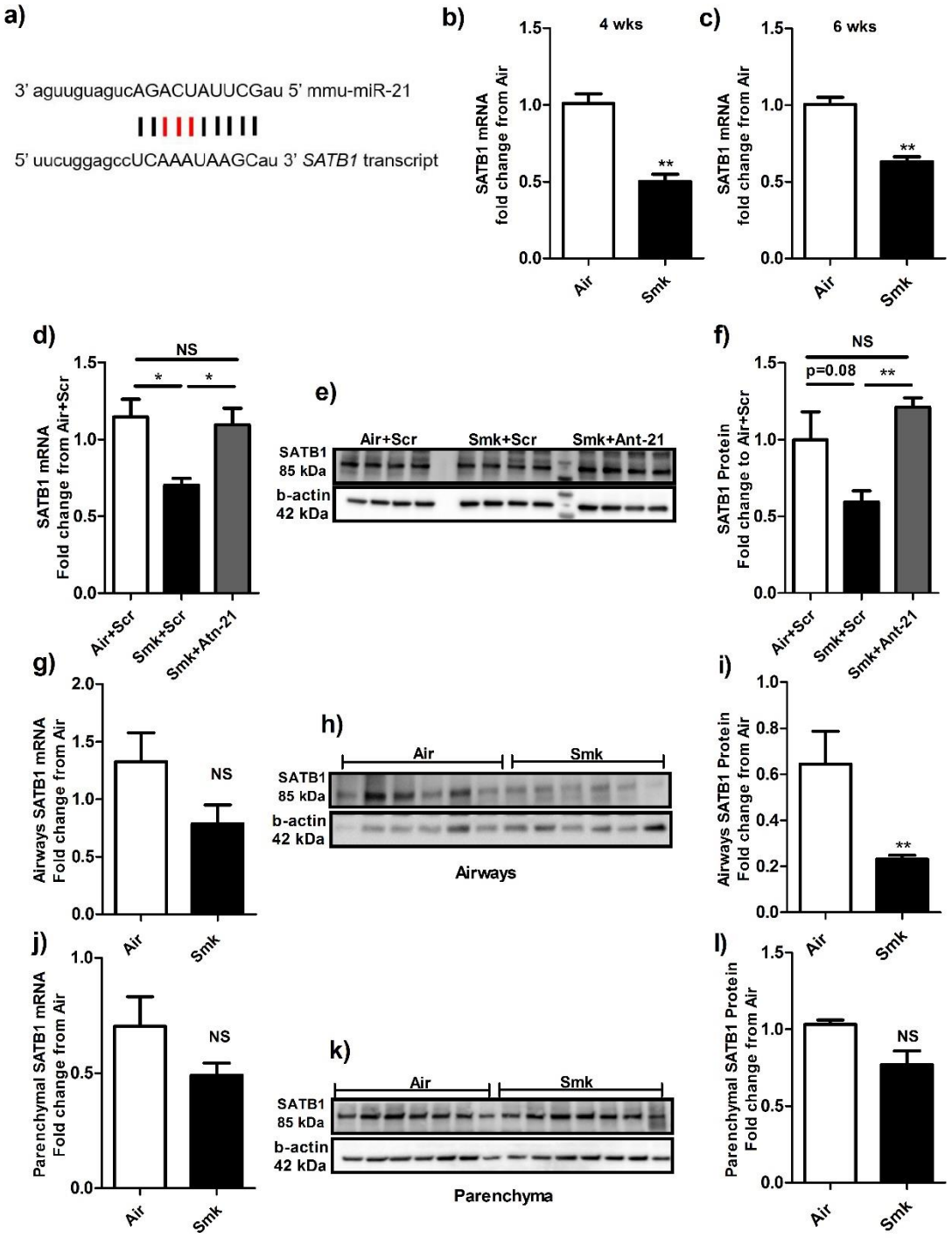
Figure 5.4: Cigarette smoke (CS)-induced miR-21 expression selectively affects the expression of canonical target genes in experimental chronic obstructive pulmonary disease (COPD). BALB/c mice were exposed to CS via the nose-only, or to normal air, and treated intranasally with miR-21-specific antagomir (Ant-21) or scrambled (Scr) antagomir once a week for 8 weeks. **(a-c)** Anti-fibrotic genes sprouty homolog 1 (SPRY1), SPRY2, programmed cell death 4 (PDCD4). **(d-f)** Tissue inhibitor of metalloproteinase (TIMP3), reversion-inducing-cysteine-rich protein with kazal motifs (RECK) and phosphatase tensin homolog (PTEN) mRNA expression in whole lung tissue was assessed by qPCR and normalised to hypoxanthine-guanine phosphoribosyltransferase (HPRT). mRNA are presented as relative abundance compared to normal air-exposed controls. n=5-6 mice/group. Data are presented as means ± s.e.m. NS; not significant *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001

compared to normal air-exposed controls. Data were analysed with One-way ANOVA using Sidak post-test.

5.2.5 CS-induced lung miR-21 targets SATB1 in experimental COPD

The 3'UTR of SATB1 possesses a putative miR-21 binding site **(Figure 5.5a)** and SATB1 is a validated target of miR-21 (220). Thus, CS-induced miR-21 may exert its effects in experimental COPD through inhibition of SATB1. Lung SATB1 mRNA expression was significantly decreased in CS-exposed mice at 4 and 6 weeks compared with normal air-exposed mice **(Figure 5.5 (b-c))**, and in CS-exposed, Scr-treated mice (Smk+Scr) compared to normal air-exposed controls (Air+Scr) at 8 weeks **(Figure 5.5d)**. Treatment with Ant-21 during CS exposure completely restored SATB1 levels back to baseline levels observed in normal air-exposed controls. Analysis of SATB1 protein revealed that levels trended towards reduction with CS-exposure in Scr treated controls ($p=0.08$), and were restored with Ant-21 treatment **(Figure 5.5 (e-f))**. We next assessed SATB1 mRNA and protein levels in the airways and parenchyma. SATB1 mRNA showed trends towards reduced levels in airways and SATB1 protein was significantly suppressed in airways in CS-exposed groups **(Figure 5.5 (g-i))**. SATB1 mRNA and protein both showed trends towards reduction in parenchyma **(Figure 5.5 (i-l))**. We then assessed SATB1 levels in whole lung sections using IF. SATB1 protein was localized primarily in the airway epithelium with some evidence of positive staining in the parenchyma **(Figure 5.5 (m-n))**. With CS-exposure SATB1 protein decreased in both airways and parenchyma in Scr-treated compared with normal air-exposed controls. Ant-21 treatment increased SATB1 protein in both the airways and parenchyma (Smk+Ant-21).

These data show that concomitant with increased miR-21 expression there is a reduction in its target SATB1 that is reversed in both the airways and parenchyma with Ant-21 treatment.


a)
3' aguuguagucAGACUAUUCGau 5' mmu-miR-21
5' uucuggagccUCAAAUAAGCau 3' SATB1 transcript
b)
4 wks
SATB1 mRNA fold change from Air
Air
Smk
c)
6 wks
SATB1 mRNA fold change from Air
Air
Smk
d)
NS
SATB1 mRNA Fold change from Air+Scr
Air+Scr
Smk+Scr
Smk+Atn-21
e)
Air+Scr
Smk+Scr
Smk+Ant-21
SATB1 85 kDa
b-actin 42 kDa
f)
NS
p=0.08
SATB1 Protein Fold change to Air+Scr
Air+Scr
Smk+Scr
Smk+Ant-21
g)
Airways SATB1 mRNA Fold change from Air
NS
Air
Smk
h)
Air
Smk
SATB1 85 kDa
b-actin 42 kDa
Airways
i)
Airways SATB1 Protein Fold change from Air
Air
Smk
j)
Parenchymal SATB1 mRNA Fold change from Air
NS
Air
Smk
k)
Air
Smk
SATB1 85 kDa
b-actin 42 kDa
Parenchyma
l)
Parenchymal SATB1 Protein Fold change from Air
NS
Air
Smk

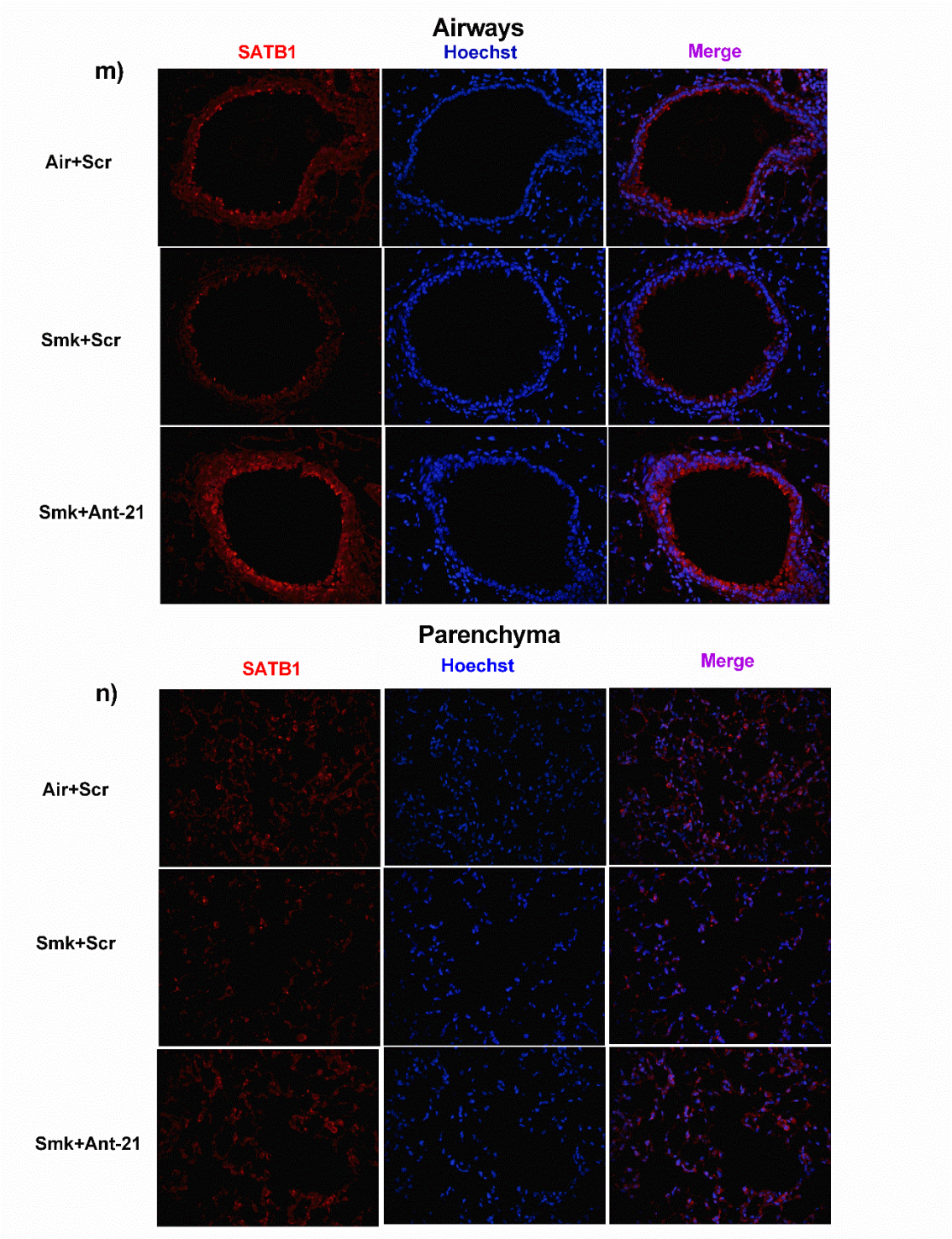


Figure 5.5: Cigarette smoke (CS)-induced miR-21 expression reduces SATB1 transcript and protein levels in experimental chronic obstructive pulmonary disease (COPD). a) Seed sequence of miR-21 binding to the 3' UTR of SATB1. BALB/c mice were exposed to CS *via* the nose only, or normal air, for 4 and 6 weeks. **(b** and **c)**

SATB1 mRNA expression in whole lung was assessed by qPCR and normalised to hypoxanthine-guanine phosphoribosyltransferase (HPRT). BALB/c mice were exposed to CS *via* the nose-only or to normal air and treated intranasally once a week with either Ant-21 or scrambled (scr) control (2.5 kg/mg) for 8 weeks. **d)** SATB1 mRNA expression in whole lung tissue was assessed by qPCR and normalised to HPRT. **(e** and **f)** Whole lung SATB1 protein levels were evaluated by immunoblot and densitometry**. ((g**-**i)** and **(j-k))** SATB1 mRNA expression and protein levels were assessed in airways and parenchyma respectively by qPCR and normalised to HPRT and by immunoblot and densitometry. Representative photomicrographs (40x magnification) of immunofluorescence detection of SATB1 in **(m)** airways and **(n)** parenchyma. n=4-8, 5-6, 4 and 6-8 mice/group for **(b** and **c), d) (e, f, m** and **n)** and **(g-l)** respectively. Data are presented as means ± s.e.m. NS; not significant, *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 compared to normal air-exposed controls. Data **(b, c,** and **g-l)** and **(d** and **f)** were analysed with two-tailed Mann Whitney test and One-way ANOVA using Sidak post-test respectively.

5.2.6 In pulmonary tissue, airway epithelium expresses higher SATB1 mRNA than parenchymal tissue.

We assessed the relative expression of SATB1 in normal air-exposed and CS-exposed airways and parenchyma at 8 weeks of CS-exposure. SATB1 expression was significantly higher in both normal air-exposed and CS-exposed airway epithelium than normal air-exposed and CS-exposed parenchyma respectively.

This data demonstrate that SATB1 mRNA is more abundant in airways than parenchyma.

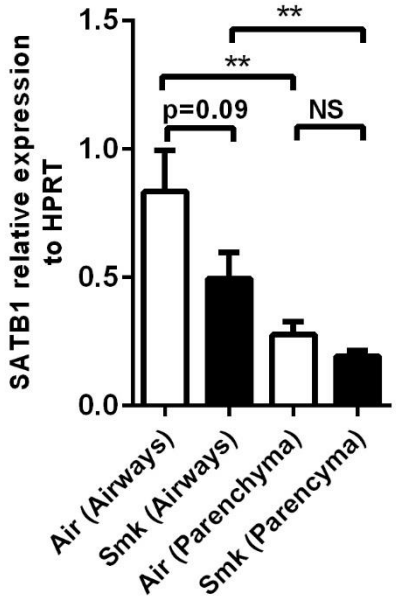


Figure 5.6: SATB1 expression is higher in airway epithelium than parenchyma in both normal air and cigarette smoke (CS)-exposed groups. BALB/c mice were exposed to CS or normal air *via* the nose-only for 8 weeks. SATB1 relative expression was assessed in blunt dissected airways and parenchyema and normalised to hypoxanthine-guanine phosphoribosyltransferase (HPRT). mRNA are presented as relative expression compared to normal air-exposed controls. n=5-6 mice/group. Data are presented as means ± s.e.m. NS; not significant, **P<0.01 compared to controls. Data were analysed with two-tailed Mann Whitney test.

5.2.7 S100A9 expression and NF-κB activity are increased in experimental COPD, and are suppressed by inhibiting miR-21 expression

S100A8 and S100A9 are pro-inflammatory mediators and potent neutrophil chemoattractants that are involved in the pathogenesis of several chronic inflammatory diseases. We therefore assessed lung mRNA expression of S100A8 and S100A9 in experimental COPD. With CS-exposure the expression of S100A9 was significantly increased in Scr-treated (Smk+Scr) compared to normal air-exposed controls **(Air+Scr; Figure 5.7a)**. In contrast, S100A8 expression was not altered **(Figure 5.7b)**. Treatment with Ant-21 reduced both S100A8 and S100A9 expression (Smk+Ant-21). Next we

determined S100A9 and S100A8 expression in the airways and parenchyma. Consistent with the data from whole lung tissue, S100A9 mRNA was up-regulated in CS-exposed airways and parenchyma **(Figure 5.7 (c-d))**, however, S100A8 expression was not affected **(Figure 5.7(e-f))**. We next evaluated NF-κB activity. With CS-exposure, NF-κB activity increased in Scr-treated controls and treatment with Ant-21 completely inhibited NF-κB activity back to baseline levels observed in normal air-exposed controls **(Figure 5.7g)**.

Collectively these data show that CS exposure increased S100A9 expression and NF-κB activity that are inhibited with Ant-21 treatment.

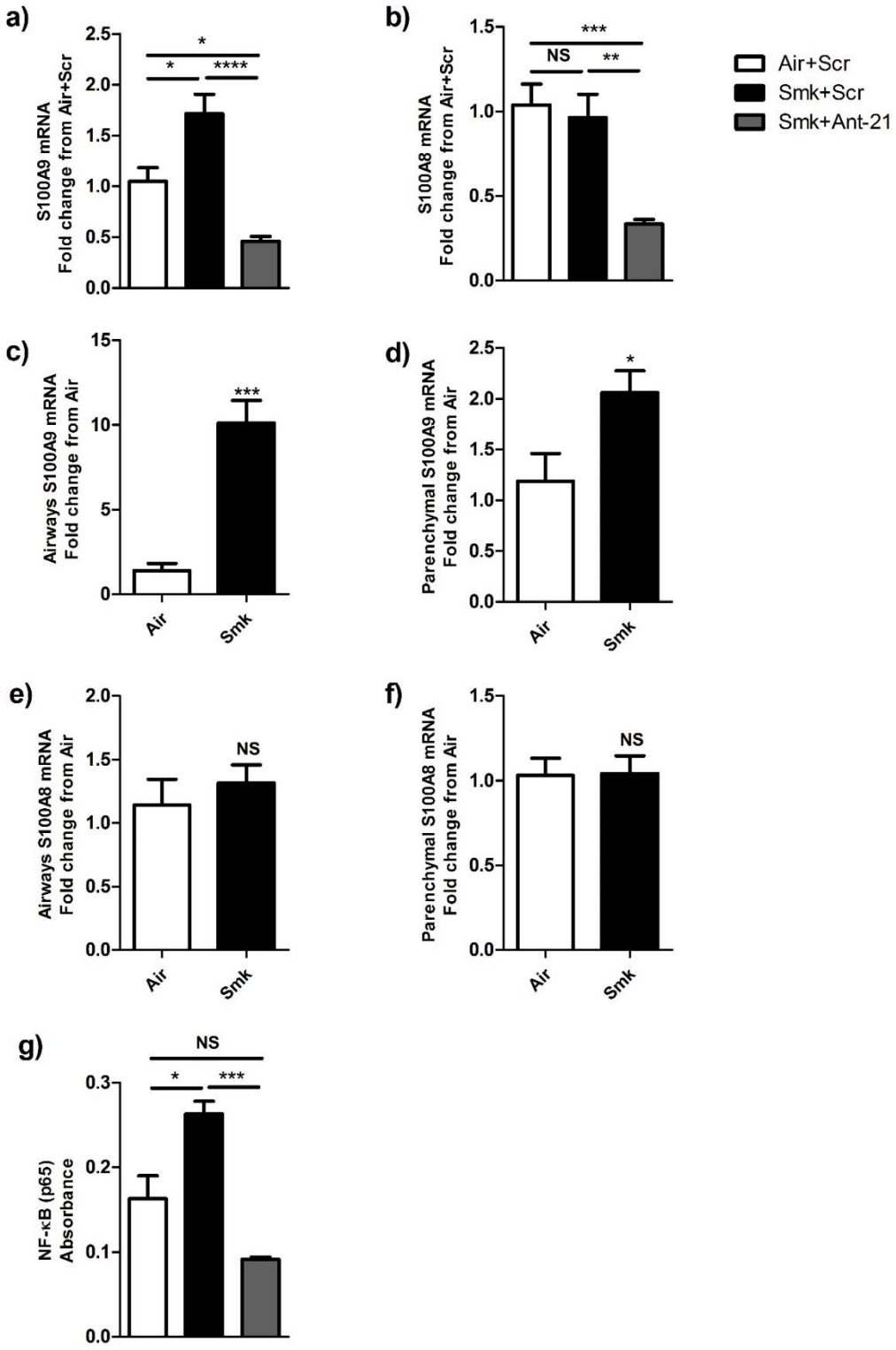

a)
S100A9 mRNA
Fold change from Air+Scr
2.5
2.0
1.5
1.0
0.5
0.0
*
*
****
b)
S100A8 mRNA
Fold change from Air+Scr
1.5
1.0
0.5
0.0
***
NS
**
Air+Scr
Smk+Scr
Smk+Ant-21
c)
Airways S100A9 mRNA
Fold change from Air
15
10
5
0
***
Air
Smk
d)
Parenchymal S100A9 mRNA
Fold change from Air
2.5
2.0
1.5
1.0
0.5
0.0
*
Air
Smk
e)
Airways S100A8 mRNA
Fold change from Air
2.0
1.5
1.0
0.5
0.0
NS
Air
Smk
f)
Parenchymal S100A8 mRNA
Fold change from Air
1.5
1.0
0.5
0.0
NS
Air
Smk
g)
NF-κB (p65)
Absorbance
0.3
0.2
0.1
0.0
NS
*
***

Figure 5.7: Cigarette smoke (CS)-induced miR-21 expression attenuates S100A9 transcript and NF-κB (p65) activity in experimental chronic obstructive pulmonary disease (COPD). BALB/c mice were exposed to CS *via* the nose-only or to normal air and treated intranasally once a week with either Ant-21 or scrambled (scr) control (2.5 kg/mg) for 8 weeks. S100A9 and S100A8 mRNA expression in whole lung tissue **(a, b)** or airways **(c, e)** and parenchyma **(d, f)** was assessed and by qPCR normalised to hypoxanthine-guanine phosphoribosyltransferase (HPRT). **g)** NF-κB (p65) activity in whole lung homoganates was quantified using DNA-binding ELISA. n=5-6, 6-8 and 4 mice/ group for **(a-b), (c-f)** and **g)** respectively. Data are presented as means ± s.e.m. NS; not significant, *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 compared to normal air-exposed controls. Data **(a-b** and **g)** and **(c-f)** were analysed with One-way ANOVA using Sidak post-test and two-tailed Mann Whitney test respectively.

5.2.8 Inhibition of CS-induced miR-21 does not affect pAKT levels in experimental chronic obstructive pulmonary disease (COPD).

miR-21 also regulates NF-κB activation through its target PTEN and its downstream regulator AKT. To determine miR-21 and the downstream regulator pAKT induced activation of NF-κB, we assessed the protein levels of pAKT with Ant-21 treatment. CS-exposed, Scr-treated (Smk+Scr) group showed reduced pAKT levels compared to air-exposed, Scr-treated controls. However, the levels of pAKT were not restored with Ant-21 treatment in (Smk+Ant-21) group **(Air+Scr; Figure 5.8 (a** and **b))**.

Thus this data suggests that miR-21 inhibition does not affect pAKT levels in experimental COPD.

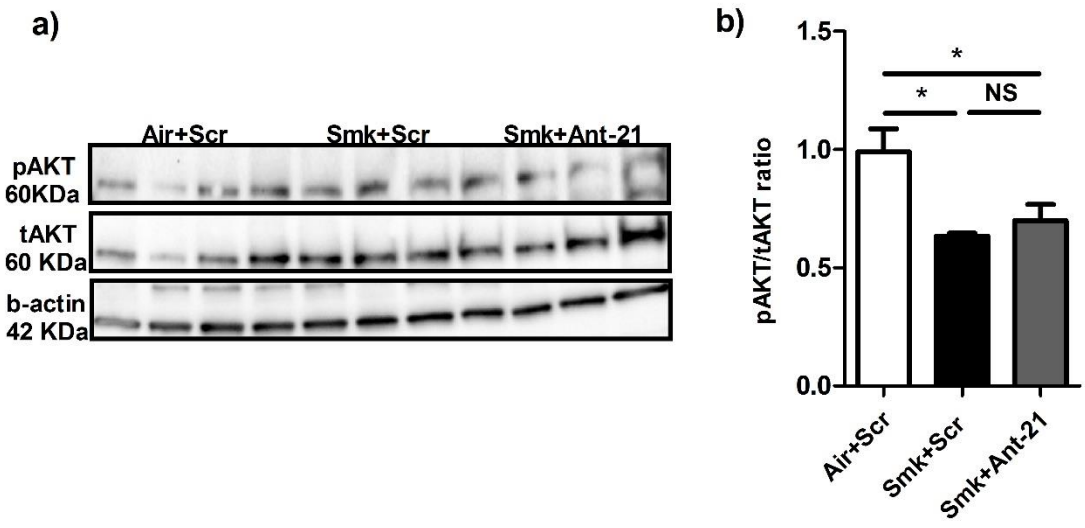


Figure 5.8: Cigarette smoke (CS) induced miR-21 reduces pAKT levels in experimental chronic obstructive pulmonary disease (COPD). BALB/c mice were exposed to CS *via* the nose-only or normal air and treated intranasally once a week with either Ant-21 or scrambled (scr) control (2.5 kg/mg) for 8 weeks. **(a and b)** whole lung pAKT protein levels were evaluated by immunoblot and densitometry. n=3-4 mice/group. Data are presented as means ± s.e.m. NS; not significant, *P<0.05 compared to controls. Data was analysed with One-way ANOVA using Sidak post-test.

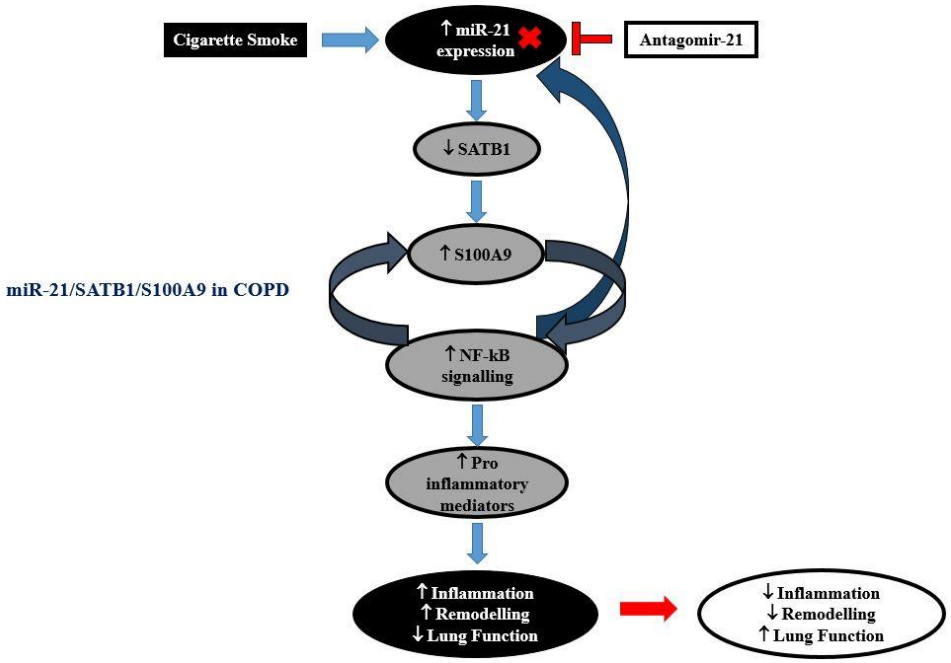


Figure 5.9: miR-21 mediated inflammatory pathways in chronic obstructive pulmonary disease (COPD). Cigarette smoke exposure increases miR-21 expression. Increased miR-21 expression inhibits SATB1 promoting S100A9 stimulation and induction of NF-κB activity thereby inducing inflammation, airway remodelling, and impairing lung function in COPD. Inhibition of smoke induced miR-21 with Ant-21 reverses these events by restoring SATB1 which in turn suppresses S100A9 mediated increases in NF-κB activity reducing inflammation and small airway remodelling and improving lung function in COPD.

5.3 DISCUSSION

Here we demonstrate that CS exposure induces a miR-21/SATB1/S100A9/NF-κB axis in the lungs that promotes the cardinal features of COPD, including chronic airway inflammation, small airway remodelling, and impaired lung function (72, 162, 254-257). We show that increased expression of miR-21 occurred throughout CS exposure in experimental COPD. We then used a highly specific and potent miR-21 inhibitor, Ant-21, to define a previously unrecognized role for miR-21, and its downstream target SATB1 in experimental COPD. Ant-21 treatment restored SATB1 levels with

concomitant reductions in S100A9 expression and NF-κB activity resulting in decreased pulmonary inflammation, small airway remodelling and improved lung function in experimental COPD. This study advances the understanding of the pro-inflammatory role of miR-21 in COPD and identifies its potential for therapeutic targeting.

Ours is the first study to identify an association between miR-21 expression in the lung and its potential as a therapeutic target. Increased miR-21 expression has been linked with several inflammatory diseases, including COPD (317). However, the role of miR-21 in immune responses is complex due in part to its range of potential targets as well as its expression in different cell types and its ability to induce both pro-inflammatory and anti-inflammatory effects. For instance miR-21 is associated with inflammation in immune cells (315), but also with senescence in endothelial cells (325). For its known activity, the increase in miR-21 expression in response to CS may contribute to disease pathogenesis by activating immune responses, increasing pro-inflammatory mediators (315, 326) and reducing tissue repair leading to remodelling (327). In this study we demonstrate persistent increase in miR-21 in the lungs of CS-exposed experimental model of COPD and at the late phase of disease we observed increased miR-21 expression in both airway epithelium and parenchyma. This indicates a potential role of miR-21 in the pathogenesis of COPD. Our data are consistent with a study by Xie *et al*., (317), which demonstrates that CS exposure results in elevated lung miR-21 expression in rats as well as in the serum of COPD patients (317). However, their study was limited to the observation of increased miR-21 expression. Whereas we demonstrated a pathogenic role for miR-21 in the induction of inflammation, remodelling and impaired lung function in COPD, and that this occurs through the inhibition of SATBI and induction of S100A9 and NF-KB.

To investigate the role of miR-21 in the pathogenesis of COPD, we inhibited CS-induced miR-21 expression in experimental COPD with Ant-21. Through *in situ* hybridisation (ISH) we reconfirmed increase in miR-21 and also its reduction with Ant-21 treatment, in both the airways and parenchyma. Inhibition of CS-induced miR-21 in the

lungs reduced airway inflammation, suggesting that miR-21 promotes inflammation in COPD. This was demonstrated by the decrease in inflammatory cell numbers in BALF, reduced expression of pro-inflammatory mediators CXCL2, chemokine (C-C motif) ligand 7 (CCL7), granulocyte-macrophage colony-stimulating factor (GM-CSF), and matrix metalloproteinase12 (MMP12) and reduced TNFα and CXCL1 levels in the lungs. TNFα, CXCL1 and CXCL2 are potent neutrophil attractants (296, 298). Mercer *et al.*, demonstrated that CCL7 induces accumulation of neutrophils in the lungs of naïve mice (56). MMP12 also regulates neutrophilic inflammation in the lungs of CS-exposed mice (297). Thus, with the inhibition of CS-induced miR-21 in our COPD model, these neutrophil chemoattractants were reduced thereby decreasing neutrophil influx in BALF suggesting that miR-21 potentially mediates neutrophilic inflammation in COPD.

We observed decreased expression of some miR-21 targets in the lung during experimental COPD, including SPRY1, SPRY2, RECK, PDCD4 but not of others such as TIMP3 and PTEN However, despite the physiological effects of treatment with Ant-21, there was no restoration of expression of SPRY1, SPRY2, RECK, PDCD4. In fact, the expression of TIMP3, RECK and PTEN were further decreased by treatment. Similarly, in a study on rectal cancer, analysis of RNA-sequencing data did not show an inverse correlation of miR-21 and its targets PTEN, SPRY2 and PDCD4 (328). This is because miRs not only induce suppression but can also activate the expression of their target genes (329). Studies have shown that miR mediated suppression or activation of target genes is associated with effector molecules known as ribonucleoproteins (miRNP) (330). Kedde *et al.*, showed that miR mediated suppression is negated due to the binding of dead end 1 (Dnd1) to the sites of target genes (331) and showed that binding of the regulator called pumilio, increased miR-mediated suppression of the target mRNAs (332).

The role of miR-21 is well characterised in remodelling diseases such as idiopathic pulmonary small airway remodelling (327). miR-21 promotes cardiac and hepatic small airway remodelling by negatively regulating anti-fibrotic SPRY1 (333),

SPRY2 (334), and PDCD4 (335). In this study, we demonstrated that CS-induced miR-21 expression promotes collagen deposition around small airways albeit through a currently unknown mechanism since Ant-21 treatment had no effect on the expression of these anti-fibrotic genes.

We next assessed the effects of depleting CS-induced miR-21 on impaired lung function. Treatment with Ant-21 improved lung function in experimental COPD by decreasing lung compliance and FVC, increasing trans-pulmonary resistance and elastance, resulting in reduced work of breathing. This improvement of impaired lung function can be linked to the reduction in inflammation and small airway remodelling (90, 336). However, with the suppression of miR-21, we did not observe restoration of parenchymal tissue destruction and improvement in emphysematous condition.

SATB1 is a target of miR-21 (220) and gene expression microarray analysis of our model of experimental COPD showed that *SATB1* is down regulated (2.6 fold) in CS-exposed mice compared to normal air-exposed controls (unpublished data). With increased miR-21 from 4 weeks to 8 weeks of CS exposure, we also showed concomitant decreases in SATB1 expression suggesting that miR-21-dependent, SATB1-mediated effects are involved in experimental COPD. Here we show that chronic CS exposure decreases lung SATB1 mRNA and protein levels in both airway and parenchymal compartments. This is consistent with reduced SATB1 expression following increased miR-21 expression in other situations (328, 337). We also show that SATB1 expression is more enriched in the airway epithelium compared with parenchyma and Selinger *et al.*, have previously reported that decreased SATB1 levels are an indicator of poor prognosis in squamous cell carcinoma (228). SATB1 potentially regulates a large number of immune functions. SATB1 deficient mice exhibit de-repression of ~2% of a subset of genes in thymocytes, including proto-oncogenes, cytokines and cytokine receptor genes, and apoptosis-related genes (338). These effects are likely to be induced through SATB1-dependent epigenetic regulation since SATB1 can acetylate histone H3 (339), which is implicated in the pathogenesis of COPD and other lung

diseases (340). Thus, it is likely that miR-21-dependent regulation of SATB1 levels in the airway epithelium play important roles in the pathogenesis of COPD.

We demonstrate that targeted inhibition of CS-induced miR-21 expression results in decreased airway inflammation and small airway remodelling in experimental COPD. Importantly, these effects were associated with a concomitant increase in lung SATB1 levels. In a recent study, liver small airway remodelling in rats was reduced by over-expressing SATB1 (341), suggesting that decreased SATB1 may promote pro-fibrotic responses. Ant-21 treatment during CS exposure did not alter the expression of anti-fibrotic genes with putative binding sites for miR-21 but increased SATB1 expression. Thus, in experimental COPD, CS-induced small airway remodelling may not be induced by miR-21 regulated anti-fibrotic genes and is potentially mediated by decreased SATB1 in airway epithelium. This may be due to the fact that SATB1 is a transcription factor regulating the expression of many genes, which can potentially mediate inflammation and small airway remodelling in CS-induced COPD.

Since CS-induced miR-21 expression induces airway inflammation in experimental COPD, and Ant-21 treatment restores lung SATB1 levels, we interrogated the role of potential downstream pro-inflammatory mediators. SATB1 activity can negatively regulate S100A8 and S100A9 responses (324), and SATB1-null mice have increased S100A8 and S100A9 expression (342, 343). Significantly, S100A9 is a reported potential biomarker for several inflammatory diseases such as rheumatoid arthritis (344), severe neutrophilic asthma (345), myocardial infarction (346) and Alzheimer's disease (347). Thus, we next assessed lung S100A8 and S100A9 expression during CS-induced experimental COPD. We are the first to show that CS-induced increase in miR-21 expression is associated with a concomitant decrease in SATB1 and increase in S100A9 activity in experimental COPD and observe this in both the airways and parenchyma. Significantly, S100A9 levels are elevated in bronchial epithelial cells (348) and BALF of COPD patients (349), which implicates increased S100A9 levels in pathogenesis. In the current study CS exposure did not alter the

expression of S100A8, suggesting that the co-expression of S100A8 and S100A9 is not essential for the functioning of S100A9. A similar observation was reported by Xu and Geczy (350) where they showed that murine macrophages stimulated with LPS, IFN-ɣ or TNF did not co-induce the expression of S100A9 with S100A8. Targeted inhibition of lung miR-21 during CS exposure increased SATB1 levels, and decreased S100A9 expression, back to baseline normal air-exposed control levels. These data suggest that S100A9 expression is negatively regulated by SATB1 responses in COPD.

We, and others, have demonstrated that CS exposure induces NF-κB activation (255, 351). Interestingly, elevated S100A9 responses can induce NF-κB activation in a TLR4- and RAGE-dependent manner, and results in increased pro-inflammatory responses (63, 352, 353). NF-κB responses can also induce the expression of S100A9 (322) and miR-21 (321), suggesting that the miR-21/SATB1/S100A9/NF-κB axis **(Figure 5.9)** we have identified, has the capacity to self-perpetuate inflammatory responses. NF-κB inhibition studies have been carried out to limit inflammatory responses. While some observed decrease in CS-induced inflammation (354) others used a chronic CS-exposed mouse model to show that inhibiting NF-κB or kB kinase-2(IKK2) has minor effects on CS-induced airway inflammation (355). We show that depleting CS-induced miR-21 to basal levels restores SATB1 expression with associated reductions in S100A9 expression and activated NF-κB levels resulting in reduced inflammation, small airway remodelling and improved lung function in experimental COPD. There are other targets of miR-21 such as PTEN which can mediate NF-κB activation through AKT. We did not observe changes in the expression of PTEN in CS-exposed mice. The levels pAKT, which is a downstream regulator of NF-κB (318) was decreased with CS-exposure but there was no change in pAKT levels with Ant-21 inhibition of miR-21. Thus we show that S100A9 is the likely activator and effector of NF-κB mediated inflammation in CS-induced experimental COPD. Nonetheless there are other targets of miR-21 that could potentially mediate NF-κB activation and further studies should explore this.

In summary, our study demonstrates for the first time that CS-induced miR-21 promotes airway inflammation and small airway remodelling and impairs lung function in experimental COPD. Inhibition of miR-21 to basal levels has broad therapeutic relevance to CS-induced COPD and may be more effective than targeting downstream mediators such as NF-κB. Although these findings advocate a novel mechanistic pathway in CS-induced experimental COPD, further functional studies with recombinant SATB1 or siRNA against S100A9 would be interesting, to determine if similar findings will be observed. Additional studies have to be undertaken to translate the current prophylactic regime into a therapeutic regime. Thus this study identifies miR-21 as a novel potential therapeutic target in the treatment of CS-induced COPD.

CHAPTER 6: ROLE OF miR-135b AND miR-146b IN THE PATHOGENESIS OF EXPERIMENTAL CHRONIC OBSTRUCTIVE PULMONARY DISEASE

6.1 INTRODUCTION

We have identified miR-135b and miR-146b to be up-regulated in microarray-based miR profiling studies in experimental COPD. The role of miR-135b in the pathogenesis and/or exacerbation of COPD is not completely characterised. Halappanavar *et.al,* recently demonstrated that the expression of miR-135b is increased in the lungs of mice exposed to CS (238) and titanium dioxide nanoparticles (356). The authors then used IL-1R1 knockout mice to show that miR-135b can also inhibit CS-induced, IL-1R1-dependent, acute inflammation through a negative feedback loop (238). In contrast, in a study on non-small-cell lung cancer, the authors demonstrated that miR-135b is NF-κB-regulated indicating the potential association of the miR-135b with inflammatory responses (357), suggesting that miR-135b may play an important role in COPD pathogenesis that needs further investigation.

Importantly, some predicted mRNA targets of miR-135b, such as hypoxia inducible factor (HIF) 1α, are known to be involved in the pathogenesis of COPD. The transcription factor, HIF1α is known to regulate the expression of VEGF (358) which is reduced in COPD patients (359-361). Increase in HIF1α level also induces the expression of numerous genes reversing tissue injury (362). However, its levels are decreased in patients with COPD, suggesting that it may play an important role in protecting against COPD (358, 363). Sirtuin (SIRT) 1 is another putative target of miR-135b. SIRT1 plays an important role in regulating the protease: anti-protease balance between matrix metalloproteinase (MMP)-9 and tissue inhibitor of matrix metalloproteinases (TIMP)1 (364). Significantly, SIRT1 levels are markedly reduced in patients with COPD and altered SIRT1 activity is strongly implicated in the development of emphysema (364, 365). miR-135b can also target transforming growth factor-beta

receptor (TGF-βR)2 and bone morphogenetic protein receptor (BMPR)2 (366). TGF-β/BMP signalling regulates numerous functions relating to cell survival, apoptosis, immune responses and remodelling (367). Collectively, these studies show that miR-135b is linked to several key immunomodulatory mechanisms that are involved in COPD and implicate its dysfunction in the pathogenesis of this disease.

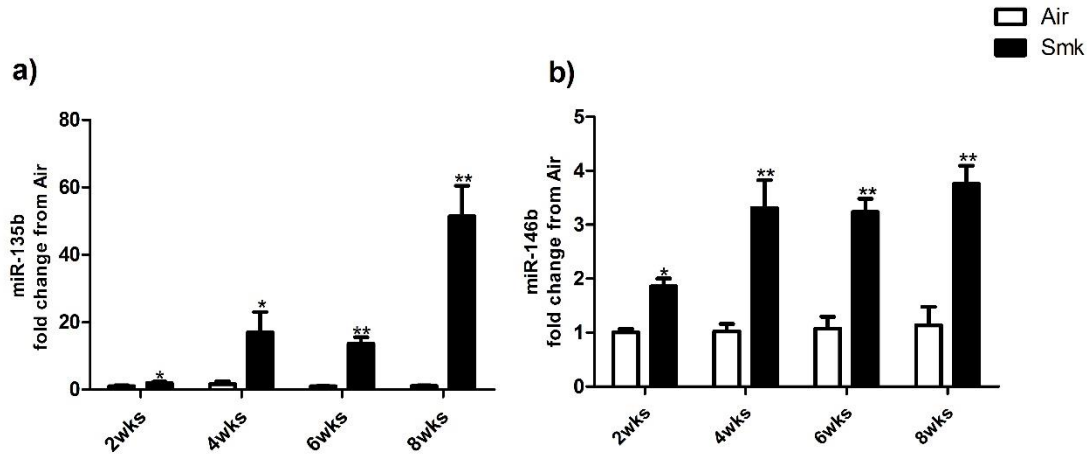
miR-146b is associated with inflammation and negatively regulate innate immune responses (368) by targeting the signal transducers IL-1 receptor associated kinase (IRAK)1 and tumour necrosis factor receptor-associated factor (TRAF)6 (368). While the role of miR-146b in COPD is unknown its expression can be induced in cultured human lung epithelial cells (369, 370), airway smooth muscle cells (371), and endothelial cells (372). These miR-146b expressing cells are known to play important roles in COPD, which suggests a potential role for miR-146b in COPD pathogenesis.

In this study, we investigated the roles of CS-induced miR-135b and miR-146b in the pathogenesis of experimental COPD. miR-135b and miR-146b expression were increased with acute and chronic CS-exposure and were implicated in the development of disease. CS-induced increases in the expression of miR-135b coincided with decreased expression of its target BMPR2 and in association with increased macrophage infiltration and collagen deposition. Targeted depletion of miR-135b during CS exposure decreased neutrophilic airway inflammation as well as emphysema-like alveolar enlargement. Inhibition of miR-146b resulted in a restoration of IRAK1 and TRAF6 expression and these effects were associated with decreases in small airway and emphysema-like alveolar enlargement, and improved lung function in experimental COPD.

6.2 RESULTS

6.2.1 CS-exposures induces persistent increase in miR-135b and miR-146b expression in experimental COPD

Based on the microarray-based miR profiling described in chapter 2, we isolated total lung RNA isolated from BALB/c mice exposed to CS or normal air for 2, 4, 6 or 8



weeks. Both miR-135b and miR-146b were significantly up-regulated throughout the time course of CS-exposure, compared to normal air-exposed controls. Increased miR-135b and miR-146b expression was validated by qPCR at 2, 4, 6, and 8 weeks of CS exposure **(Figure 6.1 (a** and **b))**.

Thus, acute and chronic CS exposure increases miR-135b and 146b expression in the lungs in experimental COPD.

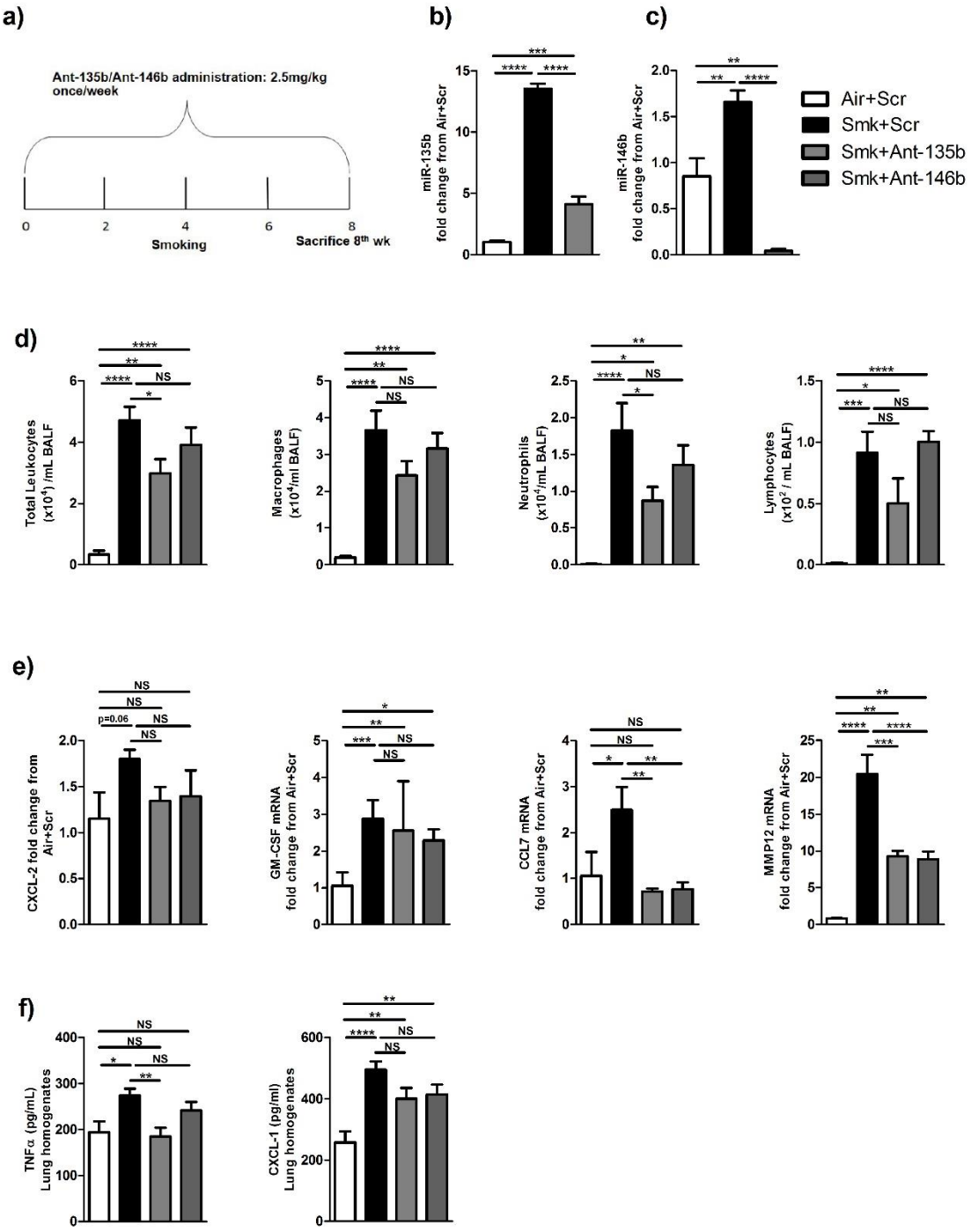
Figure 6.1: Cigarette smoke (CS) exposure increases lung miR-135b and miR-146b expression in experimental chronic obstructive pulmonary disease (COPD). BALB/c mice were exposed to CS *via* the nose-only, or normal air, for 2, 4, 6 and 8 weeks. **a)** miR-135b and **b)** miR-146b expression was normalised to the geometric mean of the housekeeping controls small nuclear RNA (SnRNA) U6 and small nucleolar RNA (SnoRNA) U49. miR-135b and miR-146b expression is expressed as fold change from normal air-exposed controls. n = 4-8 mice/group. Data are presented as means ± s.e.m, NS = not significant; *P<0.05; **P<0.01 compared to normal-air-exposed controls. Data were analysed by two-tailed Mann Whitney test.

6.2.2 CS-induced airway inflammation is reduced by inhibition of miR-135b, but not with miR-146b expression, in experimental COPD

We next investigated the roles of miR-135b and miR-146b in experimental COPD. CS-exposed mice were administered Ant-135b, Ant-146b or scrambled antagomir (i.n.) once a week for eight weeks **(Figure 6.2a)**, and pulmonary inflammation was assessed. Scr-treated (Smk+Scr) mice had increased expression of miR-135b and miR-146b in lungs compared to normal air-exposed, Scr-treated controls at 8 weeks **(Figure 6.2 (b** and **c))**. Treatment with Ant-135b and Ant-146b significantly reduced miR-135b **(Smk+Ant-135b; Figure 6.2b)** and miR-146b **(Smk+Ant-146b; Figure 6.2c)** expression, respectively, compared to CS-exposed, Scr-treated (Smk+Scr) controls. Ant-146b treatment inhibited miR-146b expression to below baseline levels in normal air-exposed controls.

We then assessed the role and potential for therapeutic targeting of increased miR-135b and miR-146b expression in experimental COPD. Ant-135b treatment suppressed CS-induced airway inflammation by reducing the numbers of total leukocytes and neutrophils, and there was a non-statistically significant trend towards suppression of macrophages and lymphocyte numbers **(Figure 6.2d)**. Treatment with Ant-146b had no effect on the number of inflammatory cells present in the airways in experimental COPD.

Next, we assessed the expression of CS-induced lung pro-inflammatory mediators. CXCL2 and the COPD related factor, GM-CSF were not significantly reduced by treatment with Ant-135b or Ant-146b (although there were trends towards suppression), but both the treatments resulted in substantial attenuation of CCL7 and MMP-12 expression **(Figure 6.2e).** TNFα levels were significantly decreased with Ant-135b treatment, but not with miR-146b inhibition. Interestingly, CXCL1 levels were not significantly suppressed by either treatment **(Figure 6.2f).**



Collectively, these data demonstrate that CS-induced miR-135b expression but not miR-146b expression in the lungs, plays an important role in promoting airway inflammation in experimental COPD.

Figure 6.2: Cigarette smoke (CS)-induced miR-135b but not miR-146b expression promotes pulmonary inflammation in experimental chronic obstructive pulmonary

disease (COPD). a) BALB/c mice were exposed to CS *via* the nose-only, or normal air, and treated intranasally with miR-135b-specific (Ant-135b) or miR-146b-specific (Ant-146b) antagomir or scrambled (Scr) antagomir once a week for 8 weeks. miR-135b and miR-146b expression was normalised to the geometric mean of the housekeeping controls (SnRNA) U6 and (SnoRNA) U49. miR-135b, miR-146b and mRNA are presented as fold change from normal air-exposed controls. **b)** miR-135b and **c)** miR-146b expression in the lungs. **d)** Total leukocyte, macrophage, neutrophil and lymphocyte numbers in bronchoalveolar lavage fluid. **e)** (C-X-C motif) ligand 2 (CXCL2), granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokine (C-C motif) ligand (CCL) 7 and matrix metalloproteinase (MMP) 12 expression in whole lung homogenates. **f)** Tumour necrosis factor (TNF) α and CXCL1 protein levels in lung homogenates. n = 5-6 mice/group. Data are presented as means ± s.e.m. NS= not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 compared to normal air-exposed controls. Data were analysed with One-way ANOVA (uncorrected Fishers LSD test)

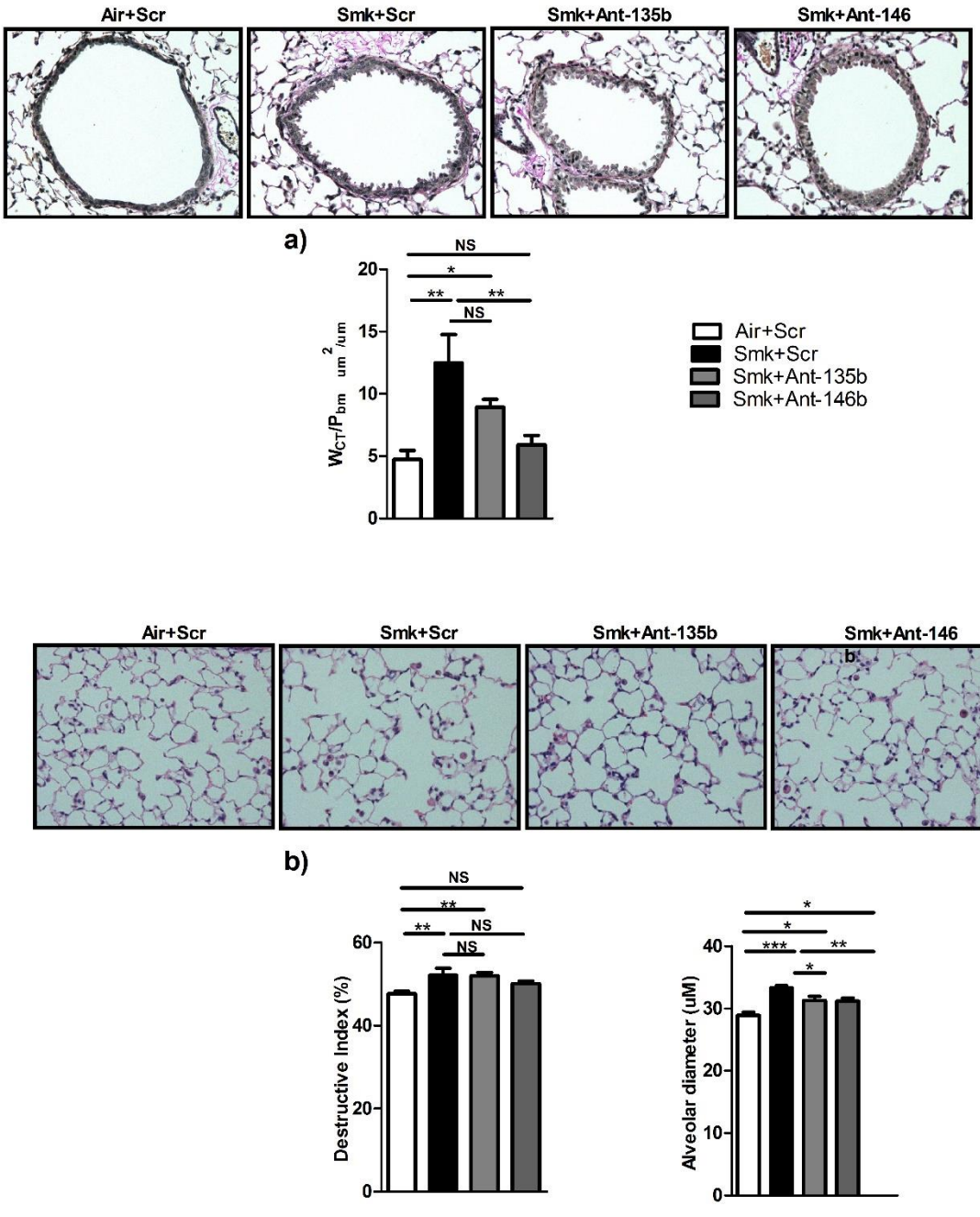
6.2.3 CS-induced miR-135b and miR-146b expression promotes small airway remodelling and emphysema in experimental COPD

We next assessed the roles of miR-135b and miR-146b in the induction of the pathophysiological features of experimental COPD. Treatment with Ant-135b trended towards inducing a significant decrease in collagen deposition around the airways, compared to Scr-treated, CS-exposed controls (Smk+Scr). Treatment with Ant-146b completely suppressed CS-induced collagen deposition around the airways to close to baseline levels observed in normal air-exposed controls **(Figure 6.3a**).

We next assessed the effects of Ant-135b and Ant-146b treatments on CS-induced, emphysema-like alveolar enlargement. Scr-treated (Smk+Scr) mice had increased emphysema-like alveolar enlargement. Significantly, treatment with Ant-135b and Ant-146b completely inhibited CS-induced emphysema **(Figure 6.3b)**.

We next examined the effects of treatment on lung function. Treatment with Ant-135b suppressed work of breathing and treatment with Ant-146b broadly attenuated the impairment of lung function with reductions in total lung capacity (TLC), forced vital capacity (FVC) and work of breathing **(Figure 6.3c)**.

Collectively, these data demonstrate that CS exposure induces small airway remodelling, emphysema and impaired lung function in experimental COPD through



miR-135b- and miR-146b-dependent mechanisms, which can be targeted therapeutically.

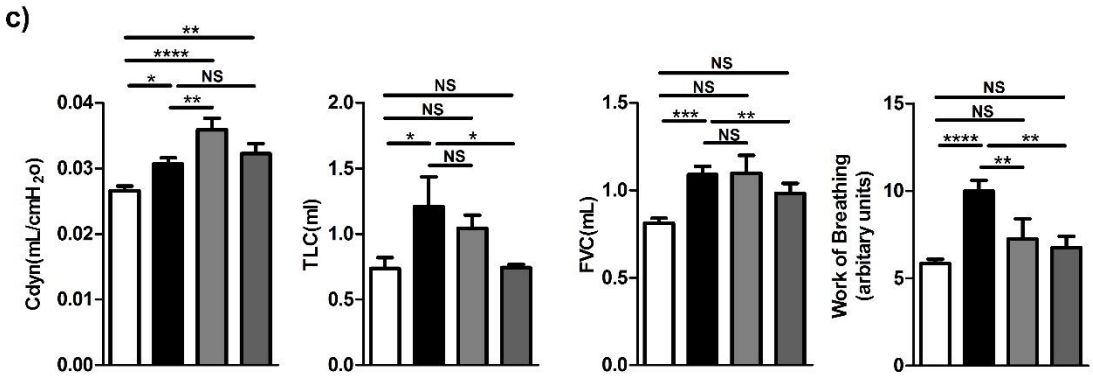


Figure 6.3: Cigarette smoke (CS)-induced miR-135b and miR-146b expression promotes small airway remodelling, emphysema and impaired lung function in experimental chronic obstructive pulmonary disease (COPD). BALB/c mice were exposed to CS *via* the nose-only, or normal air, for eight weeks and were treated intranasally with miR-135b-specific antagomir (Ant-135b) or miR-146b-specific antagomir (Ant-146b) or scrambled (Scr) antagomir once a week during CS exposure. **a)** Area of collagen deposition (μm^2; pink stain) per perimeter of basement membrane (µm) in Verhoff-Van Gieson-stained lung sections (scale bar = 40 µm). **b)** Emphysema-like alveolar enlargement was assessed using mean linear intercept and destructive index techniques**. c)** Lung function was assessed in terms of total lung capacity (TLC), forced vital capacity (FVC) and Work of Breathing. n = 4-6 mice/group. Data are presented as means ± s.e.m. NS = not significant; *P<0.05; **P<0.01; ****P<0.0001 compared to normal air-exposed controls. Data were analysed with One-way ANOVA (uncorrected Fishers LSD test).

6.2.4 Inhibition of CS-induced miR-135b and miR-146b expression selectively inhibits some canonical targets in experimental COPD

miRs modulate their target gene expression post-transcriptionally through gene silencing. Expression of the downstream targets of miRs were assessed to identify their roles in the pathogenesis of CS-induced experimental COPD.

CS exposure had no effect on the mRNA expression of SIRT1 in the lungs but significantly decreased the expression of bone morphogenetic protein receptor (BMPR)2, compared to normal air-exposed controls **(Figure 6.4a).** Treatment with Ant-135b during CS exposure in fact decreased the expression of SIRT1 and had no effect on the expression of BMPR2. We also assessed the expression of another target VEGF, which is known to be regulated by the miR-135b. CS exposure trended towards suppressing VEGF expression in the lungs and treatment with Ant-135b trended towards increasing its expression in experimental COPD.

Next, we assessed the expression of IRAK1 and TRAF6, which are inflammatory mediators and known targets of miR-146b. The increase in miR-146b expression in CS-exposed controls was associated with a non-significant trend towards reduction in IRAK1 expression and TRAF6 expression was not affected. Treatment with Ant-146b restored the expression of IRAK1 and TRAF6 in experimental COPD **(Figure 6.4b).**

Thus the increase in expression of miR135b and 146b in CS-induced experimental COPD was linked to the reduced expression of their targets, and this controls pulmonary inflammation, small airway remodelling and emphysema in experimental COPD.

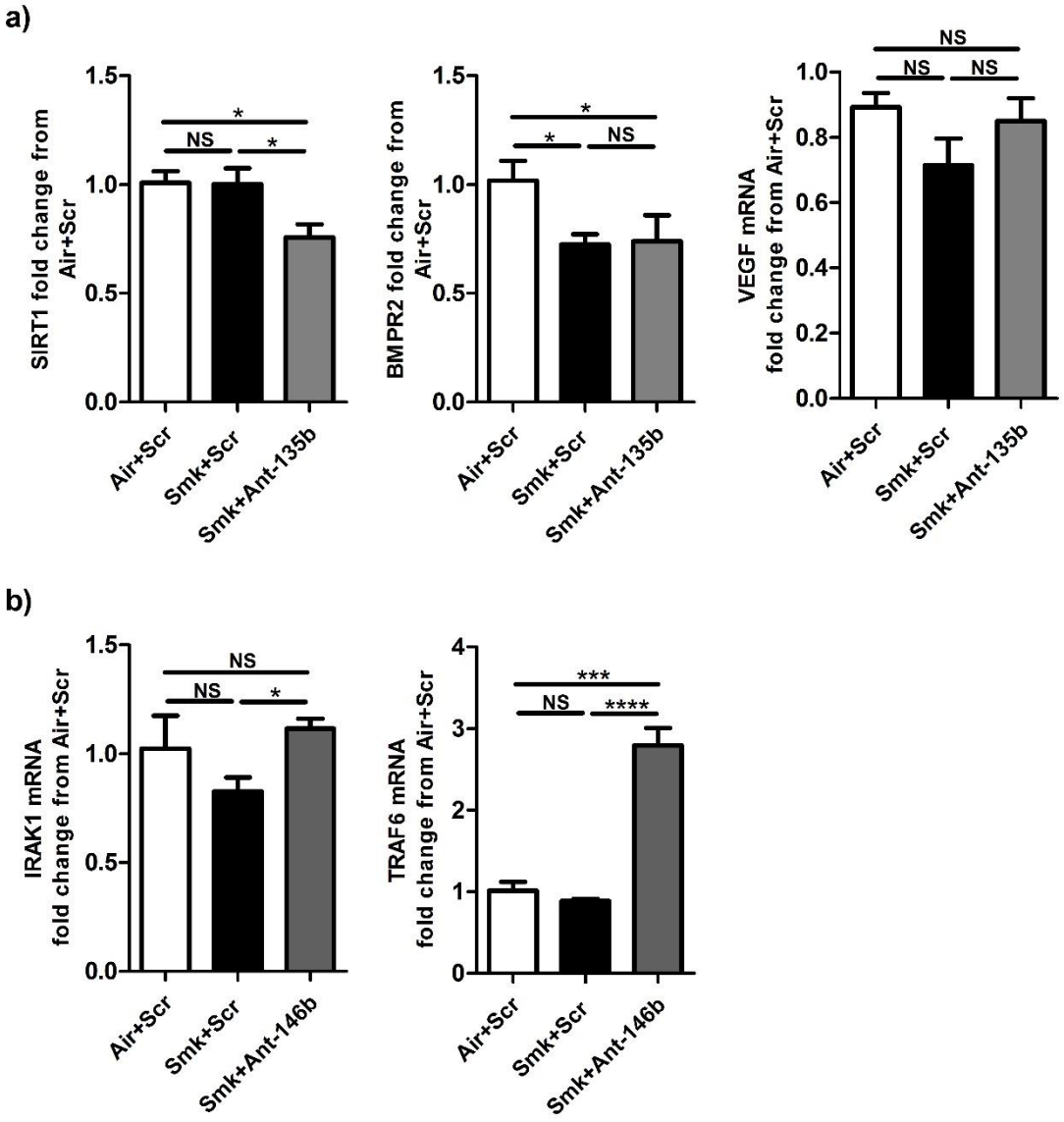


Figure 6.4: Cigarette smoke (CS)-induced miR-135b and miR-146b expression selectively affect the expression of some putative target genes in experimental chronic obstructive pulmonary disease (COPD). BALB/c mice were exposed to CS *via* the nose-only, or normal air, for eight weeks and were treated intranasally with miR-135b-specific antagomir (Ant-135b) or miR-146b-specific antagomir (Ant-146b) or scrambled (Scr) antagomir once a week during CS exposure. **a)** Lung mRNA expression of Sirtuin (SIRT)1, bone morphogenetic protein receptor type II (BMPR2), vascular endothelial growth factor (VEGF), and **b)** Lung mRNA expression of interleukin-1 receptor-associated kinase (IRAK)1 and TNF receptor associated factor (TRAF)6 were

normalised to hypoxanthine-guanine phosphoribosyltransferase (HPRT). mRNA expression presented as fold change from normal air-exposed controls. n=3-6 mice/group. Data are presented as means ± s.e.m. NS= not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 compared to normal air-exposed controls. Data were analysed with One-way ANOVA (uncorrected Fishers LSD test)

6.3 DISCUSSION

In this study, we assessed roles for CS-induced miR-135b and miR-146b in an experimental model of COPD that recapitulates the key features of human COPD. The disease features involved include chronic pulmonary inflammation, small airway remodelling, emphysema and impaired lung function (72, 162, 254-257). We show that increased expression of miR-135b and miR-146b in the lungs may be important in the development of experimental COPD. We used highly specific and potent miR-135b and miR-146b inhibitors, Ant-135b and Ant-146b, respectively, to assess the functional roles of these miRs in the development of experimental COPD. We showed that pulmonary inflammation, and alveolar enlargement were inhibited with Ant-135b treatment and airway remodelling and alveolar enlargement were suppressed with with Ant-146b treatment. We then identified that BMPR2, and TRAF6 are targets of miR-135b and miR146b in experimental COPD. Thus inhibiting these miRs may be potential therapies in the treatment of COPD.

CS induced a sustained increase in the expression of miR-135b and miR-146b in the lungs from 2 to 8 weeks of exposure, and this effect was most pronounced at 8 weeks. Both the miRs are over-expressed in various cancers such as lung and breast cancers (357, 373-375). The persistent increase of these miRs throughout the CS-exposure suggests a potential role in COPD development. Therefore to identify their roles in the pathogenesis of COPD, we inhibited CS-induced miR-135b and miR-146b with Ant-135b and Ant-146b, respectively, in experimental COPD. Treatment with Ant-135b decreased CS-induced neutrophilic inflammation, suggesting that this process is

under the control of miR-135b. Importantly, this agrees with findings published by Halappanavar *et al*., and Matsuyama *et al*., (376), they showed that miR-135b induces neutrophilic inflammation in IL-1R1 knockout mice (238). This reduction in neutrophil infiltration in BALF with the inhibition of miR-135b may be potentially due to decreases in the expression of neutrophil chemoattractants CCL7 and MMP12 and TNFα levels that we and others showed (56, 296, 297). Treatment with Ant-135b had no effect on the number of macrophages present in BALF, which may be due to the persistent expression of the potent macrophage chemoattractant GM-CSF (70). In a recent study the authors have shown that reduced BMPR2 expression induces increases in GM-CSF levels, resulting in increased macrophage recruitment (377). Thus, it is possible that failure to restore BMPR2 expression by treatment with Ant-135b may be a reason for the increased macrophages infiltration in experimental COPD.

The inflammatory cell numbers in BALF were not significantly suppressed with the inhibition of miR-146b in our experimental COPD. Although the expression of the neutrophil chemoattractants CCL7 and MMP12 were reduced there was no changes in the expression of CXCL2 and GM-CSF, and protein levels of TNFα and CXCL1. Treatment with Ant-146b increased the expression of IRAK1 and TRAF6 during experimental COPD, however, this did not translate into significant changes to airway inflammation. These data suggest that CS-induced airway inflammation in our model of experimental COPD is not dependent on miR-146b or the regulation of its target transcripts IRAK1 and TRAF6.

Treatment with Ant-135b trended towards decreasing CS-induced collagen deposition around airways. This disagrees with a recent report that suggests that miR-135b may be protective in small airway remodelling. The authors demonstrated that fibroblasts from systemic sclerosis patients show reduced miR-135b expression. Through *in vitro* experiments they showed that reduced miR-135b expression upregulates STAT6 which mediates IL-13 induced increase in fibrosis (378). They also showed that miR-135b expression is reduced in a bleomycin-induced model of skin

fibrosis. However, BMPR2, a target of miR-135b, belonging to the remodelling regulator family TGF-β, is reduced in our model of experimental COPD and in patients with moderate COPD (379). Several studies have shown that reduced BMPR2 levels are associated with exaggerated TGFβ signalling and have implicated BMPR2 in fibrogenesis in idiopathic pulmonary fibrosis and pulmonary hypertension (380-382). However expression of BMPR2 was not restored with Ant-135b treatment. Thus, failure to restore BMPR2 expression may be a reason for the deficient suppression of airway remodelling observed with the inhibition of miR-135b in experimental COPD.

Several studies have implicated increased miR-146b expression in the development of fibrosis in various diseases (383-386). In a bleomycin-induced model of pulmonary fibrosis, miR-146b expression was elevated with concomitant reductions in the levels of forkhead box O3 (387), which has been linked with pulmonary fibrosis (388). Further to this, we have shown that with the inhibition of miR-146b expression, collagen deposition around small airways were reduced back to baseline levels, indicating that miR-146b promotes small airway remodelling in lungs. We also showed reduced expression of the pro-fibrotic factor MMP12 (303, 304) with the inhibition of miR-146b expression. However the exact mechanism through which miR-146b mediates small airway remodelling is not clear and needs to be elucidated with further studies.

Increase in emphysema-like alveolar enlargement with CS-exposure was significantly reduced with both Ant-135b and Ant-146b treatments, suggesting that these miRs play potentially overlapping roles in the development of emphysema. The mechanism through which these miRs induce emphysema remains to be determined and may involve increased expression of the pro-emphysema factor MMP12 (304-306). However, simultaneous investigation of these miRs with a combination of respective antagomirs may be valuable in determining the functional roles of these miRs in the development of emphysema.

We have also shown that CS exposure does not alter the expression of pro-emphysema factors SIRT1 (364, 365) and VEGF (359, 361) in experimental COPD.

Interestingly, VEGF expression trended towards a reduction following CS exposure and an increase following treatment with Ant-135b, with miR-135b inhibition. Thus, increases in miR-135b expression may lead to reduced HIFα levels, resulting in decreased VEGF expression, which may be a contributing factor in the development of emphysema. Therefore, inhibiting CS-induced miR-135b expression may be beneficial in reducing emphysema-like alveolar enlargement.

Work of breathing is the energy required to inhale oxygen or exhale carbon dioxide. In COPD, work of breathing is increased as more energy is required to overcome the resistance to airflow in lungs (389), which is the result of the combination of increased inflammation, small airway remodelling and emphysema (390, 391). With the inhibition of miR-135b and miR-146b expression, the work of breathing was significantly reduced in combination with the decrease in small airway remodelling and emphysema.

Thus, in our CS-induced experimental COPD, it is possible that the increase in miR-146b expression in lungs may be a part of an anti-inflammatory response to cigarette smoke. We have shown that chronic CS exposure trends towards reducing the expression of the NFκB-activated genes IRAK1 and TRAF6. Interestingly, treatment with Ant-146b had no effect on the infiltration of inflammatory cells in lungs but protected against the development of collagen deposition around the small airways and emphysema. Thus, these data show that the persistent increase in miR-146b expression in our experimental COPD may not be associated with anti-inflammatory responses but plays an important role in promoting airway remodelling and emphysema.

In summary, our study demonstrates for the first time that CS-induced miR-135b promotes neutrophilic airway inflammation and emphysema in experimental COPD. We also show that CS-induced miR-146b promotes airway remodelling and emphysema that is associated with impaired lung function in experimental COPD. We identify BMPR2, a known target of miR-135b, as a potential mediator of macrophage recruitment that may play a role in the development of small airway remodelling and emphysema through a VEGF-dependent mechanism in experimental COPD. Thus, therapeutic targeting of lung

miR-135b and miR-146b may be an effective strategy in the treatment of COPD. Further exploration of the miR-dependent mechanisms identified in this study may assist in the development of more specific therapies that target the processes that drive the development of COPD.

CHAPTER 7: COMBINED TARGETING OF CIGARETTE SMOKE-INDUCED miRS IN EXPERIMENTAL COPD

7.1 INTRODUCTION

In Chapters **4-6** we showed that CS-induced miR-9, -21, -135b and -146b, play important roles in the pathogenesis of COPD. In chapters 4 and 5 we showed that CS-induced miR-9 and miR-21 promote airway inflammation and small airway remodelling in experimental COPD. Treatment with miR-9- and miR-21-specific antagomirs, Ant-9 and Ant-21 led to reduced airway inflammation and collagen deposition, and improved lung function **[(Figures (4.2c)** and **(4.3 a** and **c))** and **(Figures (5.2e-h)** and **(5.3 a** and **c))]**. In chapter 6 we also showed that CS-induced miR-135b and miR-146b promote emphysema-like alveolar enlargement and collagen deposition around small airways in experimental COPD. Treatment with Ant-135b and Ant-146b during CS exposure resulted in reduced collagen deposition around the small airways, suppressed emphysema-like alveolar enlargement, and improved lung function in experimental COPD (**(Figures 6.2d)** and **(6.3b** and **d))**. The effects of inhibiting CS-induced miRs expression have been summarised in the table 7.1.

Table 7.1: Summary table showing the effects of inhibiting CS-induced increases in miR expression through miR-specific antagomirs

miRs inhibited	Airway inflammation	Small airway Fibrosis	Emphysema	Lung function
Ant-9	+++	+++	-	++
Ant -21	+++	+++	-	++
Ant -135b	++	+	++	+
Ant -146b	-	+++	++	++

+++ Reduced to basal levels

++ Significant reduction compared with CS-exposed group

+ Non significant reduction (trend towards reduction)

- No change

In this study, we assessed the effects of treating CS-exposed mice with different combinations of two miR-specific antagomirs that, when administered alone, resulted in the suppression of key features of experimental COPD. We simultaneously targeted combinations of two miRs that were shown to regulate different features of COPD [(i.e.(miR-9 + miR-135b), (miR-9 + miR-146b), (miR-21+ miR-135b), and (miR-21+ miR-146b)] using miR-specific antagomirs in our nose-only CS induced model of experimental COPD (72, 162, 254-257). Interestingly, all combined Ant-miR treatments decreased CS-induced airway inflammation and only combined Ant-21 and Ant-146b treatment suppressed CS-induced collagen deposition and decreased emphysema-like alveolar enlargement. These data highlight the therapeutic potential of simultaneously targeting miR-21 and miR-146b as a novel approach in the treatment of CS-induced COPD.

7.2 RESULTS

7.2.1 Treatment with combinations of two miR-specific antagomirs simultaneously decreases the expression of target miRs in experimental COPD

We first assessed the effects of combined miR-specific antagomir treatments on the simultaneous inhibition of targeted miRs. CS-exposed mice were treated with Ant-9+135b, Ant-9+146b, Ant-21+135b, Ant-21+146b antagomirs and CS- or normal air-exposed mice were treated with scrambled (Scr) antagomir once a week for eight weeks in experimental COPD **(Figure 7.1a)**. Treatments with combined antagomirs lead to significant decreases in the expression of targeted miRs **(Figure 7.1b-e).** CS-induced miR-9 expression was reduced in both Ant-9+135b- and Ant-9+146b-treated groups **(Figure 7.1b)**, compared to Scr-treated, CS-exposed controls. CS-induced miR-21 expression was reduced back to baseline levels in both Ant-21+135b- and Ant-21+146b-treated groups **(Figure 7.1c)**. CS-induced miR-135b expression was decreased in both Ant-9+135b- and Ant-21+135b-treated groups (**Figure 7.1d**), compared to Scr-treated, CS-exposed controls. Finally, CS-induced miR-146b expression was reduced back to baseline levels in both Ant-9+146b- and Ant-21+146b-treated groups **(Figure 7.1d)**, compared to Scr-treated, CS-exposed controls.

Collectively, these data demonstrate that treatment with combined miR-specific antagomirs effectively inhibits the CS-induced expression of target miRs in the lungs during experimental COPD.

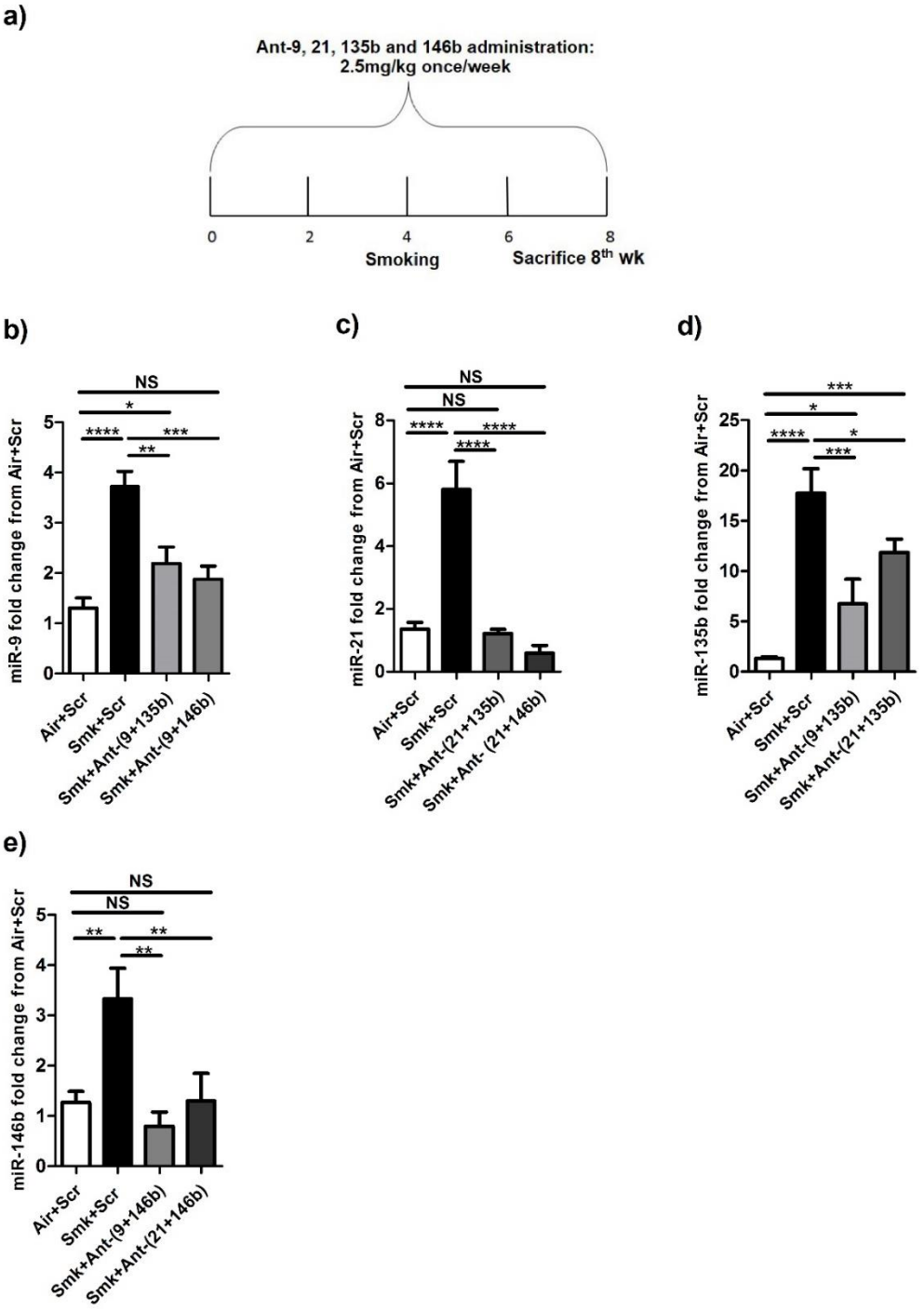


Figure 7.1: Treatment with combinations of two miR-specific antagomirs simultaneously decreases the expression of target miRs in experimental chronic obstructive pulmonary disease (COPD). a) BALB/c mice were exposed to cigarette smoke (CS) *via* the nose-only, or normal air, for eight weeks and treated with combinations of miR-specific antagomirs (Ant); Ant-(9+135b), Ant-(9+146b), Ant-(21+135b), Ant-(21+146b), or scrambled (Scr) antagomir intranasally once a week during CS exposure. miR expression was determined in whole lung tissue by qPCR. **b)** miR-9 expression in Ant-(9+135b)- and Ant-(9+146b)-treated groups, **c)** miR-21 expression in Ant-(21+135b)- and Ant-(21+146b)-treated groups, **d)** miR-135b expression in Ant-(9+135b)- and Ant-(21+135b)-treated groups, and **e)** miR-146b expression in Ant-(9+146b)- and Ant-(21+146b)-treated groups. miR expression was initially normalised to the geometric mean of the housekeeping controls small nuclear RNA U6 (snRNA U6) and small nucleolar RNA U49 (snoRNA U49). miR expression data is presented as fold change compared to normal air-exposed controls. Data (n = 3-6 mice/group) are presented as means ± S.E.M. NS: not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 compared to normal air-exposed controls. Data were analysed with One-way ANOVA (uncorrected Fishers LSD test).

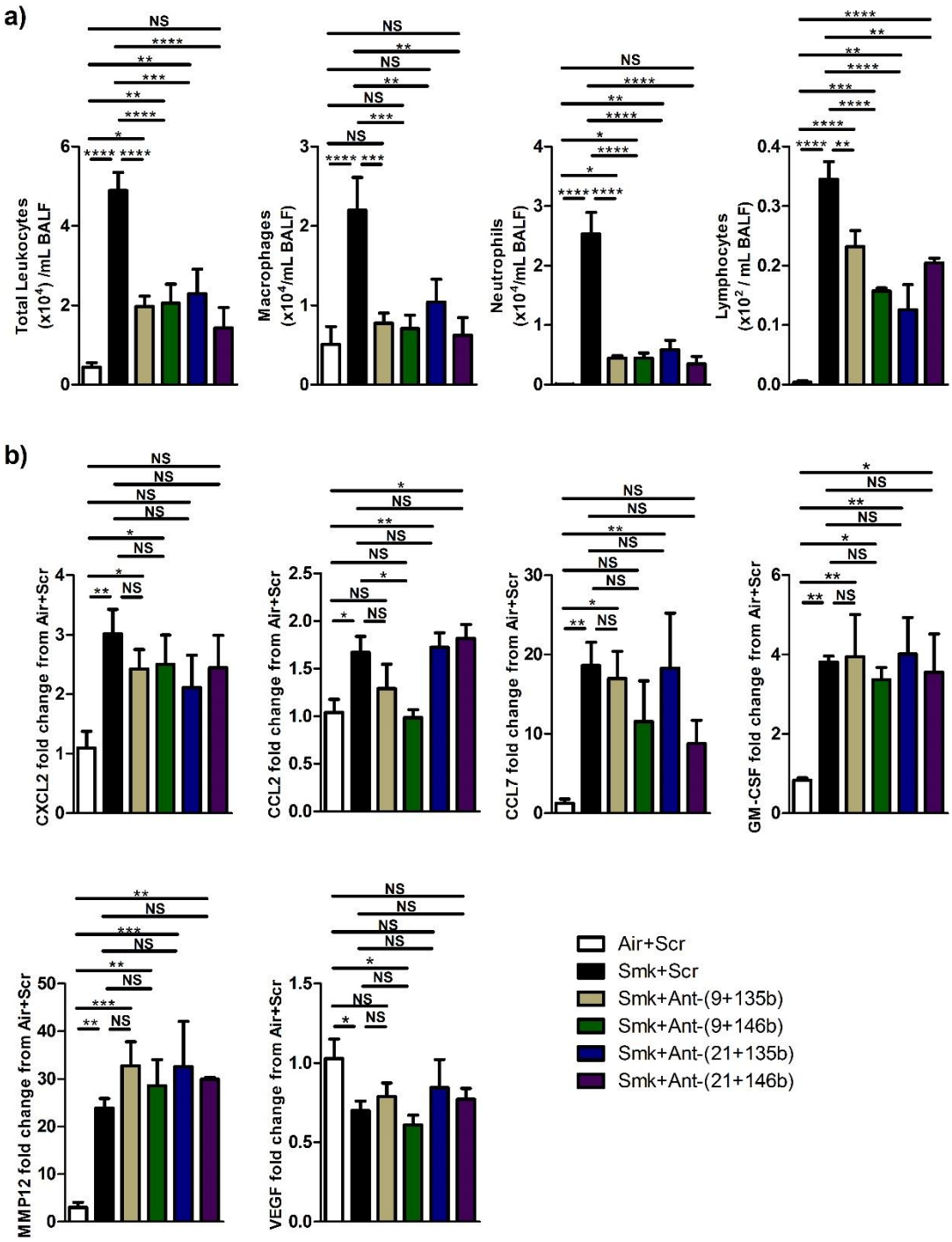
7.2.2 Treatment with combinations of two miR-specific antagomirs reduces CS-induced airway inflammation in experimental COPD.

We next assessed the therapeutic potential of combined antagomir treatment in experimental COPD. When targeted individually, miR-9, -21, -135b and -146b were found to be important for CS-induced pulmonary inflammation or emphysema. Significantly, treatment of CS-exposed mice with all combinations of miR-specific antagomirs tested resulted in decreased total inflammatory cell numbers in the airways (macrophages, neutrophils and lymphocytes; **(Figure 7.2a)**.

We then measured the lung mRNA expression of key pro-inflammatory factors that were identified in the previous chapters. CS exposure increased the expression of CXCL2, CCL2, CCL7, GM-CSF and MMP12 in scrambled (Scr) antagomir-treated mice (Smk+Scr) compared to Scr-treated, air-exposed controls **(Figure 7.2b).** In contrast, CS exposure decreased the expression of VEGF in the lungs (Smk+Scr) compared to normal air-exposed controls (Air+Scr). Ant-21+135b and Ant-21+146b treatment induced a non-significant decrease when compared to Scr-treated, CS-exposed controls (Smk+Scr). However, lung CXCL2 expression in groups treated with these antagomir combinations Ant-(21+135b and Ant-21+146b was not significantly increased from Scr-treated, air-exposed control levels (Air+Scr). Treatment with Ant-9+135b had similar effects on the expression of CCL2 in the lung. Interestingly, treatment with Ant-9+146b decreased the expression of CCL2 in experimental COPD. No treatment combination altered the CS-induced expression of CCL7 in the lungs, however, treatment with Ant-21+146b trended towards decreasing its expression compared to Scr-treated, CS-exposed controls. No treatment combination had any effect on the expression of CS-induced GM-CSF, MMP12 and VEGF in the lungs in experimental COPD.

We next assessed the protein levels of TNFα and CXCL1 in lung homogenates. Only Ant-21+146b treatment significantly reduced TNFα levels, whereas both Ant-21+135b and Ant-21+146b treatments significantly reduced CXCL1 levels, compared to Scr-treated, air-exposed control levels **(Figure 7.2c).**

Collectively, these data demonstrate that combined inhibition of CS-induced miRs reduces airway inflammation. With Ant-(21+146b) treatment, the levels of lung pro-inflammatory mediators TNFα and CXCL1 which play important roles in neutrophilic inflammation, were reduced, decreasing CS-induced inflammation in experimental COPD.


a)
Total Leukocytes (x10⁴) /mL BALF
Macrophages (x10⁴/mL BALF)
Neutrophils (x10⁴/mL BALF)
Lymphocytes (x10² / mL BALF)
b)
CXCL2 fold change from Air+Scr
CCL2 fold change from Air+Scr
CCL7 fold change from Air+Scr
GM-CSF fold change from Air+Scr
MMP12 fold change from Air+Scr
VEGF fold change from Air+Scr
Air+Scr
Smk+Scr
Smk+Ant-(9+135b)
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Smk+Ant-(21+135b)
Smk+Ant-(21+146b)

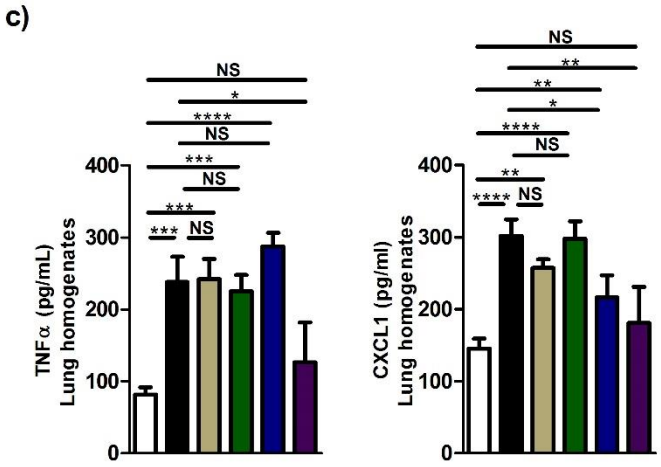
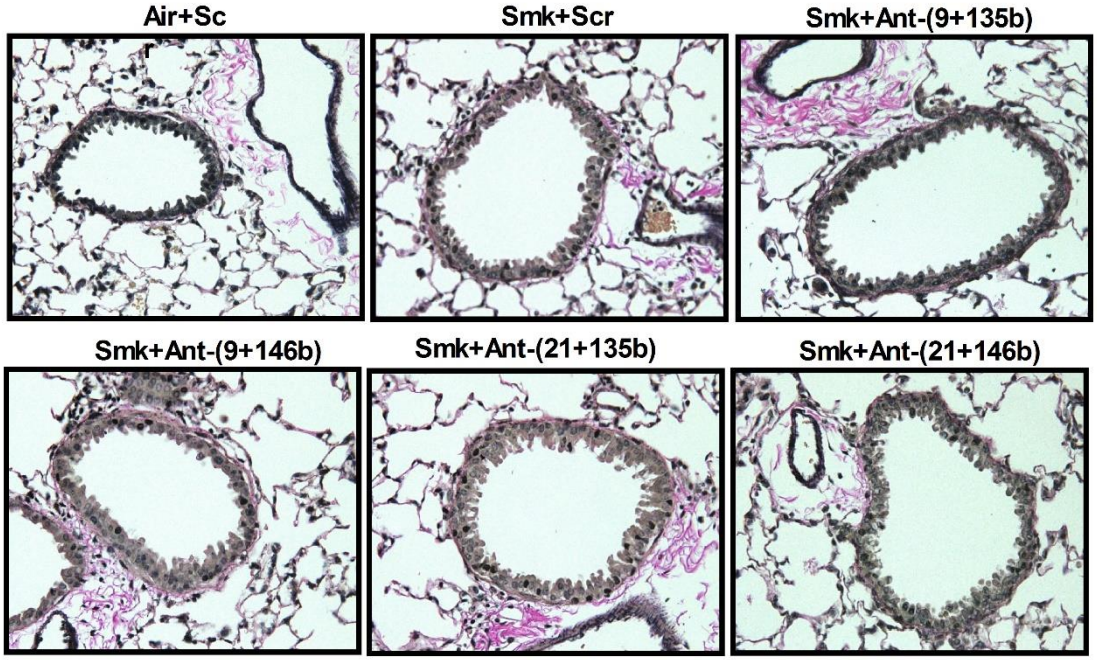


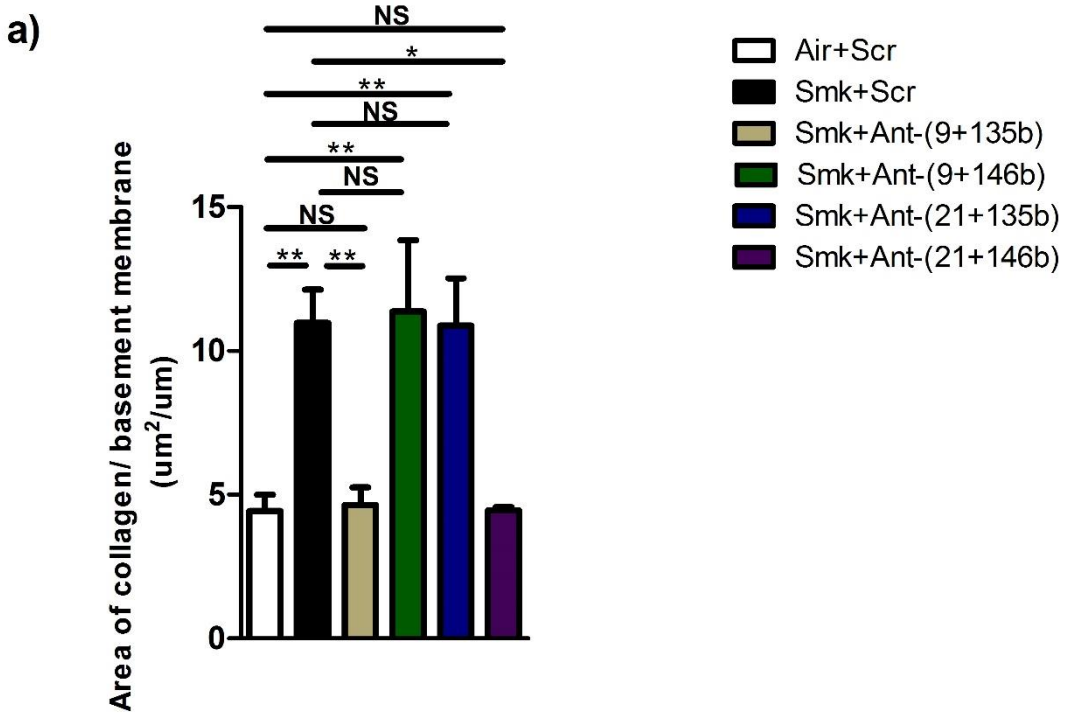
Figure 7.2: Treatment with combinations of two miR-specific antagomirs decreases cigarette smoke (CS)-induced airway inflammation in experimental chronic obstructive pulmonary disease (COPD). BALB/c mice were exposed to cigarette smoke (CS) *via* the nose-only, or normal air, for eight weeks and treated with combinations of miR-specific antagomirs (Ant); Ant-(9+135b), Ant-(9+146b), Ant-(21+135b), Ant-(21+146b), or scrambled (Scr) antagomir intranasally once a week during CS exposure. **a)** Total leukocyte, macrophage, neutrophil and lymphocyte numbers in bronchoalveolar lavage fluid. **b)** CXCL)2, chemokine (C-C motif) ligand (CCL)2, CCL7, granulocyte-macrophage colony-stimulating factor (GM-CSF), matrix metalloproteinase (MMP)12 and vascular endothelial growth factor (VEGF) expression. **c)** Tumour necrosis factor (TNF) α and CXCL1 protein levels in lung homogenates quantified by ELISA. Data (n = 3-6 mice/group) are presented as means ± S.E.M. NS: not significant; *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 compared to normal air-exposed controls. Data were analysed with One-way ANOVA (uncorrected Fishers LSD test).

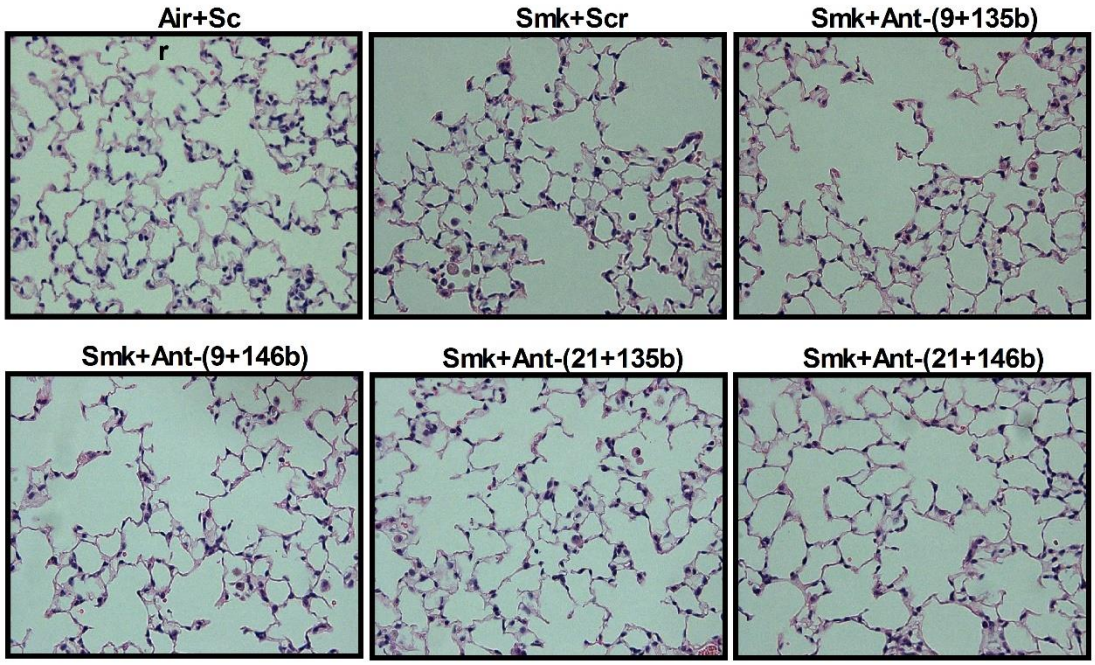
7.2.3 Combined targeting of CS-induced miR-21 and miR-146b expression suppresses small airway remodelling and emphysema-like alveolar enlargement in experimental COPD.

We next assessed the effects of combined inhibition of CS-induced miRs on key disease features of experimental COPD. Significantly, treatment with Ant-9+135b and Ant-21+146b lead to complete suppression of CS-induced collagen deposition around the small airways compared to Scr-treated, CS-exposed controls **(Figure 7.3a)**. No other treatment combinations affected collagen deposition around the airways Treatment with Ant-21+135b suppressed CS-induced emphysema-like alveolar enlargement, and treatment with Ant-21+146b trended towards reducing this effect **(Figure 7.3b),** compared to Scr-treated, CS-exposed controls. With CS-exposure lungs were more compliant compared with the Scr treated, normal air-exposed controls (Air+Scr) and treatment with only Ant (21+146b) reduced lung compliance in experimental COPD **(Figure 7.3c).**

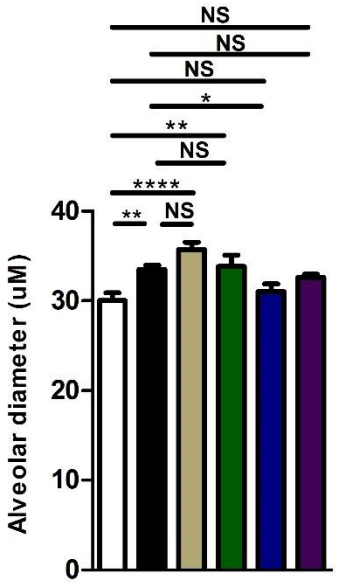
Collectively, CS-exposed mice showed increased small airway remodelling, and emphysema-like alveolar enlargement and impaired lung function compared to normal air-exposed controls. Significantly, combined treatment with Ant-21+146b resulted in small airway remodelling and emphysema-like alveolar enlargement and improved lung function in experimental COPD.

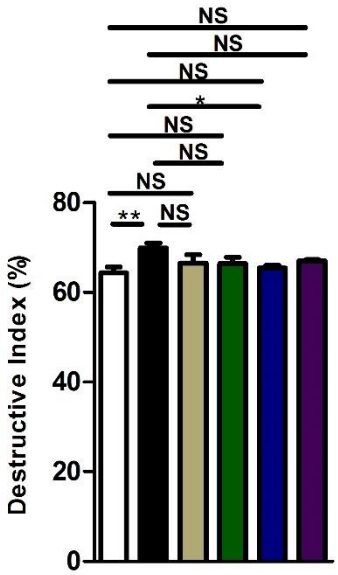

Air+Sc
Smk+Scr
Smk+Ant-(9+135b)
Smk+Ant-(9+146b)
Smk+Ant-(21+135b)
Smk+Ant-(21+146b)


a)
NS
*
**
NS
**
NS
NS
** **
15
10
5
0
Area of collagen/ basement membrane (um²/um)
Air+Scr
Smk+Scr
Smk+Ant-(9+135b)
Smk+Ant-(9+146b)
Smk+Ant-(21+135b)
Smk+Ant-(21+146b)


Air+Sc
r
Smk+Scr
Smk+Ant-(9+135b)
Smk+Ant-(9+146b)
Smk+Ant-(21+135b)
Smk+Ant-(21+146b)

b)


NS
NS
NS
*
**
NS
****
**
NS
40
30
20
10
0
Alveolar diameter (uM)


NS
NS
NS
*
NS
NS
NS
**
NS
80
60
40
20
0
Destructive Index (%)

c)

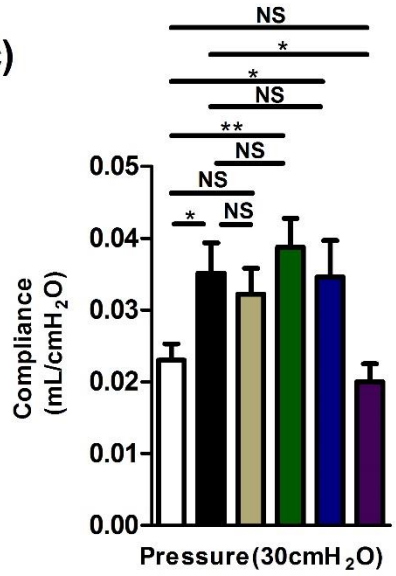

NS
*
*
NS
**
NS
NS
*
NS
0.05
0.04
0.03
0.02
0.01
0.00
Compliance (mL/cmH2O)
Pressure(30cmH2O)

Figure 7.3: Combined targeting of cigarette smoke (CS)-induced miR-21 and miR-146b expression suppresses small airway remodelling and emphysema-like alveolar enlargement in experimental chronic obstructive pulmonary disease (COPD). BALB/c mice were exposed to cigarette smoke (CS) *via* the nose-only, or normal air, for eight weeks and treated with combinations of miR-specific antagomirs (Ant); Ant-(9+135b), Ant-(9+146b), Ant-(21+135b), Ant-(21+146b), or scrambled (Scr) antagomir intranasally once a week during CS exposure. **a)** Area of collagen deposition (μm^2) per perimeter (μm) of basement membrane in Verhoff-Van Gieson-stained (pink) lung sections (scale bar = 40 μm). **b)** Alveolar size and tissue destruction was assessed using mean linear intercept and destructive index techniques, respectively**. c)** Lung function was assessed in terms of lung compliance (at 30cmH_2O). Data (n = 3-6 mice/group) are presented as means ± S.E.M. NS = not significant; *P<0.05; **P<0.01; ****P<0.0001 compared to normal air-exposed controls. Data were analysed with One-way ANOVA (uncorrected Fishers LSD test).

Table 7.2: Summary table showing the expected and detected effects of inhibiting the expression of combinations of two CS-induced miRs using miR-specific antagomirs.

miRs inhibited	Airway inflammation		Small airway Fibrosis		Emphysema		Lung function	
	Expected	Detected	Expected	Detected	Expected	Detected	Expected	Detected
Ant-(9+135b)	+++	+	+++	+++	++	-	++	-
Ant-(9+146b)	++	+	+++	-	++	-	++	-
Ant-(21+135b)	+++	+	+++	-	++	+++	++	-
Ant-(21+146b)	++	+	+++	+++	++	+	++	++

+++ Reduced to basal levels

++ Significant reduction compared with CS-exposed group

+ Non significant reduction (trend towards reduction)

- No change

7.3 DISCUSSION

Here we investigated the effects of simultaneously targeting combinations of two CS-induced miRs in our model of experimental COPD, which recapitulates the key features of COPD in humans (72, 162, 254-257). Using this model, we previously showed that CS-induced airway inflammation is induced by miR-9- and miR-21-dependent mechanisms **(Figures 4.2c and 5.2e-h)**. We also showed that CS-induced airway remodelling was induced by miR-9-, miR-21-, miR-135b- and mir-146b-dependent mechanisms **(Figure 5.3a, 5.3a and 6.3a)** and emphysema-like alveolar enlargement was induced by miR-135b and miR-146b-dependent mechanisms **(Figures 6.3a** and **b)** in experimental COPD. In this study, we assessed the effects of simultaneous inhibition of combinations of four CS-induced miRs in the lungs that we previously showed to mediate airway inflammation and emphysema in experimental COPD. Combined inhibition of these miRs, [Ant-(9+135b), Ant-(9+146b), Ant-(21+135b)

and Ant-(21+146b)] led to suppression of some, but not all, key features of experimental COPD. Combined inhibition of CS-induced miR-21 and miR-146b substantially reduced airway inflammation, airway remodelling and emphysema-like alveolar enlargement in experimental COPD.

Combined treatment with Ant-9 and Ant-135b suppressed CS-induced airway inflammation. These effects were similar to that observed with individual depletion of these miRs **(Figures 4.2c** and **6.2d)**. miR-9 is known to be induced in macrophages and neutrophils (213, 295) and with Ant-(9+135b) treatment, macrophages influx was completely reduced back base line levels observed in normal air-exposed mice. This is similar to the study (295) which shows that increased miR-9 expression in macrophages is associated with steroid resistant inflammation. Interestingly, combined treatment with Ant-9 and Ant-135b did not completely suppress CS-induced airway neutrophil and lymphocyte numbers back to baseline levels. miR-9 expression is rapidly induced in neutrophils (213) and is also known to increase the production of CD 4(+) T cells (392). Thus the incomplete suppression of miR-9 expression with Ant-(9+135b) treatment may have resulted in the persistent levels of neutrophil and lymphocyte numbers. Additionally miR-135b promotes Th-17 cell differentiation (376), and these cells induce neutrophil chemoattractants such as CXCL1, CCL7 (393). Thus, incomplete suppression of miR-135b expression with Ant-9+135b treatment may promote persistent production of neutrophil chemoattractants such as CXCL2, CCL7, TNFα and CXCL1 (68, 296-298). This may have inadvertently resulted in the increased neutrophil numbers observed in the airways.

We next assessed the effect of Ant-9+135b treatment on airway remodelling. Importantly, CS-induced airway remodelling was completely suppressed by combined treatment with Ant-9+135b. This effect is similar to that observed with individual Ant treatments, Ant-9 completely suppressed airway remodelling and miR-135b inhibition resulted in a trend towards decreased airway remodelling **(Figures 4.3a** and **6.3a)**. miR-9 and miR-135b are known to target the fibrosis-associated factors platelet-derived

growth factor receptor (PDGFR)β2 (302) and bone morphogenetic protein receptor type II (BMPR2) (366), however, Ant-9+135b treatment had no effect on the expression of these factors (data not shown). It is likely that the suppression of CS-induced airway remodelling following Ant-9+135b treatment resulted from the cumulative effect of both the miRs on other fibrosis-associated target genes such as transforming growth factor-beta receptors, (TGFβR) 1 and 2 (301, 366). Interestingly, combined treatment did not suppress CS-induced alveolar enlargement. This may be due to persistent expression of pro-emphysema factors such as MMP12. In COPD, impaired lung function is associated with increased airway inflammation, airway remodelling and emphysema (72, 79, 90, 394, 395). However, in spite of augmented pulmonary inflammation and increase in emphysema, concurrent depletion of these miRs lead to trending reduction in compliance. This decrease in compliance is probably due to reduced airway remodelling (90). Thus, targeting CS-induced miR-9 and miR-135b individually might be more effective than combined inhibition of these miRs in COPD.

Combined treatment with Ant-9 and Ant-146b reduced CS-induced inflammatory cell numbers in BALF, compared to Scr-treated, CS-exposed controls. Treatment reduced the expression of the monocyte chemoattractant CCL2, which may explain the reduction in macrophage numbers observed (396, 397). The reduction in CCL2 expression may have resulted from the inhibition of CS-induced miR-146b and this is similar to a previous report on human retinal pigment epithelial cells stimulated with IL-1β that exhibited increases in the expression of miR-146a and miR-146b (398). Importantly, this study linked increased expression of miR-146b with increased CCL2 expression, suggesting that miR-146b may mediate macrophage recruitment in experimental COPD through a CCL2-dependent mechanism. Surprisingly, combined treatment with Ant-9 and Ant-146b had no effect on CS-induced airway remodelling, alveolar enlargement or lung compliance. This shows that the combined treatment of Ant-9 and Ant-146b is not as effective as individual Ant treatments in experimental COPD.

The CS-induced expression of miR-21 and miR-135b was decreased by treatment with Ant-21+135b in experimental COPD, with the levels of miR-21 returned to baseline. Following treatment, CS-induced miR-135b expression remained higher than normal air-exposed controls and this effect was comparable to that of the individual miR-135b depletion study. Ant-21+135b treatment decreased inflammatory cell numbers in the BALF and completely suppressed macrophage numbers back to baseline levels. Similar to Ant-9+136b treatment, combined Ant-21+135b treatment only partially suppressed the numbers of neutrophils and lymphocytes in the BAL compared to air-exposed controls. This potentially resulted from incomplete suppression of CS-induced miR-135b, like with Ant-9+135b treatment. Decreased expression of the neutrophil chemoattractants CXCL2 and CXCL1 may partly explain the suppression of airway inflammation following treatment. There were no changes in the expression of the miR-21 target, special AT rich sequence binding protein (SATB)1 that we previously showed **(Figure 5.5d)** to play an important role in inducing inflammatory responses (data not shown). Interestingly, treatment with Ant-21+135b did not alter CS-induced collagen deposition around the airways and had no effects on the expression of its known targets SMAD7, sprouty homolog (SPRY)1, SPRY2, programmed cell death (PDCD)4 and BMPR2 (327, 333-335, 380). This indicates that other pro-fibrotic factors may be involved in promoting small airway remodelling and the persistent increase in airway remodelling with Ant-9+135b treatment may due to the incomplete suppression of both these miRs. Significantly, combined treatment reduced alveolar enlargement and tissue destruction compared to both CS-exposed and normal air-exposed controls. We show that Ant-21+135b treatment trended toward increased VEGF expression (359, 361) which is an important pro-emphysema factor, but the expression of another pro-emphysema factor MMP12 (304-306) was not altered. Treatment with Ant-21+135b only partially suppressed pulmonary inflammation and was associated with no change in lung compliance.

Complete inhibition of miR-21 and miR-146b expression with Ant-21+146b treatment led to complete suppression of macrophage and neutrophil numbers in the airways back to baseline levels observed in normal air-exposed controls. Since miR-21 is important in inducing neutrophilic inflammation (259) , and we showed suppressed levels of TNFα and CXCL1 with Ant-(21+146b) treatment. It is likely that CS-induced, miR-21- and miR-146b-mediated regulation of SATB1 induces neutrophilic inflammation through a SATB1/S100A9/NF-κB-dependent mechanism as shown previously with the inhibition of miR-21 by itself **(chapter 5)**. Similar to the inhibition of miR-21 and miR-146b individually **(figure 5.4a** and **6.3a)**, CS-induced airway remodelling was completely reduced. Interestingly, like with Ant-21 treatment, the expression of the aforementioned anti-fibrotic gene targets of miR-21 were not altered. These data suggest that the reduction in CS-induced airway remodelling following combined treatment with Ant-(21+146b) may not be dependent on the suppression of these miR-21 targets and the mechanism by which the CS-induced miR-21and miR-146b promotes airway remodelling needs to be characterised. Moreover Ant-(21+146b) treatment trended towards suppressing CS-induced emphysema-like alveolar enlargement. Significantly, Ant-(21+146b) treatment suppressed pulmonary inflammation and airway remodelling also showed trending decrease in emphysema-like alveolar enlargement, and were associated with complete reduction in lung compliance (72, 79, 90, 394, 395).

We thus demonstrated that in suppressing features of COPD, the combined inhibition of miRs was not as effective as individual miR depletion. A summary of the predicted and detected effects of inhibiting CS-induced combined miRs in repressing various aspects of COPD are listed in table 7.2. Nonetheless, we have shown that inhibiting CS-induced miR-21 and miR-146b together may be more beneficial in COPD. However, before the selection of miRs for combined inhibition, it is important to better understand the role of individual miRs and their target genes in disease development and progression. Further studies may be required to investigate the interactions between the various miR-related signalling pathways. Importantly, it would also be necessary to

assess any potential detrimental effects of the various miRs considered for combined inhibition.

In summary, we demonstrate for the first time that inhibition of specific combinations of CS-induced miRs are effective for suppressing some features of experimental COPD. Combinatorial treatments exerted different effects on features of disease when compared to inhibition of each miR on its own. This may have resulted from off-target effects induced by simultaneous inhibition of different miRs. Significantly, all combinatorial treatments tested decreased CS-induced airway inflammation. However, treatments had varied effects on the expression of key pro-inflammatory mediators in the lung in experimental COPD. Combined inhibition of CS-induced miR-21 and miR-146b expression partially reduced pulmonary inflammation, suppressed airway remodelling, decreased alveolar enlargement and improved lung function, suggesting that targeting these miRs together may be the most effective of the combinations tested for the treatment of COPD. Additional studies are required to fully characterise the precise mechanisms that are controlled by CS-induced miRs-21 and -146b to promote the development of COPD.

CHAPTER 8: GENERAL DISCUSSION AND CONCLUSION

8.1 Significance of research

Chronic obstructive pulmonary disease (COPD) is a progressive and disabling chronic airway disease that is characterised by irreversible, or incompletely reversible, airflow obstruction (10, 11). COPD is a leading cause of global morbidity and mortality (399) and cigarette smoke (CS) is the key risk factor among others such as burning of wood and combustion of coal (2). Importantly, current therapies only target the symptoms of disease and do not prevent disease progression irrespective of smoking cessation (182, 183). Thus, there is an urgent requirement for the development of new therapies. Significantly, development has been hampered by the lack of understanding of the mechanisms that underpin the development of COPD.

MicroRNAs (miRs) have been identified to play crucial roles in many biological processes such as cell differentiation, growth and apoptosis (192). miRs potently inhibit gene expression at the post-transcriptional level (192) and can regulate approximately 60% of all protein-coding genes in humans (400). Increasing evidence now shows that miRs play important roles in regulating immune responses (200). Importantly, deregulation of miR expression is implicated in the development of several diseases such as cancers and chronic inflammatory diseases (315, 316). However, the understanding of their relative contributions to the pathogenesis of COPD is limited. We sought to identify the key CS-induced miRs and miR-dependent mechanisms, and assess their potential for therapeutic targeting, in the development of experimental COPD.

We used our nose-only smoke exposure mouse model of COPD to investigate the mechanisms that are involved in COPD development. In this model, mice were exposed to the smoke of 12 cigarettes twice per day delivered as tightly controlled puffs interspersed with short rest periods (72). This CS exposure regimen is equivalent to

smoking one packet of cigarettes per day for humans. Eight weeks of CS exposure in mice induces many of the characteristic features of human COPD, including chronic pulmonary inflammation, small airway remodelling, emphysema, and impaired lung function (72, 162, 254-257). Most of the animal models described in the literature to date are acute, subacute or whole body CS exposure models that induce acute inflammatory responses but not the features of chronic inflammation and airway remodeling. Moreover, chronic models (401, 402) may take over more than 6 months to develop the key features of the disease. Whereas our model, recapitulates the cardinal features of human COPD in a shorter time frame. The shorter models improves the turnaround time for investigating potential mechanisms that underpin the disease pathogenesis. Thus our model enables to better understand and identify the mechanisms involved in COPD development.

Here we show that CS alters the expression of miRs and our studies suggest that CS-induced miRs are involved in the pathogenesis of COPD. These studies further the understanding of the roles of CS-induced expression of miR-9,-21,-135b, and -146b in the lungs in disease development and highlight the therapeutic potential of targeting these miRs in experimental COPD.

8.2 Microarray-based miR expression profiling in experimental COPD

In order to examine the miR-dependent mechanisms that may drive the development of experimental COPD we used the microarray-based miR profiling as a screening platform to identify miRs that are dysregulated by CS exposure at different stages of experimental COPD (i.e. in disease initiation, development and progression). We identified more than 60 miRs that exhibit altered expression following CS exposure in experimental COPD. Notably, we validated the expression of four CS-induced miRs (miR-9, miR-21, miR-135b and miR-146b) that are upregulated throughout the time course of smoke exposure. However, at the 8th week of CS exposure, altered expression of many of the miRs identified by microarray analysis were not validated by qPCR. The

reduced overlap of results between the two techniques is not uncommon (270, 271). This may have resulted from inadvertent, non-specific hybridisation of miR probes to similar miR sequences and because of higher specificity and sensitivity of qPCR assays over microarrays (273, 274). Recent advances in high-throughput profiling technologies and the introduction of next generation sequencing (NGS) approaches, such as RNA-Seq, can overcome the limitations of microarray-based profiling techniques (403). NGS-based methods can detect and identify known and novel miRs, and have the sensitivity to identify miRs that differ by a single nucleotide(404). However, these techniques are often prohibitively expensive and require significant computational support and infrastructure for data analysis (403). Importantly, to meet the requirements for small scale research platforms, affordable NGS approaches are also now becoming available and these are not limited by a library of known probes and thus novel miRs can be identified (405). Such approaches may be more suitable for profiling differentially dysregulated miRs with improved accuracy and sensitivity in various disease models. Therefore, to identify novel miRs in disease models, affordable NSG approaches may be an attractive strategy.

Collectively, this study shows that CS-exposure indeed dysregulates a range of miRs in the lungs in experimental COPD.

In the following chapters (3, 4, 5 and 6) using miR-specific antagomirs (Ant), we investigated the roles of CS-induced miRs- 9, -21, -135b and -146b in COPD development.

8.3 Role of miR-9 in the pathogenesis of COPD

Here, we examined the role of CS-induced miR-9 in the pathogenesis of experimental COPD. We performed miR-9 inhibition studies and identified a pathogenic role for miR-9 highlighting the therapeutic potential of targeting this miR in CS-induced experimental COPD. miR-9 expression is rapidly induced in neutrophils and macrophages following exposure to pro-inflammatory cytokines, such as TNFα and IL-1β, or upon stimulation with lipopolysaccharides (LPS) through a MyD88 and NF-κB-

dependent mechanisms (213). However, miR-9 directly targets the p50 subunit of NF-κB and downregulates its expression (213), suggesting a feedback control of innate immune responses by miR-9. Likewise, we showed that inhibition of CS-induced miR-9 suppressed neutrophil numbers in the airways, decreasing lung CXCL1 levels and thus reduced airway inflammation. Notably, in a study on osteoarthritic (OA) chondrocytes (406), Makki *et al*., showed that monocyte chemotactic protein induced protein (MCPIP) 1 directly binds to IL-6 mRNA and reduces IL-6 expression (406). In this study, IL-1β-stimulated OA chondrocytes exhibited increased miR-9 expression, which suppressed the expression of anti-inflammatory factor MCPIP1. This led to increased IL-6 expression and resulted in exaggerated inflammatory responses. Another study on human OA cartilage and bone tissue demonstrated (407), increased miR-9 expression in human primary chondrocytes, which resulted in decreased TNFα production through IL-1β-dependent mechanism. Furthermore, increased miR-9 expression was shown to inhibit the secretion of matrix metalloproteinase (MMP) 13 suggesting that miR-9 may play a protective role in OA tissue. Collectively, these data show that miR-9 plays diverse and both anti- and pro-inflammatory roles in OA tissue whereas in lungs with exposure to CS, miR-9 expression was increased and showed a pro-inflammatory role in our CS-induced experimental COPD.

miR-9 is known to play a critical role in neurogenesis and neural differentiation (408) and is differentially expressed in brain tumours. Its expression is increased in glioblastoma (409) and reduced in medulloblastoma (410). miR-9 exerts both oncogenic and tumour suppressive effects and its expression is increased in cancers such as breast, colon and non-small cell lung cancers (411-413). However, reduced expression of miR-9 is observed in colorectal cancer and in leukemia (414, 415). Thus, aberrant expression of miR-9 in various cancerous tissues suggests that miR-9 dysregulation is associated with oncogenic processes.

In this study, we demonstrated for the first time that CS exposure increases lung miR-9 expression. Inhibition of miR-9 expression, resulted in increased expression of its

putative target NRF2 and the expression of its known target SOCS5 (286) restored. This resulted in decreased pulmonary inflammation, small airway remodelling and improvement in impaired lung function in experimental COPD **(Figure 8.1)**. This study thus identifies CS-induced miR-9 as novel therapeutic target in COPD. However, further studies are required to improve the understanding of the role of miR-9 in COPD.

In our experimental COPD model, we showed CS-induced increase in miR-9 expression in lung parenchyma and with the inhibition of the miR, a trend toward decrease in alveolar enlargement was observed. Therefore, it may be important to examine the cellular source and expression pattern of miR-9 through *in situ* hybridisation (ISH) to determine if the expression of miR-9 is increased in lung parenchyma. Furthermore, it may be important to elucidate the mechanisms that lead to increased lung NRF2 levels observed following inhibition of miR-9 expression. This may be examined using *in vitro* reporter assays such as luciferase assays (416), which may provide insights into the miR-9-dependent, NRF2-mediated regulation of oxidative stress responses in experimental COPD. miR-9 inhibition, increased NRF2 levels but the levels of a known oxidative stress biomarker, 8-isoprostane, were not altered. 8-isoprostane is produced by the oxidation of phospholipids and assessment of its levels may indicate the level of oxidative stress in the tissue. However, with the increase in anti-oxidant responsive transcription factor NRF2 levels, we did not observe changes in 8-isoprostane levels. Therefore, further interrogation is required to assess if the increased NRF2 levels signify increased NRF2 activity. Additionally oxidative stress levels can be measured with indicators of nitrogen free radicals such as nitrotyrosine, which is protein nitration marker (118), or 4-hydroxy-2-nonenal (4-HNE)/HNE (119), which is a stable lipid peroxidation marker. Importantly, we showed that CS-induced increase in miR-9 expression resulted in suppressed lung parenchyma SOCS5 levels which is a known target of miR-9 (286). Furthermore, we also demonstrated that treatment with Ant-9 restored SOCS5 levels in whole lung samples potentially decreasing cytokine signalling through the inhibition of JAK/STAT pathway in experimental COPD (417). A recent study

showed increased levels of phosphorylated STAT1 in lung parenchyma samples of COPD patients compared with smokers and non-smokers without COPD, suggesting of a miR-9-dependent SOCS5- mediated regulation of STAT1 in COPD pathogenesis (314). Thus, further studies are required to investigate the levels of STAT1 and miR-9 induced STAT1 responses in our experimental COPD.

8.4 miR-21-mediated suppression of SATB1 induces S100A9/NF-κB and COPD pathogenesis

Here we identified a previously unrecognised role for miR-21 in COPD pathogenesis. We demonstrate that CS exposure induces a miR-21/SATB1/S100A9/NF-κB axis in the lungs that promotes the cardinal features of COPD, including chronic airway inflammation, small airway remodelling, and impaired lung function. miR-21 is expressed in numerous cell types (418) and its expression is increased in cancers and inflammatory diseases (315, 316). miR-21 is the most highly expressed miR in a range of human cancers such as glioblastoma, lung cancers, and breast, liver and cervical cancers (419-424). miR-21 is considered as a *bona fide* onco-miR as it induces cellular proliferation, reduces apoptosis and increases migration and invasion of cancer cells (425).

miR-21 plays a pleotropic role in inflammatory responses and is involved in both anti- and pro-inflammatory responses (215, 318). In this study we showed that CS exposure chronically increases lung miR-21 expression in experimental COPD. We also showed that increased miR-21 expression correlates with worsened lung function in current smokers, COPD patients and in CS-induced experimental COPD. We demonstrated a novel miR-21-dependent pro-inflammatory pathway in CS-induced experimental COPD, which agrees with many studies showing the pro-inflammatory role of miR-21 such as psoriasis, colitis and cardiovascular diseases (426-428). However, in peritonitis and cerebrovascular diseases, miR-21 is shown to induce anti-inflammatory responses and overexpression of miR-21 in LPS stimulated macrophages, attenuated

TNFα and IL-6 levels reducing the pro-inflammatory responses (429, 430). Thus, increased miR-21 expression can exert pro- or anti-inflammatory effects depending on the stimulus, cell type, tissue localisation, disease state, and target genes affected.

In our study, we show that increased expression of miR-21 is associated with the suppression of its known target SATB1 (220) which is a transcription factor with repressive functions (323). We show that decreased SATB1 levels induces simultaneous increases in the expression of the pro-inflammatory mediator, S100A9, and levels of NF-κB activation. Inhibition of CS-induced miR-21 expression with Ant-21 restored SATB1 expression which was associated with a concomitant reduction in S100A9 expression and NF-κB activity. This reduced the lung levels of the pro-inflammatory mediators TNFα and CXCL1 and resulted in the suppression of key features of COPD. Thus, we demonstrate that CS induces a miR-21/SATB1/S100A9/NF-κB axis **(Figure 8.1)** which promotes the characteristic features of COPD and highlights the importance of therapeutically targeting miR-21. We thus show a novel mechanistic pathway in CS-induced COPD development and identify miR-21 as a novel potential therapeutic target in the treatment of COPD.

miR-21 is known to promote pro-inflammatory, fibrotic responses through the regulation of its target genes, including SPRY1 and 2, PDCD4, and PTEN (259, 333-335). However, in our study, despite the physiological effects of the inhibition of CS-induced miR-21 expression there was no restoration of the expression of these known miR-21 targets. Importantly, these factors are also known to modulate inflammatory and fibrotic responses that are strongly involved in the development of COPD. Thus, it is not clear if the changes in the expression of these target genes were identified accurately in our experimental COPD. Therefore, it would be interesting to examine the functional levels of these targets in the lungs. Our findings warrant further exploration of the specific roles of miR-21 targets such as SPRY1 and 2, PDCD4, and PTEN in the development of COPD. Also additional functional studies with recombinant SATB1 or siRNA against S100A9 are needed, to determine if similar findings as that of miR-21 inhibition will be

observed. Further, it is also important to confirm that SATB1 directly regulates S100A9 through electrophoretic mobility shift or chromatin immunoprecipitation assays to show protein-DNA interactions between SATB1 and S100A9. We also showed increased S100A9 expression with miR-21 inhibition, however, it will be important to confirm whether this change occurs at the protein level.

8.5 Role of miR-135b and miR-146b in the pathogenesis of experimental COPD

Here we demonstrated that CS-induced miR-135b promotes neutrophilic airway inflammation and emphysema. We also showed that CS-induced miR-146b promotes airway fibrosis and emphysema that are associated with impaired lung function in experimental COPD. miR-135b is also a known onco-miR and is implicated in the pathogenesis of several cancers such as breast and lung cancers (357, 431, 432). Increased expression of miR-135b is also associated with poor lung cancer prognosis (357). Increased miR-135b expression in cancers suppresses the tumour suppressor proteins such as TGFβR2, BMPR2, and thus play a crucial role in tumour cell differentiation invasion and metastasis (366, 432). Importantly, miR-135b is also an NF-κB-induced gene (357). In a model of CS-induced lung inflammation, miR-135b expression was shown to decrease the pro-inflammatory responses through a feedback loop mediated by IL-1R1 dependent mechanism (238). However, in anaplastic large cell lymphoma, STAT3 activation elevated miR-135b expression and attenuation of miR-135b was shown to decrease IL-17 induced inflammatory responses (376). This suggests that the overexpression of miR-135b may be important in mediating pro-inflammatory responses.

The miR-146 family of miRs are implicated in the regulation of immune signalling pathways (368). miR-146b is known to fine tune TLR-dependent IRAK1 and TRAF6 signalling and thus regulates innate immune responses (368). Importantly, increased miR-146b expression induces both oncogenic and tumour suppressor effects. In a study on breast cancer patients, increased miR-146b expression positively correlated with

patient survival (433). Xiang et al., also showed that in normal mammary cells, miR-146b expression is rapidly induced in a STAT3-dependent manner. This increase in miR-146b expression was shown to inhibit NF-κB-dependent IL-6 production, thereby decreasing STAT3 activation which led to cancer suppression. But in breast cancer cells, induction of miR-146b was impaired, leading to increased NF-κB-dependent IL-6 signalling and increase in oncogenic properties of the cells such as invasion and metastasis (433). In contrast, in papillary thyroid carcinoma (PTC), miR-146b overexpression was an indicator of poor prognosis in PTC patients (434). In these patients, increased miR-146b expression was associated with cell metastasis and invasion. Collectively, these reports suggest that in STAT3 activated cancers, increase in miR-146b expression promotes tumour suppressor effects and in PTC, increase in miR-146b expression is associated with augmented oncogenic properties. Thus miR-146b plays varied roles which are dependent on context such as stimulus, and tissue localisation.

In this study, we showed that inhibition of CS-induced miR-135b and miR-146b suppressed key disease features of experimental COPD. We demonstrated that inhibition of CS-induced miR-135b expression, suppressed neutrophils numbers reducing neutrophil chemo-attractants CCL7 (56) expression and TNFα (296) levels resulting in reduced airway inflammation. This is similar to the findings published by Halappanavar *et al.*, (238) and Matsuyama et al., (376) where they showed that miR-135b induces neutrophilic inflammation. Increase in miR-135b expression has been shown to inhibit tumour suppressor BMPR2 levels which in turn increases the oncogenic properties (432). BMPR2 is known to induce macrophage production and is also an anti-fibrotic factor (380-382). Importantly, we showed that increase in CS-induced miR-135b suppressed BMPR2 expression and may thus promote increased macrophage infiltration in airways and airway remodelling in experimental COPD. We also showed that CS-induced increase in miR-135b may promote emphysema-like alveolar enlargement through a VEGF-HIF1α dependent mechanism (358). We also showed that CS-induced increase in miR-146b, expression promotes the key features of experimental COPD.

Inhibition of CS-induced miR-146b expression restores IRAK1 and TRAF6 expression, which are known to regulate the innate immune responses (368) and thus may mediate reduced airway remodelling and emphysema in our CS-induced experimental COPD. Thus, this study identifies the therapeutic potential of targeting lung miR-135b and miR-146b in the treatment of COPD **(Figure 8.1)**.

Further studies are required to elucidate the mechanism through which CS-induced miR-135b and miR-146b promote the development of experimental COPD. Inhibition of both miR-135b and miR-146b, suppressed alveolar enlargement in experimental COPD. Therefore it would be interesting to assess the expression pattern of these miRs in parenchyma to determine if the miRs are increased in lung parenchyma in experimental COPD. Further interrogation in *in vitro* systems using siRNA against BMPR2 may be needed to determine if cells deficient in BMPR2, when exposed to cigarette smoke extract, induce increase in macrophage numbers. Additional experiments to examine the lung levels of BMPR2, HIF1α and VEGF are required. Additionally, *in vivo* experiments with recombinant BMPR2 and VEGF may be interesting to determine if similar findings as that of miR-135b inhibition will be observed. Further studies are also required to elucidate the downstream mechanisms through which CS-induced miR-146b promotes the key features of experimental COPD.

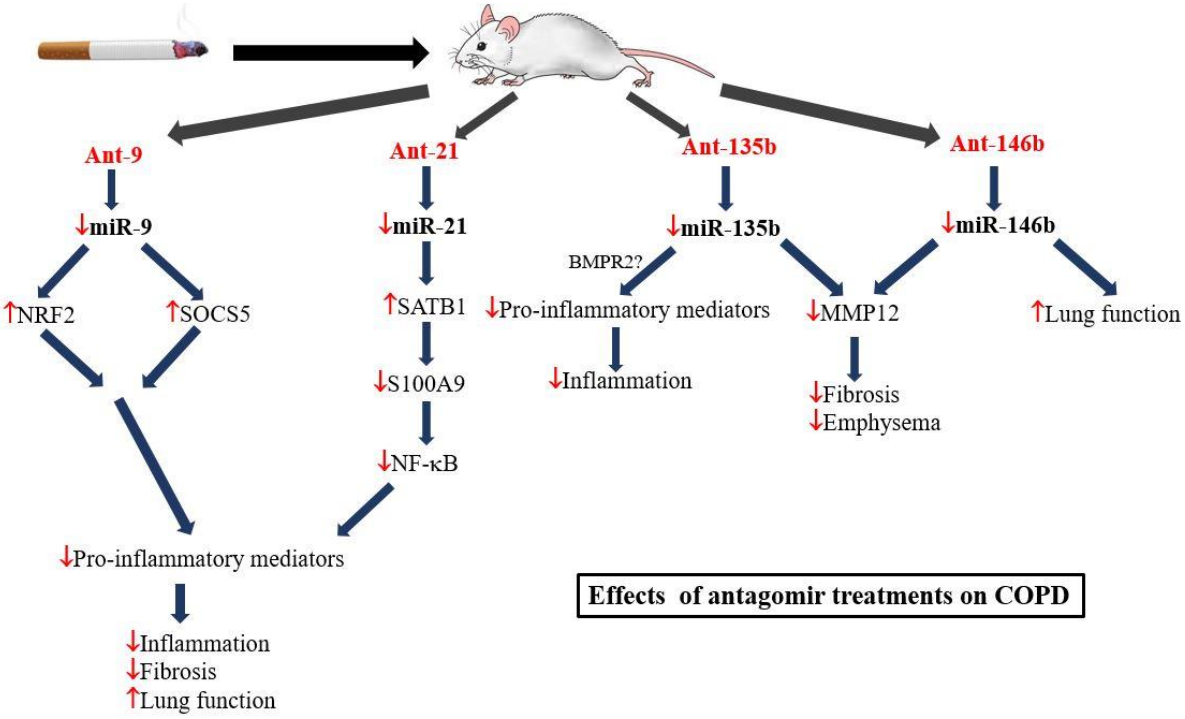


Figure 8.1 The effects of Ants -9, -21, -135b and -146b on various pathways in chronic obstructive pulmonary disease (COPD)

Cigarette smoke exposure increases miRs -9,-21-135b and -146b expression. Inhibition of CS-induced miR-9 with Ant-9 increases NRF2 and SOCS5 which suppresses inflammation, fibrosis and restores lung function in COPD. Inhibition of CS-induced miR-21 with Ant-21 restores SATB1 which in turn suppresses S100A9 mediated increases in NF-κB activity reducing inflammation and fibrosis and improves lung function in COPD. CS-induced miR-135b and miR-146b potentially play overlapping roles in the development of emphysema as their inhibition with Ant-135b and146b suppresses MMP12 which in-turn reduces fibrosis and emphysema.

8.6 Combined targeting of CS-induced miRs in experimental COPD

Here, we investigated the effects of simultaneously targeting pair-wise combinations of four CS-induced miRs (miR-9, -21, -135b and -146b) that when inhibited on their own (Chapter 3, 4 and 5) reduced key features of experimental COPD. We demonstrate that inhibition of specific combinations of these miRs only suppressed some features of experimental COPD. Combined inhibition of CS-induced miR-9 and miR-135b partially decreased airway inflammation and suppressed airway remodelling. Treatment had no effect on CS-induced emphysema and impaired lung function. Simultaneous inhibition of miR-9 and miR-146b, also partially reduced airway inflammation and did not show reduction in any other key features of COPD. Combined inhibition of miR-21 and miR-135b partially reduced CS-induced airway inflammation and completely suppressed emphysema-like alveolar enlargement. Notably, combined inhibition of CS-induced miR-21 and miR-146b expression reduced airway inflammation, suppressed airway remodelling, partially suppressed emphysema and improved lung function, suggesting that targeting these miRs together may be the most effective of the combinations tested for the treatment of COPD. Likewise recent studies have demonstrated that inhibiting a miR in combination with other drugs may be an effective strategy in treatment of cancer (435, 436). These studies showed that such combinatorial treatments may reduce cancer metastasis and invasion through synergistic action of miR inhibition and the drug. However, additional studies are required to fully characterise the mechanisms through which CS-induced miR-21 and -146b promote the development of COPD.

8.7 Future directions

We have effectively shown the knockdown of CS-induced increase in miRs in the whole lung, however our study suggests that there may be differences in the expression of miRs across different cell types or tissues (airways and parenchyma) of lung. Therefore, further experiments are required to inhibit the expression of miRs specifically

across various cell types or tissues to determine miR-induced cell type/tissue specific roles in COPD development. Although in our studies CS-induced increases in miR expression was suppressed we were unable to show the restoration of mRNA expression of some of the predicted targets. This could be explained by examining the functional levels of these targets or luciferase reporter assays could be employed to show miRNA-mRNA interactions or pull down assays could be performed to isolate miRNA-mRNA complexes. Furthermore, we successfully demonstrated a correlation between miRs and their target genes and also showed miR-regulated mechanistic pathways mediating COPD development. However, to conclusively determine the role of miRs in mediating COPD pathogenesis, additional *in vivo* studies with recombinant proteins for the predicted target or siRNA against the down-stream target genes are required.

8.8 Conclusion

In conclusion, our studies have identified the functional and pathogenic roles of four CS-induced miRs that are chronically upregulated in the lung in experimental COPD. Using our established mouse model of CS-induced experimental COPD and miR-specific inhibitors we showed that CS-induced miRs promote airway inflammation and remodelling, emphysema-like alveolar enlargement and impaired lung function. Inhibition of CS-induced miR-9 and miR-21 suppresses airway inflammation and remodelling, indicating a potential pathogenic role for the miRs in the development of COPD. Our studies have demonstrated the importance of NRF2 and SOCS5 in mediating the lung pathologies in COPD. We have also identified a novel miR-21/SATB1/S100A9/NF-κB-dependent inflammatory signalling axis that plays an important role in the pathogenesis of experimental COPD, suggesting that miR-21 plays a pro-inflammatory role in COPD development. We also showed that inhibition of CS-induced miR-135b and miR-146b, suppresses airway remodelling and emphysema-like alveolar enlargement. This indicates that miR-135b and miR-146b play potentially overlapping roles in mediating COPD pathogenesis. We also identified miR-135b and miR-146b targets, BMPR2,

VEGF, IRAK1 and TRAF6 associated with CS-induced airway remodelling and emphysema-like alveolar enlargement. Furthermore, we showed that the combined inhibition of CS-induced miR-21 and miR-146b reduces the key features of the disease, suggesting that inhibiting CS-induced miR-21 and miR-146b together may be more beneficial than inhibiting these miRs individually in COPD.

Thus, targeting CS-induced miRs, either alone or in combination, may be a novel and promising therapeutic strategy, as miRs simultaneously modulate numerous target genes that are involved in several pivotal molecular pathways in the development of COPD, increasing the chances of effective treatment.

8. REFERENCES

1. Snider GL. Nosology for our day: its application to chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2003;167(5):678-83.
2. Snider GL. Chronic obstructive pulmonary disease: risk factors, pathophysiology and pathogenesis. *Annu Rev Med.* 1989;40(411-29.
3. Lokke A, Lange P, Scharling H, Fabricius P, and Vestbo J. Developing COPD: a 25 year follow up study of the general population. *Thorax.* 2006;61(11):935-9.
4. Lundback B, Lindberg A, Lindstrom M, Ronmark E, Jonsson AC, Jonsson E, Larsson LG, Andersson S, Sandstrom T, and Larsson K. Not 15 but 50% of smokers develop COPD?--Report from the Obstructive Lung Disease in Northern Sweden Studies. *Respiratory medicine.* 2003;97(2):115-22.
5. Ko FW, and Hui DS. Air pollution and chronic obstructive pulmonary disease. *Respirology.* 2012;17(3):395-401.
6. Balmes J, Becklake M, Blanc P, Henneberger P, Kreiss K, Mapp C, Milton D, Schwartz D, Toren K, and Viegi G. American Thoracic Society Statement: Occupational contribution to the burden of airway disease. *Am J Respir Crit Care Med.* 2003;167(5):787-97.
7. McClearn GE, Svartengren M, Pedersen NL, Heller DA, and Plomin R. Genetic and environmental influences on pulmonary function in aging Swedish twins. *Journal of gerontology.* 1994;49(6):264-8.
8. Salvi SS, and Barnes PJ. Chronic obstructive pulmonary disease in non-smokers. *Lancet (London, England).* 2009;374(9691):733-43.
9. Wain LV, Shrine N, Miller S, Jackson VE, Ntalla I, Soler Artigas M, Billington CK, Kheirallah AK, Allen R, Cook JP, et al. Novel insights into the genetics of smoking behaviour, lung function, and chronic obstructive pulmonary disease (UK BiLEVE): a genetic association study in UK Biobank. *The Lancet Respiratory medicine.* 2015;3(10):769-81.

10. Chung KF, and Adcock IM. Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. *Eur Respir J.* 2008;31(6):1334-56.
11. Hogg JC. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. *Lancet (London, England).* 2004;364(9435):709-21.
12. Keely S, Talley NJ, and Hansbro PM. Pulmonary-intestinal cross-talk in mucosal inflammatory disease. *Mucosal immunology.* 2012;5(1):7-18.
13. Molfino NA, and Jeffery PK. Chronic obstructive pulmonary disease: Histopathology, inflammation and potential therapies. *Pulmonary Pharmacology & Therapeutics.* 2007;20(5):462-72.
14. Hogg JC. Pathophysiology of airflow limitation in chronic obstructive pulmonary disease. *Lancet.* 2004;364(9435):709-21.
15. Saettaa GTRZM. Pathogenesis and Pathology of COPD *Respiration; international review of thoracic diseases.* 200168):117–28.
16. Taylor JD. COPD and the response of the lung to tobacco smoke exposure. *Pulm Pharmacol Ther.* 2010;23(5):376-83.
17. GOLD. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease 2016. Available from: http://www.goldcopd.org/.
18. Woodruff PG, Barr RG, Bleecker E, Christenson SA, Couper D, Curtis JL, Gouskova NA, Hansel NN, Hoffman EA, Kanner RE, et al. Clinical Significance of Symptoms in Smokers with Preserved Pulmonary Function. *The New England journal of medicine.* 2016;374(19):1811-21.
19. World Health Organization. Global Burden of Disease (GBD) 2002 estimates. In: World Health Report 2004. Geneva: WHO, 2004
20. Halbert RJ, Natoli JL, Gano A, Badamgarav E, Buist AS, and Mannino DM. Global burden of COPD: systematic review and meta-analysis. *Eur Respir J.* 2006;28(3):523-32.

21. Welfare AIoHa. Chronic respiratory diseases in Australia: their prevalence, consequences and prevention. 2005. .Report no. AIHW Cat. No. PHE 63. Canberra: AIHW(

22. How much is spent on COPD? (AIHW). Available from: http://www.aihw.gov.au/copd/expenditure.

23. Gosselink JV, Hayashi S, Elliott WM, Xing L, Chan B, Yang L, Wright C, Sin D, Pare PD, Pierce JA, et al. Differential expression of tissue repair genes in the pathogenesis of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2010;181(12):1329-35.

24. Burgel P-R, Bourdin A, Chanez P, Chabot F, Chaouat A, Chinet T, de Blic J, Devillier P, Deschildre A, Didier A, et al. Update on the roles of distal airways in COPD. *European Respiratory Review.* 2011;20(119):007-22.

25. Salazar LM, and Herrera AM. Fibrotic response of tissue remodeling in COPD. *Lung.* 2011;189(2):101-9.

26. Hogg JC, and Timens W. The Pathology of Chronic Obstructive Pulmonary Disease. *Annual Review of Pathology: Mechanisms of Disease.* 2009;4(1):435-59.

27. Amir Sharafkhaneh475–4771 NAH, and Victor Kim2. Pathogenesis of Emphysema. *Proc Am Thorac Soc.* 2008;5(475–7.

28. Mapel DW, Dalal AA, Johnson PT, Becker LK, and Hunter AG. Application of the new GOLD COPD staging system to a US primary care cohort, with comparison to physician and patient impressions of severity. *International journal of chronic obstructive pulmonary disease.* 2015;10(1477-86.

29. Yoshida T, and Tuder RM. Pathobiology of cigarette smoke-induced chronic obstructive pulmonary disease. *Physiol Rev.* 2007;87(3):1047-82.

30. Demedts IK, Demoor T, Bracke KR, Joos GF, and Brusselle GG. Role of apoptosis in the pathogenesis of COPD and pulmonary emphysema. *Respiratory Research.* 2006;7(53-10.

31. MacNee W. Pathogenesis of chronic obstructive pulmonary disease. *Proc Am Thorac Soc.* 2005;2(4):258-66; discussion 90-1.

32. Yoshikawa T, Dent G, Ward J, Angco G, Nong G, Nomura N, Hirata K, and Djukanovic R. Impaired neutrophil chemotaxis in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2007;175(5):473-9.

33. Sarir H, Henricks PAJ, van Houwelingen AH, Nijkamp FP, and Folkerts G. Cells, mediators and Toll-like receptors in COPD. *European Journal of Pharmacology.* 2008;585(2-3):346-53.

34. Hongwei Y, De Boer WI, and Rahman I. Targeting Lung Inflammation: Novel Therapies for the Treatment of COPD. *Current Respiratory Medicine Reviews.* 2008;4(1):57-68.

35. J. M. Lofdahl JW, C. M. Sköld. Different inflammatory cell pattern and macrophage phenotype in chronic obstructive pulmonary disease patients, smokers and non-smokers. *Clinical and Experimental Immunology.* 2006;145(428–37.

36. Di Stefano A, Capelli A, Lusuardi M, Balbo P, Vecchio C, Maestrelli P, Mapp CE, Fabbri LM, Donner CF, and Saetta M. Severity of airflow limitation is associated with severity of airway inflammation in smokers. *Am J Respir Crit Care Med.* 1998;158(4):1277-85.

37. Russell RE, Culpitt SV, DeMatos C, Donnelly L, Smith M, Wiggins J, and Barnes PJ. Release and activity of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol.* 2002;26(5):602-9.

38. Gorska K, Maskey-Warzechowska M, and Krenke R. Airway inflammation in chronic obstructive pulmonary disease. *Current opinion in pulmonary medicine.* 2010;16(2):89-96.

39. Mortaz E, Kraneveld AD, Smit JJ, Kool M, Lambrecht BN, Kunkel SL, Lukacs NW, Nijkamp FP, and Folkerts G. Effect of cigarette smoke extract on dendritic cells and their impact on T-cell proliferation. *PLoS One.* 2009;4(3):e4946.

40. Diahn-Warng P, Kang-Cheng S, and Lung-Yu L. Airway Inflammation and Airflow Limitation in COPD. *Current Respiratory Medicine Reviews.* 2006;2(4):419-26.

41. Pettersen CA, and Adler KB. Airways inflammation and COPD: epithelial-neutrophil interactions. *Chest.* 2002;121(5 Suppl):142S-50S.

42. Barnes PJ. New Concepts in Chronic Obstructive Pulmonary Disease. *Annual Review of Medicine.* 2003;54(1):113-29.

43. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nature Reviews Immunology.* 2008;8(3):183-92.

44. Tosi MF, Stark JM, Smith CW, Hamedani A, Gruenert DC, and Infeld MD. Induction of ICAM-1 expression on human airway epithelial cells by inflammatory cytokines: effects on neutrophil-epithelial cell adhesion. *Am J Respir Cell Mol Biol.* 1992;7(2):214-21.

45. Lopez-Campos JL, Calero C, Arellano-Orden E, Marquez-Martin E, Cejudo-Ramos P, Ortega Ruiz F, and Montes-Worboys A. Increased levels of soluble ICAM-1 in chronic obstructive pulmonary disease and resistant smokers are related to active smoking. *Biomarkers in medicine.* 2012;6(6):805-11.

46. Tsoumakidou M, Tzanakis N, Kyriakou D, Chrysofakis G, and Siafakas NM. Inflammatory cell profiles and T-lymphocyte subsets in chronic obstructive pulmonary disease and severe persistent asthma. *Clinical & Experimental Allergy.* 2004;34(2):234-40.

47. Maestrelli P, Saetta M, Mapp CE, and Fabbri LM. Remodeling in response to infection and injury. Airway inflammation and hypersecretion of mucus in smoking subjects with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2001;164(10 Pt 2):S76-80.

48. Willemse BW, ten Hacken NH, Rutgers B, Postma DS, and Timens W. Association of current smoking with airway inflammation in chronic obstructive pulmonary disease and asymptomatic smokers. *Respir Res.* 2005;6(38.

49. Stockley RA. Neutrophils and the pathogenesis of COPD. *Chest.* 2002;121(5 Suppl):151S-5S.

50. Zhihui HE, Yan C, Ping C, Guobao WU, and Shan CAI. Local inflammation occurs before systemic inflammation in patients with COPD. *Respirology.* 2010;15(3):478-84.

51. Panina P, Mariani M, and D'Ambrosio D. Chemokine Receptors in Chronic Obstructive Pulmonary Disease (COPD). *Current drug targets.* 2006;7(6):669-74.

52. Larsson K. Aspects on pathophysiological mechanisms in COPD. *Journal of Internal Medicine.* 2007;262(3):311-40.

53. Tetley TD. Inflammatory cells and chronic obstructive pulmonary disease. *Current drug targets Inflammation and allergy.* 2005;4(6):607-18.

54. Keatings VM, Collins PD, Scott DM, and Barnes PJ. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med.* 1996;153(2):530-4.

55. Vlahos R, Bozinovski S, Jones JE, Powell J, Gras J, Lilja A, Hansen MJ, Gualano RC, Irving L, and Anderson GP. Differential protease, innate immunity, and NF-kappaB induction profiles during lung inflammation induced by subchronic cigarette smoke exposure in mice. *American journal of physiology Lung cellular and molecular physiology.* 2006;290(5):L931-45.

56. Mercer PF, Williams AE, Scotton CJ, Jose RJ, Sulikowski M, Moffatt JD, Murray LA, and Chambers RC. Proteinase-activated receptor-1, CCL2, and CCL7 regulate acute neutrophilic lung inflammation. *Am J Respir Cell Mol Biol.* 2014;50(1):144-57.

57. Chan JK, Roth J, Oppenheim JJ, Tracey KJ, Vogl T, Feldmann M, Horwood N, and Nanchahal J. Alarmins: awaiting a clinical response. *The Journal of clinical investigation.* 2012;122(8):2711-9.

58. Ehrchen JM, Sunderkotter C, Foell D, Vogl T, and Roth J. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *Journal of leukocyte biology.* 2009;86(3):557-66.

59. Foell D, Wittkowski H, Vogl T, and Roth J. S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. *Journal of leukocyte biology.* 2007;81(1):28-37.

60. Heizmann CW, Fritz G, and Schafer BW. S100 proteins: structure, functions and pathology. *Frontiers in bioscience : a journal and virtual library.* 2002;7(d1356-68.

61. Chen B, Miller AL, Rebelatto M, Brewah Y, Rowe DC, Clarke L, Czapiga M, Rosenthal K, Imamichi T, Chen Y, et al. S100A9 induced inflammatory responses are mediated by distinct damage associated molecular patterns (DAMP) receptors in vitro and in vivo. *PLoS One.* 2015;10(2):e0115828.

62. Li M, Guo L, Wang H, Wang T, Shen Y, Liao Z, Wen F, and Chen L. RAGE-ligands axis: A new 'driving force' for cigarette smoke-induced airway inflammation in COPD? *Respirology.* 2015;20(6):998-9.

63. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, van Zoelen MA, Nacken W, Foell D, van der Poll T, Sorg C, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat Med.* 2007;13(9):1042-9.

64. Kuipers MT, Vogl T, Aslami H, Jongsma G, van den Berg E, Vlaar AP, Roelofs JJ, Juffermans NP, Schultz MJ, van der Poll T, et al. High levels of S100A8/A9 proteins aggravate ventilator-induced lung injury via TLR4 signaling. *PLoS One.* 2013;8(7):e68694.

65. Gray RD, MacGregor G, Noble D, Imrie M, Dewar M, Boyd AC, Innes JA, Porteous DJ, and Greening AP. Sputum proteomics in inflammatory and suppurative respiratory diseases. *Am J Respir Crit Care Med.* 2008;178(5):444-52.

66. Holmgaard DB, Mygind LH, Titlestad I, Madsen H, Pedersen SS, Mortensen OH, and Pedersen C. Calprotectin--a marker of mortality in COPD? Results from a prospective cohort study. *Copd.* 2013;10(5):581-7.

67. Barnes PJ. Alveolar macrophages as orchestrators of COPD. *Basic Science Review.* 2004;1(1):59-70.

68. Hamilton JA. GM-CSF in inflammation and autoimmunity. *Trends in immunology.* 2002;23(8):403-8.

69. Saha S, Doe C, Mistry V, Siddiqui S, Parker D, Sleeman M, Cohen ES, and Brightling CE. Granulocyte-macrophage colony-stimulating factor expression in induced sputum and bronchial mucosa in asthma and COPD. *Thorax.* 2009;64(8):671-6.

70. Xing Z, Ohkawara Y, Jordana M, Graham F, and Gauldie J. Transfer of granulocyte-macrophage colony-stimulating factor gene to rat lung induces eosinophilia, monocytosis, and fibrotic reactions. *The Journal of clinical investigation.* 1996;97(4):1102-10.

71. Vlahos R, Bozinovski S, Chan SP, Ivanov S, Linden A, Hamilton JA, and Anderson GP. Neutralizing granulocyte/macrophage colony-stimulating factor inhibits cigarette smoke-induced lung inflammation. *Am J Respir Crit Care Med.* 2010;182(1):34-40.

72. Beckett EL, Stevens RL, Jarnicki AG, Kim RY, Hanish I, Hansbro NG, Deane A, Keely S, Horvat JC, Yang M, et al. A new short-term mouse model of chronic obstructive pulmonary disease identifies a role for mast cell tryptase in pathogenesis. *J Allergy Clin Immunol.* 2013;131(3):752-62.

73. Southworth T, Metryka A, Lea S, Farrow S, Plumb J, and Singh D. IFN-gamma synergistically enhances LPS signalling in alveolar macrophages from COPD patients and controls by corticosteroid-resistant STAT1 activation. *British journal of pharmacology.* 2012;166(7):2070-83.

74. Laskin DL, Sunil VR, Gardner CR, and Laskin JD. Macrophages and Tissue Injury: Agents of Defense or Destruction? *Annual Review of Pharmacology and Toxicology.* 2011;51(1):267-88.

75. Takanashi S, Hasegawa Y, Kanehira Y, Yamamoto K, Fujimoto K, Satoh K, and Okamura K. Interleukin-10 level in sputum is reduced in bronchial asthma, COPD and in smokers. *Eur Respir J.* 1999;14(2):309-14.

76. Zhang L, Cheng Z, Liu W, and Wu K. Expression of interleukin (IL)-10, IL-17A and IL-22 in serum and sputum of stable chronic obstructive pulmonary disease patients. *Copd.* 2013;10(4):459-65.

77. Cosio MG, and Majo J. Inflammation of the airways and lung parenchyma in COPD: role of T cells. *Chest.* 2002;121(5 Suppl):160S-5S.

78. O'Shaughnessy TC, Ansari TW, Barnes NC, and Jeffery PK. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. *Am J Respir Crit Care Med.* 1997;155(3):852-7.

79. Saetta M, Baraldo S, Corbino L, Turato G, Braccioni F, Rea F, Cavallesco G, Tropeano G, Mapp CE, Maestrelli P, et al. CD8+ve cells in the lungs of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 1999;160(2):711-7.

80. Baraldo S, Turato G, Badin C, Bazzan E, Beghe B, Zuin R, Calabrese F, Casoni G, Maestrelli P, Papi A, et al. Neutrophilic infiltration within the airway smooth muscle in patients with COPD. *Thorax.* 2004;59(4):308-12.

81. Traves SL, and Donnelly LE. Th17 Cells in Airway Diseases. *Current molecular medicine.* 2008;8(5):416-26.

82. Bathoorn E, Kerstjens H, Postma D, Timens W, and MacNee W. Airways inflammation and treatment during acute exacerbations of COPD. *International journal of chronic obstructive pulmonary disease.* 2008;3(2):217-29.

83. Sheree Brozyna JA, Greg Hodge, Judith Nairn, Mark Holmes, Paul N. Reynolds, Sandra Hodge. Chemotactic Mediators of Th1 T-cell Trafficking in Smokers and COPD Patients. *Journal of Chronic Obstructive Pulmonary Disease.* 2009;6(4-16.

84. Plataki M, Tzortzaki E, Rytila P, Demosthenes M, Koutsopoulos A, and Siafakas NM. Apoptotic mechanisms in the pathogenesis of COPD. *International journal of chronic obstructive pulmonary disease.* 2006;1(2):161-71.

85. Hizawa N, Kawaguchi M, Huang SK, and Nishimura M. Role of interleukin-17F in chronic inflammatory and allergic lung disease. *Clinical & Experimental Allergy.* 2006;36(9):1109-14.

86. Caramori G, Casolari P, Cavallesco GN, Giuffre S, Adcock I, and Papi A. Mechanisms involved in lung cancer development in COPD. *Int J Biochem Cell Biol.* 2010.

87. Hong SC, and Lee SH. Role of th17 cell and autoimmunity in chronic obstructive pulmonary disease. *Immune network.* 2010;10(4):109-14.

88. Kim V, Rogers TJ, and Criner GJ. New concepts in the pathobiology of chronic obstructive pulmonary disease. *Proc Am Thorac Soc.* 2008;5(4):478-85.

89. Caramori G, Casolari P, Di Gregorio C, Saetta M, Baraldo S, Boschetto P, Ito K, Fabbri LM, Barnes PJ, Adcock IM, et al. MUC5AC expression is increased in bronchial submucosal glands of stable COPD patients. *Histopathology.* 2009;55(3):321-31.

90. Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO, et al. The nature of small-airway obstruction in chronic obstructive pulmonary disease. *The New England journal of medicine.* 2004;350(26):2645-53.

91. Gosman MM, Willemse BW, Jansen DF, Lapperre TS, van Schadewijk A, Hiemstra PS, Postma DS, Timens W, and Kerstjens HA. Increased number of B-cells in bronchial biopsies in COPD. *Eur Respir J.* 2006;27(1):60-4.

92. van der Strate BW, Postma DS, Brandsma CA, Melgert BN, Luinge MA, Geerlings M, Hylkema MN, van den Berg A, Timens W, and Kerstjens HA. Cigarette smoke-induced emphysema: A role for the B cell? *Am J Respir Crit Care Med.* 2006;173(7):751-8.

93. John-Schuster G, Hager K, Conlon TM, Irmler M, Beckers J, Eickelberg O, and Yildirim AO. Cigarette smoke-induced iBALT mediates macrophage activation in a B cell-dependent manner in COPD. *American journal of physiology Lung cellular and molecular physiology.* 2014;307(9):L692-706.

94. Vassallo R, Walters PR, Lamont J, Kottom TJ, Yi ES, and Limper AH. Cigarette smoke promotes dendritic cell accumulation in COPD; a Lung Tissue Research Consortium study. *Respir Res.* 2010;11(45.

95. Harada H, Imamura M, Okunishi K, Nakagome K, Matsumoto T, Sasaki O, Tanaka R, Yamamoto K, and Dohi M. Upregulation of lung dendritic cell functions in elastase-induced emphysema. *International archives of allergy and immunology.* 2009;149 Suppl 1(25-30.

96. Bracke KR, D'Hulst A I, Maes T, Moerloose KB, Demedts IK, Lebecque S, Joos GF, and Brusselle GG. Cigarette smoke-induced pulmonary inflammation and emphysema are attenuated in CCR6-deficient mice. *Journal of immunology (Baltimore, Md : 1950).* 2006;177(7):4350-9.

97. Botelho FM, Gaschler GJ, Kianpour S, Zavitz CC, Trimble NJ, Nikota JK, Bauer CM, and Stampfli MR. Innate immune processes are sufficient for driving cigarette smoke-induced inflammation in mice. *Am J Respir Cell Mol Biol.* 2010;42(4):394-403.

98. Givi ME, Akbari P, Boon L, Puzovic VS, Bezemer GF, Ricciardolo FL, Folkerts G, Redegeld FA, and Mortaz E. Dendritic cells inversely regulate airway

inflammation in cigarette smoke-exposed mice. *American journal of physiology Lung cellular and molecular physiology.* 2016;310(1):L95-102.

99. Sorheim IC, Gulsvik, A, Bakke, P. S, Brogger, J. C, Grydeland, T. B, Silverman, E. K. Genetics in chronic obstructive pulmonary disease. *Tidsskr Nor Laegeforen.* 2009;129(20):2104-7.

100. Lin JL, and Thomas PS. Current perspectives of oxidative stress and its measurement in chronic obstructive pulmonary disease. *COPD.* 2010;7(4):291-306.

101. Daheshia M. Pathogenesis of chronic obstructive pulmonary disease (COPD). *Clinical & Applied Immunology Reviews.* 2005;5(5):339-51.

102. John R. Spurzem SIR. Pathogenesis of COPD. *From Seminars in Respiratory and Critical Care Medicine* 2005;26(2):142-253.

103. Rangasamy T, Misra V, Zhen L, Tankersley CG, Tuder RM, and Biswal S. Cigarette smoke-induced emphysema in A/J mice is associated with pulmonary oxidative stress, apoptosis of lung cells, and global alterations in gene expression. *American journal of physiology Lung cellular and molecular physiology.* 2009;296(6):L888-900.

104. MacNee W. Oxidative stress and lung inflammation in airways disease. *European Journal of Pharmacology.* 2001;429(1-3):195-207.

105. Rahman I. Oxidative Stress and Gene Transcription in Asthma and Chronic Obstructive Pulmonary Disease: Antioxidant Therapeutic Targets. *Current Drug Targets - Inflammation & Allergy.* 2002;1(3):291-315.

106. Yao H, and Rahman I. Current concepts on oxidative/carbonyl stress, inflammation and epigenetics in pathogenesis of chronic obstructive pulmonary disease. *Toxicol Appl Pharmacol.* 2011.

107. Barnes PJ. Role of HDAC2 in the Pathophysiology of COPD. *Annual Review of Physiology.* 2009;71(1):451-64.

108. Bowman RV, Wright CM, Davidson MR, Savarimuthu Francis SM, Yang IA, and Fong KM. Epigenomic targets for the treatment of respiratory disease. *Expert Opinion on Therapeutic Targets.* 2009;13(6):625-40.

109. Yao H, and Rahman I. Role of histone deacetylase 2 in epigenetics and cellular senescence: implications in lung inflammaging and COPD. *American journal of physiology Lung cellular and molecular physiology.* 2012;303(7):L557-66.

110. Groutas WC, Dou D, and Alliston KR. Neutrophil elastase inhibitors. *Expert Opinion on Therapeutic Patents.*0(0):1-16.

111. Chanas SA, Jiang Q, McMahon M, McWalter GK, McLellan LI, Elcombe CR, Henderson CJ, Wolf CR, Moffat GJ, Itoh K, et al. Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione S-transferase Gsta1, Gsta2, Gstm1, Gstm2, Gstm3 and Gstm4 genes in the livers of male and female mice. *The Biochemical journal.* 2002;365(Pt 2):405-16.

112. Itoh K, Wakabayashi N, Katoh Y, Ishii T, Igarashi K, Engel JD, and Yamamoto M. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes & development.* 1999;13(1):76-86.

113. Cho HY, Reddy SP, Debiase A, Yamamoto M, and Kleeberger SR. Gene expression profiling of NRF2-mediated protection against oxidative injury. *Free radical biology & medicine.* 2005;38(3):325-43.

114. Goven D, Boutten A, Lecon-Malas V, Marchal-Somme J, Amara N, Crestani B, Fournier M, Leseche G, Soler P, Boczkowski J, et al. Altered Nrf2/Keap1-Bach1 equilibrium in pulmonary emphysema. *Thorax.* 2008;63(10):916-24.

115. Sussan TE, Rangasamy T, Blake DJ, Malhotra D, El-Haddad H, Bedja D, Yates MS, Kombairaju P, Yamamoto M, Liby KT, et al. Targeting Nrf2 with the triterpenoid CDDO-imidazolide attenuates cigarette smoke-induced emphysema and cardiac dysfunction in mice. *Proc Natl Acad Sci U S A.* 2009;106(1):250-5.

116. Iizuka T, Ishii Y, Itoh K, Kiwamoto T, Kimura T, Matsuno Y, Morishima Y, Hegab AE, Homma S, Nomura A, et al. Nrf2-deficient mice are highly susceptible to cigarette smoke-induced emphysema. *Genes to cells : devoted to molecular & cellular mechanisms.* 2005;10(12):1113-25.

117. Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, Yamamoto M, Petrache I, Tuder RM, and Biswal S. Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. *The Journal of clinical investigation.* 2004;114(9):1248-59.

118. Ahsan H. 3-Nitrotyrosine: A biomarker of nitrogen free radical species modified proteins in systemic autoimmunogenic conditions. *Human immunology.* 2013;74(10):1392-9.

119. Rahman I, van Schadewijk AA, Crowther AJ, Hiemstra PS, Stolk J, MacNee W, and De Boer WI. 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2002;166(4):490-5.

120. Montuschi P, Collins JV, Ciabattoni G, Lazzeri N, Corradi M, Kharitonov SA, and Barnes PJ. Exhaled 8-isoprostane as an in vivo biomarker of lung oxidative stress in patients with COPD and healthy smokers. *Am J Respir Crit Care Med.* 2000;162(3 Pt 1):1175-7.

121. Ichinose M, Sugiura H, Yamagata S, Koarai A, and Shirato K. Increase in reactive nitrogen species production in chronic obstructive pulmonary disease airways. *Am J Respir Crit Care Med.* 2000;162(2 Pt 1):701-6.

122. Psarras S, Caramori G, Contoli M, Papadopoulos N, and Papi A. Oxidants in Asthma and in Chronic Obstructive Pulmonary Disease (COPD). *Current Pharmaceutical Design.* 2005;11(16):2053-62.

123. Carol A. Pettersen KBA. Airways Inflammation and COPD Epithelial-Neutrophil Interactions. *Chest.* 2002;121(142-50.

124. Ohbayashi H. Neutrophil elastase inhibitors as treatment for COPD. *Expert opinion on investigational drugs.* 2002;11(7):965-80.

125. Sang QX, Muroski ME, Roycik MD, Newcomer RG, Van den Steen PE, Opdenakker G, Monroe HR, and Sahab ZJ. Matrix Metalloproteinase-9/Gelatinase B is a Putative Therapeutic Target of Chronic Obstructive Pulmonary Disease and Multiple Sclerosis. *Current Pharmaceutical Biotechnology.* 2008;9(1):34-46.

126. Tetley TD. Inflammatory Cells and Chronic Obstructive Pulmonary Disease. *Current Drug Targets - Inflammation & Allergy.* 2005;4(6):607-18.

127. Tetley TD. Inflammatory Cells and Chronic Obstructive Pulmonary Disease. *Current Drug Targets - Inflammation & Allergy.* 2005;4(607-18.

128. Bracke K, Cataldo D, Maes T, Gueders M, Noel A, Foidart JM, Brusselle G, and Pauwels RA. Matrix metalloproteinase-12 and cathepsin D expression in pulmonary macrophages and dendritic cells of cigarette smoke-exposed mice. *International archives of allergy and immunology.* 2005;138(2):169-79.

129. Maria Plataki ET, Paula Rytila, Anastassios Koutsopoulos, . Apoptotic mechanisms in the pathogenesis of COPD. *International Journal of COPD.* 20062):161–71.

130. Morissette MC, Parent J, and Milot J. Alveolar epithelial and endothelial cell apoptosis in emphysema: what we know and what we need to know. *International journal of chronic obstructive pulmonary disease.* 2009;4(19-31.

131. Park J-W, Ryter SW, and Choi AMK. Functional Significance of Apoptosis in Chronic Obstructive Pulmonary Disease. *COPD: Journal of Chronic Obstructive Pulmonary Disease.* 2007;4(4):347-53.

132. Hodge S, Hodge G, Holmes M, and Reynolds PN. Apoptosis in COPD. *Current Respiratory Medicine Reviews.* 2005;1(1):33-41.

133. Ingel K Demedts TD, Ken R Bracke, Guy F Joos, Guy G Brusselle. Role of apoptosis in the pathogenesis of COPD and pulmonary emphysema. *Respiratory Research.* 2006;7(53).

134. Elmore S. Apoptosis: a review of programmed cell death. *Toxicologic pathology.* 2007;35(4):495-516.

135. Sharafkhaneh A, Hanania NA, and Kim V. Pathogenesis of emphysema: from the bench to the bedside. *Proc Am Thorac Soc.* 2008;5(4):475-7.

136. Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J, and Voelkel NF. Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *The Journal of clinical investigation.* 2000;106(11):1311-9.

137. Aoshiba K, Rennard SI, and Spurzem JR. Cell-matrix and cell-cell interactions modulate apoptosis of bronchial epithelial cells. *The American journal of physiology.* 1997;272(1 Pt 1):L28-37.

138. Aoshiba K, Yokohori N, and Nagai A. Alveolar wall apoptosis causes lung destruction and emphysematous changes. *Am J Respir Cell Mol Biol.* 2003;28(5):555-62.

139. Tuder RM, Zhen L, Cho CY, Taraseviciene-Stewart L, Kasahara Y, Salvemini D, Voelkel NF, and Flores SC. Oxidative stress and apoptosis interact and cause emphysema due to vascular endothelial growth factor receptor blockade. *Am J Respir Cell Mol Biol.* 2003;29(1):88-97.

140. Demura Y, Taraseviciene-Stewart L, Scerbavicius R, Tuder RM, and Voelkel NF. N-acetylcysteine treatment protects against VEGF-receptor blockade-related emphysema. *Copd.* 2004;1(1):25-32.

141. Knox AJ, Stocks J, and Sutcliffe A. Angiogenesis and vascular endothelial growth factor in COPD. *Thorax.* 2005;60(2):88-9.

142. Kranenburg AR, de Boer WI, Alagappan VK, Sterk PJ, and Sharma HS. Enhanced bronchial expression of vascular endothelial growth factor and

receptors (Flk-1 and Flt-1) in patients with chronic obstructive pulmonary disease. *Thorax.* 2005;60(2):106-13.

143. Mizushima N, and Komatsu M. Autophagy: renovation of cells and tissues. *Cell.* 2011;147(4):728-41.

144. Zhang J. Autophagy and Mitophagy in Cellular Damage Control. *Redox biology.* 2013;1(1):19-23.

145. Levine B, and Kroemer G. Autophagy in the pathogenesis of disease. *Cell.* 2008;132(1):27-42.

146. Levine B, Mizushima N, and Virgin HW. Autophagy in immunity and inflammation. *Nature.* 2011;469(7330):323-35.

147. Ryter SW, and Choi AM. Autophagy in lung disease pathogenesis and therapeutics. *Redox biology.* 2015;4(215-25.

148. An CH, Wang XM, Lam HC, Ifedigbo E, Washko GR, Ryter SW, and Choi AM. TLR4 deficiency promotes autophagy during cigarette smoke-induced pulmonary emphysema. *American journal of physiology Lung cellular and molecular physiology.* 2012;303(9):L748-57.

149. Chen ZH, Kim HP, Sciurba FC, Lee SJ, Feghali-Bostwick C, Stolz DB, Dhir R, Landreneau RJ, Schuchert MJ, Yousem SA, et al. Egr-1 regulates autophagy in cigarette smoke-induced chronic obstructive pulmonary disease. *PLoS One.* 2008;3(10):e3316.

150. Hwang JW, Chung S, Sundar IK, Yao H, Arunachalam G, McBurney MW, and Rahman I. Cigarette smoke-induced autophagy is regulated by SIRT1-PARP-1-dependent mechanism: implication in pathogenesis of COPD. *Archives of biochemistry and biophysics.* 2010;500(2):203-9.

151. Ryter SW, Lee SJ, and Choi AM. Autophagy in cigarette smoke-induced chronic obstructive pulmonary disease. *Expert Rev Respir Med.* 2010;4(5):573-84.

152. Kim HP, Wang X, Chen ZH, Lee SJ, Huang MH, Wang Y, Ryter SW, and Choi AM. Autophagic proteins regulate cigarette smoke-induced apoptosis: protective role of heme oxygenase-1. *Autophagy.* 2008;4(7):887-95.

153. Chen ZH, Lam HC, Jin Y, Kim HP, Cao J, Lee SJ, Ifedigbo E, Parameswaran H, Ryter SW, and Choi AM. Autophagy protein microtubule-associated protein 1 light chain-3B (LC3B) activates extrinsic apoptosis during cigarette smoke-induced emphysema. *Proc Natl Acad Sci U S A.* 2010;107(44):18880-5.

154. Zhu L, Barrett EC, Xu Y, Liu Z, Manoharan A, and Chen Y. Regulation of Cigarette Smoke (CS)-Induced Autophagy by Nrf2. *PLoS One.* 2013;8(4):e55695.

155. Nakken B, Alex P, Munthe L, Szekanecz Z, and Szodoray P. Immune-regulatory mechanisms in systemic autoimmune and rheumatic diseases. *Clinical & developmental immunology.* 2012;2012(957151.

156. Rutgers SR, Postma DS, ten Hacken NH, Kauffman HF, van Der Mark TW, Koeter GH, and Timens W. Ongoing airway inflammation in patients with COPD who do not currently smoke. *Thorax.* 2000;55(1):12-8.

157. Grumelli S, Corry DB, Song LZ, Song L, Green L, Huh J, Hacken J, Espada R, Bag R, Lewis DE, et al. An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema. *PLoS medicine.* 2004;1(1):e8.

158. Feghali-Bostwick CA, Gadgil AS, Otterbein LE, Pilewski JM, Stoner MW, Csizmadia E, Zhang Y, Sciurba FC, and Duncan SR. Autoantibodies in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2008;177(2):156-63.

159. Saetta M, Turato G, Maestrelli P, Mapp CE, and Fabbri LM. Cellular and structural bases of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2001;163(6):1304-9.

160. Kirkham PA, Caramori G, Casolari P, Papi AA, Edwards M, Shamji B, Triantaphyllopoulos K, Hussain F, Pinart M, Khan Y, et al. Oxidative stress-induced antibodies to carbonyl-modified protein correlate with severity of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2011;184(7):796-802.

161. Lee SH, Goswami S, Grudo A, Song LZ, Bandi V, Goodnight-White S, Green L, Hacken-Bitar J, Huh J, Bakaeen F, et al. Antielastin autoimmunity in tobacco smoking-induced emphysema. *Nat Med.* 2007;13(5):567-9.

162. Hsu AC, Starkey MR, Hanish I, Parsons K, Haw TJ, Howland LJ, Barr I, Mahony JB, Foster PS, Knight DA, et al. Targeting PI3K-p110alpha Suppresses Influenza Virus Infection in Chronic Obstructive Pulmonary Disease. *Am J Respir Crit Care Med.* 2015;191(9):1012-23.

163. Hurst JR, Vestbo J, Anzueto A, Locantore N, Mullerova H, Tal-Singer R, Miller B, Lomas DA, Agusti A, Macnee W, et al. Susceptibility to exacerbation in chronic obstructive pulmonary disease. *The New England journal of medicine.* 2010;363(12):1128-38.

164. Maria Tsoumakidou aNMS. Novel insights into the aetiology and pathophysiology of increased airway inflammation during COPD exacerbations. *Respiratory Research.* 2006;7(80):1-10.

165. Saetta M, Di Stefano A, Maestrelli P, Turato G, Ruggieri MP, Roggeri A, Calcagni P, Mapp CE, Ciaccia A, and Fabbri LM. Airway eosinophilia in chronic bronchitis during exacerbations. *Am J Respir Crit Care Med.* 1994;150(6 Pt 1):1646-52.

166. Saetta M, Di Stefano A, Maestrelli P, Turato G, Mapp CE, Pieno M, Zanguochi G, Del Prete G, and Fabbri LM. Airway eosinophilia and expression of interleukin-5 protein in asthma and in exacerbations of chronic bronchitis. *Clin Exp Allergy.* 1996;26(7):766-74.

167. Qiu Y, Zhu J, Bandi V, Atmar RL, Hattotuwa K, Guntupalli KK, and Jeffery PK. Biopsy neutrophilia, neutrophil chemokine and receptor gene expression in

severe exacerbations of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2003;168(8):968-75.

168. Dekhuijzen PN, Aben KK, Dekker I, Aarts LP, Wielders PL, van Herwaarden CL, and Bast A. Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 1996;154(3 Pt 1):813-6.

169. Agusti AG, Villaverde JM, Togores B, and Bosch M. Serial measurements of exhaled nitric oxide during exacerbations of chronic obstructive pulmonary disease. *Eur Respir J.* 1999;14(3):523-8.

170. Eagan TM, Ueland T, Wagner PD, Hardie JA, Mollnes TE, Damas JK, Aukrust P, and Bakke PS. Systemic inflammatory markers in COPD: results from the Bergen COPD Cohort Study. *Eur Respir J.* 2010;35(3):540-8.

171. Larsson K. Inflammatory markers in COPD. *Clinical Respiratory Journal.* 2008;2(84-7.

172. Gagnon P, Guenette JA, Langer D, Laviolette L, Mainguy V, Maltais F, Ribeiro F, and Saey D. Pathogenesis of hyperinflation in chronic obstructive pulmonary disease. *International journal of chronic obstructive pulmonary disease.* 2014;9(187-201.

173. Aoshiba K, and Nagai A. Differences in airway remodeling between asthma and chronic obstructive pulmonary disease. *Clinical reviews in allergy & immunology.* 2004;27(1):35-43.

174. Ramos FL, Krahnke JS, and Kim V. Clinical issues of mucus accumulation in COPD. *International journal of chronic obstructive pulmonary disease.* 2014;9(139-50.

175. Pini L, Pinelli V, Modina D, Bezzi M, Tiberio L, and Tantucci C. Central airways remodeling in COPD patients. *International journal of chronic obstructive pulmonary disease.* 2014;9(927-32.

176. Hogg JC, McDonough JE, and Suzuki M. Small airway obstruction in COPD: new insights based on micro-CT imaging and MRI imaging. *Chest.* 2013;143(5):1436-43.

177. Stewart JI, and Criner GJ. The small airways in chronic obstructive pulmonary disease: pathology and effects on disease progression and survival. *Current opinion in pulmonary medicine.* 2013;19(2):109-15.

178. Burgel P-R. The role of small airways in obstructive airway diseases. *European Respiratory Review.* 2011;20(119):023-33.

179. Francis SM, Larsen JE, Pavey SJ, Bowman RV, Hayward NK, Fong KM, and Yang IA. Expression profiling identifies genes involved in emphysema severity. *Respir Res.* 2009;10(81.

180. Cerveri I, and Brusasco V. Revisited role for mucus hypersecretion in the pathogenesis of COPD. *European Respiratory Review.* 2010;19(116):109-12.

181. Osadnik CR, McDonald CF, and Holland AE. Clinical issues of mucus accumulation in COPD. *International journal of chronic obstructive pulmonary disease.* 2014;9(301-2.

182. Barnes PJ. Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease. *J Allergy Clin Immunol.* 2013;131(3):636-45.

183. Theron AJ, Steel HC, Tintinger GR, Feldman C, and Anderson R. Can the anti-inflammatory activities of beta2-agonists be harnessed in the clinical setting? *Drug design, development and therapy.* 2013;7(1387-98.

184. Caramori G, and Adcock I. Pharmacology of airway inflammation in asthma and COPD. *Pulmonary Pharmacology & Therapeutics.* 2003;16(5):247-77.

185. P.J. Barnes RAS. COPD: current therapeutic interventions and future approaches. *Eur Respir J.* 2005;1084–1106(25).

186. Dunsmore SE. Treatment of COPD: a matrix perspective. *International journal of chronic obstructive pulmonary disease.* 2008;3(1):113-22.

187. Barnes PJ. Strategies for Novel COPD Therapies. *Pulmonary Pharmacology & Therapeutics.* 1999;12(2):67-71.

188. Prospects for new drugs for chronic obstructive pulmonary disease. *Lancet (London, England).* 2004;364(9438):985-96.

189. Spina D. Pharmacology of novel treatments for COPD: are fixed dose combination LABA/LAMA synergistic? *European clinical respiratory journal.* 2015;2(

190. SÃ©guin RM, and Ferrari N. Emerging oligonucleotide therapies for asthma and chronic obstructive pulmonary disease. *Expert opinion on investigational drugs.* 2009;18(10):1505-17.

191. Wang Z. Springer-Verlag-Berlin Heidelberg 2009; 2009.

192. Foster PS, Plank M, Collison A, Tay HL, Kaiko GE, Li J, Johnston SL, Hansbro PM, Kumar RK, Yang M, et al. The emerging role of microRNAs in regulating immune and inflammatory responses in the lung. *Immunological reviews.* 2013;253(1):198-215.

193. Dai R, and Ahmed SA. MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases. *Translational Research.* 2011;157(4):163-79.

194. Liu X, Sempere LF, Guo Y, Korc M, Kauppinen S, Freemantle SJ, and Dmitrovsky E. Involvement of microRNAs in lung cancer biology and therapy. *Translational Research.* 2011;157(4):200-8.

195. XIA Wei CGSN. **Progress in miRNA target prediction and identification**. *Science in China Series C: Life Sciences.* 2009;52(12):**1123-30**.

196. Bowman RV, Wright CM, Davidson MR, Francis SM, Yang IA, and Fong KM. Epigenomic targets for the treatment of respiratory disease. *Expert Opin Ther Targets.* 2009;13(6):625-40.

197. Felekkis K, Touvana E, Stefanou C, and Deltas C. microRNAs: a newly described class of encoded molecules that play a role in health and disease. *Hippokratia.* 2010;14(4):236-40.

198. Pieter Bas Kwak SI, and Yukihide Tomari. The microRNA pathway and cancer. *Official Journal of Japanese Cancer Association(Cancer Science).* 2010;101(11):2309–15.

199. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* 2004;116(2):281-97.

200. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell.* 2009;136(2):215-33.

201. Jinbiao MA YH. Post-transcriptional regulation of miRNA biogenesis and functions. *Front Biol.* 2001;5(1):32-40.

202. Shao-Yao Ying DCC, Shi-Lung Lin. The MicroRNA (miRNA): Overview of the RNA Genes that Modulate Gene Function. *Mol Biotechnol.* 200838):257–68.

203. Tang G, Reinhart BJ, Bartel DP, and Zamore PD. A biochemical framework for RNA silencing in plants. *Genes & development.* 2003;17(1):49-63.

204. Oglesby IK, McElvaney NG, and Greene CM. MicroRNAs in inflammatory lung disease--master regulators or target practice? *Respir Res.* 2010;11(148.

205. **Alberto Izzotti GAC, Vernon E. Steele, Carlo M. Croce, and Silvio De Flora**. **Relationships of microRNA expression in mouse lung with age and exposure to cigarette smoke and light**. ***The FASEB Journal****.* 2009;23(3243-50.

206. Tam W. MicroRNAs: Tiny but Not Trivial. *Current genomics.* 2004;5(3):191-205.

207. *Jacek Krol IL, Witold Filipowicz*. The widespread regulation of microRNA biogenesis, function and decay. *Nature genetics.* 2010;11(597-610.

208. Yang RB, Mark MR, Gray A, Huang A, Xie MH, Zhang M, Goddard A, Wood WI, Gurney AL, and Godowski PJ. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature.* 1998;395(6699):284-8.

209. Scaffidi P, Misteli T, and Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature.* 2002;418(6894):191-5.

210. Hennessy EJ, Parker AE, and O'Neill LA. Targeting Toll-like receptors: emerging therapeutics? *Nature reviews Drug discovery.* 2010;9(4):293-307.

211. O'Neill LA, Sheedy FJ, and McCoy CE. MicroRNAs: the fine-tuners of Toll-like receptor signalling. *Nature reviews Immunology.* 2011;11(3):163-75.

212. Pauley KM, and Chan EK. MicroRNAs and their emerging roles in immunology. *Annals of the New York Academy of Sciences.* 2008;1143(226-39.

213. Bazzoni F, Rossato M, Fabbri M, Gaudiosi D, Mirolo M, Mori L, Tamassia N, Mantovani A, Cassatella MA, and Locati M. Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. *Proc Natl Acad Sci U S A.* 2009;106(13):5282-7.

214. O'Connell RM, Taganov KD, Boldin MP, Cheng G, and Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. *Proc Natl Acad Sci U S A.* 2007;104(5):1604-9.

215. Sheedy FJ, Palsson-McDermott E, Hennessy EJ, Martin C, O'Leary JJ, Ruan Q, Johnson DS, Chen Y, and O'Neill LA. Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. *Nature immunology.* 2010;11(2):141-7.

216. Taganov KD, Boldin MP, Chang KJ, and 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.* 2006;103(33):12481-6.

217. O'Connell RM, Chaudhuri AA, Rao DS, and Baltimore D. Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc Natl Acad Sci U S A.* 2009;106(17):7113-8.

218. Yao R, Ma YL, Liang W, Li HH, Ma ZJ, Yu X, and Liao YH. MicroRNA-155 modulates Treg and Th17 cells differentiation and Th17 cell function by targeting SOCS1. *PLoS One.* 2012;7(10):e46082.

219. Crispin JC, and Tsokos GC. IL-17 in systemic lupus erythematosus. *Journal of biomedicine & biotechnology.* 2010;2010(943254.

220. Beyer M, Thabet Y, Muller RU, Sadlon T, Classen S, Lahl K, Basu S, Zhou X, Bailey-Bucktrout SL, Krebs W, et al. Repression of the genome organizer SATB1 in regulatory T cells is required for suppressive function and inhibition of effector differentiation. *Nature immunology.* 2011;12(9):898-907.

221. Ahlfors H, Limaye A, Elo LL, Tuomela S, Burute M, Gottimukkala KV, Notani D, Rasool O, Galande S, and Lahesmaa R. SATB1 dictates expression of multiple genes including IL-5 involved in human T helper cell differentiation. *Blood.* 2010;116(9):1443-53.

222. Notani D, Gottimukkala KP, Jayani RS, Limaye AS, Damle MV, Mehta S, Purbey PK, Joseph J, and Galande S. Global regulator SATB1 recruits beta-catenin and regulates T(H)2 differentiation in Wnt-dependent manner. *PLoS biology.* 2010;8(1):e1000296.

223. Li QQ, Chen ZQ, Xu JD, Cao XX, Chen Q, Liu XP, and Xu ZD. Overexpression and involvement of special AT-rich sequence binding protein 1 in multidrug resistance in human breast carcinoma cells. *Cancer science.* 2010;101(1):80-6.

224. Huang B, Zhou H, Wang X, and Liu Z. Silencing SATB1 with siRNA inhibits the proliferation and invasion of small cell lung cancer cells. *Cancer cell international.* 2013;13(1):8.

225. Chu SH, Ma YB, Feng DF, Zhang H, Zhu ZA, Li ZQ, and Jiang PC. Upregulation of SATB1 is associated with the development and progression of glioma. *Journal of translational medicine.* 2012;10(149.

226. Han HJ, Russo J, Kohwi Y, and Kohwi-Shigematsu T. SATB1 reprogrammes gene expression to promote breast tumour growth and metastasis. *Nature.* 2008;452(7184):187-93.

227. Al-Sohaily S, Henderson C, Selinger C, Pangon L, Segelov E, Kohonen-Corish MR, and Warusavitarne J. Loss of special AT-rich sequence-binding protein 1

(SATB1) predicts poor survival in patients with colorectal cancer. *Histopathology.* 2014;65(2):155-63.

228. Selinger CI, Cooper WA, Al-Sohaily S, Mladenova DN, Pangon L, Kennedy CW, McCaughan BC, Stirzaker C, and Kohonen-Corish MR. Loss of special AT-rich binding protein 1 expression is a marker of poor survival in lung cancer. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer.* 2011;6(7):1179-89.

229. Kowalczyk AE, Krazinski BE, Godlewski J, Grzegrzolka J, Kiewisz J, Kwiatkowski P, Sliwinska-Jewsiewicka A, Dziegiel P, and Kmiec Z. SATB1 is Down-regulated in Clear Cell Renal Cell Carcinoma and Correlates with miR-21-5p Overexpression and Poor Prognosis. *Cancer genomics & proteomics.* 2016;13(3):209-17.

230. Ezzie ME, Crawford M, Cho JH, Orellana R, Zhang S, Gelinas R, Batte K, Yu L, Nuovo G, Galas D, et al. Gene expression networks in COPD: microRNA and mRNA regulation. *Thorax.* 2012;67(2):122-31.

231. Christenson SA, Brandsma CA, Campbell JD, Knight DA, Pechkovsky DV, Hogg JC, Timens W, Postma DS, Lenburg M, and Spira A. miR-638 regulates gene expression networks associated with emphysematous lung destruction. *Genome medicine.* 2013;5(12):114.

232. Savarimuthu Francis SM, Davidson MR, Tan ME, Wright CM, Clarke BE, Duhig EE, Bowman RV, Hayward NK, Fong KM, and Yang IA. MicroRNA-34c is associated with emphysema severity and modulates SERPINE1 expression. *BMC genomics.* 2014;15(88.

233. Akbas F, Coskunpinar E, Aynaci E, Oltulu YM, and Yildiz P. Analysis of serum micro-RNAs as potential biomarker in chronic obstructive pulmonary disease. *Exp Lung Res.* 2012;38(6):286-94.

234. Van Pottelberge GR, Mestdagh P, Bracke KR, Thas O, van Durme YM, Joos GF, Vandesompele J, and Brusselle GG. MicroRNA expression in induced sputum of

smokers and patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2011;183(7):898-906.

235. Pinkerton M, Chinchilli V, Banta E, Craig T, August A, Bascom R, Cantorna M, Harvill E, and Ishmael FT. Differential expression of microRNAs in exhaled breath condensates of patients with asthma, patients with chronic obstructive pulmonary disease, and healthy adults. *J Allergy Clin Immunol.* 2013;132(1):217-9.

236. Ji J, von Scheele I, Bergstrom J, Billing B, Dahlen B, Lantz AS, Larsson K, and Palmberg L. Compartment differences of inflammatory activity in chronic obstructive pulmonary disease. *Respir Res.* 2014;15(104.

237. Sato T, Liu X, Nelson A, Nakanishi M, Kanaji N, Wang X, Kim M, Li Y, Sun J, Michalski J, et al. Reduced miR-146a increases prostaglandin E(2)in chronic obstructive pulmonary disease fibroblasts. *Am J Respir Crit Care Med.* 2010;182(8):1020-9.

238. Halappanavar S, Nikota J, Wu D, Williams A, Yauk CL, and Stampfli M. IL-1 receptor regulates microRNA-135b expression in a negative feedback mechanism during cigarette smoke-induced inflammation. *Journal of immunology (Baltimore, Md : 1950).* 2013;190(7):3679-86.

239. Sun X, Icli B, Wara AK, Belkin N, He S, Kobzik L, Hunninghake GM, Vera MP, Blackwell TS, Baron RM, et al. MicroRNA-181b regulates NF-kappaB-mediated vascular inflammation. *The Journal of clinical investigation.* 2012;122(6):1973-90.

240. Li D, Liu X, Lin L, Hou J, Li N, Wang C, Wang P, Zhang Q, Zhang P, Zhou W, et al. MicroRNA-99a inhibits hepatocellular carcinoma growth and correlates with prognosis of patients with hepatocellular carcinoma. *J Biol Chem.* 2011;286(42):36677-85.

241. Wang Z. The guideline of the design and validation of MiRNA mimics. *Methods in molecular biology (Clifton, NJ).* 2011;676(211-23.

242. van Rooij E, Purcell AL, and Levin AA. Developing microRNA therapeutics. *Circ Res.* 2012;110(3):496-507.

243. Peacock H, Fucini RV, Jayalath P, Ibarra-Soza JM, Haringsma HJ, Flanagan WM, Willingham A, and Beal PA. Nucleobase and ribose modifications control immunostimulation by a microRNA-122-mimetic RNA. *Journal of the American Chemical Society.* 2011;133(24):9200-3.

244. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, Chang TC, Vivekanandan P, Torbenson M, Clark KR, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell.* 2009;137(6):1005-17.

245. Susanna Obad COdS, Andreas Petri, Markus Heidenblad, Oliver Broom, Cristian Ruse, Cexiong Fu, Morten Lindow, Jan Stenvang, Ellen Marie Straarup, Henrik Frydenlund Hansen, Troels Koch, Darryl Pappin, Gregory J Hannon & Sakari Kauppinen. Silencing of microRNA families by seed-targeting tiny LNAs. *Narute Genetics.* 2011;43(4):371-9.

246. Montano M. MicroRNAs: miRRORS of health and disease. *Translational Research.* 2011;157(4):157-62.

247. van Rooij E. The art of microRNA research. *Circ Res.* 2011;108(2):219-34.

248. P Trang JWaFS. MicroRNAs as potential cancer therapeutics. *Oncogene.* 2009;27(52-7.

249. Mattes J, Yang M, and Foster PS. Regulation of microRNA by antagomirs: a new class of pharmacological antagonists for the specific regulation of gene function? *Am J Respir Cell Mol Biol.* 2007;36(1):8-12.

250. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, and Stoffel M. Silencing of microRNAs in vivo with 'antagomirs'. *Nature.* 2005;438(7068):685-9.

251. Janssen HL, Reesink HW, Lawitz EJ, Zeuzem S, Rodriguez-Torres M, Patel K, van der Meer AJ, Patick AK, Chen A, Zhou Y, et al. Treatment of HCV infection

by targeting microRNA. *The New England journal of medicine.* 2013;368(18):1685-94.

252. Agostini M, and Knight RA. miR-34: from bench to bedside. *Oncotarget.* 2014;5(4):872-81.

253. Czech MP. MicroRNAs as Therapeutic Targets. *The new england journal o f medicine.* 2006;254(11):1194-5.

254. Hansbro PM, Hamilton MJ, Fricker M, Gellatly SL, Jarnicki AG, Zheng D, Frei SM, Wong GW, Hamadi S, Zhou S, et al. Importance of mast cell Prss31/transmembrane tryptase/tryptase-gamma in lung function and experimental chronic obstructive pulmonary disease and colitis. *J Biol Chem.* 2014;289(26):18214-27.

255. Haw TJ, Starkey MR, Nair PM, Pavlidis S, Liu G, Nguyen DH, Hsu AC, Hanish I, Kim RY, Collison AM, et al. A pathogenic role for tumor necrosis factor-related apoptosis-inducing ligand in chronic obstructive pulmonary disease. *Mucosal immunology.* 2015.

256. Franklin BS, Bossaller L, De Nardo D, Ratter JM, Stutz A, Engels G, Brenker C, Nordhoff M, Mirandola SR, Al-Amoudi A, et al. The adaptor ASC has extracellular and 'prionoid' activities that propagate inflammation. *Nature immunology.* 2014;15(8):727-37.

257. Tay HL, Kaiko GE, Plank M, Li J, Maltby S, Essilfie AT, Jarnicki A, Yang M, Mattes J, Hansbro PM, et al. Antagonism of miR-328 increases the antimicrobial function of macrophages and neutrophils and rapid clearance of non-typeable Haemophilus influenzae (NTHi) from infected lung. *PLoS pathogens.* 2015;11(4):e1004549.

258. Plank MW, Maltby S, Tay HL, Stewart J, Eyers F, Hansbro PM, and Foster PS. MicroRNA Expression Is Altered in an Ovalbumin-Induced Asthma Model and Targeting miR-155 with Antagomirs Reveals Cellular Specificity. *PLoS One.* 2015;10(12):e0144810.

259. Kim RY, Horvat JC, Pinkerton JW, Starkey MR, Essilfie AT, Mayall JR, Nair PM, Hansbro NG, Jones B, Haw TJ, et al. MicroRNA-21 drives severe, steroid-insensitive experimental asthma by amplifying phosphoinositide 3-kinase-mediated suppression of histone deacetylase 2. *J Allergy Clin Immunol.* 2017;139(2):519-32.

260. Asquith KL, Horvat JC, Kaiko GE, Carey AJ, Beagley KW, Hansbro PM, and Foster PS. Interleukin-13 promotes susceptibility to chlamydial infection of the respiratory and genital tracts. *PLoS pathogens.* 2011;7(5):e1001339.

261. Liu G CM, Jarnicki AG, Hsu ACY, Nair P, Haw TJ, Fricker M, Gellatly SL, Kim RY, Inman MD, Tjin G, Wark PA, Walker M, Horvat JC, Oliver BG, Argraves WS, Knight DA, Burgess J, Hansbro PM. Fibulin-1 regulates the pathogenesis of respiratory diseases. *J Clin Invest Insight 2016.*

262. D'Hulst A I, Maes T, Bracke KR, Demedts IK, Tournoy KG, Joos GF, and Brusselle GG. Cigarette smoke-induced pulmonary emphysema in scid-mice. Is the acquired immune system required? *Respir Res.* 2005;6(147.

263. Singh S, Amin AV, and Loke YK. Long-term use of inhaled corticosteroids and the risk of pneumonia in chronic obstructive pulmonary disease: a meta-analysis. *Archives of internal medicine.* 2009;169(3):219-29.

264. Halpin DM, Gray J, Edwards SJ, Morais J, and Singh D. Budesonide/formoterol vs. salmeterol/fluticasone in COPD: a systematic review and adjusted indirect comparison of pneumonia in randomised controlled trials. *Int J Clin Pract.* 2011;65(7):764-74.

265. Ameres SL, and Zamore PD. Diversifying microRNA sequence and function. *Nature reviews Molecular cell biology.* 2013;14(8):475-88.

266. Costa LA, da Silva IC, Mariz BA, da Silva MB, Freitas-Ribeiro GM, and de Oliveira NF. Influence of smoking on methylation and hydroxymethylation levels in global DNA and specific sites of KRT14, KRT19, MIR-9-3 and MIR-137 genes of oral mucosa. *Archives of oral biology.* 2016;72(56-65.

267. Lewis BP, Burge CB, and Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell.* 2005;120(1):15-20.

268. John B, Enright AJ, Aravin A, Tuschl T, Sander C, and Marks DS. Human MicroRNA targets. *PLoS biology.* 2004;2(11):e363.

269. Dweep H, Sticht C, and Gretz N. In-Silico Algorithms for the Screening of Possible microRNA Binding Sites and Their Interactions. *Current genomics.* 2013;14(2):127-36.

270. Landi MT, Zhao Y, Rotunno M, Koshiol J, Liu H, Bergen AW, Rubagotti M, Goldstein AM, Linnoila I, Marincola FM, et al. MicroRNA expression differentiates histology and predicts survival of lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2010;16(2):430-41.

271. Baffa R, Fassan M, Volinia S, O'Hara B, Liu CG, Palazzo JP, Gardiman M, Rugge M, Gomella LG, Croce CM, et al. MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets. *The Journal of pathology.* 2009;219(2):214-21.

272. Pritchard CC, Cheng HH, and Tewari M. MicroRNA profiling: approaches and considerations. *Nature reviews Genetics.* 2012;13(5):358-69.

273. Yin JQ, Zhao RC, and Morris KV. Profiling microRNA expression with microarrays. *Trends in biotechnology.* 2008;26(2):70-6.

274. Zhao B, Ding S, Li W, and Jin Y. Hybridization kinetics analysis of an oligonucleotide microarray for microRNA detection. *Acta biochimica et biophysica Sinica.* 2011;43(7):551-5.

275. Pryor WA, and Stone K. Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. *Annals of the New York Academy of Sciences.* 1993;686(12-27; discussion -8.

276. Hellermann GR, Nagy SB, Kong X, Lockey RF, and Mohapatra SS. Mechanism of cigarette smoke condensate-induced acute inflammatory response in human bronchial epithelial cells. *Respir Res.* 2002;3(22.

277. Morrison D, Rahman I, Lannan S, and MacNee W. Epithelial permeability, inflammation, and oxidant stress in the air spaces of smokers. *Am J Respir Crit Care Med.* 1999;159(2):473-9.

278. Rahman I, and MacNee W. Role of oxidants/antioxidants in smoking-induced lung diseases. *Free radical biology & medicine.* 1996;21(5):669-81.

279. Rahman I, and MacNee W. Role of transcription factors in inflammatory lung diseases. *Thorax.* 1998;53(7):601-12.

280. Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochemical and biophysical research communications.* 1997;236(2):313-22.

281. Itoh K, Tong KI, and Yamamoto M. Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. *Free radical biology & medicine.* 2004;36(10):1208-13.

282. Suzuki M, Betsuyaku T, Ito Y, Nagai K, Nasuhara Y, Kaga K, Kondo S, and Nishimura M. Down-regulated NF-E2-related factor 2 in pulmonary macrophages of aged smokers and patients with chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol.* 2008;39(6):673-82.

283. Kumar V, Kumar S, Hassan M, Wu H, Thimmulappa RK, Kumar A, Sharma SK, Parmar VS, Biswal S, and Malhotra SV. Novel chalcone derivatives as potent Nrf2 activators in mice and human lung epithelial cells. *Journal of medicinal chemistry.* 2011;54(12):4147-59.

284. Hilton DJ, Richardson RT, Alexander WS, Viney EM, Willson TA, Sprigg NS, Starr R, Nicholson SE, Metcalf D, and Nicola NA. Twenty proteins containing a

C-terminal SOCS box form five structural classes. *Proc Natl Acad Sci U S A.* 1998;95(1):114-9.

285. Starr R, Willson TA, Viney EM, Murray LJ, Rayner JR, Jenkins BJ, Gonda TJ, Alexander WS, Metcalf D, Nicola NA, et al. A family of cytokine-inducible inhibitors of signalling. *Nature.* 1997;387(6636):917-21.

286. Zhuang G, Wu X, Jiang Z, Kasman I, Yao J, Guan Y, Oeh J, Modrusan Z, Bais C, Sampath D, et al. Tumour-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway. *The EMBO journal.* 2012;31(17):3513-23.

287. Seki Y, Hayashi K, Matsumoto A, Seki N, Tsukada J, Ransom J, Naka T, Kishimoto T, Yoshimura A, and Kubo M. Expression of the suppressor of cytokine signaling-5 (SOCS5) negatively regulates IL-4-dependent STAT6 activation and Th2 differentiation. *Proc Natl Acad Sci U S A.* 2002;99(20):13003-8.

288. Kario E, Marmor MD, Adamsky K, Citri A, Amit I, Amariglio N, Rechavi G, and Yarden Y. Suppressors of cytokine signaling 4 and 5 regulate epidermal growth factor receptor signaling. *J Biol Chem.* 2005;280(8):7038-48.

289. Nicholson SE, Metcalf D, Sprigg NS, Columbus R, Walker F, Silva A, Cary D, Willson TA, Zhang JG, Hilton DJ, et al. Suppressor of cytokine signaling (SOCS)-5 is a potential negative regulator of epidermal growth factor signaling. *Proc Natl Acad Sci U S A.* 2005;102(7):2328-33.

290. de Boer WI, Hau CM, van Schadewijk A, Stolk J, van Krieken JH, and Hiemstra PS. Expression of epidermal growth factors and their receptors in the bronchial epithelium of subjects with chronic obstructive pulmonary disease. *American journal of clinical pathology.* 2006;125(2):184-92.

291. Ganesan S, Unger BL, Comstock AT, Angel KA, Mancuso P, Martinez FJ, and Sajjan US. Aberrantly activated EGFR contributes to enhanced IL-8 expression in COPD airways epithelial cells via regulation of nuclear FoxO3A. *Thorax.* 2013;68(2):131-41.

292. Richter A, O'Donnell RA, Powell RM, Sanders MW, Holgate ST, Djukanovic R, and Davies DE. Autocrine ligands for the epidermal growth factor receptor mediate interleukin-8 release from bronchial epithelial cells in response to cigarette smoke. *Am J Respir Cell Mol Biol.* 2002;27(1):85-90.

293. Fricker M, Deane A, and Hansbro PM. Animal models of chronic obstructive pulmonary disease. *Expert opinion on drug discovery.* 2014;9(6):629-45.

294. Conickx G MP, Avila Cobos F, Verhamme FM, Maes T, Vanaudenaerde BM, Seys LJM, Lahousse L, Hsu AC, Kim RY, Wark PA, Hansbro PM, Joos GF, Vandesompele J, Bracke KR, Brusselle, G. MicroRNA profiling reveals a role for microRNA-218-5p in the pathogenesis of chronic obstructive pulmonary disease. *Revision to American Journal of Respiratory and Critical Care Medicine.*

295. Li JJ, Tay HL, Maltby S, Xiang Y, Eyers F, Hatchwell L, Zhou H, Toop HD, Morris JC, Nair P, et al. MicroRNA-9 regulates steroid-resistant airway hyperresponsiveness by reducing protein phosphatase 2A activity. *J Allergy Clin Immunol.* 2015;136(2):462-73.

296. Barnes PJ. Mediators of chronic obstructive pulmonary disease. *Pharmacological reviews.* 2004;56(4):515-48.

297. Churg A, Wang RD, Tai H, Wang X, Xie C, Dai J, Shapiro SD, and Wright JL. Macrophage metalloelastase mediates acute cigarette smoke-induced inflammation via tumor necrosis factor-alpha release. *Am J Respir Crit Care Med.* 2003;167(8):1083-9.

298. Thatcher TH, McHugh NA, Egan RW, Chapman RW, Hey JA, Turner CK, Redonnet MR, Seweryniak KE, Sime PJ, and Phipps RP. Role of CXCR2 in cigarette smoke-induced lung inflammation. *American journal of physiology Lung cellular and molecular physiology.* 2005;289(2):L322-8.

299. Elomaa O, Sankala M, Pikkarainen T, Bergmann U, Tuuttila A, Raatikainen-Ahokas A, Sariola H, and Tryggvason K. Structure of the human macrophage

MARCO receptor and characterization of its bacteria-binding region. *J Biol Chem.* 1998;273(8):4530-8.

300. Harvey CJ, Thimmulappa RK, Sethi S, Kong X, Yarmus L, Brown RH, Feller-Kopman D, Wise R, and Biswal S. Targeting Nrf2 signaling improves bacterial clearance by alveolar macrophages in patients with COPD and in a mouse model. *Science translational medicine.* 2011;3(78):78ra32.

301. Fierro-Fernandez M, Busnadiego O, Sandoval P, Espinosa-Diez C, Blanco-Ruiz E, Rodriguez M, Pian H, Ramos R, Lopez-Cabrera M, Garcia-Bermejo ML, et al. miR-9-5p suppresses pro-fibrogenic transformation of fibroblasts and prevents organ fibrosis by targeting NOX4 and TGFBR2. *EMBO reports.* 2015;16(10):1358-77.

302. Wang L, Ma L, Fan H, Yang Z, Li L, and Wang H. MicroRNA-9 regulates cardiac fibrosis by targeting PDGFR-beta in rats. *Journal of physiology and biochemistry.* 2016.

303. Churg A, Wang R, Wang X, Onnervik PO, Thim K, and Wright JL. Effect of an MMP-9/MMP-12 inhibitor on smoke-induced emphysema and airway remodelling in guinea pigs. *Thorax.* 2007;62(8):706-13.

304. Hautamaki RD, Kobayashi DK, Senior RM, and Shapiro SD. Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science.* 1997;277(5334):2002-4.

305. Churg A, Wang RD, Tai H, Wang X, Xie C, and Wright JL. Tumor necrosis factor-alpha drives 70% of cigarette smoke-induced emphysema in the mouse. *Am J Respir Crit Care Med.* 2004;170(5):492-8.

306. Tanino M, Betsuyaku T, Takeyabu K, Tanino Y, Yamaguchi E, Miyamoto K, and Nishimura M. Increased levels of interleukin-8 in BAL fluid from smokers susceptible to pulmonary emphysema. *Thorax.* 2002;57(5):405-11.

307. Yamada K, Asai K, Nagayasu F, Sato K, Ijiri N, Yoshii N, Imahashi Y, Watanabe T, Tochino Y, Kanazawa H, et al. Impaired nuclear factor erythroid 2-related

factor 2 expression increases apoptosis of airway epithelial cells in patients with chronic obstructive pulmonary disease due to cigarette smoking. *BMC pulmonary medicine.* 2016;16(1):27.

308. Garbin U, Fratta Pasini A, Stranieri C, Cominacini M, Pasini A, Manfro S, Lugoboni F, Mozzini C, Guidi G, Faccini G, et al. Cigarette smoking blocks the protective expression of Nrf2/ARE pathway in peripheral mononuclear cells of young heavy smokers favouring inflammation. *PLoS One.* 2009;4(12):e8225.

309. Sekine T, Hirata T, Mine T, and Fukano Y. Activation of transcription factors in human bronchial epithelial cells exposed to aqueous extracts of mainstream cigarette smoke in vitro. *Toxicology mechanisms and methods.* 2016;26(1):22-31.

310. Singh A, Rangasamy T, Thimmulappa RK, Lee H, Osburn WO, Brigelius-Flohe R, Kensler TW, Yamamoto M, and Biswal S. Glutathione peroxidase 2, the major cigarette smoke-inducible isoform of GPX in lungs, is regulated by Nrf2. *Am J Respir Cell Mol Biol.* 2006;35(6):639-50.

311. Antczak A, Piotrowski W, Marczak J, Ciebiada M, Gorski P, and Barnes PJ. Correlation between eicosanoids in bronchoalveolar lavage fluid and in exhaled breath condensate. *Disease markers.* 2011;30(5):213-20.

312. Szulakowski P, Crowther AJ, Jimenez LA, Donaldson K, Mayer R, Leonard TB, MacNee W, and Drost EM. The effect of smoking on the transcriptional regulation of lung inflammation in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 2006;174(1):41-50.

313. Hodge-Bell KC, Lee KM, Renne RA, Gideon KM, Harbo SJ, and McKinney WJ. Pulmonary inflammation in mice exposed to mainstream cigarette smoke. *Inhalation toxicology.* 2007;19(4):361-76.

314. Yew-Booth L, Birrell MA, Lau MS, Baker K, Jones V, Kilty I, and Belvisi MG. JAK-STAT pathway activation in COPD. *Eur Respir J.* 2015;46(3):843-5.

315. Lu TX, Munitz A, and Rothenberg ME. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. *Journal of immunology (Baltimore, Md : 1950).* 2009;182(8):4994-5002.

316. Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, and Allgayer H. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene.* 2008;27(15):2128-36.

317. Xie L, Wu M, Lin H, Liu C, Yang H, Zhan J, and Sun S. 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. *Molecular bioSystems.* 2014;10(5):1072-81.

318. Iliopoulos D, Jaeger SA, Hirsch HA, Bulyk ML, and Struhl K. STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. *Molecular cell.* 2010;39(4):493-506.

319. Brown V, Elborn JS, Bradley J, and Ennis M. Dysregulated apoptosis and NFkappaB expression in COPD subjects. *Respir Res.* 2009;10(24.

320. Di Stefano A, Caramori G, Oates T, Capelli A, Lusuardi M, Gnemmi I, Ioli F, Chung KF, Donner CF, Barnes PJ, et al. Increased expression of nuclear factor-kappaB in bronchial biopsies from smokers and patients with COPD. *Eur Respir J.* 2002;20(3):556-63.

321. Zhou R, Hu G, Gong AY, and Chen XM. Binding of NF-kappaB p65 subunit to the promoter elements is involved in LPS-induced transactivation of miRNA genes in human biliary epithelial cells. *Nucleic Acids Res.* 2010;38(10):3222-32.

322. Nemeth J, Stein I, Haag D, Riehl A, Longerich T, Horwitz E, Breuhahn K, Gebhardt C, Schirmacher P, Hahn M, et al. S100A8 and S100A9 are novel nuclear factor kappa B target genes during malignant progression of murine and human liver carcinogenesis. *Hepatology (Baltimore, Md).* 2009;50(4):1251-62.

323. Seo J, Lozano MM, and Dudley JP. Nuclear matrix binding regulates SATB1-mediated transcriptional repression. *J Biol Chem.* 2005;280(26):24600-9.

324. Swindell WR, Johnston A, Xing X, Little A, Robichaud P, Voorhees JJ, Fisher G, and Gudjonsson JE. Robust shifts in S100a9 expression with aging: a novel mechanism for chronic inflammation. *Scientific reports.* 2013;3(1215.

325. Zhu S, Deng S, Ma Q, Zhang T, Jia C, Zhuo D, Yang F, Wei J, Wang L, Dykxhoorn DM, et al. MicroRNA-10A* and MicroRNA-21 modulate endothelial progenitor cell senescence via suppressing high-mobility group A2. *Circ Res.* 2013;112(1):152-64.

326. Fabbri M, Paone A, Calore F, Galli R, Gaudio E, Santhanam R, Lovat F, Fadda P, Mao C, Nuovo GJ, et al. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proc Natl Acad Sci U S A.* 2012;109(31):E2110-6.

327. Liu G, Friggeri A, Yang Y, Milosevic J, Ding Q, Thannickal VJ, Kaminski N, and Abraham E. miR-21 mediates fibrogenic activation of pulmonary fibroblasts and lung fibrosis. *J Exp Med.* 2010;207(8):1589-97.

328. Lopes-Ramos CM, Habr-Gama A, Quevedo Bde S, Felicio NM, Bettoni F, Koyama FC, Asprino PF, Galante PA, Gama-Rodrigues J, Camargo AA, et al. Overexpression of miR-21-5p as a predictive marker for complete tumor regression to neoadjuvant chemoradiotherapy in rectal cancer patients. *BMC medical genomics.* 2014;7(68.

329. Vasudevan S, Tong Y, and Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. *Science.* 2007;318(5858):1931-4.

330. Steitz JA, and Vasudevan S. miRNPs: versatile regulators of gene expression in vertebrate cells. *Biochemical Society transactions.* 2009;37(Pt 5):931-5.

331. Kedde M, Strasser MJ, Boldajipour B, Oude Vrielink JA, Slanchev K, le Sage C, Nagel R, Voorhoeve PM, van Duijse J, Orom UA, et al. RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. *Cell.* 2007;131(7):1273-86.

332. Kedde M, van Kouwenhove M, Zwart W, Oude Vrielink JA, Elkon R, and Agami R. A Pumilio-induced RNA structure switch in p27-3' UTR controls miR-221 and miR-222 accessibility. *Nature cell biology.* 2010;12(10):1014-20.

333. Bronnum H, Andersen DC, Schneider M, Sandberg MB, Eskildsen T, Nielsen SB, Kalluri R, and Sheikh SP. miR-21 promotes fibrogenic epithelial-to-mesenchymal transition of epicardial mesothelial cells involving Programmed Cell Death 4 and Sprouty-1. *PLoS One.* 2013;8(2):e56280.

334. Zhao J, Tang N, Wu K, Dai W, Ye C, Shi J, Zhang J, Ning B, Zeng X, and Lin Y. MiR-21 simultaneously regulates ERK1 signaling in HSC activation and hepatocyte EMT in hepatic fibrosis. *PLoS One.* 2014;9(10):e108005.

335. Zhang Z, Zha Y, Hu W, Huang Z, Gao Z, Zang Y, Chen J, Dong L, and Zhang J. The autoregulatory feedback loop of microRNA-21/programmed cell death protein 4/activation protein-1 (MiR-21/PDCD4/AP-1) as a driving force for hepatic fibrosis development. *J Biol Chem.* 2013;288(52):37082-93.

336. McDonough JE, Yuan R, Suzuki M, Seyednejad N, Elliott WM, Sanchez PG, Wright AC, Gefter WB, Litzky L, Coxson HO, et al. Small-airway obstruction and emphysema in chronic obstructive pulmonary disease. *The New England journal of medicine.* 2011;365(17):1567-75.

337. Yang S, Banerjee S, Freitas A, Cui H, Xie N, Abraham E, and Liu G. miR-21 regulates chronic hypoxia-induced pulmonary vascular remodeling. *American journal of physiology Lung cellular and molecular physiology.* 2012;302(6):L521-9.

338. Alvarez JD, Yasui DH, Niida H, Joh T, Loh DY, and Kohwi-Shigematsu T. The MAR-binding protein SATB1 orchestrates temporal and spatial expression of multiple genes during T-cell development. *Genes & development.* 2000;14(5):521-35.

339. Cai S, Han HJ, and Kohwi-Shigematsu T. Tissue-specific nuclear architecture and gene expression regulated by SATB1. *Nature genetics.* 2003;34(1):42-51.

340. Sundar IK, Nevid MZ, Friedman AE, and Rahman I. Cigarette smoke induces distinct histone modifications in lung cells: implications for the pathogenesis of COPD and lung cancer. *Journal of proteome research.* 2014;13(2):982-96.

341. He J, Gong J, Ding Q, Tan Q, Han P, Liu J, Zhou Z, Tu W, Xia Y, Yan W, et al. Suppressive effect of SATB1 on hepatic stellate cell activation and liver fibrosis in rats. *FEBS letters.* 2015;589(12):1359-68.

342. Balamotis MA, Tamberg N, Woo YJ, Li J, Davy B, Kohwi-Shigematsu T, and Kohwi Y. Satb1 ablation alters temporal expression of immediate early genes and reduces dendritic spine density during postnatal brain development. *Molecular and cellular biology.* 2012;32(2):333-47.

343. Fessing MY, Mardaryev AN, Gdula MR, Sharov AA, Sharova TY, Rapisarda V, Gordon KB, Smorodchenko AD, Poterlowicz K, Ferone G, et al. p63 regulates Satb1 to control tissue-specific chromatin remodeling during development of the epidermis. *The Journal of cell biology.* 2011;194(6):825-39.

344. Obry A, Lequerre T, Hardouin J, Boyer O, Fardellone P, Philippe P, Le Loet X, Cosette P, and Vittecoq O. Identification of S100A9 as biomarker of responsiveness to the methotrexate/etanercept combination in rheumatoid arthritis using a proteomic approach. *PLoS One.* 2014;9(12):e115800.

345. Lee TH, Jang AS, Park JS, Kim TH, Choi YS, Shin HR, Park SW, Uh ST, Choi JS, Kim YH, et al. Elevation of S100 calcium binding protein A9 in sputum of neutrophilic inflammation in severe uncontrolled asthma. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology.* 2013;111(4):268-75.e1.

346. Morrow DA, Wang Y, Croce K, Sakuma M, Sabatine MS, Gao H, Pradhan AD, Healy AM, Buros J, McCabe CH, et al. Myeloid-related protein 8/14 and the risk of cardiovascular death or myocardial infarction after an acute coronary syndrome in the Pravastatin or Atorvastatin Evaluation and Infection Therapy:

Thrombolysis in Myocardial Infarction (PROVE IT-TIMI 22) trial. *American heart journal.* 2008;155(1):49-55.

347. Horvath I, Jia X, Johansson P, Wang C, Moskalenko R, Steinau A, Forsgren L, Wagberg T, Svensson J, Zetterberg H, et al. Pro-inflammatory S100A9 Protein as a Robust Biomarker Differentiating Early Stages of Cognitive Impairment in Alzheimer's Disease. *ACS chemical neuroscience.* 2015.

348. Baines KJ, Hsu AC, Tooze M, Gunawardhana LP, Gibson PG, and Wark PA. Novel immune genes associated with excessive inflammatory and antiviral responses to rhinovirus in COPD. *Respir Res.* 2013;14(15.

349. Lorenz E, Muhlebach MS, Tessier PA, Alexis NE, Duncan Hite R, Seeds MC, Peden DB, and Meredith W. Different expression ratio of S100A8/A9 and S100A12 in acute and chronic lung diseases. *Respiratory medicine.* 2008;102(4):567-73.

350. Xu K, and Geczy CL. IFN-gamma and TNF regulate macrophage expression of the chemotactic S100 protein S100A8. *Journal of immunology (Baltimore, Md : 1950).* 2000;164(9):4916-23.

351. Marwick JA, Kirkham PA, Stevenson CS, Danahay H, Giddings J, Butler K, Donaldson K, Macnee W, and Rahman I. Cigarette smoke alters chromatin remodeling and induces proinflammatory genes in rat lungs. *Am J Respir Cell Mol Biol.* 2004;31(6):633-42.

352. Hofmann MA, Drury S, Fu C, Qu W, Taguchi A, Lu Y, Avila C, Kambham N, Bierhaus A, Nawroth P, et al. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell.* 1999;97(7):889-901.

353. Riva M, Kallberg E, Bjork P, Hancz D, Vogl T, Roth J, Ivars F, and Leanderson T. Induction of nuclear factor-kappaB responses by the S100A9 protein is Toll-like receptor-4-dependent. *Immunology.* 2012;137(2):172-82.

354. Rajendrasozhan S, Hwang JW, Yao H, Kishore N, and Rahman I. Anti-inflammatory effect of a selective IkappaB kinase-beta inhibitor in rat lung in response to LPS and cigarette smoke. *Pulm Pharmacol Ther.* 2010;23(3):172-81.

355. Li YT, He B, Wang YZ, and Wang J. Effects of intratracheal administration of nuclear factor-kappaB decoy oligodeoxynucleotides on long-term cigarette smoke-induced lung inflammation and pathology in mice. *Respir Res.* 2009;10(79.

356. Halappanavar S, Jackson P, Williams A, Jensen KA, Hougaard KS, Vogel U, Yauk CL, and Wallin H. Pulmonary response to surface-coated nanotitanium dioxide particles includes induction of acute phase response genes, inflammatory cascades, and changes in microRNAs: a toxicogenomic study. *Environmental and molecular mutagenesis.* 2011;52(6):425-39.

357. Lin CW, Chang YL, Chang YC, Lin JC, Chen CC, Pan SH, Wu CT, Chen HY, Yang SC, Hong TM, et al. MicroRNA-135b promotes lung cancer metastasis by regulating multiple targets in the Hippo pathway and LZTS1. *Nature communications.* 2013;4(1877.

358. Yasuo M, Mizuno S, Kraskauskas D, Bogaard HJ, Natarajan R, Cool CD, Zamora M, and Voelkel NF. Hypoxia inducible factor-1alpha in human emphysema lung tissue. *Eur Respir J.* 2011;37(4):775-83.

359. Kasahara Y, Tuder RM, Cool CD, Lynch DA, Flores SC, and Voelkel NF. Endothelial cell death and decreased expression of vascular endothelial growth factor and vascular endothelial growth factor receptor 2 in emphysema. *Am J Respir Crit Care Med.* 2001;163(3 Pt 1):737-44.

360. Suzuki M, Betsuyaku T, Nagai K, Fuke S, Nasuhara Y, Kaga K, Kondo S, Hamamura I, Hata J, Takahashi H, et al. Decreased airway expression of vascular endothelial growth factor in cigarette smoke-induced emphysema in mice and COPD patients. *Inhalation toxicology.* 2008;20(3):349-59.

361. Thaikoottathil JV, Martin RJ, Zdunek J, Weinberger A, Rino JG, and Chu HW. Cigarette smoke extract reduces VEGF in primary human airway epithelial cells. *Eur Respir J.* 2009;33(4):835-43.

362. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, et al. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature.* 1998;394(6692):485-90.

363. To M, Yamamura S, Akashi K, Charron CE, Barnes PJ, and Ito K. Defect of adaptation to hypoxia in patients with COPD due to reduction of histone deacetylase 7. *Chest.* 2012;141(5):1233-42.

364. Yao H, Hwang JW, Sundar IK, Friedman AE, McBurney MW, Guarente L, Gu W, Kinnula VL, and Rahman I. SIRT1 redresses the imbalance of tissue inhibitor of matrix metalloproteinase-1 and matrix metalloproteinase-9 in the development of mouse emphysema and human COPD. *American journal of physiology Lung cellular and molecular physiology.* 2013;305(9):L615-24.

365. Yao H, Chung S, Hwang JW, Rajendrasozhan S, Sundar IK, Dean DA, McBurney MW, Guarente L, Gu W, Ronty M, et al. SIRT1 protects against emphysema via FOXO3-mediated reduction of premature senescence in mice. *The Journal of clinical investigation.* 2012;122(6):2032-45.

366. Bhinge A, Poschmann J, Namboori SC, Tian X, Jia Hui Loh S, Traczyk A, Prabhakar S, and Stanton LW. MiR-135b is a direct PAX6 target and specifies human neuroectoderm by inhibiting TGF-beta/BMP signaling. *The EMBO journal.* 2014;33(11):1271-83.

367. Guo X, and Wang XF. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell research.* 2009;19(1):71-88.

368. Akira S, and Takeda K. Toll-like receptor signalling. *Nature reviews Immunology.* 2004;4(7):499-511.

369. Perry MM, Moschos SA, Williams AE, Shepherd NJ, Larner-Svensson HM, and Lindsay MA. Rapid changes in microRNA-146a expression negatively regulate the IL-1beta-induced inflammatory response in human lung alveolar epithelial cells. *Journal of immunology (Baltimore, Md : 1950).* 2008;180(8):5689-98.

370. Perry MM, Williams AE, Tsitsiou E, Larner-Svensson HM, and Lindsay MA. Divergent intracellular pathways regulate interleukin-1beta-induced miR-146a and miR-146b expression and chemokine release in human alveolar epithelial cells. *FEBS letters.* 2009;583(20):3349-55.

371. Comer BS, Camoretti-Mercado B, Kogut PC, Halayko AJ, Solway J, and Gerthoffer WT. MicroRNA-146a and microRNA-146b expression and anti-inflammatory function in human airway smooth muscle. *American journal of physiology Lung cellular and molecular physiology.* 2014;307(9):L727-34.

372. Echavarria R, Mayaki D, Neel JC, Harel S, Sanchez V, and Hussain SN. Angiopoietin-1 inhibits toll-like receptor 4 signalling in cultured endothelial cells: role of miR-146b-5p. *Cardiovascular research.* 2015;106(3):465-77.

373. Arigoni M, Barutello G, Riccardo F, Ercole E, Cantarella D, Orso F, Conti L, Lanzardo S, Taverna D, Merighi I, et al. miR-135b coordinates progression of ErbB2-driven mammary carcinomas through suppression of MID1 and MTCH2. *The American journal of pathology.* 2013;182(6):2058-70.

374. Al-Ansari MM, and Aboussekhra A. miR-146b-5p mediates p16-dependent repression of IL-6 and suppresses paracrine procarcinogenic effects of breast stromal fibroblasts. *Oncotarget.* 2015;6(30):30006-16.

375. Geraldo MV, Yamashita AS, and Kimura ET. MicroRNA miR-146b-5p regulates signal transduction of TGF-beta by repressing SMAD4 in thyroid cancer. *Oncogene.* 2012;31(15):1910-22.

376. Matsuyama H, Suzuki HI, Nishimori H, Noguchi M, Yao T, Komatsu N, Mano H, Sugimoto K, and Miyazono K. miR-135b mediates NPM-ALK-driven oncogenicity

and renders IL-17-producing immunophenotype to anaplastic large cell lymphoma. *Blood.* 2011;118(26):6881-92.

377. Sawada H, Saito T, Nickel NP, Alastalo TP, Glotzbach JP, Chan R, Haghighat L, Fuchs G, Januszyk M, Cao A, et al. Reduced BMPR2 expression induces GM-CSF translation and macrophage recruitment in humans and mice to exacerbate pulmonary hypertension. *J Exp Med.* 2014;211(2):263-80.

378. O'Reilly S, Ciechomska M, Fullard N, Przyborski S, and van Laar JM. IL-13 mediates collagen deposition via STAT6 and microRNA-135b: a role for epigenetics. *Scientific reports.* 2016;6(25066.

379. Llinas L, Peinado VI, Ramon Goni J, Rabinovich R, Pizarro S, Rodriguez-Roisin R, Barbera JA, and Bastos R. Similar gene expression profiles in smokers and patients with moderate COPD. *Pulm Pharmacol Ther.* 2011;24(1):32-41.

380. Chen NY, Collum SD, Luo F, Weng T, Le TT, Hernandez AM, Philip K, Molina J, Garcia-Morales LJ, Cao Y, et al. Macrophage Bone Morphogenic Protein Receptor 2 (BMPR2) depletion in Idiopathic Pulmonary Fibrosis (IPF) and Group III Pulmonary Hypertension. *American journal of physiology Lung cellular and molecular physiology.* 2016:ajplung.00142.2016.

381. Guignabert C, Tu L, Girerd B, Ricard N, Huertas A, Montani D, and Humbert M. New molecular targets of pulmonary vascular remodeling in pulmonary arterial hypertension: importance of endothelial communication. *Chest.* 2015;147(2):529-37.

382. Takahashi H, Goto N, Kojima Y, Tsuda Y, Morio Y, Muramatsu M, and Fukuchi Y. Downregulation of type II bone morphogenetic protein receptor in hypoxic pulmonary hypertension. *American journal of physiology Lung cellular and molecular physiology.* 2006;290(3):L450-8.

383. Ge S, Wang X, Xie J, Yi X, and Liu F. Deep sequencing analysis of microRNA expression in porcine serum-induced hepatic fibrosis rats. *Annals of hepatology.* 2014;13(4):439-49.

384. Ha JS, and Jung HR. Up-regulation of MicroRNA 146b is Associated with Myelofibrosis in Myeloproliferative Neoplasms. *Annals of clinical and laboratory science.* 2015;45(3):308-14.

385. Jiang X, Ning Q, and Wang J. Angiotensin II induced differentially expressed microRNAs in adult rat cardiac fibroblasts. *The journal of physiological sciences : JPS.* 2013;63(1):31-8.

386. Wang J, Wang Y, Han J, Li Y, Xie C, Xie L, Shi J, Zhang J, Yang B, Chen D, et al. Integrated analysis of microRNA and mRNA expression profiles in the left atrium of patients with nonvalvular paroxysmal atrial fibrillation: Role of miR-146b-5p in atrial fibrosis. *Heart rhythm : the official journal of the Heart Rhythm Society.* 2015;12(5):1018-26.

387. Jing Chai ZL, Bin Zhou, Honglin Zhu, Xiaoyun Xie, Xiaoxia Zuo. miR-146b expression is upregulated in the lung of pulmonary fibrosis mice. *Int J Clin Exp Pathol*

2016;9(2):464-72.

388. Nho RS, Peterson M, Hergert P, and Henke CA. FoxO3a (Forkhead Box O3a) deficiency protects Idiopathic Pulmonary Fibrosis (IPF) fibroblasts from type I polymerized collagen matrix-induced apoptosis via caveolin-1 (cav-1) and Fas. *PLoS One.* 2013;8(4):e61017.

389. Fleury B, Murciano D, Talamo C, Aubier M, Pariente R, and Milic-Emili J. Work of breathing in patients with chronic obstructive pulmonary disease in acute respiratory failure. *The American review of respiratory disease.* 1985;131(6):822-7.

390. Loring SH, Garcia-Jacques M, and Malhotra A. Pulmonary characteristics in COPD and mechanisms of increased work of breathing. *Journal of applied physiology (Bethesda, Md : 1985).* 2009;107(1):309-14.

391. Marchand E, Gayan-Ramirez G, De Leyn P, and Decramer M. Physiological basis of improvement after lung volume reduction surgery for severe emphysema: where are we? *Eur Respir J.* 1999;13(3):686-96.

392. Thiele S, Wittmann J, Jack HM, and Pahl A. miR-9 enhances IL-2 production in activated human CD4(+) T cells by repressing Blimp-1. *European journal of immunology.* 2012;42(8):2100-8.

393. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nature immunology.* 2005;6(11):1133-41.

394. Donaldson GC, Seemungal TA, Patel IS, Bhowmik A, Wilkinson TM, Hurst JR, Maccallum PK, and Wedzicha JA. Airway and systemic inflammation and decline in lung function in patients with COPD. *Chest.* 2005;128(4):1995-2004.

395. Ishii T, Abboud RT, Wallace AM, English JC, Coxson HO, Finley RJ, Shumansky K, Pare PD, and Sandford AJ. Alveolar macrophage proteinase/antiproteinase expression in lung function and emphysema. *Eur Respir J.* 2014;43(1):82-91.

396. Davies C, Rhodes JA, Barnes P, and Donnelly L. Elevated CCL2 responses in COPD and attenuation by selective chemokine receptor antagonists. *European Respiratory Journal.* 2015;46(suppl 59).

397. de Boer WI, Sont JK, van Schadewijk A, Stolk J, van Krieken JH, and Hiemstra PS. Monocyte chemoattractant protein 1, interleukin 8, and chronic airways inflammation in COPD. *The Journal of pathology.* 2000;190(5):619-26.

398. Kutty RK, Nagineni CN, Samuel W, Vijayasarathy C, Jaworski C, Duncan T, Cameron JE, Flemington EK, Hooks JJ, and Redmond TM. Differential regulation of microRNA-146a and microRNA-146b-5p in human retinal pigment epithelial cells by interleukin-1beta, tumor necrosis factor-alpha, and interferon-gamma. *Molecular vision.* 2013;19(737-50.

399. Lozano R, Naghavi M, Foreman K, Lim S, Shibuya K, Aboyans V, Abraham J, Adair T, Aggarwal R, Ahn SY, 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 (London, England).* 2012;380(9859):2095-128.

400. Friedman RC, Farh KK, Burge CB, and Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome research.* 2009;19(1):92-105.

401. Barreiro E, del Puerto-Nevado L, Puig-Vilanova E, Perez-Rial S, Sanchez F, Martinez-Galan L, Rivera S, Gea J, Gonzalez-Mangado N, and Peces-Barba G. Cigarette smoke-induced oxidative stress in skeletal muscles of mice. *Respiratory physiology & neurobiology.* 2012;182(1):9-17.

402. Gosker HR, Langen RC, Bracke KR, Joos GF, Brusselle GG, Steele C, Ward KA, Wouters EF, and Schols AM. Extrapulmonary manifestations of chronic obstructive pulmonary disease in a mouse model of chronic cigarette smoke exposure. *Am J Respir Cell Mol Biol.* 2009;40(6):710-6.

403. Gunaratne PH, Coarfa C, Soibam B, and Tandon A. miRNA data analysis: next-gen sequencing. *Methods in molecular biology (Clifton, NJ).* 2012;822(273-88.

404. Sun Z, Evans J, Bhagwate A, Middha S, Bockol M, Yan H, and Kocher JP. CAP-miRSeq: a comprehensive analysis pipeline for microRNA sequencing data. *BMC genomics.* 2014;15(423.

405. Glenn TC. Field guide to next-generation DNA sequencers. *Molecular ecology resources.* 2011;11(5):759-69.

406. Makki MS, Haseeb A, and Haqqi TM. MicroRNA-9 promotion of interleukin-6 expression by inhibiting monocyte chemoattractant protein-induced protein 1 expression in interleukin-1beta-stimulated human chondrocytes. *Arthritis & rheumatology (Hoboken, NJ).* 2015;67(8):2117-28.

407. Jones SW, Watkins G, Le Good N, Roberts S, Murphy CL, Brockbank SM, Needham MR, Read SJ, and Newham P. The identification of differentially expressed microRNA in osteoarthritic tissue that modulate the production of TNF-

alpha and MMP13. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society.* 2009;17(4):464-72.

408. Zhao C, Sun G, Li S, and Shi Y. A feedback regulatory loop involving microRNA-9 and nuclear receptor TLX in neural stem cell fate determination. *Nature structural & molecular biology.* 2009;16(4):365-71.

409. Munoz JL, Bliss SA, Greco SJ, Ramkissoon SH, Ligon KL, and Rameshwar P. Delivery of Functional Anti-miR-9 by Mesenchymal Stem Cell-derived Exosomes to Glioblastoma Multiforme Cells Conferred Chemosensitivity. *Molecular therapy Nucleic acids.* 2013;2(e126.

410. Ferretti E, De Smaele E, Po A, Di Marcotullio L, Tosi E, Espinola MS, Di Rocco C, Riccardi R, Giangaspero F, Farcomeni A, et al. MicroRNA profiling in human medulloblastoma. *International journal of cancer.* 2009;124(3):568-77.

411. Ma L, Young J, Prabhala H, Pan E, Mestdagh P, Muth D, Teruya-Feldstein J, Reinhardt F, Onder TT, Valastyan S, et al. miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nature cell biology.* 2010;12(3):247-56.

412. Lu MH, Huang CC, Pan MR, Chen HH, and Hung WC. Prospero homeobox 1 promotes epithelial-mesenchymal transition in colon cancer cells by inhibiting E-cadherin via miR-9. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2012;18(23):6416-25.

413. Xu T, Liu X, Han L, Shen H, Liu L, and Shu Y. Up-regulation of miR-9 expression as a poor prognostic biomarker in patients with non-small cell lung cancer. *Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico.* 2014;16(5):469-75.

414. Bandres E, Agirre X, Bitarte N, Ramirez N, Zarate R, Roman-Gomez J, Prosper F, and Garcia-Foncillas J. Epigenetic regulation of microRNA expression in colorectal cancer. *International journal of cancer.* 2009;125(11):2737-43.

415. Senyuk V, Zhang Y, Liu Y, Ming M, Premanand K, Zhou L, Chen P, Chen J, Rowley JD, Nucifora G, et al. Critical role of miR-9 in myelopoiesis and EVI1-induced leukemogenesis. *Proc Natl Acad Sci U S A.* 2013;110(14):5594-9.

416. Miranda KC, Huynh T, Tay Y, Ang YS, Tam WL, Thomson AM, Lim B, and Rigoutsos I. A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. *Cell.* 2006;126(6):1203-17.

417. Alexander WS. Suppressors of cytokine signalling (SOCS) in the immune system. *Nature reviews Immunology.* 2002;2(6):410-6.

418. Lagos-Quintana M, Rauhut R, Lendeckel W, and Tuschl T. Identification of novel genes coding for small expressed RNAs. *Science.* 2001;294(5543):853-8.

419. Papagiannakopoulos T, Shapiro A, and Kosik KS. MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. *Cancer research.* 2008;68(19):8164-72.

420. Zhang X, Wang C, Shan S, Liu X, Jiang Z, and Ren T. TLR4/ROS/miRNA-21 pathway underlies lipopolysaccharide instructed primary tumor outgrowth in lung cancer patients. *Oncotarget.* 2016.

421. Gao Y, Cai Q, Huang Y, Li S, Yang H, Sun L, Chen K, and Wang Y. MicroRNA-21 as a potential diagnostic biomarker for breast cancer patients: a pooled analysis of individual studies. *Oncotarget.* 2016.

422. Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volinia S, Liu CG, Alder H, et al. MicroRNA signatures in human ovarian cancer. *Cancer research.* 2007;67(18):8699-707.

423. Han Y, Xu GX, Lu H, Yu DH, Ren Y, Wang L, Huang XH, Hou WJ, Wei ZH, Chen YP, et al. Dysregulation of miRNA-21 and their potential as biomarkers for the diagnosis of cervical cancer. *Int J Clin Exp Pathol.* 2015;8(6):7131-9.

424. Xu G, Zhang Y, Wei J, Jia W, Ge Z, Zhang Z, and Liu X. MicroRNA-21 promotes hepatocellular carcinoma HepG2 cell proliferation through repression of mitogen-activated protein kinase-kinase 3. *BMC cancer.* 2013;13(469.

425. Medina PP, Nolde M, and Slack FJ. OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. *Nature.* 2010;467(7311):86-90.

426. Guinea-Viniegra J, Jimenez M, Schonthaler HB, Navarro R, Delgado Y, Concha-Garzon MJ, Tschachler E, Obad S, Dauden E, and Wagner EF. Targeting miR-21 to treat psoriasis. *Science translational medicine.* 2014;6(225):225re1.

427. Shi C, Liang Y, Yang J, Xia Y, Chen H, Han H, Yang Y, Wu W, Gao R, and Qin H. MicroRNA-21 knockout improve the survival rate in DSS induced fatal colitis through protecting against inflammation and tissue injury. *PLoS One.* 2013;8(6):e66814.

428. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, and Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology.* 2007;133(2):647-58.

429. Barnett RE, Conklin DJ, Ryan L, Keskey RC, Ramjee V, Sepulveda EA, Srivastava S, Bhatnagar A, and Cheadle WG. Anti-inflammatory effects of miR-21 in the macrophage response to peritonitis. *Journal of leukocyte biology.* 2016;99(2):361-71.

430. Feng J, Li A, Deng J, Yang Y, Dang L, Ye Y, Li Y, and Zhang W. miR-21 attenuates lipopolysaccharide-induced lipid accumulation and inflammatory response: potential role in cerebrovascular disease. *Lipids in health and disease.* 2014;13(27.

431. Hua K, Jin J, Zhao J, Song J, Song H, Li D, Maskey N, Zhao B, Wu C, Xu H, et al. miR-135b, upregulated in breast cancer, promotes cell growth and disrupts the cell cycle by regulating LATS2. *International journal of oncology.* 2016;48(5):1997-2006.

432. Li J, Liang H, Bai M, Ning T, Wang C, Fan Q, Wang Y, Fu Z, Wang N, Liu R, et al. miR-135b Promotes Cancer Progression by Targeting Transforming Growth Factor Beta Receptor II (TGFBR2) in Colorectal Cancer. *PLoS One.* 2015;10(6):e0130194.

433. Xiang M, Birkbak NJ, Vafaizadeh V, Walker SR, Yeh JE, Liu S, Kroll Y, Boldin M, Taganov K, Groner B, et al. STAT3 induction of miR-146b forms a feedback loop to inhibit the NF-kappaB to IL-6 signaling axis and STAT3-driven cancer phenotypes. *Science signaling.* 2014;7(310):ra11.

434. Chou CK, Yang KD, Chou FF, Huang CC, Lan YW, Lee YF, Kang HY, and Liu RT. Prognostic implications of miR-146b expression and its functional role in papillary thyroid carcinoma. *The Journal of clinical endocrinology and metabolism.* 2013;98(2):E196-205.

435. He X, Li J, Guo W, Liu W, Yu J, Song W, Dong L, Wang F, Yu S, Zheng Y, et al. Targeting the microRNA-21/AP1 axis by 5-fluorouracil and pirarubicin in human hepatocellular carcinoma. *Oncotarget.* 2015;6(4):2302-14.

436. Zhang S, Han L, Wei J, Shi Z, Pu P, Zhang J, Yuan X, and Kang C. Combination treatment with doxorubicin and microRNA-21 inhibitor synergistically augments anticancer activity through upregulation of tumor suppressing genes. *International journal of oncology.* 2015;46(4):1589-600.