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MECHANISMS OF INFLAMMATORY SIGNALLING IN CHRONIC LUNG DISEASES:

Transcriptomics & metabolomics approaches

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ABSTRACT

Sarcoidosis, asthma and chronic obstructive pulmonary disease (COPD) are inflammatory pulmonary diseases with many different clinical phenotypes. A central characteristic of all three diseases is the altered airway function due to inflammation. In sarcoidosis this is believed to be caused by a yet unidentified substance(s), in COPD largely by exposure to inhaled toxicants, e.g. smoking and in asthma due to an excessive reaction to an often harmless environmental substance. Long-term inflammation may cause tissue damage and in some cases irreversible destruction and remodelling. The current absence of a cure for these diseases points out a need to define the pathologic mechanisms in order to personalise treatment.

In this dissertation we investigated the above mentioned diseases using global screening methods in combination with validation techniques. We investigated intracellular wingless/integrated (WNT)-signalling in bronchoalveolar lavage (BAL) and epithelial cells in sarcoidosis. Oxylipin–levels were measured in BAL fluid of asthmatics. And global screening for miRNA and mRNA expression in pulmonary inflammatory cells in COPD, as well as miRNA in extracellular exosomes from both asthmatics and COPD patients, were performed.

WNTs are lipoglycoproteins, important in several cellular functions such as proliferation and differentiation, which by binding to membrane receptors starts an intrecellular signalling cascade involving β-catenin. The results revealed clear differences in *WNT* expression levels in sarcoidosis patients, suggesting a role for the molecules in the development of fibrosis. Oxylipins are lipids synthesised on demand from omega-3 and omega-6 unsaturated fatty acids and involved in various inflammatory processes. Differing oxylipin levels in BAL fluids of mild asthmatics were detected in response to subway air exposure, indicating a decreased protective response to noxious stimuli in individuals with lowered lung function. Exosomes are nanosised vesicles, created in the multivesicular endosomes in various cells, believed to be involved in the extracellular transport of molecules. MikroRNA (miRNA), shown to be present in exosomes, are small RNAs capable of affecting translation of proteins through the regulation of mRNA. Expression of miRNAs in both cells and exosomes and geneexpression in the BAL cells were significantly different between smoking COPD patients and healthy smokers. These combined results clearly indicates global changes in different signalling cascades and pathways, requiring global analysis of expression on multiple levels in order to elucidate mechanistic differences between the diseases.

The projects included in this dissertation are part of larger studies. In further studies we aim to integrate the results from several platforms; transcriptomics, proteomics and metabolomics with complex analysis methods in order to elucidate the similarities, and more importantly, the differences between these pulmonary diseases. By doing this we will acquire a better understanding of the pathological reasons and further our understanding of these inflammatory pulmonary diseases. This may ultimately lead to earlier and more specific diagnostic tools, and to the development of better and more personal treatment options for the patients.

LIST OF PUBLICATIONS

I. **Levänen B,** Wheelock ÅM, Eklund A, Grunewald J, Nord M
Increased Pulmonary Wnt (wingless/Integrated)-signaling in patients with Sarcoidosis. Respiratory Medicine (2011) 105, 282-291.

II. Lundström SL*, **Levänen B***, Nording M, Klepczynska-Nyström A, Sköld MC, Haeggström JZ, Grunewald J, Svartengren M, Hammock BD, Larsson B-M, Eklund A, Wheelock CE, Wheelock ÅM
Asthmatics exhibit altered oxylipin profiles compared to healthy individuals after subway air exposure. PLoS One. 2011;6(8):e23864. *These authors contributed equally.

III. **Levänen B**, Bhakta N, Torregrosa-Paredes P, Barbeau R, Hiltbrunner S, Pollack JL, Sköld CM, Svartengren M, Grunewald J, Gabrielsson S, Eklund A, Larsson B-M, Woodruff P, Erle DJ, Wheelock ÅM.
Differences in exosomal microRNAs in bronchoalveolar lavage fluid from asthmatics and healthy individuals. *Manuscript.*

IV. **Levänen B**, Cansu Z, Pollack JL, Grunewald J, Karimi R, Eklund A, Gabrielsson S, Erle DJ, Sköld CM, Wheelock ÅM
Differential expression of exosomal and cellular miRNA in healthy never-smokers, healthy smokers and COPD patients. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic acid
AEC	Airway epithelial cells
Ago	Argonaute
AHR	Airway hyperresponsiveness
Ahr	Arylhydrocarbon receptor
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
BAL	Bronchoalveolar Lavage
BALF	BAL fluid
BHL	Bilateral hilar lympadenopathy
BSA	Bovine serum albumin
CCL	CC chemokine ligand
CCSP	Clara cell secretory protein
CD	Cluster of differentiation
cDNA	Complementary DNA
CHI3L1	Chitinase-3 like-1
CK1	Casein kinase 1
CLDN1	Claudin-1
CO	Carbon monoxide
COPD	Chronic Obstructive Pulmonary Disease
COPDxS	COPD ex-smokers
Cox-2	Cyclooxygenase 2
CT	Computer tomography
CV	Coefficient of Variance
CV-ANOVA	Cross-validated ANOVA
DAB	3,3´-diaminobenzidine
DAPI	4´,6-diamidino-2-phenylindole, dihydrochloride
DNA	Deoxyribonucleic acid
DSH	Dishevelled
dsRNA	Double stranded RNA
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial mesenchymal transition
ERV	Expiratory reserve volume
FACS	Fluorescence activated cell sorting
FDR	False discovery rate
FEV_1	Forced expiratory Volume in 1 second
Fra-1	Fos-related antigen 1
FVC	Forced Vital Capacity
FZD	Frizzled receptor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSK-3β	Glycogen synthase kinase 3 β
H_2O_2	Hydrogen peroxide
HETE	Hydroxyl eicosatetraenoic acid
IC	Inspiratory capacity

ICAM-1	Intercellular adhesion molecule-1, CD54
IFN-γ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
IPF	Idiopathic Pulmonary Fibrosis
IRV	Inspiratory reserve volume
LA	Linoleic acid
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LEF	Leukocyte enhancing factor
LFA	Lymphocyte function-associated protein 1
LOD	Limit of detection
Log	Logarithmic
LOQ	Limit of quantification
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LRP	Lipoprotein related protein
LTB_4	Leukotriene B_4
MHC	Major histocompatibility complex
miRNA	microRNA
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
MVA	Multivariate Analysis
MVE	Multivesicular endosome
MΦs	Macrophages
NF-κB	Nuclear factor κ B
NNMT	Nicotinamide N-methyltransferase
NO	Nitric oxide
NO_2	Nitrogen dioxide
NSAID	Non-specific anti-inflammatory drug
O_3	Ozone
OPLS	Orthogonal projections to latent structures
PAH	Polycyclic aromatic hydrocarbons
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEF	Peak expiratory flow
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
PM	Particulate matter
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptors
Q^2	Seven fold cross-validation predictive value
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
R^2	Cumulative correlation coefficient
RHOU	Ras homolog family member U
RISC	RNA-induced silencing complex
RNA	Ribonucleic Acid

ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RV	Residual volume
SOX9	Sex determining region Y-box 9
SP	Surfactant protein
SPP1	Secreted phosphoprotein 1, osteopontin
SUS-plot	Shared and unique structures plot
TCF	T-cell factor
TGFβ	Transforming growth factor β
TIMP	Tissue inhibitors of metalloproteinases
TLC	Total lung capacity
TLR	Toll like receptors
Tm	Annealing (melting) temperature
TNFα	Tumour necrosis factor α
tRNA	Transfer RNA
TV	Tidal volume
UV	Ultraviolet
VC	Vital Capacity
VEGFA	Vascular endothelial factor A
VEGFR	Vascular endothelial factor receptor
VIP	Variable importance in the projection
WISP	WNT1 inducible signalling pathway protein 1
WNT	Wingless/Integrated
XDR	Extended dynamic range

1 INTRODUCTION

Chronic inflammatory lung diseases are an increasing global health problem [4]. In Europe, chronic obstructive pulmonary disease (COPD) currently causes the most lost work days among all respiratory illnesses [5]. In the Global Burden of Disease study, the World Health Organization (WHO) predicts that the prevalence of chronic lung disease is going to increase significantly over the next decade, and become an even more prominent cause of morbidity and disability worldwide [4]. Also the prevalence of asthma is growing globally, in particular in urban areas where polluted air is a main trigger of asthmatic exacerbations [6]. Sarcoidosis, however, is not as common a disease, with approximately 2000 new cases in Sweden yearly. The majority of patients recover, but some develop chronic disease with fibrosis and eventually respiratory failure, resulting in a mortality rate of ~5%.

In the studies described here, we investigated three chronic inflammatory lung diseases, with some common features but also distinct differences. We approached this question by combining methods screening for differences at a wider scale with more traditional techniques, investigating molecules or proteins of interest. All of these factors can aid us in our search for disease-specific differences to be used in early diagnosis and individualised treatment, and thus improved management of inflammatory pulmonary disease, ultimately finding biomarkers to be used at the clinic. Biomarkers are biomolecule representatives for biological processes and can be used to indirectly measure or determine different temporal stages in a disease process. Several biomarkers have been suggested for the use in inflammatory lung disease, but so far only few have proved useful in clinical practice [6, 7].

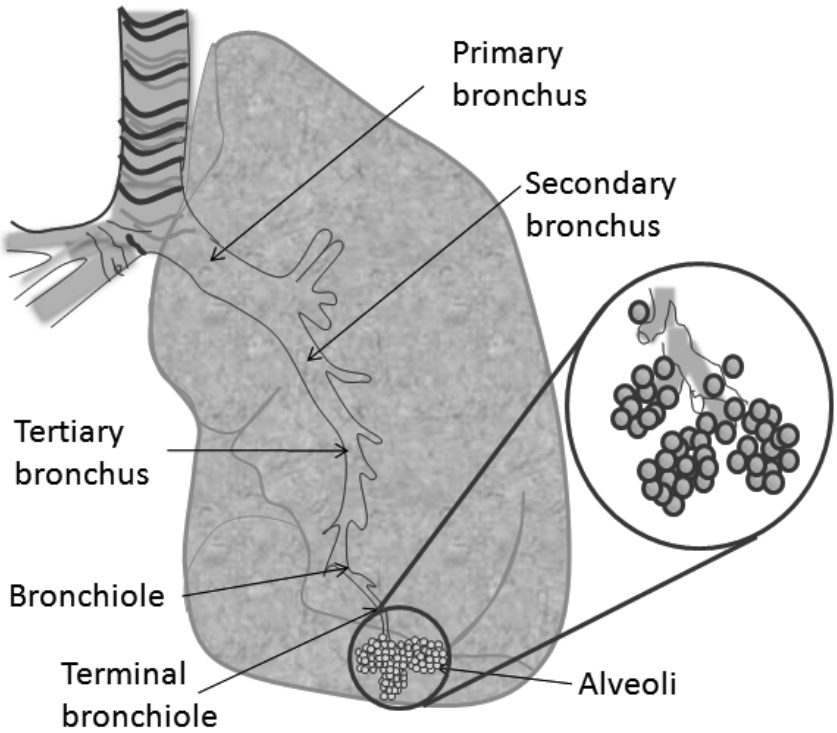

Figure 1. A schematic picture demonstrating the conducting airways from the primary bronchi to the distal alveoli.

1.1 THE RESPIRATORY SYSTEM

The respiratory system is a collective word for the anatomical system that is composed, in the order from proximal to distal, of the nasal cavities and the mouth, the trachea, bronchi, bronchioles, alveolar ducts, alveolar sacs and the alveoli (Figure 1). The main function of the respiratory system is to supply the body with oxygen and remove carbon dioxide from the tissues.

The nasal cavities play an important role as a primary barrier against particulates entering through the air, metabolising inhaled airborne particles and for humidification of the breathed air. The human lungs consist of two lobes on the left side and three lobes on the right side. The bronchioles divide, after entering the parenchyma, accordingly with three branches to the right and two to the left, one into each lobe. The main function of the alveolar cells is to provide gas exchange between the inspired air and the blood in the pulmonary circulation, which occurs in the most distal alveoli [8].

The lung is a complex organ which consists of more than 40 different cell phenotypes, organised in a complex structure which facilitates gas exchange as well as protection against the surrounding environment.

1.1.1 Pulmonary function measurements

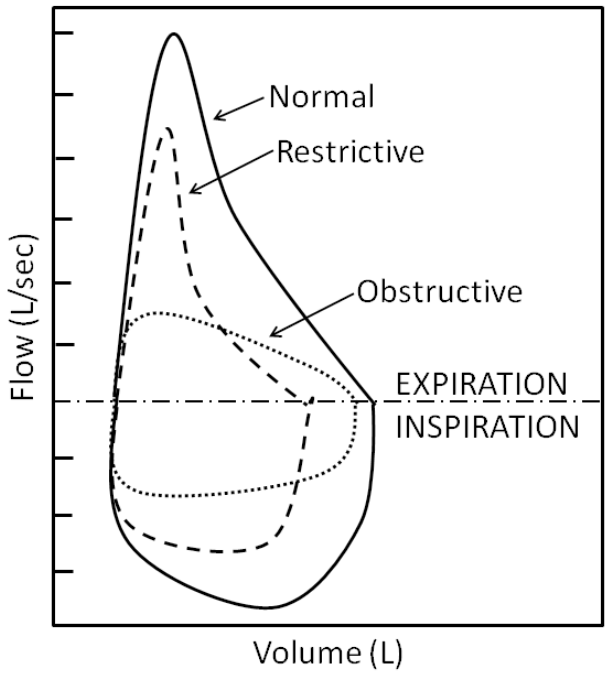

Figure 2. . Spirometry measurements demonstrating the normal tracings compared to ones from a patient with obstructive and restrictive pulmonary disease.

Pulmonary function measurements are an important indicator whether something is wrong with the function of the lungs. Spirometry is used to measure lung volume and function in order to determine whether the individual being measured has obstruction or restriction in their lung function. Obstructive lung disorders such as COPD are characterised by a reduced passage of air. The FEV_1/FVC ratio is reduced due to high airway resistance. Patients with restrictive disorders, such as fibrosis and sarcoidosis,

are prevented to expand their lungs due to an increased amount of respiratory tissue scarring, with a resulting reduction in TLC (Figure 2).

Peak expiratory flow (PEF) refers to the maximum expiratory flow rate and can be measured by a handheld flow meter. Levels are lower in patients with constricted lungs. Normal values are dependent on the patients' height, age and gender.

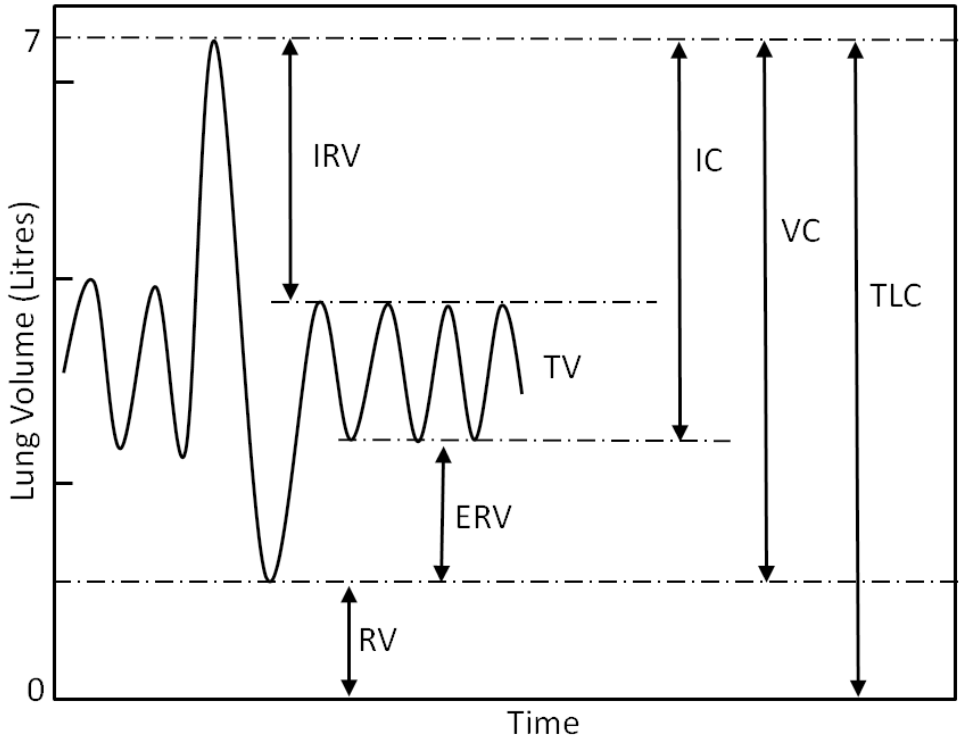

Figure 3. An illustration showing the lung volumes. TLC = total lung capacity, VC = vital capacity, IC = inspiratory capacity, TV = tidal volume, ERV = expiratory reserve volume, IRV = inspiratory reserve volume, RV = residual volume.

Forced vital capacity (FVC) measures how much air you can exhale with force after inhaling as deeply as possible. It is a combination of tidal volume (TV), expiratory reserve volume (ERV) and inspiratory reserve volume (IRV) (Figure 3).

Forced expiratory volume in 1 s (FEV_1) is the amount of air being exhaled at the end of the first second of expiration. Values between 80-120% of predicted normal values are considered healthy. FEV_1/FVC is often reported as a percentage (FEV_1%). This is reduced in obstructive diseases such asthma, COPD, emphysema and chronic bronchitis.

Residual volume (RV) is the air left in the airways after maximum exhalation. The residual volume increases quite remarkably in COPD.

Total lung capacity (TLC) is the entire volume of the lungs, including RV.

Tidal volume (TV) is the volume inspired or expired in one normal breath.

1.2 CELLS TYPES OF THE LUNG

The large bronchi contain irregularly shaped cartilaginous plates, which are absent in the smaller bronchi. The epithelium of the airways contains several types of cell and the combination of these changes along the airway. Beneath the epithelium lies the basement membrane, mostly consisting of collagen fibers and the submucosa. Beneath the basement membrane of the airway is a layer of smooth muscle. In addition, several types of inflammatory cells are present in the lungs at various stages. All of these cells are important in creating the necessary microenvironment to maintain these complex structures, and culturing of primary cells from the lung results in rapid de-differentiation of the cells. This makes the use of cell lines problematic in respiratory research.

1.2.1 The Basement membrane

The basement membrane, on top of which the epithelium is anchored, serves several important roles in the lung. It acts as a barrier between the underlying mesenchymal compartment and the surface epithelial cells, facilitates migration and regulates the phenotype of epithelial cells. It consists of collagen; type IV collagen and V laminin present in the upper layers are secreted by the epithelial cells, type III and V collagen and fibronectin synthesised by subepithelial fibroblasts [9].

1.2.2 Airway epithelium

The airway epithelium is the first barrier for airborne pollutants such as particulate matter, polycyclic aromatic hydrocarbons (PAHs) and oxidants present in cigarette smoke. In addition to the barrier function the epithelium serves a secretory function, producing a wide array of lipid mediators, chemokines, cytokines and growth factors [10-12]. Pulmonary surfactants which are composed of lipoproteins and formed by alveolar type II cells, maintains alveolar integrity, aiding pulmonary compliance (capability to expand) and plays a role in host defence. The epithelium is also an important source of arachidonic acid (AA) metabolites and ezymes such as cyclooxygenase (COX) and lipoxygenase (LOX), which are involved in mucus secretion, inflammation and smooth muscle tone [13]. Under normal conditions the airway epithelium has a capacity of regeneration and repair [14, 15]. However, during exposure and other foreign stimuli an imbalance can be created starting a disease process, which the normal system is unable to control.

There are several types of epithelial cells and the composition of the airway wall epithelium changes from the proximal to distal airway. These cells are of three main types; basal cells, ciliated and secretory cells (Figure 4). The cell types in the proximal airways are mainly ciliated columnar and mucus secreting goblet cells. The bronchioles lack cartilage and glands, have thinner walls and the epithelium here mainly consists of ciliated columnar epithelium, only a few goblet cells are present [16]. The terminal bronchioles mainly consist of ciliated cuboidal cells and Clara cells. Clara cells play an important role in exposure to cigarette smoke, as the PAHs mainly target these cells. Sampling of epithelial cells from the distal airways is a very specific technique, using fiberoptic bronchoscopy combined with fluoroscopic guidance, unfortunately not available at our clinic at the time of sample collection [17]. The epithelium in the

alveoli consists of two types of pneumocytes, Type I cells primarily being responsible for gas exchange, and Type II cells producing surfactant. The type I pneumocytes are the more common ones, making up two-thirds of the alveolar epithelial cells and covering up to 90% of the surface.

Basal cells

Basal cells are ubiquitous in the large conducting airways and there is a direct correlation between the thickness of the epithelium and the number of these cells. These cells are attached to the basement membrane and play a role in the adhesion of the columnar epithelium to the same [18]. Basal cells serve as stem cells giving rise to ciliated and mucous epithelial cells [19].

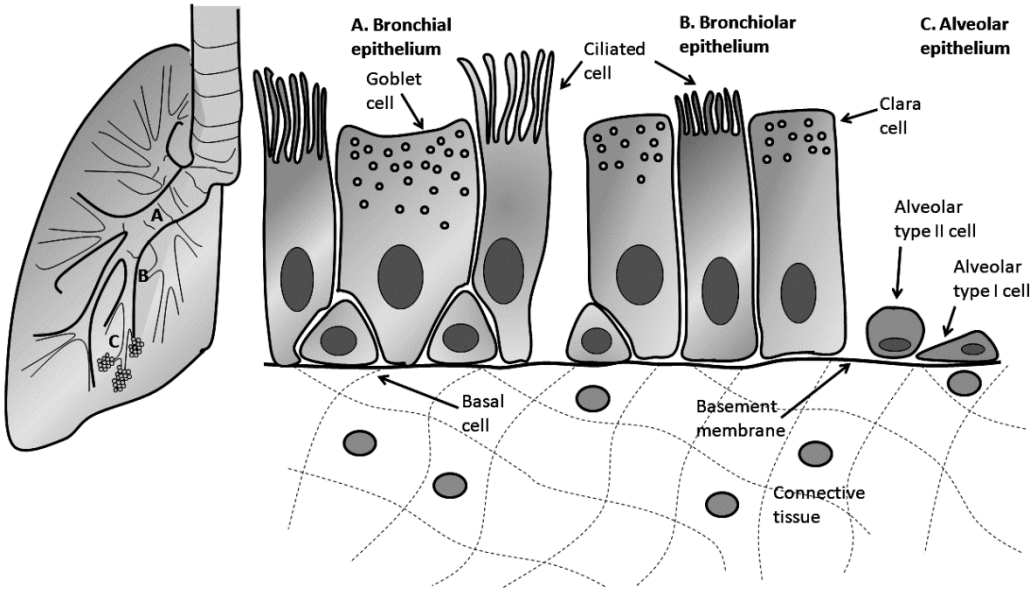

Figure 4. Illustration showing the typical cells of the bronchial, bronchiolar and alveolar epithelium.

Ciliated cells

Ciliated cells are the main type of epithelial cell in the lung and account for about half of the total number of cells. These cells are covered with up to 300 cilia/cell used to transport mucus from the lung towards the throat.

Secretory cells

Clara cells typically extend from the basement membrane to the airway lumen, have a granule filled apical bulge and are present in both the bronchial and bronchiolar airways. They are the main secretory cells of the distal airways. Clara cells produce granules of surfactants, such as Clara cell secretory protein (CCSP), which cover the surface of the bronchial epithelium. Clara cells also have a high metabolic capacity, and metabolise a range of xenobiotic compounds that enter the lung through inhalation, such as tobacco smoke [20]. In the absence of basal cells, like in the smaller airways, Clara cells are able to re-differentiate into other cell types, giving rise to other types of epithelial cells [21].

Goblet cells are characterised by secretion of mucin rich granules creating mucus for trapping foreign objects in the airways. The goblet cell appearance varies from the classical goblet shape to tall columnar cells and they have a basal nucleus [22].

Normally the mucus protects the airways, maintaining hydration and trapping particles viruses and bacteria, as well as contributing important anti-inflammatory properties. The mucosal amount can be affected by exposure to noxious stimuli such as cigarette smoke and it is common with mucous cell hyperplasia and metaplasia, with the associated hypermucosis and productive cough, in inflammatory pulmonary diseases such as COPD [22]. The composition and consistency of the mucus also changes in COPD becoming more viscous due to alterations on chloride channels and destruction of cilia.

Clara cells are capable of secreting mucins and surfactant protein (SP)-D. The arylhydrocarbon receptor (Ahr), a ligand activated transcription factor highly expressed in epithelial cells, has been implicated to mediate the effects of toxins entering the lung through the archidonic acid cascade and mucin production [23, 24]. COX-2 levels have been shown to increase upon Ahr activation [25]. The eicosanoid prostaglandin E_2 (PGE_2), derived from COX-2, can activate mucin production in the airway [26].

1.2.3 Inflammatory cells

In addition to the structural cells of the lungs, a number of other cell types, such as inflammatory cells, migrate to and reside in the epithelium. Some of these continue to migrate out into the lumen. The immune system is a collection of organs, cells and tissue, working in a tightly regulated manner to fight foreign pathogens. The immune system is further divided into the innate (non-specific) immunity and the adaptive (specific) immunity [27].

The innate immunity

The innate immunity is the first line of defence against foreign pathogens. Danger signals, such as pathogen derived molecules are recognised by pattern recognition receptors (PRRs). The best known PRRs are toll-like receptors (TLRs). When ligands, such as bacteria derived DNA, lipids or double stranded RNA (dsRNA), are bound to these receptors it leads to an intracellular cascade involving the activation of the transcription factor nuclear factor κB (NF-κB). NF-κB then translocates to the nucleus where it initiates transcription of proinflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α [28].

Neutrophils are involved in the initial response to pathogens and phagocytise foreign material. They are attracted by the cytokines IL-1β and IL-8, as well as the lipid mediator leukotriene B_4 (LTB_4). Neutrophils kill the pathogens using their intracellular reactive oxygen species (ROS) or by releasing granules containing enzymes that act in the extracellular space [29]. The levels of neutrophils are elevated in both smokers and COPD patients and a recent study shows increased levels of migration of neutrophils in smokers [30].

Eosinophils are attracted to the site of inflammation by the cytokines IL-3, IL-5 and granulocyte macrophage colony-stimulating factor (GM-CSF), as well as LTB_4, chemokine ligand (CCL) 5 and eotaxins. Their granules contain histamine and eosinophilic peroxidase and they produce the cytokines IL-4, IL-5, IL-6 and IL-13 steering the response toward a Th2 type. Therefore an increased number can be detected in asthma and allergies.

The least common granulocyte is the basophils and normally there are very low amounts in human peripheral blood. Basophils contain the anticoagulant heparin and the vasodilator histamine, allowing better blood flow through the infected tissue.

Mast cells, involved in the early allergic response, contain histamine, prostaglandin and leukotriene filled granules which are able to induce mucus secretion and bronchoconstriction [31]. Mast cells recruit other cells to the site of infection through indirect and direct methods through a number of cytokines and chemokines; (TNF-α, LTB_4, IL-8, CCL2) and are involved in activating the innate immunity through IL-10, TNF-α, transforming growth factor (TGF)-β and histamine [32-34]. Mast cells are present in the airway epithelium in asthma and can be activated through TLRs by viral products [31, 35].

Alveolar macrophages are derived from the blood monocytes and are present in the alveolar lumen. They are activated by Th1 cells, as well as through TLRs, and their main function is to phagocytise foreign material from the body. By releasing chemokines such as IL-8, they also recruit other inflammatory cells to the site of infection, and by production of interferon (IFN)-γ they induce cluster of sifferentiation (CD)8^+ responses and a Th1-type immunity [36]. After phagocytosis macrophages are capable of presenting antigens to T-cells via their major histocompatibility complex (MHC) molecules.

Adaptive immunity

The adaptive immune system is the secondary line of defence against pathogens. Among the cell types involved are T- and B-lymphocytes, both with surface receptors with high specificity. Each organism has its own unique set of immunologic memory and hence a specific combination of cells. When a cell encounters and recognises an antigen during e.g. an infection it leads to a clonal expansion of that specific cell to increase the number in order to fight the infectious agent. One subset of the cells will become activated, another will become memory cells with a faster reaction to the same antigen upon a second encounter.

T-lymhocytes are developed in the bone marrow before maturing in the thymus and entering the blood stream and the lymphoid organs. There are two main types of T-cells, depending on their cell surface co-receptors; $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T-cells. Antigens are presented to T-cells by antigen-presenting cells through MHC class I to $CD8^+$ cells and through MHC class II to $CD4^+$ cells [37].

$CD4^+$ T-helper cells are further differentiated into Th1 and Th2 types depending on what antigen they encounter and this differentiation is regulated by cytokines. IL-12 promotes differentiation into the Th1 type, and the absence of IL-12 leads to

differentiation into the Th2 type. IL-4 actively drives the differentiation towards the Th2 development [38]. Th1 cells mainly release IFN-γ, while Th2 cells mainly produce IL-4, IL-5 and IL-13.

$CD8^+$ cytotoxic T-cells are involved in the protection of the host from bacteria and virus by the induction of apoptosis and is the predominant T cell type in COPD [39]. The numbers are positively correlated to the degree of emphysema and tissue destruction [40]. $CD8^+$ T cells release proteolytic enzymes called perforin and granzymes that can cause cell death and necrosis through which they might be involved in the tissue destruction in COPD [39, 41].

Antigen producing B-cells are responsible for the humoral immune response. The production of IgE antibodies by B-cells requires production of IL-4 and IL-13 from activated T-cells.

1.3 CELLULAR SIGNALLING

Cellular signalling is a complex system governing the events in the organism. It can be regarded as communication within and between cells. Through this signalling the cells are capable of reacting to signals from their environment and respond by creating the correct response for each type of stimuli. There are several types of which I will discuss a selected few; intracellular wingless/integrated (WNT) signalling, investigated in paper I in respect to the disease process in sarcoidosis; Oxylipins, shown to play a major role in signalling in the immune and nervous system; RNAs which are essential for protein synthesis, and in particular miRNAs which are capable of regulating this process; and finally the small vesicles called exosomes, believed to be able to transport signals between cells.

1.3.1 WNT

WNT signalling plays an important regulatory role in both development of the lung and normal lung homeostasis in the adult lung. It is believed to be involved in disease processes when fine tuning of repair functions have gone astray, with the persistent activation of proliferation leading to fibrosis and tissue destruction. A role for WNT signalling in different cancers, including lung cancers, is well established and a role in other lung diseases have been suggested [42, 43].

WNTs are highly conserved lipoglycoproteins expressed in a tightly regulated spatiotemporal manner. To date, 19 WNT proteins have been identified in humans. These assign their effects through three different signalling cascades involving WNTs; the most conserved and well-characterised canonical WNT/β-catenin pathway, the WNT/CA^{2+} pathway signalling through calmodulin kinase II and protein kinase C, and finally the WNT/JNK pathway which signals through small GTPases [44]. In paper I we have focused on WNT/β-catenin signalling in Sarcoidosis, and this pathway will be the main focus.

Without a WNT ligand β-catenin is bound to a destruction complex containing the proteins AXIN and adenomatous polyposis coli (APC), which continually phosphorylates the β-catenin molecule. This process requires casein kinase I (CK1) and

glycogen synthase kinase (GSK)-3β. The phosphorylated β-catenin is thereafter ubiquitinated and degraded by proteosomes.

Binding of a WNT ligand to the frizzeld (FZD) and lipoprotein related protein (LRP) receptors on the cell surface activates a cascade where β-catenin, normally residing in the cytosol, is released from the degradation complex (Figure 5). This cascade contains the protein Dishevelled (DSH), which recruits the destruction complex of AXIN and APC to the plasma membrane and away from β-catenin. This step inhibits GSK-3β and reduces the degradation of β-catenin, allowing it to translocate to the nucleus [45].

In the nucleus β-catenin forms a complex with its DNA-binding partners known as lymphocyte enhancer factor (LEF)/T-cell factor (TCF) [46]. This complex then bind to promoters and activate target genes involved in proliferation and differentiation, such as *MMP2*, *MMP7, MMP9, VEGFA, CLDN1, CCND1, SOX9*, *RHOU, COX-2* and *WISP [47-55]*.

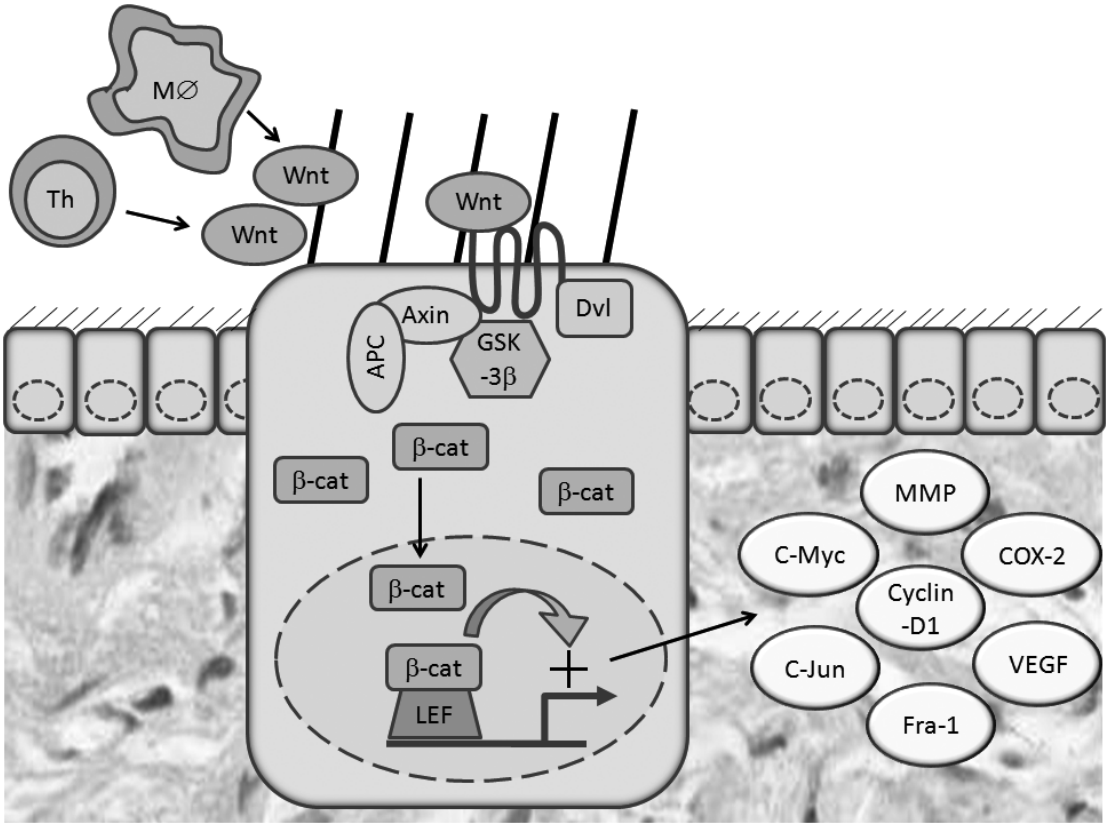

Figure 5. WNT/β-catenin signalling. We hypothesised that WNT ligands secreted from inflammatory cells in the proximity act on cell surface FZD receptors on epithelial cells to start the signalling cascade that will activate β-catenin. β-catenin translocates to the nucleus where it binds to the TCF/LEF transcription factor complex and starts the transcription of target genes.

The most well-known profibrotic cytokine TGF-β is known to cross-talk with the WNT/β-catenin pathway. These pathways can promote each other's activation and in some cases even co-regulate the same gene through TCF and Smad binding sites within the same promoter [56-60]. WNT signal activation in cell cultures with fibroblasts stimulates the cells to enhanced proliferation, migration and matrix production [61]. The initial reports of the involvement of β-catenin in fibrotic disease was the discovery of mutations of β-catenin and its negative regulator APC [62]. And the role for β-

catenin in wound healing was demonstrated by studies in mice [63]. It is today a well accepted fact that repetitive injury and repair processes of the alveolar epithelium, with an additional local inflammation, is a central pathogenic mechanism of idiopathic pulmonary fibrosis (IPF) [64]. Furthermore, there is an additional impairment of the epithelial- mesenchymal crosstalk contributing to the pathology of IPF [65]. These and other studies supported the hypothesis that WNT/β-catenin signalling is involved in the fibroproliferative part of wound healing, as well as a role in the imbalance leading to disorders with fibrosis.

In this thesis we have mainly focused on the above described canonical WNT/β-catenin pathway. Although this pathway occurs in all tissues, the genes activated are cell-type and context specific. Targets of WNT/β-catenin should therefore be investigated in individual cell types and disease states to understand the contribution of this pathway in the pathogenesis.

Potential candidates for modulating the WNT/β-catenin pathway include siRNAs, antibodies against WNTs, inhibitors of GSK-3β, activators of LRP, or blocking agents of TCF/β-catenin interactions.

The canonical WNT β-catenin pathway is down-regulated in the airway epithelium of both healthy smokers and smokers with COPD [66]. And it has been shown that activation of β-catenin attenuates the experimental emphysema in mice [67]. Abnormal regulation of the WNT pathway in the epithelium has been reported in smokers [66].

1.3.2 Oxylipins

Cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 are the main enzymes involved in oxylipin biosynthesis in human airway epithelium [13]. There are four families of eicosanoids; the prostaglandins, prostacyclins, tromboxanes and leukotrienes. They derive from either omega-3 or omega-6 and their function depending on the essential fatty acid origin. Omega-6 is more pro-inflammatory, but both are affected by non-specific anti-inflammatory drugs (NSAIDs) and aspirin through down-regulation of synthesis.

Mast cells metabolise arachidonic acid through the LOX and COX pathways and release the products leukotriene B_4 (LTB_4), prostaglandin E_2 (PGE_2) and leukotrienes such as LTC_4 [68]. PGE_2 is produced in the airway epithelium, with inhibitory effects on airway smooth muscle and mucus secretion, and is therefore described as a bronchoprotective mediator [69-72]. The complex role of PGE_2 in the airways is, however, demonstrated by its opposing role of promoting a Th-2 bias through IL-12 deficient dendritic cells [73]. The enzymes COX and LOX, as well as the products 5-hydroxy eicosatetraenoic acid (HETE), 15-HETE and 12-HETE, are present in abundance in the airway epithelium [74, 75]. Previous studies have demonstrated increased amounts of 15-HETE in the epithelium of asthmatics and patients with chronic bronchitis [76]. Interestingly, COX-2 has been shown to be a target gene of WNT signalling in mice, demonstrating the complexity of these signalling pathways with multiple levels of regulation [53, 54].

1.3.3 RNA

A series of events, called gene expression, is required for the production of specific proteins or functional RNAs. Regulation of gene expression plays a crucial role in ensuring that the correct proteins are being translated. Regulatory events of such kind have cell- and tissue-specific expression patterns. There are various types of RNAs, all with specific roles and sizes. The information encoded in a gene is used to produce a specific protein through messenger RNA (mRNA). Ribosomal RNA (rRNA) forms the basic machinery on which the protein synthesis takes place. Transfer RNA (tRNA) functions as adapter RNAs, translating the information in the mRNAs into sequences of aminoacids to produce the protein. microRNAs (miRNA) are involved in post-transciptional regulation of mRNA. All of these RNA transcripts combined constitute the trancriptome of a cell, which can be analysed using microarray based methods.

mRNA

Messenger RNA (mRNA) is a hard copy of the DNA that is being transcribed. Transcription describes a series of enzymatic processes that result in the transfer of information from the double-stranded DNA to the single-stranded mRNA, which will then be used as a template for protein translation. mRNAs are relatively unstable and easily degraded as it is only needed for a brief moment for the above described translation. Protein synthesis is local and extremely important for the regulation of gene expression.

microRNA

An ubiquitous class of RNA, microRNAs (miRNA) are relatively recently identified short (20-23 nt) endogenous, single-stranded nucleotides, derived from longer precursors called pre-miRNAs (Figure 6) [77, 78].

The main function of miRNAs is to regulate gene expression and protein translation, and thus represent an important link between proteomic and transcriptomics/mRNA data. The miRNAs are relatively stable, more so than mRNA, and are known to add to mRNA degradation levels and also affect the protein levels by dampening the translation of mRNAs [79].

Though previously this was thought to be their only regulatory effect, it has recently been discussed whether miRNAs can additionally have an up-regulatory effect on the mRNA [80]. The miRNAs are therefore a fundamental post-transcriptional layer that allows fine tuning of the regulation of gene expression. Abnormal expression patterns of miRNAs have been implicated in pulmonary fibrosis, COPD and asthma [81-84].

Two ribonuclease-III (RNAse III) enzymes, called drosha and dicer, process the mature miRNA in a step-wise manner from one arm of the precursor hairpin structure. The other strand, called the passenger strand, previously marked miRNA* and more recently named 3p, has been thought to be degraded. However, recent findings suggest that the miRNA-3p might have a functional and active contributive role in regulation [85, 86].

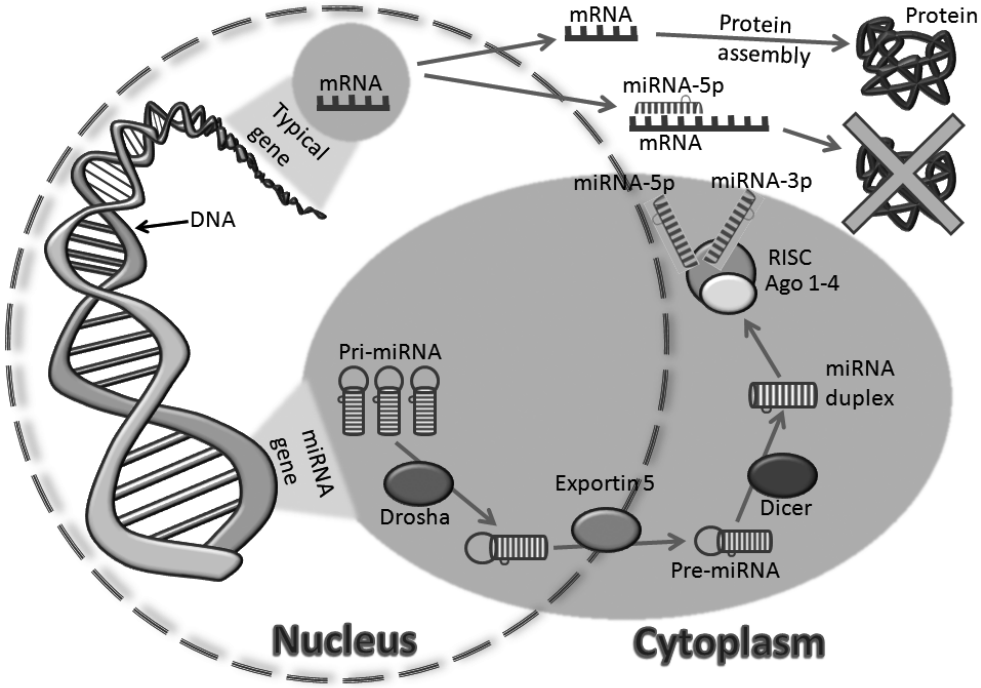

Figure 6. A simplified illustration of miRNA synthesis and method of action. miRNAs are transcribed from genes as pri-miRNAs, whereafter they are processed by two enzymes called Drosha and Dicer. Exportin 5 is involved in the transport of the pri-miRNA from the nucleus to the cytoplasm. Then the processed miRNA duplex is cleaved by a complex of RNA-induced silencing complex (RISC) and argonaute (Ago) 1-4 into two strands. These single-stranded miRNAs are capable of regulating mRNA and protein translation.

The nomenclatures of miRNAs changes as their function are elucidated. There is more to read from the name of a miRNA (example hsa-miR21a); hsa indicating it is a human miRNA, as opposed to mouse (mmu), rat (rno), schimpanzee (ptr) and so on. The number indicates the order of which they were discovered, 21 being an early discovery. Let-7, however, was one of the first miRNAs to be discovered and was therefore named prior to this system. The letter in 21a suggests that there are several closely related miRNAs. There are two mature products of hsa-miR-21a, miR-21a-5p and miR-21a-3p, which was previously labelled miR-21a*, as discussed previously.

1.3.4 Exosomes

Exosomes are nanosized (30-100nm) membrane vesicles of endosomal origin, secreted from several different celltypes; epithelial cells, mast cells, B-cells, dendritic cells, T cells, macrophages and neurons [87-96]. The exosomal protein composition and the functions appear to differ depending on from what type of cell they originate [97]. They can function in antigen delivery, tolerance induction, T-cell activation and inhibition [95]. Exosomes can contain multiple signals, both within themselves and as surface receptors. Recent research has shown that exosomes can contain functional RNA molecules including mRNA and miRNAs, which can be shuttled from one cell to another, affecting the recipient cell's protein translation (Figure 7) [98-100]. The exosomes can consequently serve as vehicles by which a cell can communicate with another by delivering RNA between them and are found in physiological fluids from which they can be extracted. Previous analyses have been done on exosomes from breast milk, saliva, blood, serum, nasal secretions, urine and BALF [101-107].

Previous studies have been conducted examining exosomes in sarcoidosis and asthma [96, 107-109]. At the time of writing no publications could be found on exosomes in COPD.

Chairoungdua et al. showed that WNT-signalling is down-regulated through the tetraspanin CD82 and CD9 control of exosomal β-catenin levels, suggesting an effect of exosomes on intracellular signalling [110].

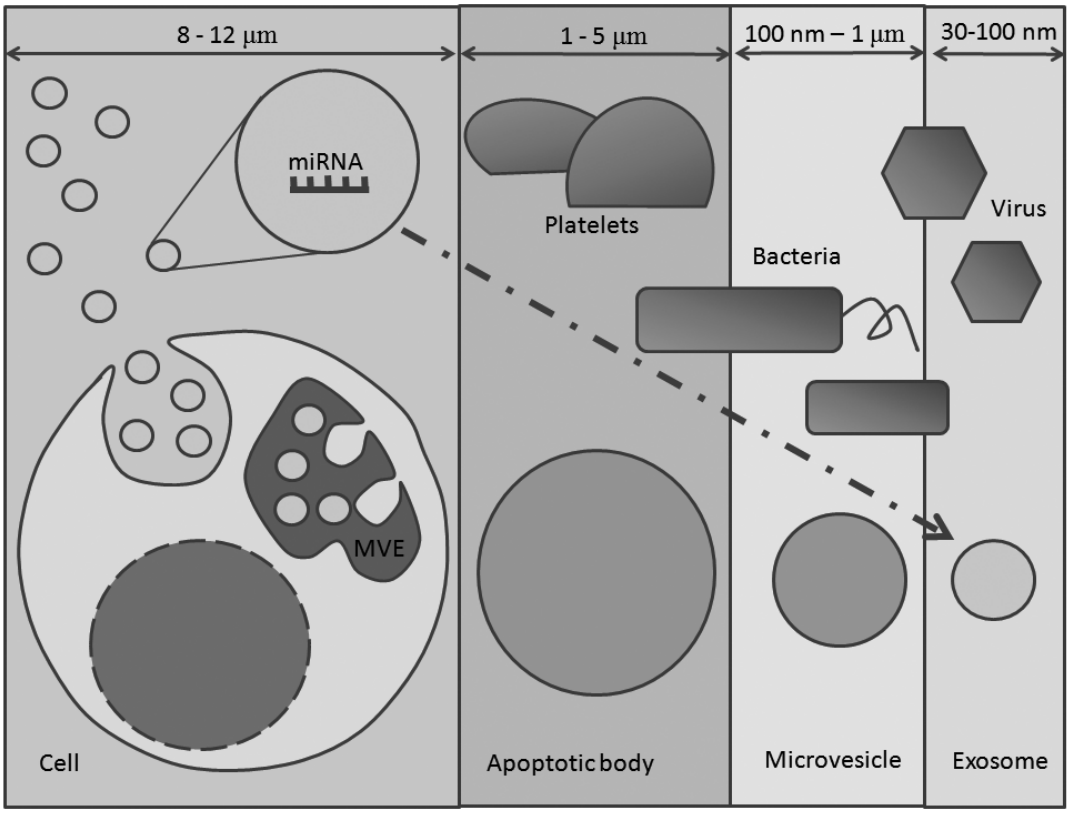

Figure 7. Size comparison of exosomes to microvesicles, apoptotic bodies, virus, bacteria, platelets and cells. Exosomes are created by invagination into multivesicular endosomes (MVE) before being released into the extracellular space. Exosomes can contain miRNA. *The image has been created with adjustments from [1, 2].*

These vesicular exosomes are not to be confused with the described exosomes involved in RNA processing.

1.4 INFLAMMATORY PULMONARY DISEASES

This dissertation includes studies of three inflammatory pulmonary diseases; sarcoidosis, asthma and COPD. Common features of these three diseases are that the causative mechanism for the disease process is mainly unknown, and that they are characterised by an ongoing inflammation in the lung leading to structural changes and remodelling. However, the disease processes are all different, as is the pathology and immune response. These are all largely heterogenous diseases with variation in the clinical manifestations, as well as in the disease outcome and treatment. In order to personalise treatment there is a pressing need to investigate and define the pathologic mechanisms in all three conditions more closely. Currently there is no cure to any of the discussed diseases and they are generally managed by oral glucocorticoids (GC) and/or bronchodilators as maintenance.

1.4.1 Sarcoidosis

Sarcoidosis is a multisystemic inflammatory disease, characterised by non-caseating granulomas in various organs, commonly affecting the lungs. As opposed to other inflammatory lung diseases, such as COPD, bronchiolitis and emphysema, sarcoidosis primarily affects non-smokers [111]. Sarcoidosis has an unknown aetiology, but is believed to be promoted by an abnormal immune response to one or a few specific, but as yet unknown, environmental antigen(s). Patients display a typical accumulation in their lungs of activated T helper ($CD4^+$) cells, and a CD4/CD8 ratio in bronchoalveolar lavage (BAL) fluid over 3.5 is considered a diagnostic indicator for sarcoidosis [112]. These lung-accumulated, activated $CD4^+$ T cells have a Th1 cytokine profile, characterized by release of IFN-γ, TNF-α and IL-2 [113, 114].

Stage	***Finding***
0	Normal chest radiograph
I	Bilateral hilar lympadenopathy (BHL)
II	BHL and pulmonary infiltrations
III	Pulmonary infiltrations (no BHL)
IV	Pulmonary fibrosis

Table 1. Chest radiographic stages in pulmonary sarcoidosis.

Chest radiographs are used, in combination with the above mentioned CD4/CD8 ratio, for diagnosis of sarcoidosis. Pulmonary involvement, enlarged lymphnodes, fibrosis and parenchymal infiltrates can be found on the chest radiographs and the disease can be divided into stages depending on the appearance of these (Table 1, Figure 8).

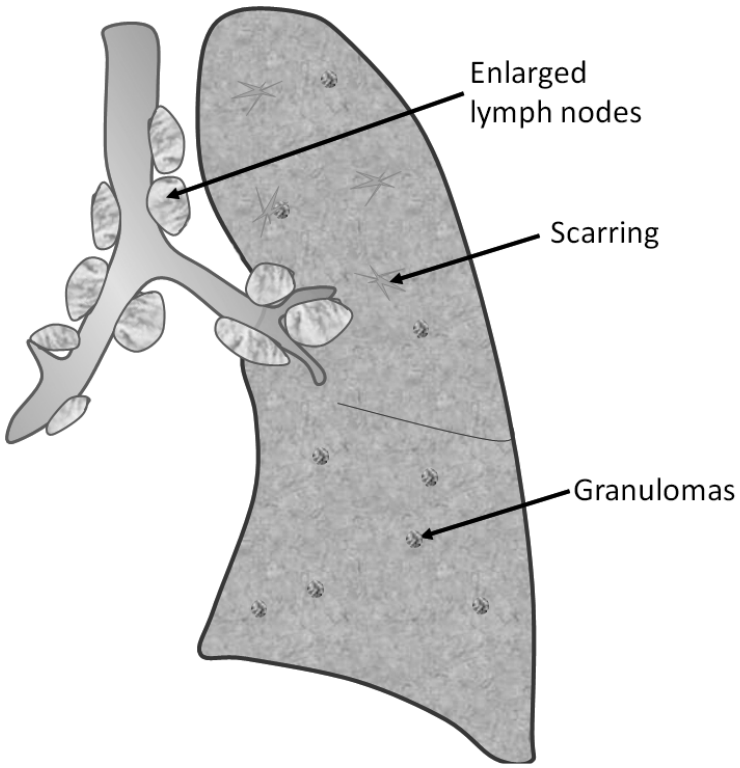

Figure 8. Sarcoidosis.

Genetic variability, especially in the form of DNA polymorphism, has been reported for specific phenotypes among patients with sarcoidosis [115-117]. One sub-group of sarcoidosis patients have Löfgren's syndrome, defined by an acute disease onset combined with fever and specific symptoms such as erythema nodosum, ankle arthritis and bilateral hilar lymphadenopathy [118]. These patients usually have a good prognosis and a relatively quickly resolving disease. Patients with a chronic disease course are referred to as non-Löfgrens patients.

It has been suggested that genetically susceptible individuals are more likely to develop sarcoidosis when exposed to some sort of infection or antigen. The exact pathogenic mechanisms have however not been resolved, and interestingly, recent microarrays on lung tissue and peripheral blood mononuclear cells (PBMC) revealed differentially expressed miRNAs in sarcoidosis patients with possible roles in the disease process [119].

1.4.2 Asthma

Asthma is a complex inflammatory pulmonary disease affecting all ages and races. Currently an estimated 300 million people are affected worldwide, with the mean prevalence of clinical asthma of 4.9% in the Scandinavian and Baltic countries (Finland, Sweden, Denmark, Norway, Estonia, Iceland, Latvia, Lithuania and Poland) [120]. In year 2001 it was estimated as the 25th leading cause of disability [120]. Asthma mortality varies largely between countries, depending much on recognition of the disease, availability of drugs and treatment.

There are many phenotypes of asthma, for example allergic (atopic) asthma and non-allergic asthma. Allergic asthma is characterised by a Th2-dominated inflammation. Both phenotypes show signs of bronchial hyper-reactivity and a reversible obstruction of airflow. Common symptoms are wheezing, shortness of breath, chest tightness and cough. The cause of asthma is not fully understood, but is believed to be caused by a combination of environmental and genetic factors.The airway inflammation in asthma is primarily localised to the proximal airways, but occasionally extend to the distal lung and parenchyma (Figure 9) [16].

Currently there is no exact clinical or laboratorial test for diagnosing asthma. Asthma is diagnosed by a combination of methods and observation of symptoms. Spirometry is used to measure airflow limitation; an increase in FEV_1 of <12% and 200 ml after administration of a bronchodilator is a sign of reversible airflow limitation in accordance to asthma. Peak expiratory flow (PEF) measurements are used to follow an individual's fluctuations of measurements. Ideally the reference point will be the patient's own previous measurements. A diurnal variation of >20% and an improvement of >20% post-bronchodilator inhalation, compared to the pre-bronchodilator measurement, indicate asthma.

Mild intermittent asthma

Symptoms are not severe and breathing problems rarely occur. The patient manages asthma attacks with inhalers. $FEV_1 > 80\%$.

Mild persistent asthma

As with mild intermittent asthma the symptoms are very mild and the patient can manage the disease by self-medicating as needed. $FEV_1 > 80\%$.

Moderate persistent asthma

Moderate asthmatics have daily symptoms and a FEV_1 60 - 80%. Daily use of β2-agonists are often required.

Severe persistent asthma

Severe asthmatics suffer from continuous symptoms, limited physical activity and a $FEV_1 < 60\%$.

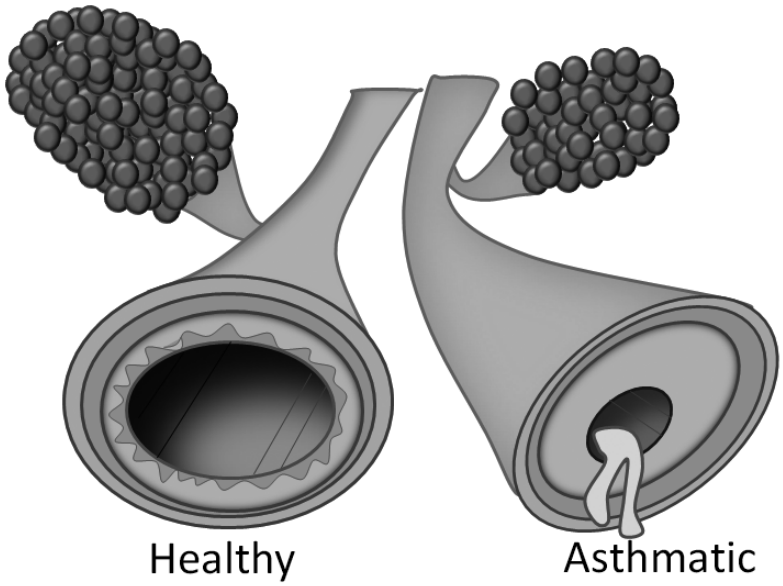

Figure 9. Asthma.

Airway hyper-responsiveness (AHR) is one central feature of asthma and can be tested by metacholine or histamine exposure, which causes a dose-dependent drop in FEV_1 values in asthmatics. Other factors, such as dry and cold air, cigarette smoke and strong smells can also cause hyper-responsiveness in asthmatics, but the mechanism is different from the metacholine exposure. Metaholine and histamine act on smooth muscle receptors, while the other stimuli mainly activate the immune system [121].

Nitric oxide (NO) concentration in the breath of asthmatics is generally elevated. This could however also be affected by what the patient has eaten recently. Skin test allergen and serum IgE measurements indicate allergies and increase the probability of an asthma diagnosis. Atopic (allergic) asthmatics are a subgroup with hypersensitivity to allergens, with abnormally high levels of the IgE antibody (for review, see [122]). The allergens, such as pollen, animal fur and house dust mite, trigger the inflammation and airway obstruction in these individuals. Other environmental irritants, such as ozone (O_3), perfume and cigarette smoke can also function as triggers.

Local accumulation of eosinophils and mast cells can be observed depending on the sub-type of the disease. Th2 cytokines; IL-4, IL-5, IL-6, IL-10 and IL-13 levels are

high, leading to an inflammation and further narrowing of the airways. There is a wide spectrum in the severity of asthma, depending on exacerbation frequency. In asthmatics cytokines derived from epithelial cells, such as IL-25 and IL-33, play a role in orchestrating the eosinophilic airway inflammation [122].

WNTs appear to be involved, through β-catenin, in regulatory functions by preventing overproduction of inflammatory cytokines such as IL-6 in lipopolysaccharide (LPS) induced responses [123].

1.4.3 Chronic obstructive pulmonary disease

Although the underlying causes of asthma and COPD are both inflammatory in origin, the aetiology and progression of the diseases are distinctly different (for review, see [124]). COPD primarily involves the peripheral bronchioles and the lung parenchyma, whereas asthma mainly affects the larger conducting airways. COPD is currently the 12th leading cause of disability worldwide [120]. The slowly progressive airflow limitation in COPD leads to disability and premature death, while the variable airflow limitation in asthma rarely progresses in severity.

A common feature in COPD is chronic bronchitis, a sputum-producing cough due to mucus hyper-secretion and inflammation. Symptoms often bother the patients for very long periods of time and is defined chronic after a total of three months in two consecutive years [125]. Chronic bronchitis is observed in both patients and smokers with normal lung function.

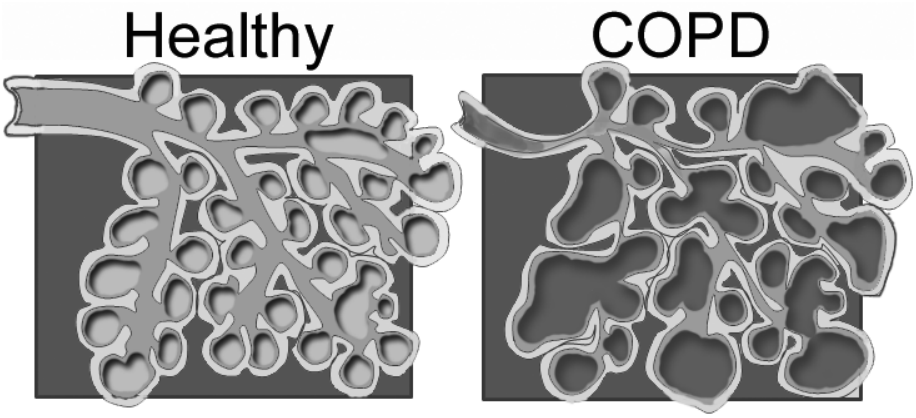

Figure 10. Alveoli in healthy and COPD lungs. The image displays the enlarged alveoli and mucus production in COPD patients.

Bronchiolitis, a constant inflammation of the bronchioles, leads to narrowing of the airways and is the main cause of obstruction [126]. Obstruction of peripheral airways due to inflammatory cell infiltration and fibrosis together with inflammatory exudates in the lumen (bronchiolitis) correlates best with the severity of the airflow obstruction in COPD [126].

Emphysema is characterised by the destruction of alveolar parenchyma and loss of alveolar septa, and can be explained as a permanent enlargement of the alveolar airspaces, due to destruction of the walls (Figure 10). This leads to a smaller total area of the alveolar epithelium, a reduced gas exchange and an elevated RV.

Infiltration of macrophages and T lymphocytes (mainly $CD8^+$) is characteristic for COPD, with an increased number of neutrophils prevalent in patients with severe disease and during exacerbations [124]. Exacerbations are acute periods of worsening of the patient's condition, often due to an infection or exposure to some environmental factor [127, 128]. COPD patients are more predisposed to infections due to the alterations of the tight junctions in the epithelium, allowing pathogens to enter the tissue more easily [129].

The number of macrophages are markedly increased (5-25 fold) in most lung compartments of COPD patients, and macrophages appear to play a central role in the pathology of COPD [130]. Airway epithelial cells are likely to play a role in the recruitment and differentiation of macrophages through the release of chemoattractants and other mediators specifically acting on macrophages. Airway epithelium changes, including altered ion channel regulation resulting in increased secretion and viscosity of mucins and defective tight junctions, stress the importance of such alterations in the pathogenesis of COPD [131].

COPD is diagnosed using spirometry to gain lung function measurements. A post-bronchodilator measurement ratio of FEV_1/FVC below 0.7 is required for COPD diagnosis. Besides smoking cessation, no efficacious treatments currently exist to prevent the progression of COPD. The severity of COPD is divided into four stages according to the Global Initiative for Obstructive Lung disease (GOLD); mild (I), moderate (II), severe (III) and very severe (IV) [132].

Stage I: Mild COPD

At stage one the patient is usually unaware of the disease and their decline in lung function. There may be mild airflow limitation, as well as some symptoms like cough or excessive mucus production. FEV_1 values are equal to or greater than 80% of the predicted normal values and FEV_1/FVC l < 70% [132].

Stage II: Moderate COPD

In moderate disease the airflow limitation worsens and more symptoms appear, such as cough, shortness of breath and sputum production. FEV_1 values are between 50-80% of the predictive normal values and $FEV_1/FVC < 70\%$ [132].

Stage III: Severe COPD

At stage III the symptoms are more prominent, with a more evident shortness of breath, higher degree of airflow limitation and recurrent exacerbations. FEV_1 is between 30-49% and $FEV_1/FVC < 70\%$ [132].

Stage IV: Very severe COPD

At the last GOLD stage the quality of life has decreased significantly with more severe symptoms. The exacerbations at this stage may be life threatening. FEV_1 levels are 30-50% and $FEV_1/FVC < 70\%$ [132].

Asthma and early stage COPD may have many symptomatic similarities, and traditionally the two diseases have been differentiated by whether or not the airway obstruction can be reversed by β2-agonists. However, this criterion is becoming more and more outdated as sub-categories of COPD with some reversibility are becoming evident [133]. This further underlines the need for better diagnostic tools.

1.5 ENVIRONMENTAL FACTORS

Environmental air pollution is potentially harmful factors in the air, such as chemicals, particulate matter (PM) and biological material such as allergens and pathogens. These can be of the solid, liquid or gaseous phases. The lung epithelium is a relatively large surface regularly in contact with the environment and a possible means of entry for these harmful agents.

Recent development of increased allergies and vulnerability to normally harmless environmental allergens supports the so called hygiene hypothesis [134]. This hypothesis suggests that, as levels of living standards increase, there is a decreased exposure to pathogenic and non-pathogenic micro-organisms. These micro-organisms usually promote the development of a normal immune response and an appropriate control of the same is refined. However, when the exposures to such infections decrease, the reactions to otherwise harmless environmental allergens increase. At the same time the levels of exposure increase due to more intense energy consumption and larger industrial sources. The human pulmonary system is exposed to, not only larger quantities, but also a more diverse mixture of pollution than before.

The most abundant pollutants are PM, O_3 and nitrogen dioxide (NO_2). PM is composed of a complex mixture of solids and/or liquids varying in size, number and chemical composition depending on the source. O_3 is a constituent of smog and is generated in a reaction between NO_2, hydrocarbons and UV-light. The main source for PM and NO_2 is combustion of fossil fuels, including traffic.

Exacerbations of airway disease can be caused by numerous factors such as allergen exposure, drugs, viral infections, changes in the weather and environmental pollution [135]. Several studies have been conducted to investigate the effects of traffic pollution on inflammatory pulmonary diseases such as asthma and COPD. Greater reductions of FEV_1 and FVC could be detected in mild to moderate asthmatics spending two hours walking on a heavily trafficked street in London compared to a walk in Hyde park [136]. Gehring et al. showed that traffic related air pollution caused an increased development of asthma and allergies early in life [137]. Andersen et al. showed in two studies that long-term exposure to traffic-related air pollution may contribute to the development of COPD and increase hospitalisation in asthmatics [138, 139].

All three above discussed diseases appear to be affected by external factors such as air pollution, allergens and antigens. Exacerbations in COPD and asthma can be caused by infections entering through the lung. In three of the publications, Paper II, III and IV, we investigated the effects of two different air pollutions; subway air and cigarette smoke.

1.5.1 Subway air

In many large cities, the subway system represents a major form of transportation for numerous individuals, yet few studies have focused on the health effects of this environment.

The pathogenesis of asthma in urban areas is affected by multiple factors including PM and oxidizing agents such as O_3 and nitric oxides present in polluted air. There are other oxidizing agents, the ones mentioned here are the main ones present in

photochemical smog and are often regulated and used as a general measure of the level of air pollution. In terms of PM, the health effects most studied to date are related to road emissions in urban areas, which have been demonstrated to play an important role in asthma morbidity.

Road traffic exhaust has previously been shown to affect the lung function, such as FEV_1 and FVC in asthmatics and in healthy individuals [136, 140]. Subway exposures have, however, not shown these results. A study investigating particles collected in the London underground showed effects on A549 cell lines [141]. A study investigating the effect of subway air on employees working both at the level of the platform and above at the entry level, could not detect any short-term effects of the exposure on the respiratory system [142]. Klepczynska Nyström et. al.detected elevated expression of the T cell activation marker CD69 on both $CD8^+$ and $CD4^+$ cells after subway exposure [143, 144]. One thing in common with these exposure studies is that they mainly investigated the effects on respiratory function, measured by spirometry, as well as cell differentials and some FACS analyses, and did not examine effects on smaller molecules with a potentially pathological outcome.

Subway air is a significant source of PM emission with physiochemical properties that differ drastically from that of road emission. Road emission PM is largely composed of PAHs, whereas subway PM generally contains more iron oxides. Given the potential for large-scale chronic exposure to subway air, and the cumulative risk for sensitive populations with compromised respiratory systems such as asthmatics, it is important to examine the effects of this system in greater detail. This study examined responses of the respiratory system to Stockholm subway air in mild intermittent asthmatics and healthy individuals (Papers II and III).

1.5.2 Cigarette smoke

Smoking is a common way of exposure of the lung to noxious stimuli. Cigarette smoke consists of $1 \times 10^9 – 5 \times 10^9$ different toxic particles per ml [145]. The particle diameter size in tobacco smoke varies from 0.16 to 1.1 μm, with similar sizes observed in different tobacco types [146]. Generally PM_1 reaches distal lung, and nanoparticles reach out in the blood stream to some extent, while PM_{10} mainly reach the proximal lung, or even get stuck in the nasal epithelium. The use of filters on cigarettes reduces the particle size slightly. Considering that the smaller particle sizes are able to cross the membranes and cross out into the bloodstream, thereby affecting the entire body this may not be a very desirable effect. Cigarettes contain for example large numbers of free radicals, certain ions, PAHs, nicotine, alkenes, acids, alcohols and esters just to name a few [145]. The exact effects of these compounds on the innate and adaptive immunity are largely unknown, but are believed to increase the inflammatory response and allergic reactions in the lungs [147].

Cigarette smoke leads to an influx of macrophages into the alveolar lumen, engulfing smoke particles and secreting matrix metalloproteases (MMPs) involved in tissue destruction.

While smoking might have a protective effect on sarcoidosis, it is the main cause of COPD [148].

2 STUDY SUBJECTS & SAMPLE COLLECTION

2.1 PATIENT DATA

2.1.1 Paper I

A total of 48 sarcoidosis patients were recruited for this study when referred to the Division of Respiratory Medicine (Karolinska University Hospital, Stockholm, Sweden) for initial diagnostic investigation. All patients had clinical features of sarcoidosis evaluated by chest radiography, symptoms and pulmonary function tests. The patients were not on corticosteroid treatment during the study. Eighteen non-smoking healthy control individuals, with normal lung function, were included in the study as controls.

The BAL recovery % was significantly lower and cell concentration was significantly higher in sarcoidosis patients. 12 out of 48 of the sarcoidosis patients had Löfgren's syndrome and 10 out of 48 were smokers. Pulmonary function tests revealed the expected differences, with lower VC%, FVC% and FEV_1 in sarcoidosis patients (more details on the data can be found in paper I, table 1). The study was approved by the local ethics committee at Karolinska Institutet (Case number: 2005/1031-31/2).

2.1.2 Papers II & III

The patient samples collected for papers II and III were part of a larger study and more detail can be found in previous publications [143, 144]. 18 healthy non-smoking volunteers and 15 intermittent asthmatics participated in the study described in paper II. A sub-group of these patients, 10 healthy and 10 asthmatics, participated in the study described in paper III.

Both populations underwent the same exposure regimen to both subway air and ambient office control environment in a randomised order, with a minimum of three weeks between. All individuals were non-smokers, non-habitual subway commuters (refraining from subway use altogether for 2 months prior to the study), had normal chest radiography, were not on corticosterioid or anti-inflammatory drug treatment and had no airway infections within 6 weeks of the study. β2-agonist use was allowed on demand.

Screening for the presence of specific immunoglobulin (Ig) E antibodies against common inhaled allergens (Phadiatop; Pharmacia-Upjohn, Uppsala, Sweden) showed that 89% of the healthy volunteers had no IgE antibodies (n=16), whereas 87% (n=13) of the asthmatics had IgE antibodies. The asthmatics also had a positive reaction to a number of the radioallergosorbents (RAST, Pharmacia-Upjohn). The study was performed out of pollen season.

All volunteers had a normal baseline FEV_1. Hyper-responsiveness in asthmatics was tested by increasing doses of metacholine, defined as ≥20% fall in FEV_1 at an accumulated inhalation dose of < 894 μg.

Lung samples were collected through bronchoscopy, described in more detail below.

All volunteers participating in the study gave their informed written consent, and the study was approved by the Stockholm Regional Ethical Board (Case numbers 2006/643-31/4 and 2007/748-31/3).

2.1.3 Paper IV

The samples were collected as part of a larger study called the COSMIC study performed at the Karolinska University Hospital encompassing a total of 120 individuals; 40 COPD patients (later divided into a subgroup of ex-smokers), 40 healthy current smokers and 40 healthy never smokers with equal numbers of each gender. The COPD group of ex-smokers were defined as individuals who had refrained from smoking for at least 2 years prior to the inclusion in the study. All subjects were matched in terms of age (45-65 years) and gender (20/20 per group). In addition, COPD and healthy smokers were matched in terms of smoking history (>10 pack years, >10 cigarettes/day the past 6 months). The use of inhaled or oral corticosteroids was not allowed and subjects had no exacerbations for the last 3 months before the study. In order to avoid acute effects of smoking on inflammatory parameters, smokers were asked to refrain from smoking 8 hrs prior to bronchoscopy, which was assessed by exhaled carbon monoxide (CO) monitoring prior to sampling as previously described [149].

The final number of patients included in the study is described in more detail in Table 1 in Paper IV. Clearly degraded RNA samples and arrays with obvious experimental problems, such as scratches or uneven hybridisation were removed from further analysis. To ensure that the investigated samples were as homogenous as possible in regards to cell composition an additional exclusion was added. Samples with a macrophage percent composition of < 85% were excluded (n = 5) to assure sufficient enrichment of the target cell population (i.e. alveolar macrophages). A final set of strict exclusion criteria specifically designed for the transcriptomics analyses were applied in order to reduce the heterogeneity of the groups and thereby increase the statistical power of the study. Low recovery of BAL fluid may result in a more proximal sampling of the bronchoalveolar tree, resulting in a different BAL cell or exosomal population. As such, subjects with a recovery < 34% were excluded (n =3) [150].

All individuals underwent a thorough clinical examination with chest radiography, computed tomography (CT), and spirometry. The healthy never and current smokers had a fully normal lung function. COPD patients had mild to moderate disease (GOLD stage I-II, FEV_1=50-100% and FEV_1/FVC < 0.7). Lung function parameters from the spirometry were calculated as post-bronchodilator percent of predicted using the European Community of Coal and Steel (ECCS) normal values [151]. All subjects also completed an additional questionnaire on previous occupational exposure, menopause, hormone replacement therapy and/or other drugs, dyspnoea, fatigue, emotional function and occurrence of productive cough.

The study was approved by the local Stockholm Regional Ethical Board (Case number 2006/959-31/1, 2007-743-32, 2008/600-32, 2010/1064-32), and all volunteers gave their informed written consent.

2.2 SAMPLES

Bronchoscopy is a technique of visually investigating the insides of the lungs, while the patient is lightly sedated. During this procedure various samples, such as cells and biopsies, can be collected and it is used for diagnostic and therapeutic purposes. Bronchoalveolar lavage (BAL), performed during bronchoscopy, is a way of investigating inflammatory pulmonary diseases. The bronchoscope, a flexible fiberoptic tube is wedged into a middle-lobe bronchus and aliquots of phosphate buffered saline (PBS) are instilled into the lung and gently aspirated. Through BAL, inflammatory cells and airway exudates are collected from the distal lung, on which we then perform a range of analyses to characterise the patient. BAL samples from healthy individuals mainly contain macrophages and a small percentage of lymphocytes. The cell differentials can be drastically different depending on which inflammatory pulmonary disease the patient is suffering from, some of the changes being quite characteristic. Also the composition of the BAL fluid, i.e. the soluble fraction remaining after the inflammatory cells have been removed from the BAL, differs drastically in terms of the composition of inflammatory mediators depending on the inflammatory state of the lung. In addition to components from the epithelial lining fluid, the BAL fluid also contains a range of protein-, peptide-, lipid and nucleic acid mediators that can be used to characterise the nature of an inflammatory response or disease.

2.2.1 BAL cells

The cells obtained in the BAL are a mixture of inflammatory cells such as macrophages, lymphocytes, neutrophils and basophils. The composition of the BAL cells mainly depends on the disease state and whether or not there is an ongoing infection. The BAL fluid CD4/CD8 T cell ratio, which is elevated in sarcoidosis patients, is used for the clinical diagnosis of sarcoidosis. Asthmatics have increased eosinophil counts. Smokers have normally a larger concentration of cells than healthy individuals, with a large amount of macrophages and normally also increased numbers of neutrophils.

2.2.2 BAL fluid

After the cells are removed by centrifugation the BAL fluid remains. This fluid contains soluble factors and smaller vesicles, such as oxylipins and exosomes respectively, which are too light to have been pelleted by the low speed centrifugation performed for removing the cells.

2.2.3 Bronchial brush biopsies

Bronchial brush biopsies are collected during the bronchoscopy by brushing the airway epithelial lining with stereological brushes which are inserted through the work canal of the bronchoscope. One brush collects approximately 1 x 10^6cells and the cell composition of these are mainly epithelial cells (90-95%), the majority being ciliated cells, with some observed differences in smokers who have increased neutrophil cell counts. The composition of the brush biopsies is mostly due to a very proximal sampling location. As demonstrated in Figure 4 the composition of cells changes along the airways.

2.2.4 Exosomes

Exosomes are present in the BALF and can be isolated through ultracentrifugation (discussed in more detail below). The strength in using BAL exosomes in the search of biomarkers is that it is less complex than serum and/or blood biomarkers. BAL samples also contribute greatly to knowledge of local immuno-inflammatory responses in both normal and disease conditions. We aim to investigate both the proteome and miRNAs present in exosomes and their role in the disease process. There are not as many miRNAs as proteins and, as mentioned above, a single miRNA may affect the translation of proteins from several genes.

2.3 SUBWAY AIR EXPOSURE

All individuals in paper II and III were exposed to both subway air and ambient office air, in a randomised order with a minimum interval of 3 weeks. The study was carried out outside of pollen season (mid-October to mid-March). The subjects were not habitual commuters and were further encouraged to refrain from using the subway during the study, avoid areas with high pollution and limit the amount of exercise at the day of exposure to the minimum.

The subway air exposures were conducted during rush hour (4-6pm) at near platform level at a central subway station in Stockholm (Odenplan). The air in the room where the subway exposure took place had a corresponding quality to the air at the platform. Breathing was controlled by allowing the subjects use an exercise bicycle in 15 min interval with a resistance of 20 L/min/m^2 body surface. This was to ensure that they would adapt mouth breathing and take deeper breaths. Control exposures were performed during a corresponding amount of time in a normal office environment with ambient clean air.

The PM levels were monitored during each exposure session. Sampling of $PM_{2.5}$ and PM_{10} were measured, meaning an upper cut-off of 2.5 and 10 μm respectively, with Harvard impactors (Air Diagnostics and Engineering Inc, Harrison, ME, USA) with telflon filters that were weighed after the session [143]. The measurements after these sessions in the subway were 77±10 and 242±40 $\mu g \cdot m^{-3}$ for $PM_{2.5}$ and PM_{10} respectively [143]. In comparison, in a road tunnel study perfomed under the same conditions and with the same equipment these levels were 64 (46–81) $\mu g \cdot m^{-3}$and 176 (130–206) $\mu g \cdot m^{-3}$ [140].

All bronchoscopies and sample collections were performed 14 h after the 2 h exposure. These time points were chosen to enable comparison with a previous study investigating inflammatory effects after exposure to road tunnel pollution [140].

3 COMMENTS ON METHODOLOGY

3.1 SAMPLE PREPARATIONS

3.1.1 Exosome ultracentrifugation

In order to isolate exosomes from BALF we applied a series of centrifugations; first 3000 x g for 30 minutes in order to remove debris, followed by an ultracentrifugation of 10.000 x g for 30 minutes (Ultracentrifuge Beckman Coulter Optima L-100 XP Ultracentrifuge with Ti45 rotor) in 4°C before filtering the supernatant through a 0.2 µm filter (DISMIC-25cs cellulose acetate, Advantec) and finally a second ultracentrifugation at 140.000 x g for 2 h at 4°C to pellet the exosomes. The pellet was resuspended directly in Nucleospin® miRNA lysis reagent (Macherey-Nagel, Düren, Germany) and frozen at -80°C for the upcoming RNA extractions. Even with a starting material of 100±1 ml BALF/patient, the resulting miRNA sample amount was very limited.

There has been some discussion about the distinction between exosomes from other vesicles, easily confused with exosomes, such as microvesicles, exosome-like vesicles and small vesicles containing virus [1]. Currently there are no specific exosome markers available, which complicate the extraction of a homogenous sample. The accepted method at the moment involves ultracentrifugation, filtration by size and using a sucrose gradient to separate the vesicles by density [152]. Exosomes float in sucrose gradients of 1.10 – 1.20 g/ml [90]. In addition, electron microscopy can be used to visually confirm the presence and purity of the exosomes. Exosomes express tetraspanins CD9, CD63, CD81 and CD 82 on their surface [153]. These can be used to ensure that a sample contain exosomes, but they are, however, not specific. These markers can be present on cells, but these have been removed from our samples and do not create complications for our experiment. Other vesicles with membranes from cells, however can also express these markers.

The limited amount of sample prevented us from performing all of the above mentioned methods to verify the sample composition. However, the ultra-centrifugation protocol used here has previously been verified in other publications [103, 107, 154].

3.1.2 RNA extractions

RNA was extracted using the Nucleospin®miRNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions, to separate small RNAs (including miRNAs) from larger RNAs before RNA integrity validation. The protein fraction was stored separately for further analysis in -70°C. RNA quality was assessed by determining UV 260/280 and 260/230 absorbance ratios obtained by the Nanodrop (Thermo Scientific, Wilmington, DE, USA), resulting in a mean 260/280 ratio of 1.7 (Median 1.57). The 260 wavelength measures the absorbance of nucleic acids, 280 measures proteins and 230 measures organic contaminants and solvents. The ratios between the selected combination of these give an indication of the purity of the measured sample. RNA size distribution was examined on RNA Pico LabChips (Agilent Technologies, Palo Alto, CA) processed on the Agilent 2100 Bioanalyzer using the small RNA electrophoresis program.

3.2 EXPERIMENTAL METHODS

3.2.1 EMSA

Electrophoretic mobility shift assay (EMSA), also referred to as gel shift assay, applied in paper I, is an in vitro method used to study protein-DNA interactions. Using a probe, a selected DNA strand of interest one can investigate whether the sequence of interest is present in a particular sample. The separation is performed over an electrophoretic charge in a polyacrylamide gel. Different molecules travel through the gel depending on their size and charge. In order to visualise the bands of probe-protein interactions, the probe can be pre-labelled with radioactivity and visualised by exposure onto a film [155]. In paper I we used an oligonucleotide with a TCF/LEF consensus binding site (5´- TGT TGT TAA GCA AAG ATC AAA GCC CGG CAG AG -3´) end-labelled with [γ32-P] ATP, as a probe. Alkaline phosphatase treatment is used to remove a 5´-phosphate from the probe of choice. Thereafter polynucleotide kinase is added to catalyse the transfer a new phosphate group, this time a radioactively pre-labelled phosphate, to the 5´-end of the probe. After purification the probe is ready to be used on the EMSA.

3.2.2 Cytocentrifugation

Cytocentrifugation is a technique to apply collected cells onto glass slides for further analyses. We used this method in paper I and paper II, for epithelial and BAL cells respectively. In both experiments a total of 60 000 cells/slide were cytocentrifuged onto Superfrost®Plus glass slides (Menzel-Gläser, Braunschweig, Germany) for further differential cell counts and immunocytochemistry.

3.2.3 Immunocytochemistry

In paper I and II we used immunocytochemistry to investigate the expression of proteins of interest in our cells. The cells, bronchial epithelial cells in paper I and BAL cells in paper II, were cytospun onto glass slides and specific antibodies (Ab) were used to target the proteins. In paper I we used the 3,3'-Diaminobenzidine (DAB) method and in paper II fluorescent labels conjugated to the secondary antibodies were used to visualise and quantify the specific binding.

The slides were first fixed in formalin or paraformaldehyde (PFA) and washed in PBS before retrieving the antigens by boiling the slides in citrate buffer. Because these were whole cells, as opposed to tissue slides, we permeabilised the cell membranes by applying ice-cold methanol on them. After another brief wash we blocked endogenous peroxidise activity using hydrogen peroxide (H_2O_2). Unspecific binding was blocked using serum or antibody dilution buffer consisting of serum, bovine serum albumin (BSA) and TritonX. The protein of interest is bound by a primary antibody through incubation overnight. Incubation with the secondary antibody is briefer.

In the fluorescent method the primary antibodies used were monoclonal antibodies against 15-LOX-1 (dilution 1:1000), peroxisome proliferator-activated receptor gamma (PPARγ; dilution1:100, Santa Cruz, sc-7196) produced in rabbit, and COX-1(dilution 1:200, Invitrogen 35–8100) and COX-2 (dilution 1:200, Invitrogen 35–8200) produced in mouse. The secondary antibody is covalently bound to a fluorescent

dye, which then requires a fluorescent microscope with lasers to visualise the location of the antigen/primary antibody binding. We used Cy5 labeled anti-rabbit sera from donkey (dilution 1:1000, Amersham 711-175-152) and Alexa488 labeled anti-mouse sera from goat (dilution 1:500– 1:1000, Molecular Probes A11001). Normal goat serum was purchased from Vector Laboratories (Burlingame, CA, USA; S-1000 and S-4000) and normal donkey serum from Sigma-Aldrich (D9663). Counterstaining was done using 4´,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen D1306).

In the case of using horseradish peroxidase a secondary antibody conjugated with biotin is used and then avidin is added, creating a complex together with biotin. A DAB substrate is applied onto the slides converting the avidin/biotin complex into a brown coloured pigment visual at the location of the protein of interest. A monoclonal β-catenin antibody was used as the primary antibody (1:100, BD Biosciences, San Jose, CA), the secondary antibody was a biotin-conjugated anti-mouse serum antibody (1:300, Vector Laboratories, Burlingame, CA). Hematoxylin was used as a counterstain.

3.2.4 Microarrays

Microarray based methods allow simultaneous semi-quantitative detection of thousands of transcripts from small samples. Usually this high-throughput method is used for hypothesis generation and the risk of cross hybridisation requires some sort of validation of the results.

The small size and high sequence homology of miRNAs create a unique challenge for hybridisation-based detection techniques, requiring a different approach from the previous designs of genomic and mRNA methods.

In this method the RNA is directly labelled with a Cy3 fluorescent dye to the 3´end of each RNA. RNA probes of interest are anchored to the array glass slide by a stilt. This probe is a synthesised sequence that will hybridise to the target RNA in the sample.
The miRNA microarray method requires some additional steps, compared to mRNA arrays, due to their smaller size.The protocol used (Agilent) adds a C residue to the 3´end during labelling of miRNAs and a G residue at the 5`end, to complement the previously added C residue, which increases the stability of binding target miRNAs [156]. The addition of these residues stabilises the annealing temperatures (Tm) of nearly all miRNAs to above 55°C. Tm is the temperature at which 50% of the oligonucleotide and its complementary strand are in duplex. The arrays we used utilise a calculated optimal probe length for several probes to work at the same Tm [157]. An additional 5´hairpin increase the target and size specificity of the miRNA. This hairpin structure destabilises binding to larger non-intended targets.

In papers III and IV we used this technique. For miRNA expression analyses the RNA was labeled with Cy3-CTP using the miRNACURY LNA microRNA power labeling kit (Exiqon, Inc, Woburn, MA), according to manufacturers protocol. Labeled RNA was hybridised to one-color Agilent custom UCSF miRNA v3.5 multi-species 8x15K Ink-jet arrays (Agilent Technologies, Palo Alto, CA) containing 894 (paper III exosomes and IV BAL cells) or 1223 (paper IV exosomes) different miRNAs. Briefly, dephosphorylation of 5´ end was done in 37°C for 30 min followed by 95°C for 5 min

to stop the enzyme reaction and denature the RNA. Dye labeling of 3´ end with fluorochrome Cy3 was performed in a thermal cycler for 3 hrs in 16°C, 15 min 65°C and kept at 4°C until the next step. The reaction was stopped by blocking agent at 100°C, thereafter samples were snap-frozen before hybridisation overnight (16 hrs) at 55°C with a rotation of 20rpm.

For the genexpression arrays (used in paper IV) RNA was amplified using the Low Input Quick Amplification Kit (Agilent Technologies) according to the manufacturer's protocol, and subsequent Cy3-CTP labeling was performed by using one-color labeling kits (Agilent Technologies). Clean-up of the labeled and amplified probe was performed (Zymo Research Corporation, Irvine, CA). The size distribution and quantity of the amplified product was assessed by Nanodrop. Equal amounts of Cy3-labeled target were hybridised to Agilent human whole-genome 4x44K Ink-jet arrays containing a total of 41,000 probes corresponding to 19,596 entrez genes. Hybridisations were performed at 65°C for 17 hours at a rotation of 10 rpm.

Arrays were washed in Agilent gene expression wash buffer 1 & 2 before scanning on the Agilent G2565BA scanner (Agilent Technologies, Palo Alto, CA) with Scan region: Agilent HD (61x21.6) and a resolution of 5um, TIFF: 16 bit, extended dynamic range (XDR): 0.10. Raw signal intensities were extracted with Feature Extraction v10.1 software (Agilent). Flagged outliers were not included in any subsequent analyses.

3.2.5 PCR

Polymerase chain reaction (PCR) is the most commonly used technique for mRNA detection and quantification. It is used for example for functional analysis of genes, DNA cloning, detection of hereditary diseases, paternity testing and forensic identification by genetic fingerprints and diagnosis of disease.

Short DNA sequences of choice can be amplified into large quantities in a short amount of time. By repeated thermal cycles of heating and cooling the method allows DNA melting and enzymatic replication. Short DNA fragments called primers, complementary to the target region of interest, are used together with an enzyme called DNA polymerase for the amplification. These are the main components of the reaction. All the newly amplified strands are used in the next cycle and the DNA is thereby exponentially amplified.

Sybr green RT-PCR

Reverse transcription (RT) means to enzymatically convert RNA into first strand complementary DNA (cDNA), the substrate or template for the PCR. The method allows semi-quantitative detection of small amounts of template in the sample of interest. Real-time PCR, also called quantitative (q)PCR or qRT-PCR) is not to be confused with reverse-transcription PCR. In real time PCR the absolute quantity of the PCR product is measured during amplification, in real-time and is often combined with reverse transcription.

In project I we used the RT-PCR method with SYBR-green fluorescence, where the amount of PCR product is related to the fluorescence of the reporter dye. The SYBR green based detection method is the least expensive PCR method. Another option is

TaqMan which requires additional primers and a quencher, making it a lot more expensive. SYBR green binds to double stranded DNA by interacting between base pairs and only fluoresces when bound. Measurements occur at the end of each cycle when the largest amount of product is present.

A house-keeping gene is selected by its stability in the measured samples and experimental, as well as diseases, conditions. The choice of house-keeping gene for paper I was based on available literature defining PSMB2 as the best choice for BAL cells at the time of the experiments [158].

After RNA extraction and measurements aliquots of total RNA were converted to cDNA using a kit called ReactionReady First strand cDNA synthesis kit (SABiosciences, Frederick, MD). We used primers for WNT5A (#PPH02410A), WNT7B (#PPH02464A), WNT7A (#PPH02465A) and the housekeeping gene PSMB2 (#PPH20109A), as well as RT2Real-Time-SYBRGreen PCR-Master Mix from SABiosciences. The reaction cycle was as follows: 95°C for 10 min, 95°C for 15 s and 60°C for 1 min, for 40 cycles in an ABI PRISM Sequence Detector 7700 (Applied Biosystems,Foster City, CA).

Nano-scale qPCR

In paper III we applied a high-throughput nano-scale technique, due to its sensitivity, to validate our microarray miRNA findings in exosomes, because of our limited amount of samples as well as due to the advancement in technology that has occurred over the few years passing between the two studies.

In brief, we applied a multiplex quantitative RT-PCR method involving the purification of multiplex PCR products followed by uniplex analysis on a microfluidics chip (Fluidigm, South San Francisco). We validated a total of 41 miRNAs selected from our microarrays findings using stem-loop reverse transcription as described previously [159].

MiRNAs and exosomes are both relatively new and unknown subjects, and there was not much information found on what house-keeping miRNA to use. Furthermore, miRNAs are not cellular samples and housekeeping genes do not exist in the same manner as for cells. The selection was therefore done using an algorithm called geNorm identifying stable endogenous miRNAs from our samples to be used for normalisation. Normalisation was performed by subtracting the mean of the cycle thresholds (Ct) for the selected house-keeping miRNAs from the Ct for a miRNA of interest, for each sample [160].

3.2.6 FACS

Flow cytometry, applied in paper III, is a technique used for counting cells and measuring expression of pre-labelled cell-surface markers. By suspending cells into solution, fluorescently labelling and passing them through a fluorescent detection monitor in a Fluorescence Activated Cell Sorter (FACS), one can determine the physical and chemical characteristics of the studied sample. In order to label the molecules of interest, specific antibodies, co-labelled with a fluorescent dye, are required. The FACS contains lasers with specific wavelengths that activate the

fluorescent dyes. These can be combined in different manner in order to sort out cells of interest expressing different markers.

3.3 STATISTICAL METHODS

In the use of statistical methods in omics and systems biology research, which is characterised by large datasets with a high number of analytes tested by relatively small group sizes, the pitfalls of using traditional univariate statistics become plentiful, as discussed in more detail below.

3.3.1 Univariate analysis

Univariate (and bivariate) methods are designed for use in smaller sets of data. When over 6 variables are compared, correction for the increased high positive rate should be considered.

In paper I we used the non-parametric Mann-Whitney test to evaluate differences seen in the EMSA and RT-PCR data. The Mann-Whitney test, sometimes called the rank sum test, is a nonparametric test that compares two unpaired groups. Non-parametric means not assuming that the data is normally distributed. It works by ranking the values from low to high, paying no attention to which group each value belongs. If two values are the same, they both get the average of the two ranks for which they tie. The smallest number gets a rank of 1, the largest number a rank of N, where N is the total number of values in the two groups. The result is generated by summing up the ranks in each group. When the total sums in each group are very different, it leads to a low p value and a high significance of the test.

Correlations between groups in paper I were performed using Spearman's rank correlations test. This test is also a non-parametric test, comparing the dependence of two variables for each other.

Analysis of fluorescent immunostaining results in paper II were done using Student's t-test. This test is used for data with a normal distribution, which was confirmed by means of Shapiro-Wilks test.

Univariate statistical analyses in paper III and IV were performed using a two-way analysis of variance (ANOVA), and p-value correction to account for a high false positive rate due to multiple hypothesis testing was performed using the false discovery rate (FDR) method according to Benjamini- Hochberg [161]. The FDR method is a way of correcting for multiple comparisons. The adjustment of the p-value is based on a fixed proportion of expected false positives among the significant results, rather than a fixed statistical power as is the case for the raw p-value.

False positives are the so called Type I errors, occurring when something is considered significant when indeed it is not. Type II errors on the other hand are called false negatives and appear when a statistical test fails to detect real differences. When using univariate methods one should consider pitfalls such as these.

The cost of correcting for type I errors is a loss of statistical power in the study, meaning the ability to detect your true positives, i.e. an increase of type II errors.

ANOVA provides a statistical test comparing, like most tests, whether or not the means of groups are equal. But in the case of ANOVA it is possible to test several groups at a time, applying the t-test to more than two groups. Repeated t-tests would result in an increased chance of committing a type I error, which ANOVA corrects for.

In paper IV a non-parametric one-way ANOVA with the Kruskal-Wallis test combined with Dunn's post test was used for evaluating clinical data between the groups. The Kruskal-Wallis ANOVA is used for statistics comparing several unrelated groups. As in the Mann-Whitney method, the Kruskal-Wallis ranks the data independently of groups. The Kruskal-Wallis test is used for three or more groups.
Because this test only tells whether there is a significant difference, not where it is, we continued with the Dunn's post-test which gave us this information. This test compares the gained sum of ranks with an expected average difference, based on the number of comparisons and groups, reporting it then as a p value.

Logarithmic $(\log)_2$ transformation of the data was applied to the data in Paper I and paper II, III and IV in order to acquire a normal distribution of the measured data. Applying a logarithmic scale hereby allows further analysis by parametric methods.

3.3.2 Multivariate analysis

An complement to univariate analysis is multivariate methods that are used for datasets with a larger number of variables (measurement points). The method decreases the dimensionality of the data to thereby aid in the interpretation and visualisation of the data. Multivariate modelling is a good alternative or complement to the univariate methods, as you avoid the problem with false positives due to a simultaneous analysis of all your variables and at the same time account for the co-variance of the dataset.

In paper II, III and IV we had large datasets with several different measurements from each patient, which required more complex analytical tools. Using multivariate methods as a complementary method alongside the primary univariate analysis of the dataset, allowed us to detect more subtle changes and interactions between variables. Using supervised models we could reveal important differences related to other factors within the datasets. The multivariate findings are often also validated by univariate statistical testing.

We performed the multivariate analyses using SIMCA P+ software v12.0.1 (Umetrics AB, Umeå, Sweden) using principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) [162].

PCA analysis is an unsupervised method where the high dimensional data is reduced to a few dimensions, called principal components in order to visualize the data more clearly. As illustrated in Figure 11 the data is visualised as a cloud of dots, with one dot representing each observation combining the data points of all the variables for that particular observation. In a model with 2 PCs, the data will be visualised as a two dimensional image instead of a multidimensional cloud which would be impossible to visualise. This could be compared to a light shining on the cloud of dots. The software

extracts components from this spread; The 1st component always being the one with the largest variance, the second largest variance becomes the 2nd component etc. The maximal number of components is the same as the number of original variables, but the number of significant variables is generally limited to a few [163].

In contrast to the more commonly used PCA modelling, OPLS analysis is a supervised method removing variation from X (descriptive variables) that are not correlated to Y (response variables). Separating this structured noise unrelated (orthogonal) to the predictive variance of interest increases the interpretability of the multivariate model, particularly in deriving the observed group separation back to the variables of interest [164].

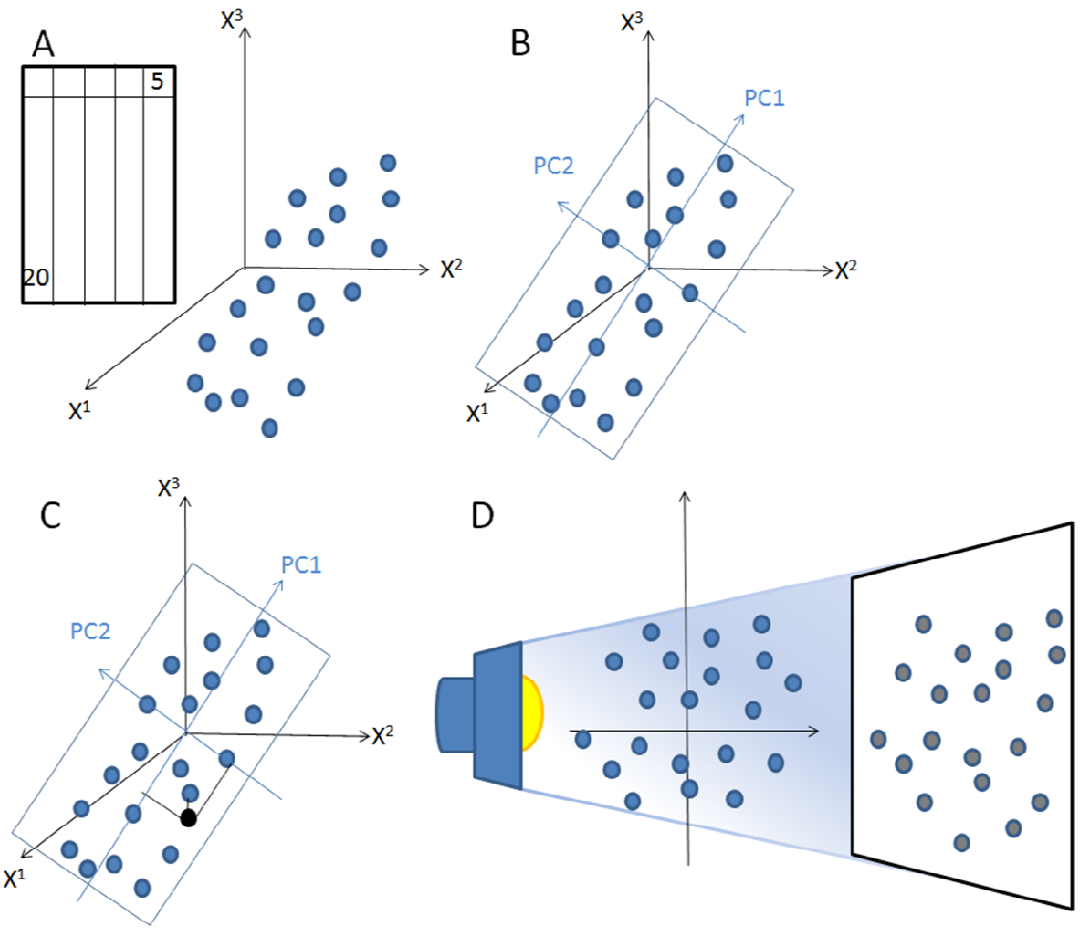

Figure 11. An illustration demonstrating the method by which algorithms creates a two-dimensional image of multiple dimensions. In the example we have a combination of 20 observations and 5 variables for each observation combined into one dot for each observation (A). The 1st principal component (PC1) is created where the variance is the largest, the 2nd (PC2) where the variance is second largest and so on (B). When all the components explaining the data are created, the data is projected onto a plane, illustrated by a black dot (C). This could be compared to shining a light onto a cloud of dots and looking at the shadow that it creates (D). *Illustration created using an original images from SIMCA as a model [163, 165].*

In order to effectively evaluate the usefulness of your model the performance is reported as cumulative correlation coefficients for the model (R^2) and predictive performance based on seven-fold cross validation calculations (Q^2), as well as cross-validated ANOVA (CV-ANOVA) p-values for the OPLS models. These are highly important as a visually convincing separation does not always indicate a good model or a high predictive power. Over-fitting data may lead to an impressive graph showing a clear-cut separation of the groups, but without biological relevance. This means that too

much of the variation in the data is explained, including noise and unrelated variation in the description of the consistent structure of interest. Over-fitting should be avoided as it lowers the prediction quality measured by the above mentioned coefficient Q^2.

Variable selection for model optimisation was performed iteratively based on the Variable Importance in the Projections (VIP) and scaled loadings of the predictive component of the OPLS models (p(corr)), using iterative selection cut-offs of VIP scores and p(corr) values, as displayed in the volcano plot used in paper III (Figure 12). The inclusion criteria applied were VIP > 1.0 (Y-axis) and absolute p(corr) > 0.5 (x-axis). Blue colored spots indicate selection in first iteration, and red dots indicate final selection following a second iteration utilising the same criteria.

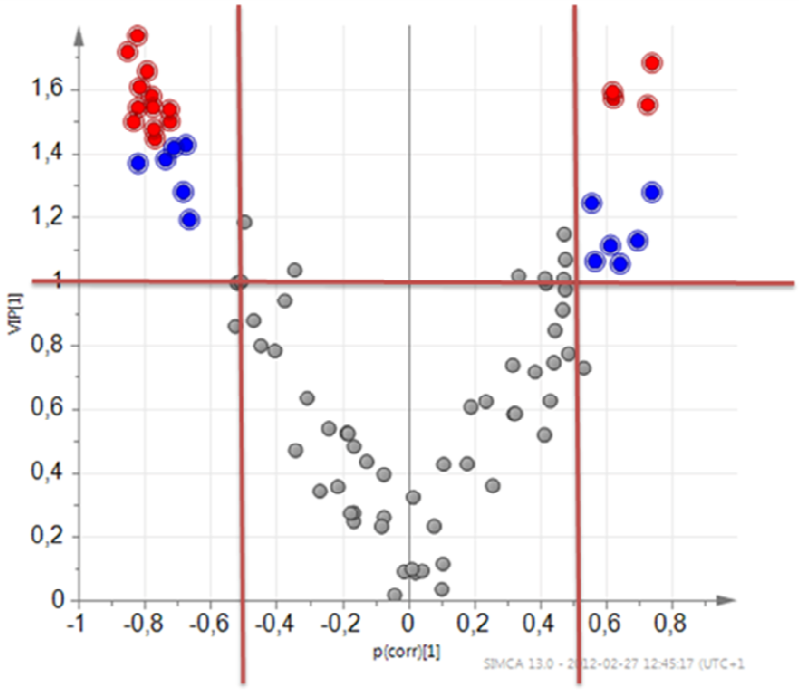

Figure 12. A volcano plot demonstrating the selection of variables based on VIP and p(corr) values for optimisation of the model.

Individual models were compared through a shared-and-unique-structures (SUS) analyses, where the scaled loadings (p(corr)) of the respective models are plotted against each other (Figure 13) [166]. In the resulting plot the shared variables will cluster along the diagonals, while variables unique for either model are found along the axes. The empty space at the center is due to removal of variables with low VIP and/or p(corr) values.

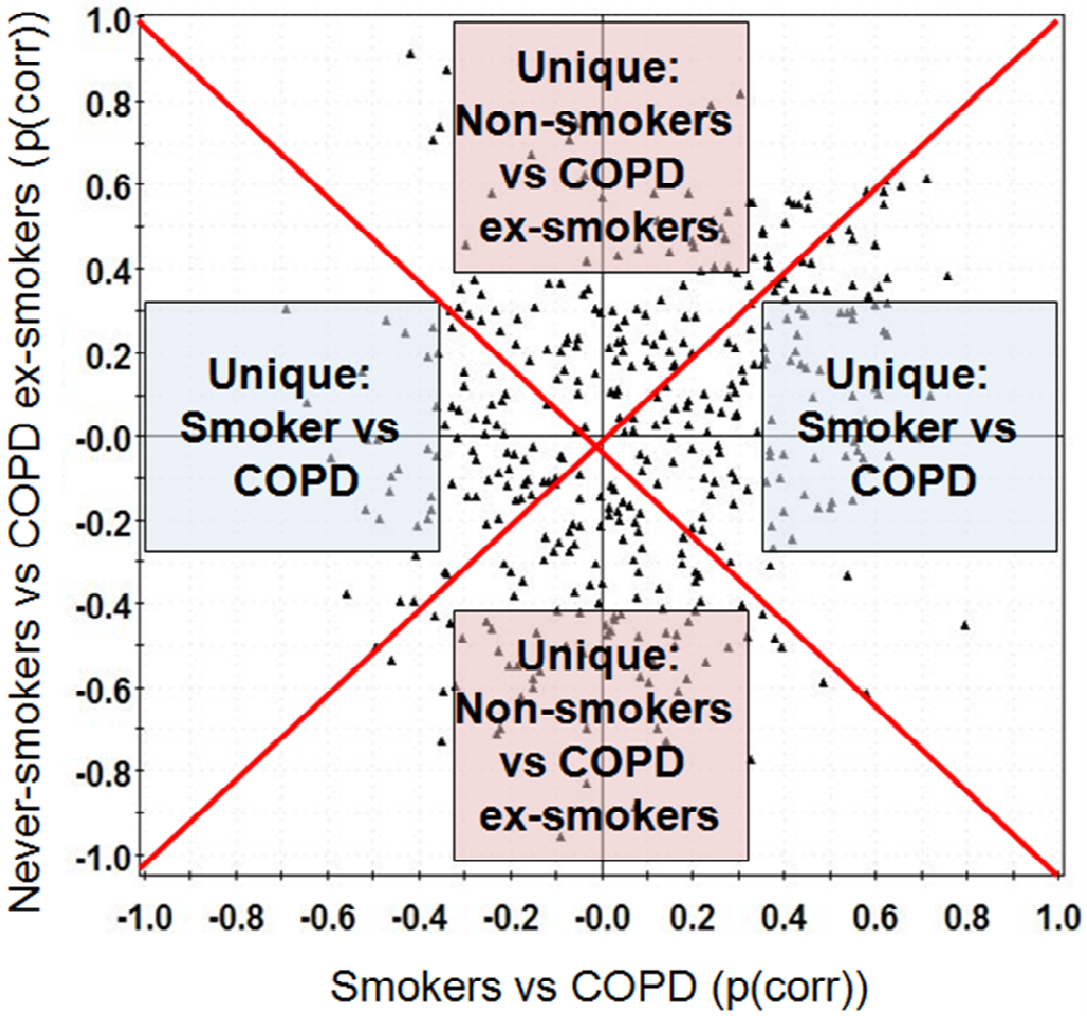

Figure 13. SUS-plot used to compare two models. P(corr) values from each model are plotted against each other. Variables around the diagonal red lines are shared structures in both models. Variables located along the axes, i.e. in the squares are unique for one of the models.

4 AIMS

The overall aims of the following projects are to, by the use of multiple platforms, elucidate differences and similarities in three common inflammatory pulmonary diseases; Sarcoidosis, asthma and COPD. In addition to results from techniques used in this dissertation; electrophoretic mobility shift assay (EMSA), semi quantitative RT-PCR, immunocytochemistry, FACS and RNA microarrays, we will expand our data with additional proteomics and metabolomics techniques. By applying complex analysis methods we strive to add to the current knowledge of inflammatory pulmonary diseases, potentially finding biomarkers and pharmaceutical targets to be used for early diagnosis and individualised treatment.

5 RESULTS & DISCUSSION

5.1 WNT SIGNALLING IN SARCOIDOSIS

As discussed in the introductory part, WNTs are important in several physiological functions such as cell proliferation, differentiation and migration. Many of the target genes of the WNT/β-catenin pathway have been shown to be up-regulated in sarcoidosis [167, 168].

Previous findings have shown WNTs to be important in the development of fibrosis in fibrotic pulmonary diseases such as idiopathic pulmonary disease (IPF) and fibrosis often develops in chronic sarcoidosis [167]. With this background we hypothesised that WNTs might play a role in sarcoidosis. More specifically we speculated that WNTs secreted from inflammatory cells in BAL, could be involved in the increased intracellular active β-catenin levels in epithelial cells and contribute to the development of fibrosis in the sarcoidosis patients. Epithelial cells express several WNT-receptors [169, 170].

In paper I we investigated the activity of β-catenin, the intracellular signalling molecule in the WNTs signalling cascade, in BAL cells and brush biopsy samples from sarcoidosis patients. We investigated the gene expression by RT-PCR in BAL cells. Using a radioactively marked probe with the known sequence of a TCF/LEF oligonucleotide on nuclear extracts prepared from the collected cells, we could by EMSA determine the presence of complementary binding sites. By immunocytochemistry we studied the nuclear expression of β-catenin in epithelial cells in patients with sarcoidosis, and found indications of an increased activation.

WNT3, -4, -7A, -7B, -10B and -11 expressions have previously been described in lung and lung cell lines [170-174]. We investigated the expression of *WNT3, WNT5A, WNT7A, WNT7B* and *WNT11* in BAL cells and found *WNT5, WNT7A* and *WNT7B* to be differentially expressed. These differences were even more pronounced when sub-dividing the patients into groups according to disease stages (Figure 14). The levels of *WNT3* and *WNT11* were below detection levels. This suggests a role for WNTs in the inflammatory process in sarcoidosis and a possible role in the formation of granulomas, which is a characteristic feature of sarcoidosis. Abnormal WNT-signalling has previously been shown in idiopathic pulmonary fibrosis, with an up-regulation of WNT7B, WNT10B and WNT1 [175]. The later stages of sarcoidosis have elevated fibrosis development (Table 1) and these results support our hypothesis of WNT involvement in this process.

Interestingly, our results indicate that the expression of WNTs in sarcoidosis may be affected by smoking status. This is supported by another study that showed that the WNT-pathway is activated by smoke in bronchial epithelial cells [176]. It would be of great interest to further evaluate if this is true also in COPD patients who smoke.
The changes in *WNT7B* expression did not reach significance, but did, however, approach significance ($p = 0.052$) when we removed smoking individuals from the

sarcoidosis group (Figure 15). Smoking has previously been indicated to have a protective effect on the development of sarcoidosis [148].

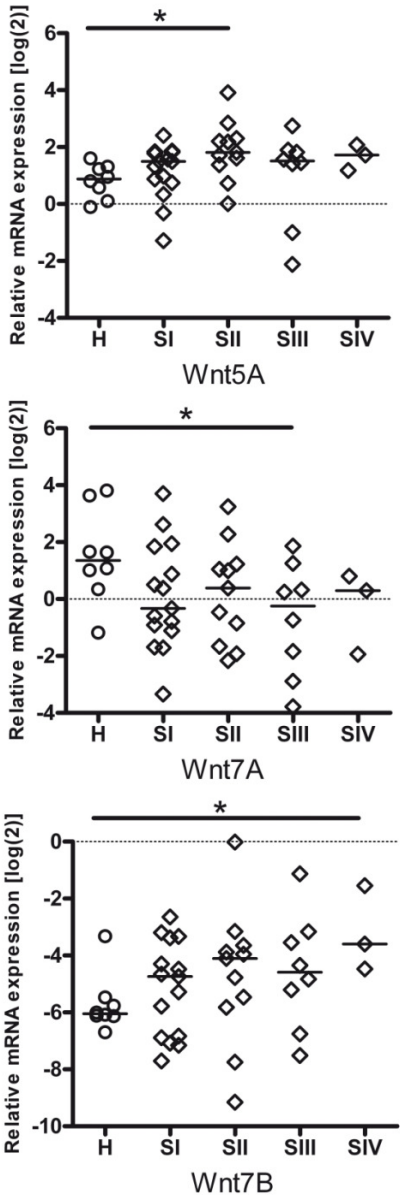

Figure 14. WNT expression correlated with the sarcoidosis chest radiographic stages. Semi-quantitiative RT-PCR levels of *WNT5A, WNT7A* and *WNT7B* mRNA in patients with sarcoidosis compared to healthy individuals. The Bars indicate median. H = healthy, S = sarcoidosis, I-IV = radiographic stages 1-4. *p<0.05.

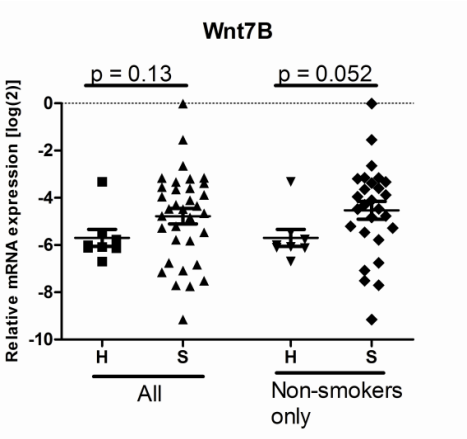

Figure 15. *WNT7B* expression in all included subjects compared to expression with non-smoking sarcoidosis patients only. Bars indicate median. H = healthy, S = sarcoidosis.

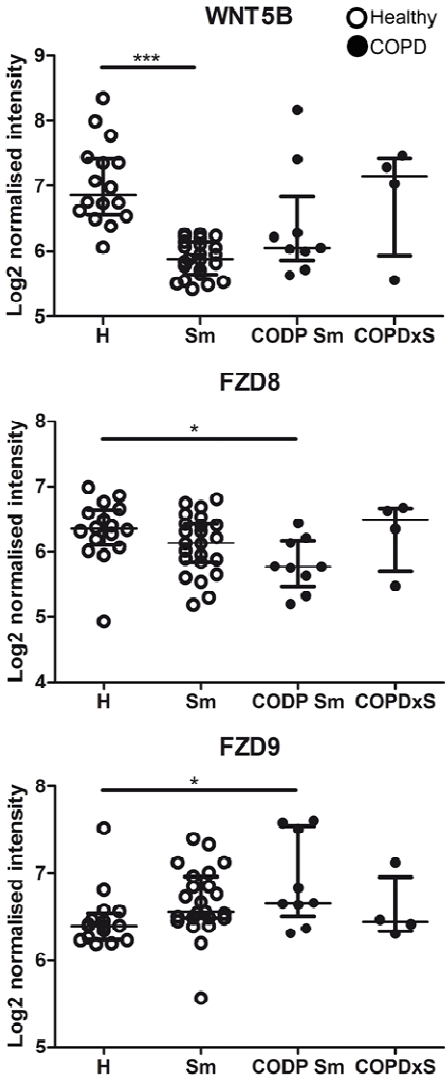

Figure 16. WNT pathway related genes differentially expressed in COPD and smokers. H = healthy, Sm = smokers, xS = ex-Smokers. Bars indicate median and interquartile range. *< 0.05, *** < 0.0001.

By performing global analyses, the involvement of WNTs in asthma and COPD can be investigated in conjunction with the hypothesis generating aspects of the omics technologies. Unpublished results from our microarray data revealed *WNT5B* and *FZD8* expression to be significantly lower in smokers and COPD smokers respectively (Figure 16). Other genes related to WNT signalling seen to be lower in smokers and COPD smokers were *WNT9*, *WNT11*, *WNT7B* and *WNT10A*, although these did not reach significance (Unpublished data). *FZD9* expression was significantly higher in COPD smokers (unpublished data).

Löfgrens' syndrome is a sub-group of sarcoidosis patients with a usually good prognosis. Twelve of the included sarcoidosis patients had Löfgren's syndrome. *WNT7B* expression between sarcoidosis patients with and without Löfgren's syndrome reached significance ($p < 0.01$) (Figure 17).

Several recent studies indicate that WNT5A and WNT7B are of importance also in asthma [177, 178].

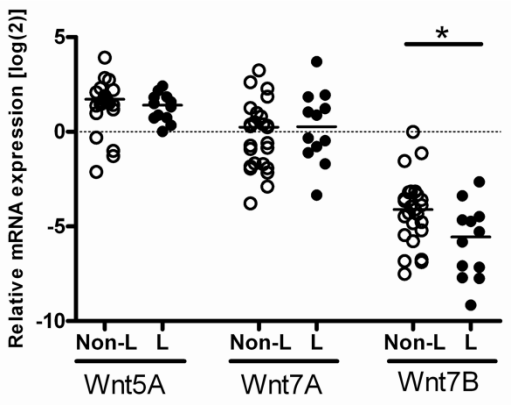

Figure 17. Correlation of WNT expression with patients with and without Löfgren's syndrome. Bars indicate median. Non-L = Non-Löfgren's, L = Löfgren's.. **p<0.001.

Epithelial-mesenchymal signalling has been implicated in IPF by several previous studies and some noted WNT/β-catenin to be involved in this process [167, 175, 179]. The inflammation in sarcoidosis involves an accumulation of T cells and macrophages, involved in the formation of granulomas. In the chronic disease this inflammation can lead to the formation of fibrosis. One possible hypothesis is that the infiltrating inflammatory cells involved in the pathology of sarcoidosis, much like in IPF, secrete mediators that start a process of epithelial-mesenchymal signalling that then becomes self-sustaining.

Epithelial mesenchymal transition (EMT), is an important process during tissue repair, which has been implicated in disease pathology for both asthma and COPD [180, 181]. Proper lung mesenchymal growth and vascular development requires WNT7B signalling [182]. Mice with disrupted WNT5 displayed abnormal morphogenesis in the distal lung [183].

Matrix metalloproteinases (MMPs) are known gene products of WNT/β-catenin signalling. TGF-β may be activated through the proteolytic activities of a number of

mediators including MMPs. MMPs are a family of enzymes that can degrade components of the extracellular matrix (ECM). Deregulation of MMP activity has been implicated in asthma, COPD and sarcoidosis [184-191],with some differences in the type of MMPs present; In COPD mainly MMP-1, -2, -8 and -9, smokers with COPD MMP-12, sarcoidosis patients MMP-8 and -9.

Recently a group investigating the expression of miRNA in lung tissue and PBMCs from patients with sarcoidosis found that many of the differentially expressed miRNAs, miRNA-25, miRNA-199-5p and miRNA-214, were involved in the regulation of TGFβ and WNT signalling [119].

This study showed for the first time the role of WNT and β-catenin signalling in the pathology of sarcoidosis. The increased WNT-signal activation could contribute to the inflammatory processes involved in the disease.

5.2 ALTERED OXYLIPIN PROFILES IN ASTHMATICS

Although several road exposures studies have been conducted; few studies have investigated the effect of subway air on lung function of sensitive individuals such as asthmatics. Considering the vast amount of people using the subway daily, it is highly important to evaluate the effects of subway air on both healthy individuals, as well as more sensitive individuals with an already compromised lung function, such as asthmatics.

Asthma is a chronic inflammatory lung disease that causes significant morbidity and mortality worldwide. Air pollutants such as PM and oxidants are important factors in causing exacerbations in asthmatics, and the source and composition of pollutants greatly affects pathological implications.

Oxylipins are compounds formed from unsaturated fatty acids that play an important regulatory role in several processes including inflammation and immunity.

In paper II we investigated responses of the respiratory system to Stockholm subway air in asthmatics and healthy individuals. Eicosanoids and other oxylipins were quantified in the distal lung to provide a measure of shifts in lipid mediators in association with exposure to subway air relative to ambient air.

Sixty-four oxylipins representing the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) metabolic pathways were screened using liquid chromatography-tandem mass spectrometry (LC-MS/MS) of BALF. The results revealed divergent oxylipin profiles in asthmatics and healthy following exposure to ambient and subway air. Significant changes were observed in nine metabolites (Figure 18).

The data plotted are normalised values used for calculation the actual difference between the exposures as a ration between subway (S) and control (C) exposures. The values are also $\log_2$ scaled. Oxylipin levels were increased in healthy individuals following exposure to subway air, whereas asthmatics evidenced decreases or no change.

Validations through immunocytochemistry staining of BAL-cells were performed for 15-LOX-1, COX-1, COX-2 and peroxisome proliferator-activated receptor gamma (PPARγ) (Figure 19). The majority of the significantly different oxylipins, all except PGE_2, are metabolites from the linoleic acid (LA) or α-LA pathways. 15-LOX is an enzyme important in the synthesis of these metabolites. PGE_2 is a COX product from the arachidonic acid metabolic pathway, and is the most well studied in regards to asthma [192]. Previous studies have indicated that some of the differentially expressed oxylipins are involved in the regulation of PPARγ, an anti-inflammatory receptor involved in several inflammatory diseases [193-196].

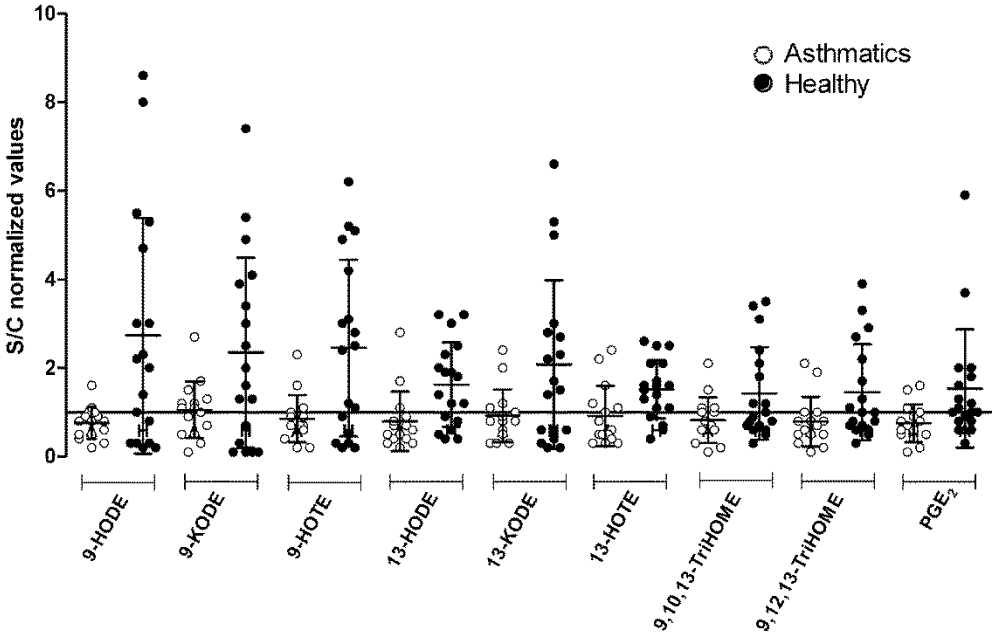

Figure 18. Alterations of the oxylipin profiles in response to subway air exposure. Significant differences ($p<0.05$) were observed in these 9 oxylipins. S/C = Subway Control. Values >1 indicate an increase, values >1 indicate a decrease. Bars indicate mean and interquartile range. Values are Log_2 transformed and baseline normalised prior to analysis, expressed as S/C = Subway/control exposure ratio [3].

The results showed no difference between asthmatics and healthy when comparing the actual differences. These were calculated as a subway/control exposure ratio on normalised values and blotted as log_2 values. When analysing the normalised data before ratio calculations, we could see a significant difference of COX-1 at baseline (control exposure) level between asthmatics and healthy (Figure 20).

The observed differences indicate that asthmatics lack a protective response to noxious stimuli that the healthy individuals show example of in their elevated levels of oxylipins in response to subway air exposure.

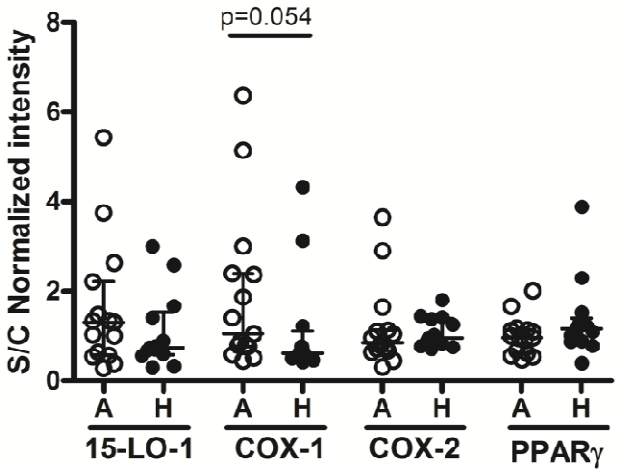

Figure 19. Immunocytochemistry results revealed a response difference approaching significance between asthmatics and healthy after subway exposure. S/C = subway/control exposure ratio, A= asthmatics, H= healthy. The bars indicate median with interquartile range [3].

Multivariate statistics were employed to interrogate acquired oxylipin and immunocytochemistry data in combination with patient clinical information. In Figure 21 multivariate OPLS analysis results are shown (A). The VIP plot reveals the degree of allergy response to be the variable with most weight in separating the two groups, asthmatics and healthy (B).

Several of the altered oxylipins have known or suspected bronchoprotective or anti-inflammatory effects, suggesting a possible reduced anti-inflammatory response in asthmatics following exposure to subway air. These observations may have ramifications for sensitive subpopulations in urban areas.

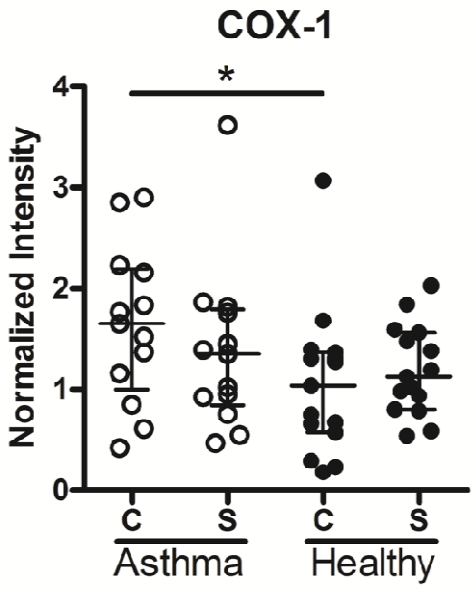

Figure 20. Analysing the subway and control exposed groups separately revealed a significant difference between baselines (control exposures) of asthmatics and healthy. *p<0.05. C = control exposure, S = subway exposure. Bars indicate median with interquartile range [3].

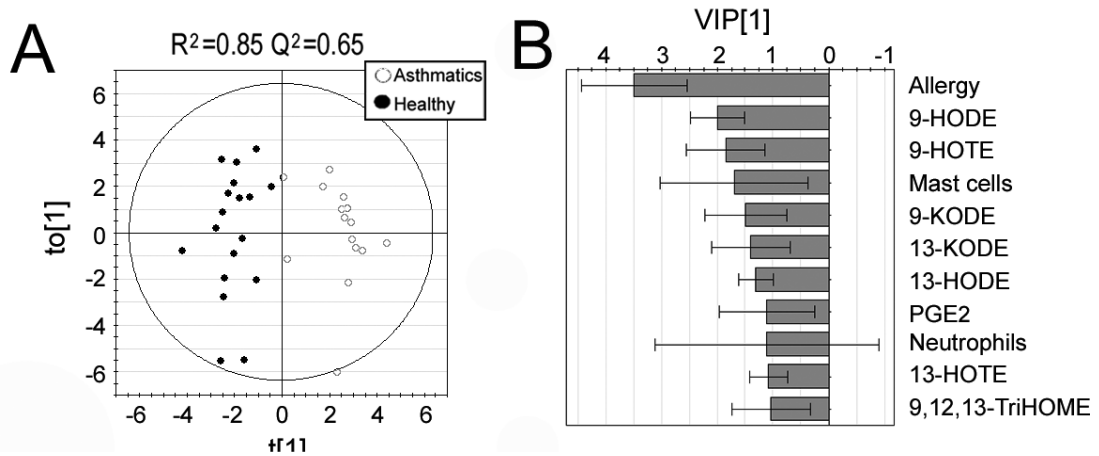

Figure 21. OPLS analysis integrating oxylipin, immunohistochemistry and clinical variables, with asthma as the y-variable. The scores plot show a strong predictive power of the model (65%), as well as a nice separation of the groups with only two exceptions (A). The VIP plot demonstrates the variables important in separating the two groups seen in the scores plot (B). Error bars indicate 95% confidence interval.

The main differences were seen at baseline, which is interesting especially as these are mild intermittent asthmatics which were symptom free at the time of the study. When exposed to subway air the differences were levelled out. This may indicate that asthmatic patients have a lowered capacity to respond to noxious stimuli.

5.3 EXOSOMAL MIRNAS IN ASTHMA AND COPD

Exosomes have previously been shown to contain miRNAs, their presence has however not been demonstrated in BALF exosomes [99, 106]. We investigated the miRNA content of exosomes collected by ultracentrifugation from BALF from asthmatics (paper III) and COPD patients (paper IV), as well as in their corresponding matched healthy controls. In paper III we had 894 different probes and in paper IV we had 1223 probes. This was due to an update of the array version from 3.5 to 3.6 between the two analyses.

Exosomes, with a potential role in the pathology of inflammatory pulmonary diseases, may serve as transport vesicles for the small RNAs and thereby affect gene expression in the target cell. Not much is currently known about the packaging of exosomes, the selection of target cells or method of uptake by target cells. Nevertheless, it has recently been shown by live-cell microscopy that exosomes are taken up by cells through the endocytic pathway involving actin [197]. Previous studies have shown uptake of exosomes through phagocytosis [198]. ICAM-1(also known as CD54) bearing exosomes can be captured by lymphocyte function-associated protein 1(LFA1), a ligand for ICAM-1, expressed on CD8+ dendritic cells and activated T cells [199, 200].

Both exosomes and miRNAs have been implicated in lung homeostasis during steady-state conditions by regulating gene expression.

In paper III we characterised the miRNA content of exosomes at baseline and in response to subway air exposure in healthy and mild intermittent asthmatics. In paper IV we investigated the miRNA content in COPD smokers, COPD ex-smokers, healthy current smokers and healthy never-smokers.

We confirmed the presence of miRNAs in exosomes from BALF in asthmatics, COPD patients, smokers and healthy individuals (Figure 22). We extracted RNA from the

exosomes in two fractions, large and small RNAs, but could not detect any large RNAs. Whether this is due to degradation or due to the fact that large RNAs are never transported by exosomes remains to be elucidated.

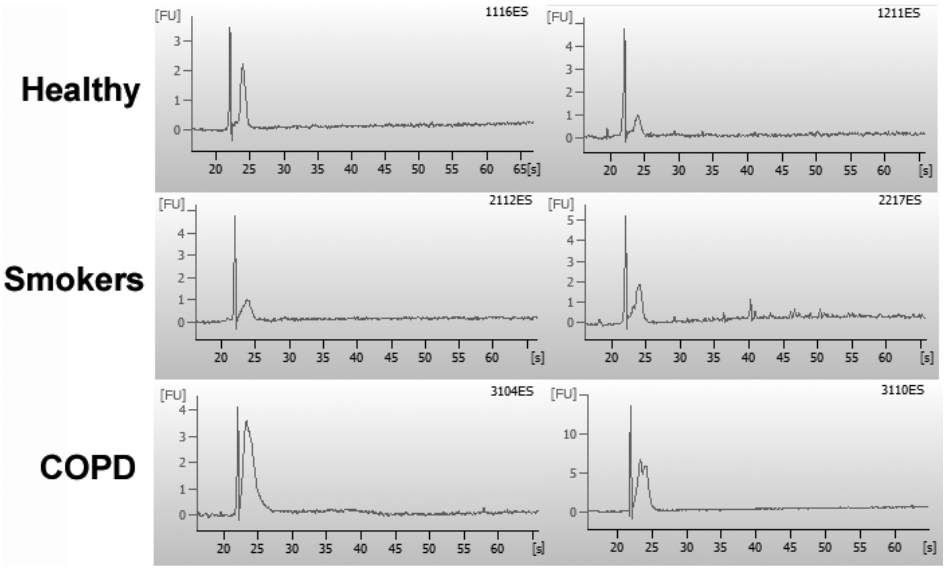

Figure 22 Bioanalyzer tracings confirming the presence of small RNAs in the exosomes samples. The first sharp peak is a marker,small RNAs as visible directly after the marker. Any large RNAs would be visible as the 18S and 28S rRNA at 40 and 45 seconds (x axis).

Significant differences in the BALF exosomal miRNA content was revealed between asthmatics and healthy controls at baseline. A subset of 16 miRNAs could be used to classify mild, non-symptomatic asthmatics from healthy individuals with 73% predictive power (Figure 23).

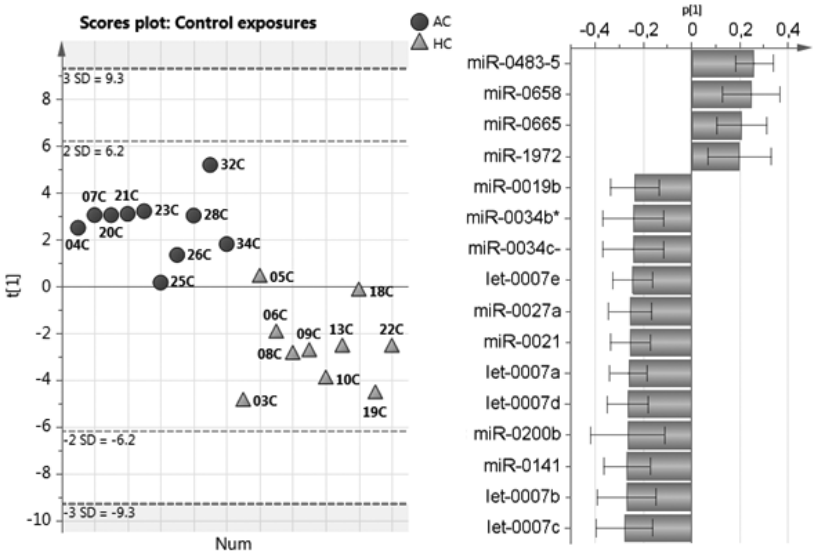

Figure 23. An OPLS scores plot demonstrating the separation of healthy and asthmatic individuals at baseline exposure (left panel). The right panel displays the 16 variables driving the separation of the two groups. The model is highly significant (CV-ANOVA = $1.4 * 10^{-5}$) and had a predictive power of 73% (R^2 =0.77, Q^2 = 0.73). HC = healthy control exposure, AC = asthma control exposure [3].

The OPLS analysis of exosomal miRNA expression in healthy smokers and smoking COPD patients revealed a significant separation between the groups (CV-ANOVA p =

0.03), but with a few apparent outliers clustering with the opposing groups resulting in an overall low predictive power (R^2 = 0.42, Q^2 = 0.30). The majority of the miRNAs were over-expressed in smoking COPD patients compared to healthy smokers; miRNA-1260, miRNA-92b, miRNA-4286, miRNA-1181, miRNA-766, let-7b and let-7c (Figure 24).

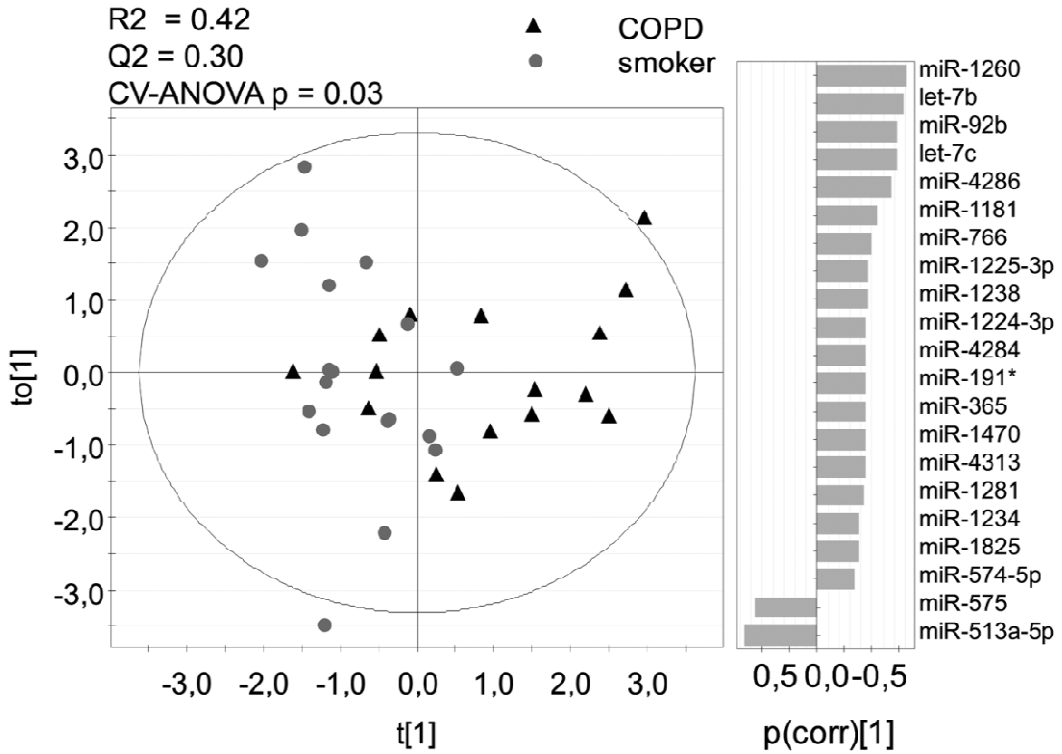

Figure 24. OPLS scores plot of exosomal miRNAs using one orthogonal factor in a model with COPD smokers and healthy smokers lead to a significant model (CV-ANOVA = 0.03) with a predictive power of 30%.

The let-7 family of miRNAs are one of the first discovered miRNAs and are known to be expressed in lung tissue and cells. Let-7c for example has been shown to be reduced in sputum of smoking COPD patients [201]. With this in mind, it is not unexpected to discover these miRNAs in both disease models. The otherwise differing set of miRNAs might be an indication of the difference between the diseases, and indeed be a clue for the involvement of miRNAs in the regulation of the specific inflammatory disease pathology in the respective diseases. In order to use miRNAs as disease biomarkers, specifically selected to distinguish one inflammatory lung disease from another, we are in search for highly specific markers only expressed in one condition.

Whether the differential expression of miRNAs from exosomes in these two diseases are due to the differences between the diseases or some other yet unknown reason remains to be elucidated. The inflammatory cell composition and reason for inflammation in these diseases are rather different. It would be of great interest to know the origin of the exosomes. Their surface composition and content varies depending on the cell type of origin and we hoped to be able to reveal the host cell by FACS analysis. Currently there are however, no specific cell surface markers for exosomes and the analysis is based on a combination of markers. Tetraspanins such as CD63, CD81 and CD9 are expressed on the surface of exosomes, these are however not specific and merely tell us that they are exosomes, not where they originate from. Moreover, the expression of tetraspanins have been shown to be affected by cigarette smoke and it is

therefore questionable whether the measurements of these can be trusted when investigating exosomes from smokers and COPD patients [153].

We measured MHC class I and II, CD54, CD63 and CD86 by FACS in the exosomes from the asthma study (paper III) and found high levels of MHC class II and CD63. The expression of CD63 supports the endosomal origin of the vesicles. The presence of high MHC class II could indicate the host cells to be antigen presenting cells. But with CD86, also expressed by antigen presenting cells, expressed only slightly above background level, this theory would need further validation. CD54 (ICAM-1) is expressed by inflammatory cells, as well as airway epithelial cells. Although the levels of both CD54 and CD86 were only slightly above background levels, there were detectable levels which might indicate that a proportion of the exosomes originated from both inflammatory and epithelial cells. Furthermore, several of the miRNAs identified in exosomes were recent discoveries and have yet unknown functions.

These two studies were not designed to be compared and one must bear in mind in the inter-comparison, that the matching is not perfect. The age differs significantly in the two study populations, with a median age of 26 years in the asthmatic study and 57 years in the COPD study.

These studies show that there are substantial differences in the exosomal miRNA profile between healthy individuals and unprovoked asthmatics with mild, stable disease, as well as individuals with COPD regardless of smoking status.

5.4 MIRNA AND MRNA EXPRESSION IN BAL CELLS IN COPD

In paper IV we also performed, in addition to the exosomal miRNA experiments discussed above, microarray analyses using 894 and 41,000 probes respectively for miRNA and mRNA analysis of BAL cells.

The results revealed significant differences of the miRNA content and gene expression between COPD smokers and healthy smokers with normal lung function. The majority of the miRNAs were up-regulated in COPD smokers both in BAL cells and exosomes. Using multivariate statistical modelling on the BAL miRNA profiles provided a significant separation of the groups (p=0.004), a number of miRNAs important in driving the separation between healthy smokers and smokers with COPD could be identified. With one orthogonal and one predictive component we could achieve a model with R^2= 0.52 and a Q^2 =0.33 (Figure 25, left panel). The most prominent miRNA variables driving the separation of healthy smokers and smoking COPD patients in the BAL samples are displayed in the VIP-plot (Figure 25, right panel). The majority were higher in COPD patients compared to smokers, including miRNA-513b, miRNA-665, miRNA-664, miRNA-494, miRNA-1972, miRNA-665, miRNA-574-5p, miRNA-1291; while a few miRNAs were found to be down-regulated due to COPD, miRNA-1260, miRNA-27b and miRNA-1274a.

Further analysis was performed creating gender-specific models; one for females and one for males, comparing smoking COPD patients to healthy smokers. The model for females was slightly stronger (R^2 = 0.35, Q^2 = 0.30, CV-ANOVA = 0.04) than the male model (R^2 = 0.2, Q^2 = 0.24, CV-ANOVA = 0.08). These differences were however not strong enough to draw any final conclusions about gender differences. Further analysis using larger groups are required.

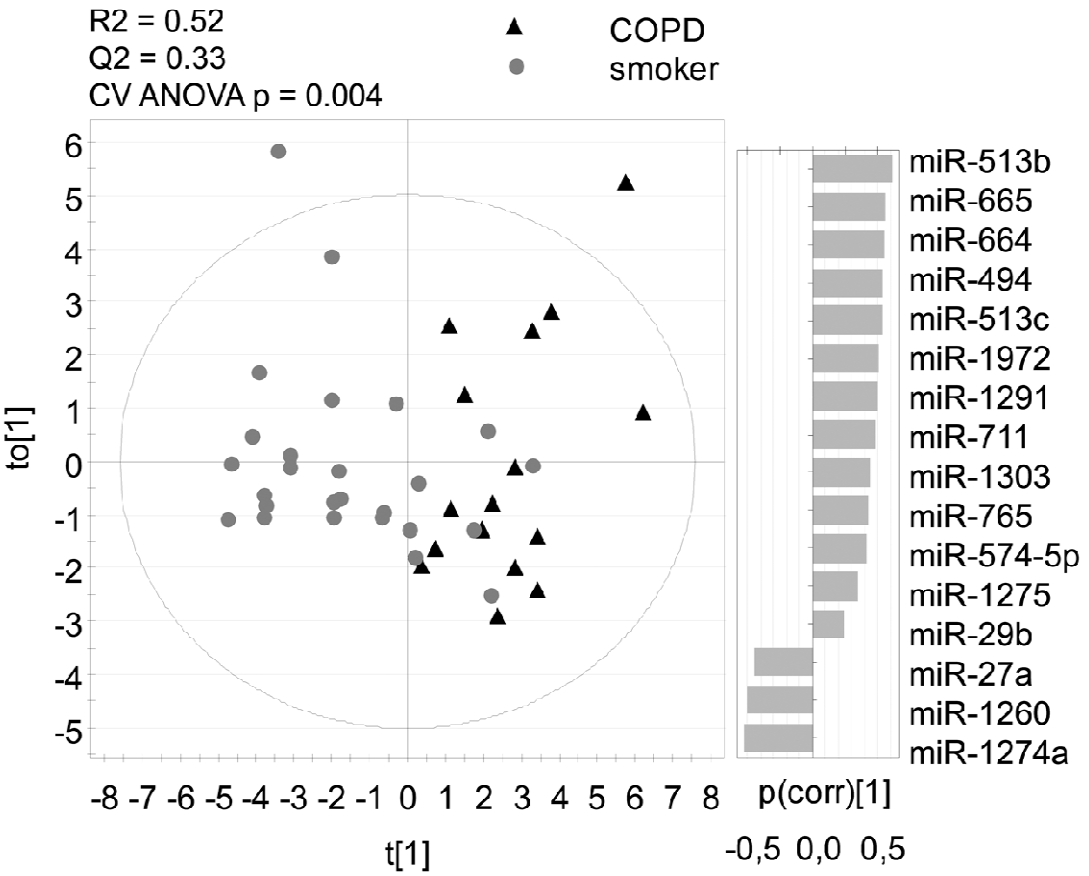

Figure 25. OPLS analysis of BAL cell miRNA expression revealed 16 miRNAs important in separating the groups of healthy smokers and COPD smokers.

We created a similar model for the non-smoking population, comparing healthy never-smokers to COPD ex-smokers, and using a SUS-plot investigated the correlation of important variables. This plot helped reveal miRNAs that were unique for disease; miRNA-1260, miRNA-24, miRNA-720, miRNA-22*, let-7b and c, miRNA-1291, miRNA-664*, miRNA-612, miRNA-198, miRNA-1183, miRNA-494.

The BAL mRNA data showed a strong OPLS model with a clear separation between healthy smokers and smoking COPD patients ($R^2 = 0.68$, $Q^2 = 0.63$, CV ANOVA $p = 1.1*10^{-6}$, Figure 26, left panel). mRNAs important for this separation were; *GPAT2, XIST, RBP1, NNMT, TNNT1, KDM5D, MMP7, IGFBP2, SPP1, ZDHHC11* and *CHI3L1* (Figure 26, right panel). In contrast to the miRNAs, the majority of these mRNAs were over-expressed in COPD.

An important factor in the COPD disease process is inflammation and this could be detected by the top variables driving the separation between the groups. Some of these, with known roles in the inflammatory process, were let-7, miRNA-29b, *SPP1, CHI3L1*, *MMP-2* and *MMP-7*. Other ones, such as *NNMT* are important in proliferation and migration of cells.

We found that MMP-7 and osteopontin (SPP1), target genes of WNT signalling, were significantly differentially expressed in COPD smokers [202, 203].

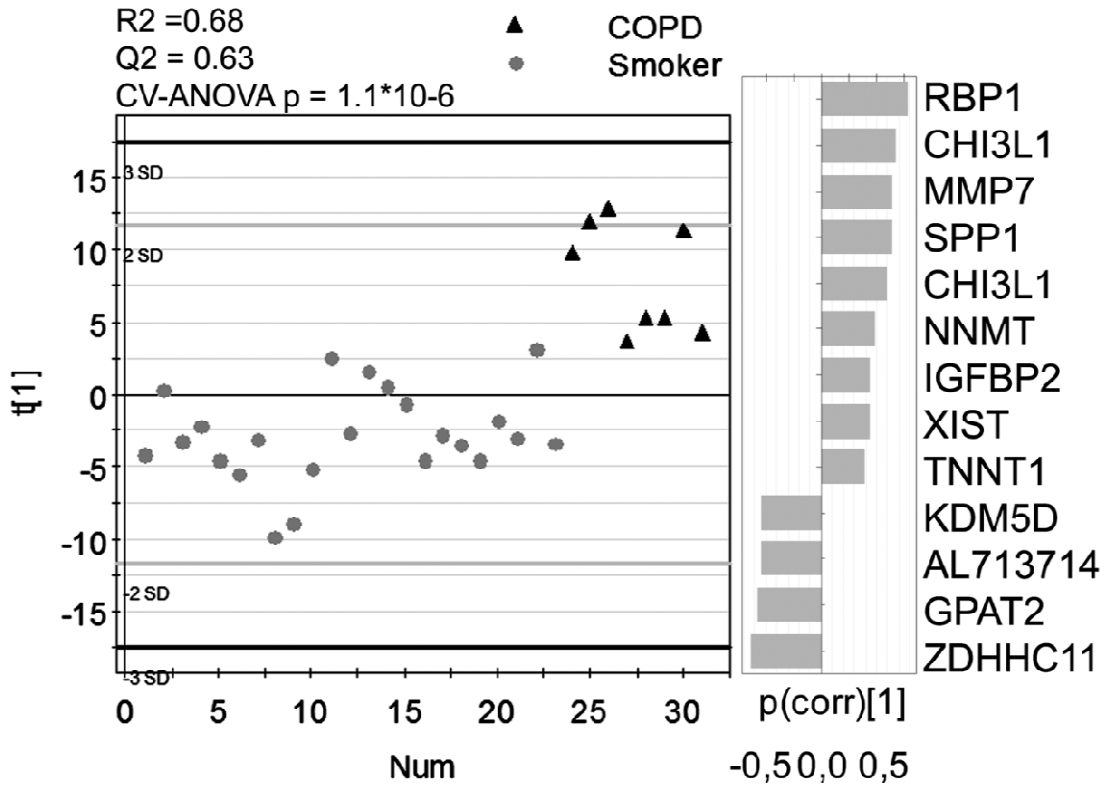

Figure 26. OPLS analysis of gene expression between healthy smokers and COPD smokers.

These results indicate clear differences in both miRNA and mRNA expression between healthy smokers and COPD smokers. The analysis method is designed to focus on disease related differences, instead of revealing the more expected smoking-related differences, by comparing the smoking population to each other separately from the non-smoking population. MiRNAs play an important role in regulating protein translation and are capable of affecting several levels at the same time, making it difficult to study their impact. Further experiments verifying our results and analysis using pathway analysis software are, therefore needed to understand the complex interplay between the differentially expressed genes and miRNAs. Integrating these data with other results, such as FACS analysis data, proteomics data will further our understanding of the pathology of COPD.

6 CONCLUDING REMARKS

This dissertation illustrates the complexity of the discussed diseases with many factors involved. Current diagnosis of the discussed inflammatory pulmonary diseases, in particular asthma and COPD is mainly based on a combination of lung function evaluation and observation of symptoms. There is no sole and exact clinical or laboratorial test available. This accentuates the need for a biomarkers used for specific and preferably early clinical markers to diagnose disease or disease phenotypes.

Treatment is also currently mainly focusing on controlling inflammation and maintaining the diseases, and there is no cure. Unspecific systemic treatment often causes side effects and this calls for new medication options, possible new targets, in order to remove or minimise this disadvantage.

The results show regulation of several pathways on multiple levels, including some inter-regulation between the studies molecules. Combining global analysis methods with complementary validation techniques in order to evaluate the similarities, or perhaps more importantly, the differences of these pulmonary diseases is the strength of this project.

Recently it has been discussed that complex diseases, such as the ones discussed here, are likely to be due to a global change of regulation involving several factors rather that just singular events. The use of global screening methods from multiple molecular levels is therefore the preferred approach to unravel the underlying pathological mechanisms. Traditional so called reductionist methods are still valuable and essential, but rather than as a starting point, perhaps better suited as validation methods after the pre-selection has been made based on the global techniques.

The combination of clinical phenotyping and omics methodology, bioinformatics tools and clinical immunology will in the larger context of the projects described here ultimately lead to an improved understanding of the cellular processes involved in the development of inflammatory pulmonary diseases. However, it should be noted that in order to fully evaluate the similarities and differences between the examined inflammatory pulmonary diseases, it might be necessary to perform all the proposed methodologies on all three diseases. Accordingly, this project represents a first step and a fraction of a larger context of elucidating the systems components underlying chronic inflammatory lung diseases.

7 FUTURE PERSPECTIVES

With large datasets from multiple platforms, and the combined study of various diseases with related symptoms and manifestations, it is crucial to integrate data in order to benefit from all the available, disparate information. The combination of data from separate studies will aid us to understand the underlying reasons and differences. This is, however, an extensive problem in bioinformatics research.

Currently there are no completed studies integrating data this way, and to this extent, although several attempts have been made. Data sets from different experimental techniques lead to a very variable set of data, requiring some sort of global scaling in order to include these in the same analysis. Integrating data like this from diverse and dynamic sources has become increasingly more demanding as the amount of data, sources and variability grows.

The studies included in this dissertation are part of larger studies. Other sets of data including proteomics and metabolomics, as well as FACS analyses in addition to all the collected clinical patient data, on the same set of patients are already available or under ways. Our future aims are to complete the missing data sets, ultimately completing the same analysis in all three diseases, further investigating miRNA and mRNA, oxylipin and WNT expression in inflammatory pulmonary diseases in combination with the results from the other platforms.

Our next challenge is therefore to integrate data from different platforms in order to interpret the entire data set as a whole. This rather arduous process requires further knowledge in complex data analysis and methodology outside the scope of this dissertation.

8 SAMMANFATTNING

Sarkoidos, astma och kronisk obstruktiv lungsjukdon (KOL) är tre inflammatoriska lungsjukdomar med varierande kliniska fenotyper. Ett gemensamt drag för alla sjukdomarna är inflammationen. Vid sarkoidos tros denna inflammtion bero på exponering för ett eller flere ännu okända ämnen, vid KOL i de flesta fall på cigarrettrökning och vid astma på en överdriven reaktion mot ofarliga substanser. Långvariga inflammationer som dessa skadar och omformar vävnaden i lungan, i vissa fall oåterkalleligt. För tillfället finns inget botemedel för någon av sjukdomarna, vilket tyder på ett behov av att definiera de patologiska mekanismerna för en mera individuell behandling.

I denna avhandling studerade vi ovannämnda sjukdomar genom en kombination av globala screeningsmetoder med valideringstekniker. Vi studerade intracellulär wingless/integrated (WNT)-signalering i bronchoalveolärlavage (BAL) celler och i epitelceller hos sarcoidos patienter. Oxylipin nivåer mättes i BAL vätska från astmatiker. Global screening av mRNA och miRNA utfördes i BAL celler från KOL patienter, samt miRNA i exosomer från både astmatiker och KOL patienter.

WNT är ett lipoglykoprotein, viktig i flera cellulära processer såsom proliferation och differentiering genom att binda till receptorer på membranet som startar en intracellulär signaleringsprocess som involverar intracellulära β-catenin. Resultaten i denna avhandling visade att det finns skillnader i *WNT* uttrycket och β-catenin aktiviteten hos sarkoidos patienter, vilket tyder på att dessa molekyler inverkar på utvecklandet av fibros. Fettämnen som kallas oxylipider syntetiseras vid behov från omega-3 och omega-6 omättade fettsyror och är inblandade i olika inflammatoriska processer. Vi upptäckte skillnader i oxylipin nivåerna i BAL vätskan hos milda astmatiker exponerade med tunnelbaneluft, vilket antyder att individer med en sänkt lunfunktion har en försvagad respons till inandade skadliga stimuleringar. Exosomer är små vesikler, tillverkade i multivesikulära endosomer i flere olika celltyper, vilka tros vara involverade i extracellulär transport av molekyler. MikroRNA (miRNA), som har visats finnas i exosomer, är korta RNA sekvenser som kan påverka genuttrycket och translationen av proteiner genom att regulera budbärar RNA (mRNA). Uttrycket av miRNA i celler och exosomer och genuttrycket i BAL celler var signifikant olika hos KOL rökare jämfört med friska rökare. Dessa kombinerade resultat indikerar på globala förändringar i olika signaleringsvägar vilket kräver omfattande studier som tar flera metoders resultat i beaktande för att klarlägga de mekanistiska skillnaderna i dessa sjukdomar.

Projekten i denna avhandling är en del av flere större studier. Vår målsättning är att integrera resultaten från flere tekniker; transcriptomik, proteomik och metabolomik genom att använda oss av komplexa analysmetoder för att klargöra de gemensamma dragen, samt olikheterna mellan dessa inflammatoriska lungsjukdomar. Genom större kännedom om bakomliggande mekanismer och orsaker för sjukdomarma, kan vi möjligen i framtiden framställa diagnosen tidigare och mera tydligt, och utveckla bättre och mera personliga behandlingsalternativ för patienterna.

9 TIIVISTELMÄ

Sarkoidoosi, astma ja keuhkoahtaumatauti ovat kolme keuhkotulehdussairautta vaihtelevilla kliinisillä fenotyypeillä. Yhteistä näillä sairauksilla on tulehdustila, joka sarkoidoosissa luullaan riippuvan vielä tuntemattoman aineen alistumisesta, keuhkoahtaumatauti johtuu yleensä tupakoinnista ja astmaatikoilla on liioiteltu reaktio vaarattomille hengitetyille aineille. Yllämainitut pitkäaikaiset tulehdukset vahingoittavat ja muuttavat keuhkokudosta, joissain tapauksissa peruuttamattomasti. Tällä hetkellä parannuskeinoa ei ole, osoittaen tarpeeseen määrittää näiden sairauksien patologiset mekanismit johtaen henkilökohtaisempaan hoitoon.

Tässä väitöskirjassa tutkittiin yllämainittua kolmea sairautta yhdistämällä laajoja seulontatutkimuskia validointi tekniikoilla. Tutkimme solun sisäistä wingless/integrated (WNT)-viestinvälitystä bronkoalveolaari (BAL) ja epiteeli soluissa sarkoidoosipotilaissa. Astmaatikkojen oxylipiiditasot mitatiin BAL nesteessä. Lähetti RNA (mRNA) ja mikroRNA (miRNA) tasoja mitatiin seulontatutkimuksilla keukoahtaumatautisten soluissa ja miRNA tasoja myös astmaatikoiden ja keuhkoahtaumatutisten exosoomeista.

WNT on lipoglykoproteiini joka on tärkeä esimerkiksi solujen kasvussa ja eriytymisessä. WNTin solunsisäinen viestinvälitys, jossa β-cateninilla on tärkeä osa, alkaa sen sitoutuneen solukalvon reseptoreihin. Tulokset tässä väitöskirjassa osoittaa erillaisuuksia *WNT* tasoissa sekä solunsisäisissä β-catenin viesteissä sarkoidoosi potilaiden BAL ja epiteelisoluissa. Oxylipidit ovat rasva-aineita, jotka syntetisoituvat tarvittaessa omega-3 ja omega-6 tyydyttymättömistä rasvahapoista ja ovat osallisia useissa tulehduksillisissa prosesseissa. Havaittiin lievien astmaatikoiden BAL nesteen oxylipiidi tasoissa eroja maanalaisen metro ilman alistuksen jälkeen, ehdottaen keuhkosairailla olevan heikennetty vastaus hengitetyille vahingollisille aineille. Eksosomi on pieni vesikkeli, valmistettu solunsisäisissä endosomeissa, monissa eri solutyypeissä, joiden uskotaan olevan osallisia solun ulkoisessa viestinnässä kantamalla molekyyli viestejä kuten miRNA:ita. MiRNAt toimii transkription jälkeisinä geeninsäätelijöinä solussa, vaikuttamalla proteiinisynteesiin mRNA:iden kautta. Meidän tuloksemme osoittivat erilaisuuksia solujen mRNA ja miRNA ekspressiotasoissa, sekä eksosoomien miRNA tasoissa tupakoivilla keuhkoahtaumatautisilla potilailla verrattuna terveisiin tupakoitsijoihin. Nämä yhdistetyt tulokset ilmaisevat muutoksia laajalla tasolla ja monessa eri viestintätapahtumassa, ehdottaen tarvetta laajaan tutkimukseen, ottaen huomioon tuloksia monista eri tekniikoista, sillä tavalla selventäen sairauksien mekanistiset erot.

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