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IMMUNOPEPTIDOMICS AND AUTOANTIGENS OF INTERSTITIAL LUNG DISEASES

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Immunopeptidomics and Autoantigens of Interstitial Lung Diseases

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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"But, Mousie, thou art no thy-lane,
In proving foresight may be vain;
The best-laid schemes o' mice an' men
Gang aft agley,
An' lea'e us nought but grief an' pain,
For promis'd joy!"

Robert Burns
(To a Mouse)

POPULAR SCIENCE SUMMARY OF THE THESIS

This doctoral thesis studies two diseases affecting the lung, more precisely sarcoidosis and idiopathic pulmonary fibrosis (IPF). Both diseases affect the lung tissue and can present with similar symptoms and findings. Sarcoidosis could be transient, but most develop a chronic disease; IPF is always chronic as well as fatal.

Despite great scientific effort, the role of the immune system in sarcoidosis and IPF remains elusive. In this thesis we approach the phenomenon where the patients' immune system attacks their own tissues or cells, known as autoimmunity, as possible marker for disease.

The overall aim is to find and characterize autoantibodies and antigens that can be related to autoimmunity or function as a marker for disease.

We mainly use patient samples from the lung or blood in our analyses. The lung samples are collected through a procedure called bronchoalveolar lavage (BAL) where a flexible instrument access the patients' airways and instils physiological salt solution which is then recollected, blood is taken by conventional venous sampling via the arm of the patient.

In several of our studies we use the powerful high-throughput method of multiplex suspension bead array (mSBA) to analyze our samples. The method uses microscopic colour coded beads and the use of antibodies/proteins to detect its counterpart.

In the first project we investigate samples from the lung, bronchoalveolar lavage (BAL) and blood from sarcoidosis patients in order to characterize the presence of inflammatory proteins. These results are then compared with healthy controls and other inflammatory diseases, such as asthma, to look at differences between what is healthy and what can be associated with disease. Two proteins (Fibronectin 1 and C-C motif chemokine 2) stood out with a stronger presence in BAL samples from sarcoidosis patients compared to controls.

In the second project we used lung and blood samples to search for self recognizing (autoimmune) antibodies with the help of the mSBA method. By utilizing the Human Atlas Project, a large library of antibodies and proteins fragments, we were able to investigate virtually any proteomic antigen target within the body. We discovered four targets displaying autoreactivity in lung and blood samples from sarcoidosis patients compared to healthy controls, especially antibodies towards the proteins Zinc finger 688 (ZNF688) and Adenosine diphosphate-ribosylation factor GTPase activating protein 1 (ARFGAP1) showed significantly higher reactivity frequency in sarcoidosis than in control samples.

In order to further understand the range of function for antibodies we investigated variations in the sugar structures (glycosylation) of the so called constant part of the antibodies, both in blood and lung samples. These structures are known to change in different inflammatory diseases, and a way of assessing the state of inflammation in the airways through a blood sample would be of great benefit to the clinic. We identified a potential marker for chronic inflammation in the lung by using the ratio between two different types of glycosylation structures in a subset of antibodies; the marker was able to separate different lung diseases to each other as well as healthy controls. This marker can thus be easily accessed through a regular venous blood sample, while still estimate the extent of inflammation in the airways.

Together with collaborators in France and Netherlands we put together a large number of samples from IPF patients; we aimed to characterize the self-recognizing (autoimmune) antibodies in lung samples and blood and compare IPF patients to sarcoidosis. Targets recognized by the autoantibodies (autoantigens) were found in a majority of samples, including healthy controls, although the healthy controls had in general a lower presence of autoreactivity and also relatively lower levels of antibodies. An antibody towards one type of collagen (collagen 5 A1) stood out with higher presence in IPF and nLS. Altogether, autoreactivity was higher in IPF and in nLS patients compared to all other groups; interesting since both groups develop fibrosis at a higher rate than the other diseases. The protein epitope of COL5A1 is proposed as an autoimmune target and/or marker of fibrosis, and is of interest for further investigation.

Currently there are few options for sarcoidosis or IPF patients when it comes to tools for prognosis or disease specific treatment; this, together with diagnostic challenges, entails the need for improved biomarkers and potential immunotherapy targets.

ABSTRACT

We investigate Sarcoidosis and Idiopathic Pulmonary Fibrosis, IPF, to find markers for early fibrosis development. The aetiologies for both diseases are unknown and there is no specific treatment, moreover, there is a lack of diagnostic biomarkers for both diagnose and disease activity. Overall, the diseases have a considerable effect on patients' physical health and quality of life. To identify patients at risk of rapid development of fibrosis, it is vital to improve patient care. We hypothesize that identification of specific antigens can help in the exposition of the pathogenesis of sarcoidosis as well as of IPF, and in the long perspective this could lead to identification of therapeutic targets.

In project I we investigated the presence of proteins in BAL and serum from sarcoidosis patients and controls to discover disease associated proteins. In total eight proteins had increased levels in sarcoidosis patients, two were the most prominent (Fibronectin 1, FN1 and C-C motif chemokine 2, CCL2) that displayed the greatest differences between cohorts. Furthermore, the protein cadherin 5 (CDH5) had a positive association to lymphocyte count in BAL, interesting since T-lymphocytes are the major cell type in sarcoidosis. Potentially this could provide a way of monitoring lymphocyte presence in the lung through blood sample.

In project II the large source of antigens from the Human Protein Atlas Project, a large library of protein fragments and antibodies that represent virtually all proteins in the body, was used to screen the immunoglobulin G specificity towards 3072 selected antigens in serum and BAL samples from patients with either of the sarcoidosis subcategories (Löfgren's syndrome (LS), non-Löfgren's sarcoidosis (nLS)), and asthma as well as healthy controls. A selected set of antigens went on to be analyzed in mSBA analysis. Sarcoidosis patients demonstrated an elevated reactivity frequencies toward Zinc finger protein 688 (ZNF688) and mitochondrial ribosomal protein L43 (MRPL43), particularly MRLP43 displayed a higher frequency in patients with non-Löfgren syndrome. Even though the protein fragment representing adenosine diphosphate-ribosylation factor GTPase activating protein 1 (ARFGAP1) showed high reactivity frequency in all sample groups, it was still significantly elevated in patients with sarcoidosis compared to the other cohorts.

In project III we used a mass spectrometry-based method to analyze and characterize the Fc regions of human IgGs in paired serum and BAL fluid. Antibodies were isolated using a fast and reliable approach via MelonGel extraction. The isolated IgGs were digested by trypsin and separated by nLC (nano liquid chromatography) as in conventional proteomics, but peptide fragmentation was performed by both high resolution HCD MS/MS (Higher-energy C-trap dissociation) and high-resolution ETD MS/MS (Electron-transfer dissociation).

We identified a candidate marker IgG_4, which corresponded well to inflammatory activity in chronic lung diseases while also correlating between BAL and serum (R^2=0.95), thus being readily available for sample collection. The IgG galactosylation marker could prove to be useful in clinical settings by monitoring chronic pulmonary inflammation status. Based on the results of project II we concluded that the Fc galactosylation status of IgG_4 could potentially be used as a serum marker for severity in pulmonary inflammation.

In project IV project, we used the mSBA approach for both sarcoidosis and IPF samples in order to evaluate similarities and differences between fibrosis associated diseases.

Autoantigens were found in a majority of patient samples, including healthy controls, although that cohort displayed a lower frequency of autoreactivity and also comparatively lower titers. Altogether, autoreactivity was higher in IPF and in nLS patients compared to all other groups; both groups included a higher fibrosis rate than the other diseases potentially linking a general presence of autoreactive antibodies to fibrosis development. Reactivity toward collagen 5A1 (COL5A1) was discovered with a statistical significant higher frequency in patients with IPF compared to all other groups apart from the nLS group. The protein epitope of COL5A1 is proposed as an autoimmune target and/or marker of fibrosis, and is of interest for further investigation.

In this thesis we have profiled the repertoire of proteins and autoantibodies in serum and BAL from patients with sarcoidosis, in addition we also investigated and compared the presence of autoreactive antibodies in patients with sarcoidosis and IPF, as well as control subjects and various comparable diseases. We have also proposed the ratio between agalactosylated and galactosylated forms of the Fc region in IgG_4 as a marker for severe chronic lung disease. These discoveries open up for more studies to characterize, and test functionality, of these autoantibodies and proteins in the setting of sarcoidosis, IPF, or inflammatory and/or pulmonary diseases.

LIST OF SCIENTIFIC PAPERS

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II. Häggmark A, Hamsten C, **Wiklundh E**, Lindskog C, Mattsson C, Andersson E, Lundberg IE, Grönlund H, Schwenk JM, Eklund A, Grunewald J*, Nilsson P*. Proteomic profiling reveals autoimmune targets in sarcoidosis. Am J Respir Crit Care Med. 2015 Mar 1;191(5):574-83.

III. Heyder T†, **Wiklundh E**†, Eklund A, James A, Grunewald J, Zubarev RA, Lundström SL. Altered Fc galactosylation in IgG_4 is a potential serum marker for chronic lung disease. ERJ Open Res. 2018 Jul 31;4(3):00033-2018.

IV. **Emil Wiklundh**, Cecilia Hellström, Anna Månberg, Elisa Pin, Coline van Moorsel, Bruno Crestani, Jan C Grutters, Peter Nilsson and Johan Grunewald. Autoantibodies against COL5A1 in Idiopathic Pulmonary Fibrosis and Sarcoidosis. *Manuscript*

*These authors share senior authorship

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ADDITIONAL PUBLICATIONS NOT INCLUDED

I. Lundström SL, Heyder T, **Wiklundh E**, Zhang B, Eklund A, Grunewald J, Zubarev RA. SpotLight Proteomics - An IgG-Enrichment Phenotype Profiling Approach with Clinical Implications. Int J Mol Sci. 2019 May 1;20(9):2157.

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LIST OF ABBREVIATIONS

Ab	Antibody
ACPA	Anti-citrullinated protein antibody
AM	Alveolar macrophages
APC	Antigen presenting cell
ARDS	Acute Respiratory Distress Syndrome
ARFGAP1	Adenosine diphosphate-ribosylation factor GTPase activating protein 1
AUC	Area under the curve
BAL	Broncoalveolar lavage
BCR	B cell receptor
BHL	Bihilar lymphadenopathy
BOS	Bronchiolitis obliterans syndrome
CBD	Chronic beryllium disease
CCL2	C-C motif chemokine 2
CD	Cluster of differentiation
CDH5	Cadherin 5
COL5A1	Collagen 5 A1
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
CV-ANOVA	Cross validated ANOVA
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DLCO	Diffusing capacity of carbon monoxide
ECM	Extra cellular matrix
ELISA	Enzyme-linked immunosorbent assay
EN	Erytema nodosum
ETD	Electron transfer dissociation
Fab	Fragment antigen binding
Fc	Fragment crystallizable

FcγR	Fc gamma receptors
FDR	False discovery rate
FEV_1	Forced expiratory volume during 1 second
FN1	Fibronectin 1
FVC	Forced vital capacity
GlNAc	N-Acetylglucosamine
HC	Healthy control
HCD	Higher-energy C-trap dissociation
HLA	Human leukocyte antigen
HRCT	High resolution computer tomography
HSPA4	Heat shock 70kDa protein 4
Ig	Immunoglobulin
IL	Interleukin
IPF	Idiopathic pulmonary fibrosis
LC	Liquid chromatography
LS	Löfgren's syndrome
MAC	Membrane attack complex
MAD	Mean Absolute Deviation
MBP	Mannan-binding protein
MFI	Median Fluorescent Intensity
MHC	Major histocompatibility complex
MRPL43	Mitochondrial ribosomal protein L43
MS	Mass spectrometry
mSBA	Multiplex Suspension Bead Array
mTORC1	Mechanistic target of rapamycin complex 1
NCOA2	Nuclear receptor coactivator 2
NK cell	Natural killer cell
nLS	Non-Löfgren's sarcoidosis
NO	Nitrous oxide

OPLS-DA	Orthogonal projections to latent structures discrimination analysis
PAMP	Pathogen associated molecular pattern
PCA	Principal component analysis
PET	Positron emitting tomography
PRR	Pathogen recognition receptor
PTM	Posttranslational modification
Q^2	Goodness of fit
R^2	Predictive power
RA	Rheumatoid arthritis
ROC	Receiver operating characteristics
RORγt	Retinoic acid receptor-related orphan receptor gamma
ROS	Reactive oxygen species
SA	Severe asthmatics
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SE	Standard error
Tbet	T-box transcription factor 21
TCR	T-cell receptor
T_{FH}	Follicular T-helper cells
T_h	T helper
TNF	Tumor nekrosis factor
Treg	Regulatory T-cells
UIP	Usual interstitial pneumonia
ZNF 688	Zinc finger 688

1 INTRODUCTION

Sarcoidosis is an inflammatory granulomatous disorder primarily affecting the lungs, though virtually any organ may be affected. Patients usually suffer from cough, chest discomfort or pain, shortness of breath and a prominent fatigue. In Sweden, approximately one third of the patients develop an acute disease that, while more rapid, has a higher frequency of remission (Löfgren's syndrome), whereas others develop a chronic disease with possible fibrosis (non-Löfgren's sarcoidosis) (1, 2). Over time, especially in patients with long term treatment, sarcoidosis has an extensive impact on the patients' wellbeing and life quality.

Just as sarcoidosis, Idiopathic pulmonary fibrosis (IPF) also belongs to the group of interstitial pneumonias, IPF is a chronic disease characterized by continuous fibrotic scarring of the lungs (3). IPF is progressive and first and foremost affects older adults, moreover IPF lead to a steady deterioration in lung function causing severe impairment of quality of life and is ultimately fatal within 2-5 years for those affected (3, 4). For both diseases the aetiology is unknown and there is no curative treatment. Moreover, there is a lack of disease markers to facilitate diagnosis and to assess disease activity, as well as to evaluate how patients respond to treatment.

By running samples from both sarcoidosis, IPF, healthy and control diseases we gathered a repertoire of autoantibodies to be used as interesting targets for further functional studies. Furthermore, the antibodies or corresponding antigens in question could be used in the clinic as a marker for IPF or sarcoidosis in diagnosis or as a prognostic marker provided further research connecting these findings to disease outcome. All of this would hold great significance for research in interstitial lung diseases as well as the patients suffering from it.

Potential biomarkers would help physicians diagnose the disease in an earlier state as well as provide drug targets in aid of treating patients (3, 4). In the short term this will give a broader understanding on how IPF and sarcoidosis patients differ in their immune status from healthy and other inflammatory diseases. The aim in the long term for this study is to provide targets which can be used for treatment.

2 BACKGROUND

2.1 THE RESPIRATORY SYSTEM

The primary function of the respiratory system is to manage the gas exchange between inhaled air and the circulatory system. Within the pulmonary tissue, oxygen and carbon dioxide exchange by passive diffusion over a concentration gradient through the thin cell layers between the capillaries and alveoli (5). The respiratory system comprise of the conducting portion, where no gas exchange occurs, and the distal respiratory tract through which respiration transpires (6). The conducting portion begins with the nasal cavity, going through trachea and bronchus to terminal bronchioles, after which the distal portion starts with respiratory bronchioles and ends in the sacs of the alveoli. Throughout the respiratory system the airways go through numerous bifurcations, decreasing airway size but increasing the total surface area, which is an important aspect in order to amplify the surface for gas exchange (6). The globus structure and great number of alveoli represents a vast area upwards to 100 m^2, consequently, the respiratory system presents one of the body's largest surface areas, and is in direct contact with an outside world of potentially harmful agents, microorganisms and pollutants etc. (7). The respiratory epithelium is crucial in the defense against pathogens, using the aid of mucous producing goblet cells and ciliated lining to expel pathogens from the airways (5). In addition to its mucociliary functions, the epithelium possesses the ability to mount an immunological response. When exposed to harmful substances, the epithelial cells lining the airways can trigger immune cells, as well as produce proinflammatory cytokines and chemokines (8-10).

Underneath the epithelial lining lies the interstitium, the space between cells, consisting of collagen I, III, and V entwined with elastin, glucosaminoglycans and proteoclycans to form a reticulate pattern (11). Its function is to support the surrounding tissue through transport of constituents and nutrients as well as for antigens and cytokines contributing in immunological responses (12). This interstitial space is frequently the location for some of the potentially difficult and debilitating pulmonary diseases, such as sarcoidosis and IPF (13, 14).

3 INNATE AND ADAPTIVE IMMUNE RESPONSE

The immunological defense is stratified into two subcategories, the innate and adaptive immune response (Figure 1). The innate is congenital and represents a fast response against common and shared structures between microbes such as pathogen-associated molecular patterns (PAMP) and damage-associated molecular pattern (DAMP) (5). It is a highly conserved function, common throughout many species and organisms (15, 16).

In contrast, the adaptive system represents a slower but more specific response as well as the capacity of immunological memory. The adaptive immune response can be further divided into the cellular response and the humoral response (15).

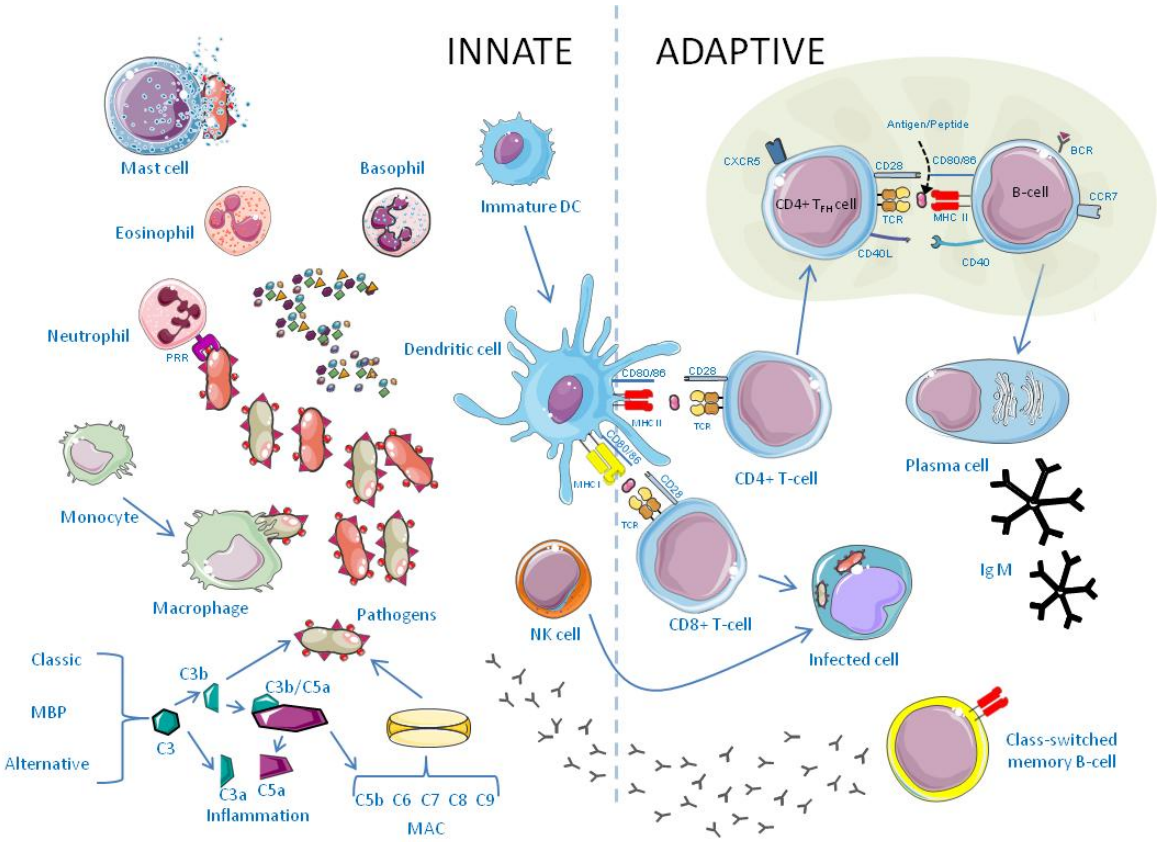

Figure 1. Overview of the immune system. Including the quicker but broader innate immunity and the slower but more specific adaptive immunity. C(x) - Complement, MAC - Membrane attack complex, MBP - Mannan-binding protein /Classic/Alternative - Complement pathway. PRR - Pathogen recognizing receptor.

3.1 INNATE IMMUNITY

The cells that make up the innate, also known as natural, immune system includes neutrophils and natural killer cells (NK-cells), granule releasing cells, for example eosinophils, basophils and mast cells as well as monocytes. Circulating monocytes can migrate into tissues can differentiate into tissue resident macrophages and dendritic cells (DCs) (15). Macrophages, neutrophils and NK-cells are phagocytic, able to engulf and degrade extracellular pathogens through phagocytosis. In contrast, dendritic cells are professional antigen presenting cells (APCs) whose function primarily is to present antigens to other leukocytes (5). Eosinophils, basophils and mast cells release antimicrobial constituents to incapacitate the pathogens (5). Macrophages and DCs together with migrating neutrophils are able to react to a harmful pathogen through recognizing PAMPs and DAMPs and subsequently release pro-inflammatory molecules. These molecules are known as interleukins (IL) 1, IL6, IL12 and tumor necrosis factor (TNF) to promote inflammation and recruit leukocytes (17-19). Pathogens invading directly into human cells, e.g. viruses, can be cleared by NK cells. The virus infected cells present viral antigens on the cell surface which can be detected by NK cells who in turn will release perforins which disrupts the cell membrane of the infected cells, causing apoptosis (20).

Another crucial component of the innate immune system is the complement activation cascade (16). Through cleaving complement proteins, the activated forms of complement protein 3 and 5 (C3a and C5a) will release additional proinflammatory cytokines. Other complement proteins form the cylindrical membrane attack complex (MAC) which attacks pathogens cell membrane rupturing bacterial cell walls (21). The native form of complement protein 3, C3b, will attach to, and identify, pathogens to more readily facilitate phagocytosis by macrophages, in a process called opsonization (15). The complement system is constant and doesn’t change over a lifetime, it can however be activated by antibodies produced by the adaptive immune system (22).

3.2 ADAPTIVE IMMUNITY

Where the innate system is general but quick in its response, the adaptive system is slower to initiate but rather more specific and potent. The main effector cells are the T- and B -lymphocytes and they are triggered by specific antigens corresponding to the individual cell's antigen receptor. Through somatic recombination of the receptor gene segments, a countless diversity of receptor specificity can be produced (15).

APCs, including DCs, macrophages and B-cells are the primary cell types that actively take up antigen, process it and present it on the cell surface, where it is recognized by antigen receptors on T-cells (TCR). The T-cells belong to the cellular, T-mediated, response, while the B-cells belong to the antibody/humoral response. The two types of lymphocytes have different actions although the fundamental function of developing antigen specificity and establishing memory is similar. An overview of the T-cell and B-cell interaction can be seen in figure 2.

Naïve T-cells are produced in the thymus after which they circulate into lymph nodes awaiting activation through specific antigen recognition. T-cells interact with APCs through the TCR via the major histocompatibility complex (MHC) on the APC surface. MHC is present in most vertebrates; the human version of MHC is known as human leukocyte antigen (HLA) (15). The most common T-cell receptor is a heterodimer consisting of two chains (alpha and beta), and each chain is composed of a constant (C) and a variable (V) region, whereas the antigen binding site consists of V-alpha and V-beta segments (15, 21). TCRs only recognize short fragments of proteins, peptides, and only when presented by the HLA molecules [29]. To detect all peptide variants, the TCR needs to accommodate an equal number of variants; this is done through germline recombination of the V(D)J segments of the variable regions (23). The HLA consists of a receptor with an antigen peptide being presented in the groove of the receptor. Depending if the origin of the antigen is from within the APC (endogenic) or outside the APC (exogenic) the antigen will be presented on HLA class I or II, respectively (15), and interact with TCRs of CD4+ or CD8+ T cells, respectively. The need for both the HLA molecule and the antigen peptide to interact with the TCR prevents the TCR from reacting to free circulating antigens. APCs will, after antigen uptake, migrate to the closest draining lymph node where resident naïve CD4+ and CD8+ waits to be activated (24). To avoid immune responses towards self-antigens, all T-cells go through a stringent selection process during maturation. In addition, a co-stimulatory signal is required during antigen

presentation to complete the activation of T-cells (25). Co-stimulation acts through APC co-stimulatory proteins CD80/CD86 interacting with the CD28 and/or CTLA4 co-receptor on the T-cell (26). Not until both signals are present will the T-cell undergo maturation and start producing IL-2 which promotes proliferation and clonal expansion (21). CD4+ cells will identify peptides from HLA class II and CD8+ will recognize peptides from HLA class I. Activated CD8+ T-cells are primarily cytotoxic and aimed at intracellular antigens, commonly virus-derived, meaning the host cell has been invaded by a pathogen (15). This causes the T-cell to induce apoptosis of the infected cell through the release of granzymes into the cell via the perforin created at the receptor interaction, effectively eliminating the intracellular pathogen (27). Activated CD4+ T-cells (T-helper cells, Th-cells) release cytokines and chemokines to attract macrophages, activate cytotoxic T-cells and enhance antibody production by B-cells, basically their primary purpose in inflammation is to aid other cell types through cytokine release. T-cells, in particularly CD4+ positive cells, can be functionally divided into subsets, called Th1 and Th2 type. Th1 is directed towards intracellular pathogens by stimulating a cellular response and Th2 against extracellular through stimulation of the humoral response, over time, additional subsets have been uncovered, adding T-regulatory cells (Treg), Th17, Th9 and Th22 to the list (28).

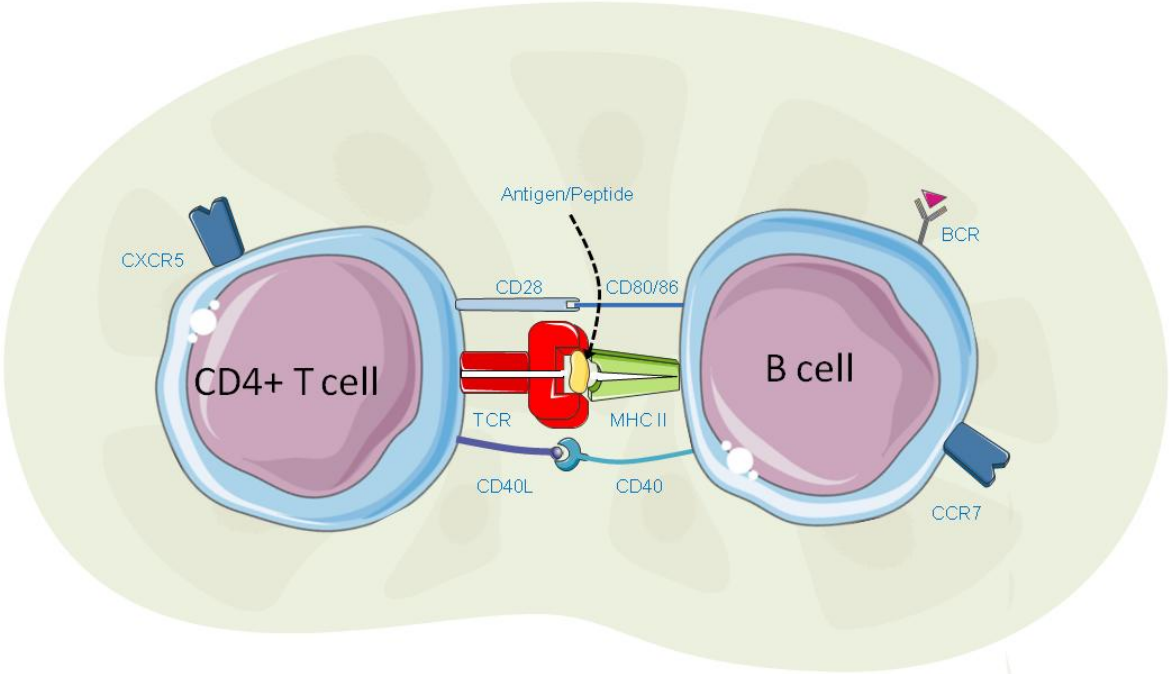

Figure 2. Schematic overview of Follicular CD4+ T cell interaction with B cells in the lymph node.

B-cells constitutes the other half of the adaptive immune system, in contrast to T-cells which in essence solely detects protein peptides, the B-cells can identify a large variety of antigenic structures e.g. proteins, lipids, polysaccharides and nucleic acids. The B-cells develop in the bone marrow where recombination of the b-cell receptor (BCR) genes takes place, a process similar to the TCR recombination. After BCR assembly in the bone marrow, each BCR being specific towards one antigen structure, the transitional B-cell will then exit the bone marrow to travel to the spleen to fully mature. The fully matured B-cell then acts as an APC, detecting pathogens through its specific BCR and presenting antigens on their HLA class II molecule (5). The B-cells resides within the peripheral lymphoid organs, commonly within the follicles of lymph nodes, there, the B-cell consume pathogens through phagocytosis which are presented on the MHC class II molecule as peptides. The B-cell then becomes activated if the peptide is in parallel recognized by a CD4+ T-cell through the TCR, in combination with a co-stimulatory signal by interaction of CD40 on the B-cell and CD40L on the T-cell (16). Subsequently, the T-cell will start to produce cytokines causing the activated B-cell to differentiate into a plasma cell, producing highly antigen specific soluble non-membrane bound BCRs known as immunoglobulins or antibodies. After clearing the infection, a small number of B-cells will remain as memory cells, able to mount a much faster response in case of a re-infection (15).

3.3 ANTIBODIES

Immunoglobulins (Ig) are Y-shaped proteins, which exists either as a part of the BCR receptor facilitating antigen recognition or produced and secreted by plasma cells after B-cell activation. The basic Ig unit constitutes of two identical heavy and light chains. The heavy chain is composed of one variable and three constant domains (IgE has four constant domains). The light chain is built of one variable and one constant domain. After enzymatic degradation the antibody is split in two parts. One part is called fragment antigen binding, (Fab), comprising the antigen binding site and the first constant domain. The second fragment, containing the constant domains two and three of the heavy chain make up the fragment crystallizable region (Fc) which interacts with the receiving cell through Fc receptors (15).

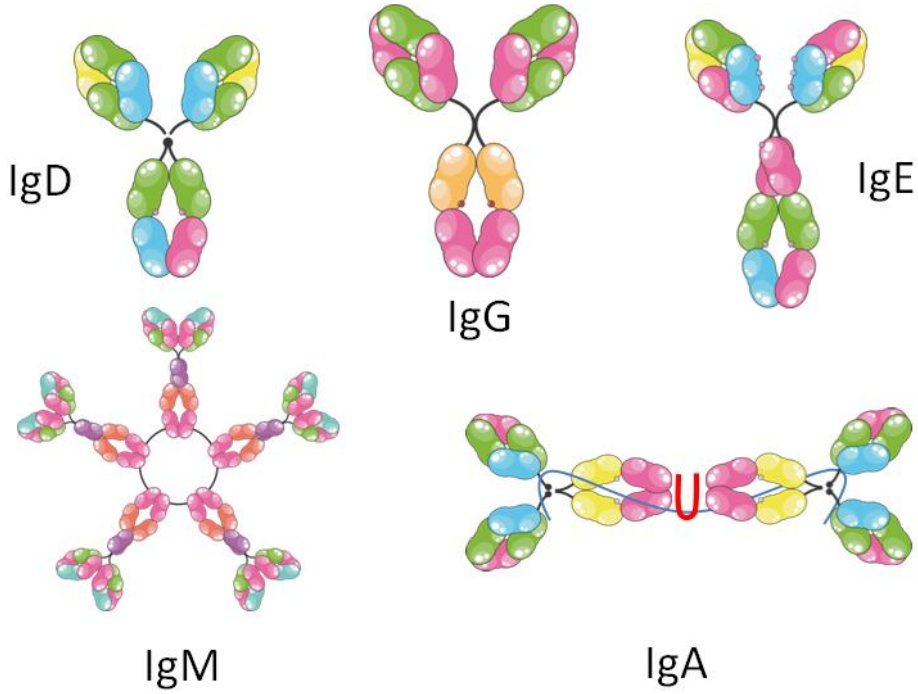

Figure 3. Antibody subsets. IgD, IgG, IgE, IgM, and IgA schematic representations.

There are five classes of immunoglobulins (Ig); IgD, IgG, IgE, IgM and IgA each with its primary function (Figure 3). The IgM molecule is composed of a pentamer, and together with IgD produced by naïve B-cells acting in early stages of humoral immune responses to eliminate pathogens (15). The IgD can stimulate production and release of antimicrobial factors in basophils and mast cells (29). Through class switching, plasma cells are able to produce the other types of Ig's, where IgG is the main isotype in the body, and is stratified into four subclasses named IgG 1-4. They are numbered in decreasing order of abundance, though their abundance does not reflect their effector functions nor their efficiency (30). The IgG molecule can exert its antimicrobial function in various ways, either through binding to the antigen/microbe, priming it for opsonization by macrophages, or binding and neutralizing toxins, or initiating antibody-dependent cellular cytotoxicity through NK-cells. The IgA exists either as a monomer or as a dimer; it resides primarily in mucosal tissue, e.g. the respiratory or intestinal tract as well as in saliva and tears, where it rapidly acts against toxins and microbes. The IgEs primary function is defense against helminths, however, it is also responsible for binding allergens and causing hypersensitivity through degranulation of mast cells (15).

3.3.1 FC-glycans

In order for the IgG antibody to facilitate effector functions, it relies on being able to identify a specific antigen through its Fab region and subsequently connect to the effector cell via the Fc gamma receptors (FcγR). While immunological effector functions serves to act as regulators of immunological homeostasis, a dysregulation can facilitate both tolerance and increased risk of infection (31).

Structural alterations through glycosylation of the Ig's Fc region can have great effect on the downstream immunological response (32, 33). Variation to the Fc glycan composition can direct the antibody's affinity towards type I or II Fc gamma receptors (FcγR) and thus shift the immunological response to either pro- or anti-inflammatory actions, as well as elicit specific actions such as opsonization or complement activation (32, 33). Generally, a more compound Fc-region will be inclined towards an anti-inflammatory response, especially if it has a high fraction of galactosylated or sialylated glycans (33-36). Furthermore, Fc-glycan changes have been demonstrated to be a potential marker for inflammation as well as being altered in autoimmune and inflammatory disease (37-40).

Alterations by galactosylation in the Fc region has been described extensively in context of autoimmunity (41-43), including the discovery of anti-citrullinated protein antibodies (ACPA) acquiring a proinflammatory Fc glycan phenotype before diagnosis in rheumatoid arthritis patients (44). In Figure 4 a representation of the IgG molecule and the position of the Fc glycans can be observed (A) including the glycan core structure with nomenclature suggested by Royle et al (B) (45).

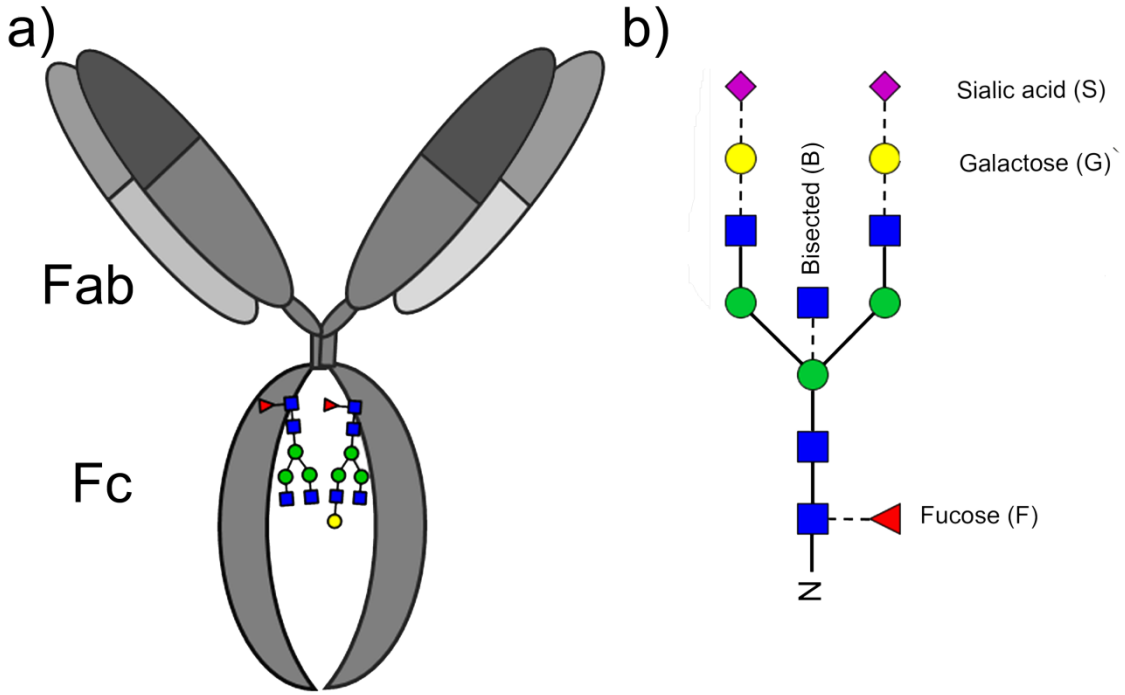

Figure 4. a) Graphic representation of the IgG molecule and subsequent position of the Fc glycan structures. b) A schematic of glycan formation with nomenclature suggested by Royle et al. (45). The core Fc glycan structure consists of a heptasaccharide constituent (denoted as A2). If the first saccharide unit is carries a fucose, the core Fc glycan is fucosylated (denoted as FA2). The core configuration can as well be bisected by an additional N-acetyl-glucosamine (GlcNAc (denoted as FA2B)). Additionally, the top glucosamine units can be lengthened with galactoses (FA2Gn, n=1 or 2) and the galactoses can be extended with sialic acids (FA2GnSn, n=1 or 2). Blue squares - GlcNAc, green circles - mannose, yellow circles - galactose (G), red triangle - fucose (F), purple diamonds - sialic acid (S). Adapted from (46).

3.4 TOLERANCE AND AUTOREACTIVITY

T- and B-cells go through a selection procedure while maturing in order to ensure they only act against potentially harmful external antigens and not towards self-antigens, this acceptance for “self” is known as central tolerance (15). The T-cells undergo positive and negative selection in the thymus while B-cells undertake a similar selection but in the bone marrow. The first control point is during generation of the TCR and BCR receptor, where inability to form a functioning receptor results in apoptosis. Next the T-cells are tried against affinity for the HLA complex, no recognition results in apoptosis due to that a nonfunctional receptor would be futile. A too strong affinity runs the risk of activation without the second co-stimulatory signal, yielding apoptosis through negative selection. Positive selection take place as the interaction between the T-cell and APC is identified with low affinity, yielding

APC survival signals (15). Note that only self-antigens (autoantigens) are tested in positive and negative selection since external potentially pathogenic peptides rarely occurs in the thymus (5). The explanation for solely low/moderate avidity T-cells surviving the selection process is that these cells could instead exhibit a high avidity for pathogenic peptides in the periphery (15).

B-cells are positively selected on the presence of a functional BCR and not its specificity (unlike T-cells). Negative selection ensues when immature B-cells interact too strong with self-antigens, this leads either to change in receptor specificity (receptor editing) or apoptosis. Anergy develops if a B-lymphocyte recognizes an antigen but at low avidity or does not receive the second co-stimulatory signal, the B-cell will then be functionally impaired. Once a functional BCR has been produced, the immature B-lymphocyte only expresses IgM. In order to mature the B-cell must co-express IgD together with IgM; this is done through recombination and splicing of the heavy chain V(D)J segment. Maturation develop either in the bone marrow or the B-cell will migrate to the spleen (15). Though lymphocytes go through a stringent selection process, one study indicated that as many as 1/5 of all circulating B cells were autoreactive, though not generating disease (47).

Although the tolerance system is highly accomplished, it can still malfunction and when tolerance is lost it can lead to autoimmunity. The prevalence in Europe for autoimmune disorders is estimated to 5%, in general, women tend to be more often afflicted than men (48, 49). It is not known why certain individuals develop autoimmunity and not others, however, risk factors such as environment, smoking, infections and gender has been observed (50-53). A strong connection with HLA composition has also been demonstrated (42-47). Additionally, autoreactivity can be triggered during infections if the pathogen's antigen exhibits similar or homologous structures as a self-peptide, a phenomenon known as molecular mimicry (54).

3.5 PULMONARY IMMUNOLOGY

In contrast to circulating leukocytes, where neutrophils are most abundant, the primary phagocytic cells present in bronchoalveolar lavage (BAL) fluid are alveolar macrophages (AM). In healthy individuals the respiratory leukocytes consists of up to 95% of AMs; lymphocytes constitutes between 1-10% and neutrophils only 1% (55). However, in the case of an infection neutrophils will migrate to, and accumulate within the lungs, by far outnumbering the AMs (56). The epithelium can also activate innate and adaptive immune responses through the release of cytokines and chemokines, including production of antimicrobial substances (10, 22). Apart from phagocytosis of hazardous particles, AMs can also release nitric oxide (NO), reactive oxygen species (ROS) and proteolytic enzymes to destroy and degrade the attacking pathogen (57). Additionally, AM together with dendritic cells (DCs) present antigens for T-cells in order to stimulate a T-cell response (58).

In general the lungs are an exposed organ given its large surface area and almost unhindered direct contact with environmental surroundings. This infers the immune system to be alert, without going to excess. In fact, both immunological hyper- and hypo-responses can lead to pathological reactions in the airways, e.g. obstructive diseases such as asthma or chronic obstructive pulmonary disease (COPD). However, it is not just the airways that can be affected, the interstitial space is frequently the site for pathogenic conditions, often of a restrictive type, including; Sarcoidosis, chronic beryllium disease (CBD), idiopathic pulmonary fibrosis (IPF) and silicosis. These diseases risk the development of fibrosis, subsequently, leading to impaired gas diffusion and gas exchange (59, 60).

3.6 COVID-19

It has been virtually impossible not to been affected by the coronavirus disease 19 (COVID-19) during the past year, thus the disease require attention in this thesis. There have been over 150 million cases and more than 3 million deaths as of beginning of May 2021 due to COVID-19 (61). The pandemic that started late 2019 and took off during the first half of 2020 has greatly affected a significant portion of the world population, and has dramatically increased hospitalization due to pneumonia and respiratory failure (62). COVID-19 is caused

by the novel virus variant severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), with symptoms ranging from asymptomatic to life-threatening sepsis and respiratory failure, although, the majority of infections result in a mild disease (62).

The SARS-CoV-2 virus enters targeted cells via binding to converting enzyme 2 (ACE2) receptor on the cell surface using the SARS-CoV-2's own surface protein spike (S), in order to infuse the cell with ribonucleic acid (RNA) for virus replication (63). The type 2 transmembrane serine protease (TMPRSS2) facilitates the fusion to host cells by cleaving the ACE2 receptor and trigger the (S) protein; both ACE2 and TMPRSS2 are ubiquitously found on alveolar type II cells located in the airways SARS-CoV-2 virus (63-65).

During the later phase of the infection the SARS-CoV-2 virus infect capillary endothelial cells causing and invasion of monocytes and neutrophils into the respiratory space, resulting in oedema consistent with early stage acute respiratory distress syndrome (ARDS), with the consequence of deteriorating oxygen exchange (66, 67).

There have been concerns that patients suffering from interstitial diseases would fare worse than others, especially for patients on immunosuppressant drugs, e.g. certain sarcoidosis patients (68). A study used a self-reporting questionnaire did demonstrate an increase in COVID-19 infection for sarcoidosis patients compared to non-sarcoidosis (69). A review of the incidence and prevalence of COVID-19 for patients (including sarcoidosis patients) on immunosuppressant drugs did not find increased prevalence for COVID-19 nor increased risk for life-threatening disease, though the data was limited (70, 71). There have also been ideas of antifibrotic therapies protecting IPF patients from COVID-19 as well as providing treatment for non-IPF patients suffering from post-COVID-19 pulmonary fibrosis (72). However, more studies are needed on the subject of interstitial lung disease and COVID-19, and considering both sarcoidosis and IPF are comparatively rare diseases there is a great risk of delay before substantial data could be acquired.

4 SARCOIDOSIS

4.1 CLINICAL FEATURES

Sarcoidosis is an inflammatory granulomatous disorder primarily affecting the lungs, though essentially any organ may be affected. Approximately two thirds of the patients develop a chronic disease with possible fibrosis development and a risk of suffering respiratory failure (73, 74). Deteriorating physical health and side effects from long term treatment impacts the life of sarcoidosis patients.

Sarcoidosis has been suggested to be composed of several separate disease entities with distinct etiologies, but also with common inflammatory pathways, e.g. resulting in the typical granuloma formation (75). One group of patients that is now known to suffer from chronic beryllium disease (CBD), caused by exposure to beryllium, was commonly misdiagnosed as sarcoidosis because of the clinical resemblance of sarcoidosis, including granuloma formation (76-78). One distinct subgroup of sarcoidosis patients, i.e. Löfgren´s syndrome (LS), characterized by its clinical phenotype with acute onset, fever, erythema nodosum (EN), bilateral ankle arthritis and bilateral hilar lymphadenopathy (BHL), could constitute a separate disease (79). Approximately one third of Swedish sarcoidosis patients have LS, which is an unusually large proportion from an international perspective (80). The nLS patient group, constituting the majority of patients, usually presents gradually and are linked to a more chronic disease course, more often with development of pulmonary fibrosis (81). In many of our studies we compare LS and nLS patients to identify markers for good prognosis (usually LS) or for a less favorable disease course (usually nLS).

4.2 EPIDEMIOLOGY

The incidence as well as prevalence of sarcoidosis varies extensively between different parts of the world, as well as ethnicity. The highest incidence can be observed in Scandinavia with 11-24 cases per100.000/year (80, 82, 83), and African Americans with 18-71 cases per 100,000/year (84-86). Incidence in Sweden was recently estimated to be 12 per 100.000/year, and the prevalence to be 160 per 100.000 (80) Some variation can also be noted between sexes where 45-60 percent of sarcoidosis cases occurs in women (87). The age of onset is on average between 40-55 years for all patients combined, though a

difference between sexes can be observed where men peak 30-50 years age and women between 50-60 years of age (80, 88, 89).

Several risk factors for developing sarcoidosis have been proposed. The most prominent is genetic susceptibility through increased risk of developing sarcoidosis for people with certain variants of Human Leukocyte Antigen (HLA) molecule (90, 91). The risk is also increased if you have a family member with the disease, increasing the risk by 2-4 times (92, 93).

While smoking is a risk factor associated with many diseases in general, and lung diseases in particular, smoking has consistently been related to a lower risk for sarcoidosis (1). Even a study collecting smoking status before diagnosis (removing bias where patients stop smoking because of the disease), smoking demonstrated a 50% lower risk of developing sarcoidosis (1, 94).

4.3 PATHOGENESIS

The complete aetiology behind sarcoidosis is not known. Current hypothesis states that susceptible individuals are exposed to one or more unknown antigen which leads to an inflammatory reaction where the immune system is unable to clear the antigen and form the base of granuloma formation. Activated macrophages can turn into epithelioid multinucleated cells under the influence of the metabolic checkpoint kinase mechanistic target of rapamycin complex 1 (mTORC1) pathway (95). This leads to a granuloma formation that could either resolve or progress. In case of progression, the granuloma is incapable of degrading the antigen and the antigen is instead encapsulated within the granuloma which continues to grow and chronic disease is developed. The granuloma consists, apart from previous mentioned epithelioid cells, also vast number of lymphocytes surrounding the core (96). The lymphocytes consists mainly of CD4+ T cells, but also a small number of CD8+ T-cells and some B-ells (97). Furthermore, an increased production of reactive oxygen species (ROS) together with increased inflammatory signals and tissue damage can be observed in chronic sarcoidosis which is believed to be cause by malfunctioning phosphorylation of mitochondrial proteins (98). In comparison with healthy individuals, dendritic cells from sarcoidosis patients produced much higher levels of TNF-α, which would indicate a self sustaining mechanism of both dendritic cells, cell

migration and granuloma formation (98). Sarcoidosis granulomas are non-caseating, meaning they are non-necrotizing, which is the opposite from granulomas in tuberculosis where macrophages do go into necrosis. Interestingly, a link between tuberculosis, or other mycobacteria, and the sarcoidosis antigen has both been suspected and dismissed by researchers; however, still there is no definite answer (99-102). An overview of the proposed pathogenesis of sarcoidosis can be seen in figure 5.

The CD4+T-cell subset is the most common cell type in sarcoidosis, they are essential for sarcoidosis progress and for granuloma formation. When stimulating the immune system T-lymphocytes are activated and start proliferating, primarily T-cells and macrophages in the alveoli which leads inflammation in the airways (1). Macrophages and dendritic cells at the site of inflammation secrete IL-12 and IL-18 which aids the T-cells in their differentiation and maturation. In turn, the activated T-cells release high levels of IL-2, IFNγ and TNF-α which promotes macrophage activation and T-cell survival (103). Furthermore, after stimulation with candidate antigens, activated T-cells manifest a Th1 type of response by producing large quantities of IFNγ in vitro (104).

The view on the role of activated lymphocytes presence in sarcoidosis patients has shifted over time, from solely CD4+Th1 type driven disease into a more complex view involving the Th17.1 phenotype. These cells are capable of producing large quantities of IFNγ, thus promoting an inflammatory process in the lungs. T helper cells differentiate into Th1 and cells producing IFNγ, i.e. Th17.1 effector cells, as well as Th17 cells (105-107). A majority of T-cells acquired from BAL expressed the unique Th1 transcription factor T-box transcription factor 21 (Tbet), although they were also found to co-express RAR-related orphan gamma receptor (RORγt) which is the transcription factor for Th17, indicating plasticity among T-cell phenotypes (104).

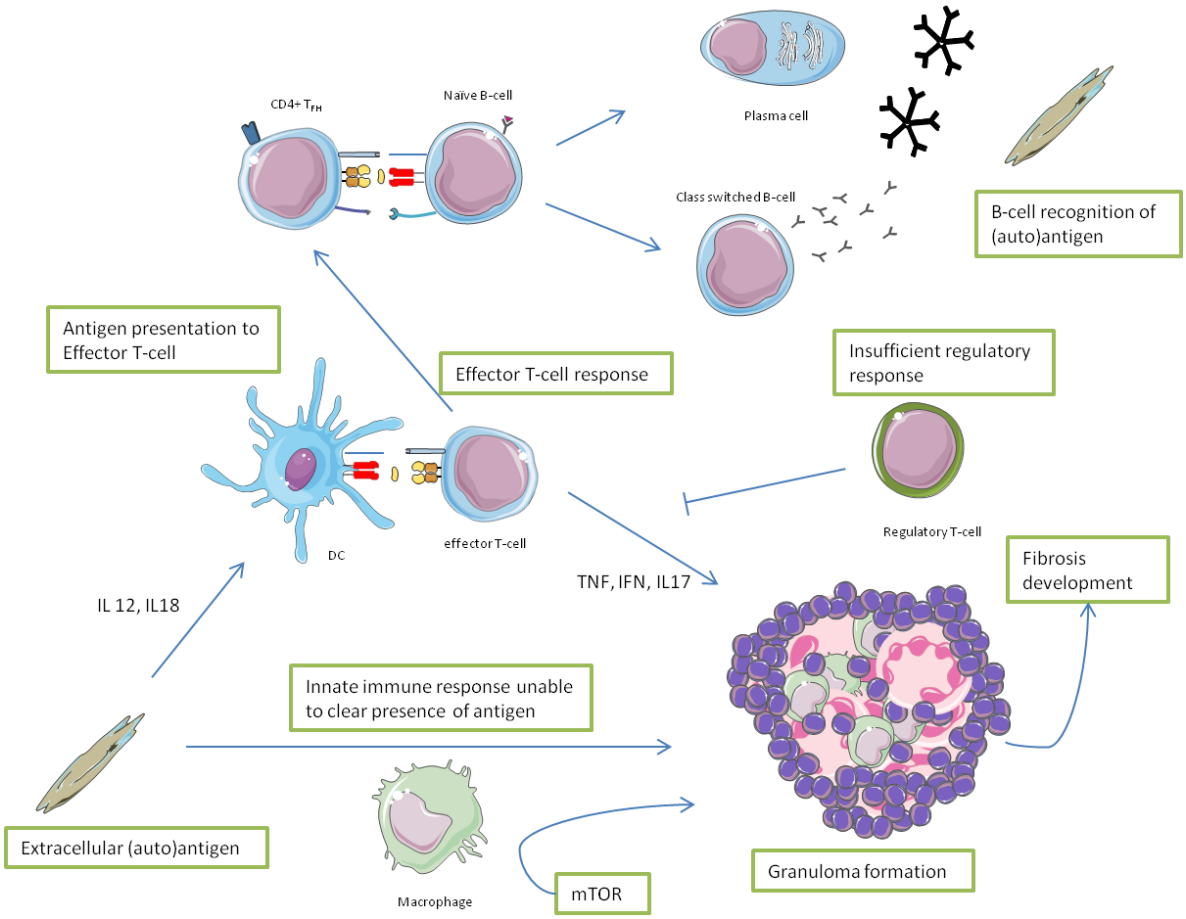

Figure 5. Overview of the pathogenesis of Sarcoidosis. An extracellular (auto)antigen in the lung triggers the activation of phagocytes such as dendritic cells (DC) and macrophages. If the phagocytes are unable to clear the antigen, they will instead encapsulate the antigen and a characteristic granuloma formation occurs. The antigen is presented to CD4+T-cells that recognize the peptide and respond with a variety of cytokines. CD4+ T-cells expressing HLA-DRB1*03 will present the antigen to B-cells resulting in antigen specific antibodies. With a coordinated T and B cell response, the granuloma can be resolved. Though in chronic stages the response is unable to resolve the granuloma and instead the granuloma progresses, resulting in chronic inflammation and risk of pulmonary fibrosis.

Still, the main effector cell in sarcoidosis is CD4+ T-cell; in fact lymphocytosis above 25% in bronchoalveolar lavage and an increased ratio between CD4/CD8 above 3.5 associate with sarcoidosis and it constitutes one diagnostic indicator (108, 109). In addition, T cells expressing TCR Vα2.3 gene segment has been found more frequently in patients with the DRB1*03 allele, most commonly patients with Löfgren's syndrome(108).

Moreover, the regulatory T-cells (Treg) are normally responsible for maintaining steady state in T-cell activity; however, in sarcoidosis there is an exaggerated T-cell activity which leads to the conclusion that the Treg response is impaired. Although, it is uncertain whether the insufficient activity is primary, or if it's secondary to prolonged antigenic stimulation (109-111).

A cornerstone in the sarcoidosis pathophysiological hypothesis is the existence of a triggering factor, an antigen. Quite a few suggestions exist, ranging from mycobacterium to autoantigens (112-115). Notably, LS patients expressing DRB1*03 were found to display immune response following stimulation with the autoantigen Vimentin (115). In line with this, several other peptides derived from proteins like lysyl-tRNA synthetase and ATP synthase were found in BAL from in DRB1*03 positive sarcoidosis patients (116, 117).

4.4 DIAGNOSIS AND TREATMENT

Sarcoidosis is diagnosed through its most prominent trait which is non-necrotizing granuloma formation in the affected organ; histological verification together with clinical findings typical for sarcoidosis with the absence of other explanation is the basis for diagnosis. Other investigations such as chest radiograph, bronchoalveolar lavage (BAL), positron emitting computer tomography (PET-CT) and spirometry can aid in the assessment of the patient. If there is a case where histopathological sampling is not possible, diagnosis can be made on a combination of classic clinical features, laboratory results and chest radiographs. One such typical finding in BAL is increased accumulation of CD4+ cells in BAL, yielding an elevated CD4+/CD8+ ratio >3.5, which is especially accurate for patients with Löfgren syndrome (118-120). Since the lung is the most commonly involved organ, a chest radiograph is standard procedure which commonly shows bihilar lymphadenopathy w/o pulmonary infiltrates. The chest radiograph is also used for staging (I-IV). The staging ranges from mild infiltrates all the way to fulminate fibrosis (121).

Bronchoscopy and BAL is routinely performed in the clinical evaluation of patients with suspected sarcoidosis, regularly giving access to inflammatory cells and mediators to be used for studies of the local inflammatory process in the lungs. Also, biopsies are obtained to search for non-necrotizing granulomas in affected tissues, typical for (but not definitively proving) sarcoidosis.

The aetiology is unknown and there is no specific treatment, current praxis uses oral corticosteroids first choice, adding/or substituting to methotrexate in patients with persistent infiltrates or severe pulmonary dysfunction (109, 122, 123). Though patients with Löfgren syndrome and the HLADRB1*03 (HLA- DR3) allele have a better outcome, with remission in 70–80% of the cases (2). Biological treatments such as Infliximab (anti-TNF-α) has been tried in patients with severe disease where all other therapies have failed, in an effort to downregulate T-cell activity, though success rates are highly individual (124, 125). As stated earlier, there is a lack of disease markers to facilitate diagnosis and to assess disease activity, as well as to evaluate how patients respond to treatment.

4.5 SARCOIDOSIS AND AUTOREACTIVITY

Autoimmunity in sarcoidosis is currently one of the more prominent concepts; the idea is supported by strong associations between certain HLA variants and LS. Furthermore, for a small percentage of patients the disease can be relapsing, whether this is a re-infection, or an autoimmune burst of the disease is unknown (126, 127). There are several instances where immunological hyperactivity rather than a deficiency will yield sarcoidosis or sarcoidosis like disease. For example, HIV positive patients undergoing anti-retroviral treatment has been shown to risk developing sarcoidosis when CD4+ T-cell counts >200 cells/µL (128). Also, some patients treated with IFNα e.g. due to chronic hepatitis C have been discovered to develop a sarcoidosis-like disease (129-131). These examples are not what you would typically expect to find in a disease maintained by an infection.

RA patients have been demonstrated to have autoreactive antibodies towards a citrullinated variant of the vimentin protein, strongly associated with disease (132, 133). Moreover, antibodies against citrullinated vimentin have been demonstrated in the lungs prior to disease onset, suggesting disease initiation in the lungs (despite a different target

organ) (134). Vimentin peptide has been implicated as a possible autoantigen in sarcoidosis, i.e. sarcoidosis patients have been shown to have a higher frequency of antibodies against vimentin in BAL compared to controls (115). The same vimentin peptide has also been eluted from AMs at several occasions in BAL samples from sarcoidosis patients (74).

Sarcoidosis is not contagious, not cured by antibiotics, and not re-activated upon long term immunosuppressive treatments like corticosteroids, methotrexate or anti-TNF-drugs, arguing against any live actively replicating micro-organism maintaining the disease.

5 IDIOPATHIC PULMONARY FIBROSIS

5.1 CLINICAL FEATURES

Idiopathic pulmonary fibrosis (IPF) belongs to the group of interstitial pneumonias; it is a chronic fibrotic disease characterized by continuous fibrotic scarring of the lungs (3). IPF is progressive and first and foremost affects older adults, moreover IPF lead to a steady deterioration in lung function causing severe impairment of quality of life and is ultimately fatal within 2-5 years for those affected (3, 4, 14). As the name suggests IPF is idiopathic, meaning its pathogenic cause remains unknown, furthermore, there is also limited knowledge of prognostic factors and effective treatments (135).

5.2 PATHOGENESIS

Throughout history IPF was considered a primarily inflammatory disease, and as such it was treated with corticosteroids. Over time it became more apparent that the antiinflammatory treatment did not improve the outcome for these patients, and eventually the concept of IPF being primarily an inflammatory disease was overturned (136). Furthermore, the standard treatment at the time with corticosteroids and azathioprine was shown not only to be ineffective but also increase the risk of death.

The pathogenesis behind IPF is still not clear, current hypothesis involves a multifaceted etiology with several genetic and environmental risk factors causing multiple microinjuries to the epithelium of the airways (137, 138). These microinjuries will subsequently lead to aberrant wound healing and excessive fibroblast-myofibroblast foci resulting in extensive amounts of extracellular matrix and remodeling (137, 138). There are also indications that shortening of telomeres and dysfunction protein telomerase could play a part in IPF pathophysiology, especially in familial cases (139-141). A general overview of the proposed pathogenesis of IPF can be viewed in figure 6.

The cytokines MMP1 and MMP7 aids in the epithelial migration, in addition, in the IPF lung the epithelial cells are highly active and produce growth factors and chemokines as well as stimulate fibroblasts the lungs (137). The epithelial cells also secrete TGFβ1, which is considered one of the key cytokines behind the IPF pathology. TGFβ1 stimulate extra cellular matrix production and help fibroblasts differentiate to myofibroblasts (138). Together, these cells form the fibroblast foci, in the foci the myofibroblasts are thought to be active in producing excessive quantities of ECM (138).

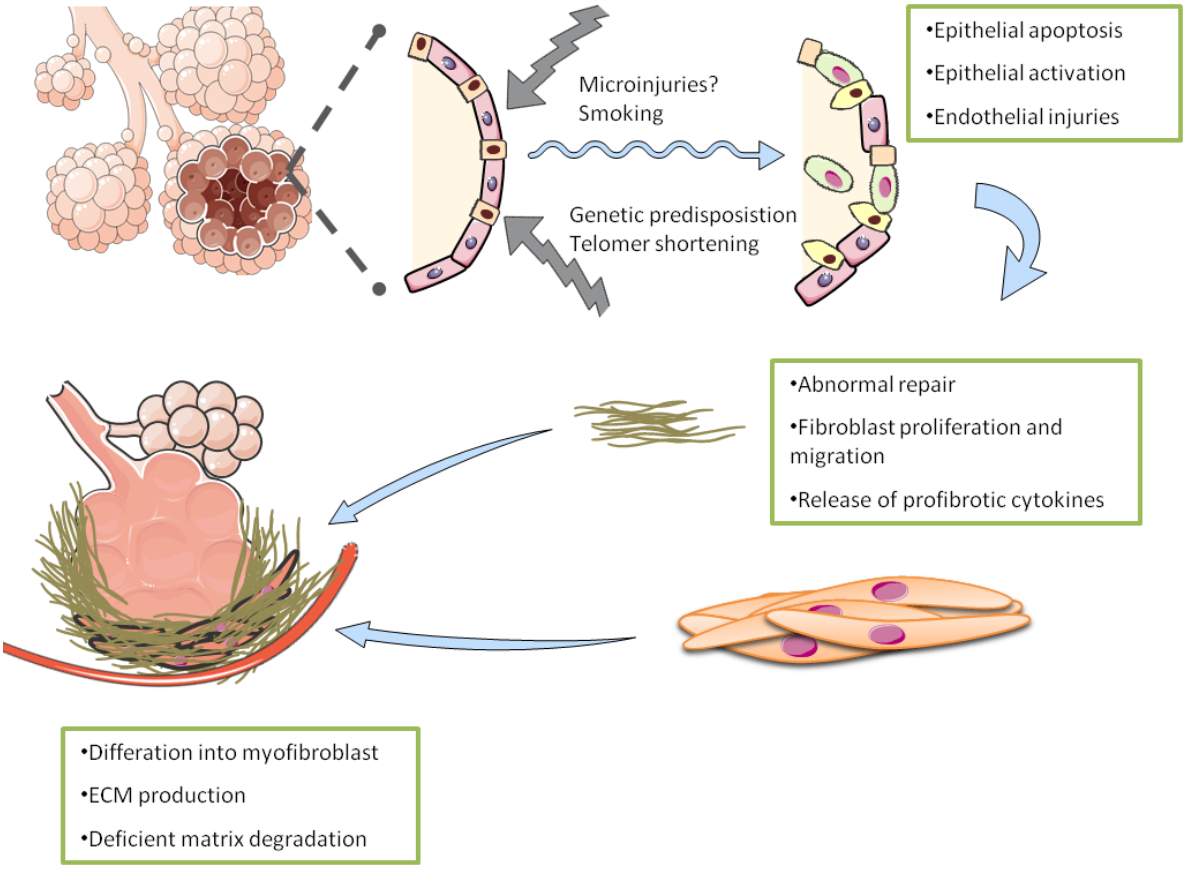

Figure 6. General overview of the IPF pathogenesis. The initiation of IPF is distinguished by both a genetic susceptibility to form progressive fibrosis together with several environmental triggers that can cause injury. Many risk factors have been indentified, smoking and aging being the two most prominent. When injuries have been sustained over time, an abnormal repair process occurs, with re-epithelialisation, migration of fibroblasts and the formation of collagen matrix. The progression results en excessive matrix formation which results in deformation of the airway structure and a steady decline in gas exchange. ECM - Extracellular matrix.

5.3 EPIDEMIOLOGY

The incidence for IPF seems to be on the rise, although there is some uncertainty if the increase is real or due to more awareness and better diagnostics (138). Additionally, incidence is higher in North America and Europe (3-18 cases per 100,000/year) compared to other parts of the world such as Asia and South America (under 4 cases per 100,000/year) (4, 142-144). IPF occurs more often in older adults, usually over the age of 70, it is rare that people under the age of 50 get the disease.

As stated earlier, several risk factors are believed to be contributing to the pathogenesis of IPF. Inhalation of particles, and more specific, cigarette smoke is strongly associated with the disease (145). Though, inhalation of other particles such as viruses, wood dust, silica and exposure to agriculture is believed to increase the risk of IPF (146-148).

5.4 DIAGNOSIS AND TREATMENT

Diagnosis of IPF is made on radiographic or histological foundation where pattern of Usual Interstitial Pneumonia (UIP) is one criterion (146, 149, 150). Patients routinely undergo High Resolution Computer Tomography (HRCT) where the UIP pattern can be observed. The same nomenclature is used for the histopathological image, though not the same criteria as for HRCT (151). Pulmonary fibrosis is the main feature of IPF, but since other diseases and drug side-effects are also able to yield fibrosis, a diagnosis of IPF is always in absence of other explanations. Because of this, it is recommended to diagnose IPF patients in multidisciplinary meetings to avoid missing any underlying cause (146). The classification of interstitial pneumonias has changed on several occasions, partly due to challenges with categorization; biomarkers would therefore be of big to help physicians diagnosing the disease in an early state (3, 4).

In the later years IPF patients has had access to two new therapies, nintedanib and pirfenidone, both of which has demonstrated to have an effect in treating IPF (152). Primarily the drugs have had an effect on slowing the disease rate down, exhibited by holding back the decline in Forced Expiratory Volume (FVC) (153, 154). In addition, some data suggest a positive effect on acute exacerbations and hospitalization (155, 156).

5.5 INFLAMMATION AND AUTOREACTIVITY

Even though IPF is not considered to be primarily an inflammatory disease, there is still a role to play for the immune system. Lymphocytes are found in the interstitium and fibrotic scars of IPF patients, as well as autoantibodies present in the serum which suggest a possible role for the immune system in either initiation or progression of IPF (142, 157). The tolerance loss in autoreactive T or B-cells to antigens resulting from debris of dying epithelial cells could drive the immunopathology of IPF (142).

Still, substantial amount of research has been done to elucidate mechanisms to either explain or reject role for immune system to no avail.

There are autoimmune diseases that have an established connection with developing pulmonary fibrosis such as systemic sclerosis (158), Sjögren's syndrome (159) and rheumatoid arthritis (160) Though if an underlying cause can be found the fibrosis by definition is no longer idiopathic. However, autoimmunity in IPF has been implicated in previous research (142), demonstrating reactivity to various potential targets such as vimentin (161-163). Although these autoimmune reactions have not been fully understood or comprehended, this provides an interesting approach to illuminate pathogenic mechanisms (164, 165). So far only small studies have been conducted, to improve chances of finding answers there is a need for a larger study looking at vastly greater numbers of potential autoimmune targets.

6 SARCOIDOSIS AND IPF

Both in Sarcoidosis and in IPF there are clinical subgroups that present with higher rate of fibrosis (sarcoidosis) and a more rapid disease progression rate (IPF) (14, 166). Moreover, it is currently impossible, within the respective disease, to distinguish the patients with worse prognosis from those with a better prognosis on an early stage. IPF and sarcoidosis share other aspects as well, humoral anomalies are frequent in both sarcoidosis and IPF (167), there are even cases of concurrent sarcoidosis and IPF (168). Specific autoantibodies or antigens could potentially influence disease outcome or at least serve as a marker of disease development (14, 167). Potential biomarkers would help physicians diagnosing the disease in an earlier state as well as provide drug targets in aid of treating patients (3, 4).

7 PROTEOMICS

Proteomics is the science and study of function of proteins and peptides, the phrase was coined more than 20 years ago (169), played off by the term proteome formulated a few years prior (170). In this paper, proteomics will only refer to proteins and peptides in the human system, though proteomics in general encompass proteins of all organisms. Proteomics is more dynamic in nature compared to s (genomics), where genes in a cell are close to invariable the proteome will alter over time and between cells. Genomics encompass the information of what potentially can occur, while proteomics describe what is happening at the present moment.

Due to posttranslational modifications (PTMs) the variability of proteins increases even further (171). The PTMs refer to the process of adding a functional group by covalent binding to the modified protein. The modifications are usually carried out by various enzymes in order to enhance or modify the function of the protein. The most common PTM is phosphorylation where a phosphate group is attached to the protein, which is a typical way of regulating enzyme activity (172). Another feature among proteins is to attach carbohydrate molecules, a procedure known as glycosylation, which can promote of affect protein folding as well as structural stability (173, 174). In project III we investigated if the glycosylation variations to the sugar moieties of the Fc region of antibodies could be related to inflammation in lung diseases.

This high variability of proteomic expression both inter- and intra-individually yield certain challenges in analyzing proteins, such as reproducibility, and accounting for protein degrading (175, 176). Previously the quantification and detection of proteins were done through RNA analyzing methods; however these were shown to be inaccurate in protein content due to inconsistent transcription of mRNA (177-179).

The ways of analyzing proteins has increased, and keeps expanding, though essentially proteomic can be divided into top-down or bottom-up approaches (180, 181). Top-down analyzes entire proteins or substantial fragments of proteins; this approach is beneficial for analyzing PTMs or isoform determination (182). Bottom-down analyzes small peptides or metabolites of proteins, which has methodological benefits over the top-down approach when it comes to protein fractionation and ionization (182). Both strategies have their advantages and disadvantages, the reason for choosing either depends on the research question. The bottom-down approach can be further categorized into targeted or non-targeted analysis. Targeted means the investigation of a known metabolite, this method is used for validation of hypotheses and thus is hypothesis driven. Whereas for non-targeted (also known as shotgun approach) the metabolites are unknown and the analysis is unbiased, this approach is better used for discovery rather than validation (183, 184). In project III we used LC-MS/MS which is a non-targeted bottom-down approach, compared to the immunoassay of bead array analysis which is closer to a targeted analysis method.

8 MATERIALS AND METHODS

This section provides an overview of certain methods and set-ups used in this thesis, as well as some additional considerations. Please review the original articles for a more detailed description of the methodologies.

8.1 ETHICAL APPROVAL AND CONSIDERATIONS

The collection and use of patients samples and information been approved by the Stockholm regional ethics committee. The ethical permissions for are **project I-III;** main application no. 2005/1031-31, complementary applications 2009/20-32, 2011/35-32, 2012/132-329. In addition to the previous applications, **project IV** requires the complementary application 2021/00802 to be able to analyze and handle patient information of IPF samples from international collaborators. The collaborators themselves have ethical approval from local ethic committees (Netherlands - BIOBANK DATA ILD R-05.08A**,** France - CPP Ile de France I: no 0911932).

Patients are admitted to the hospital and undergo a diagnostic investigation, with regard to interstitial pulmonary diseases, this usually means they perform a bronchoalveolar lavage (BAL) and biopsies, from which the material collected can be used for both clinical investigation as well as research. However, if a secondary lavage is performed for research interests only, this will be on a volunteer basis and compensated as such.

Volunteers are healthy individuals who participate in the studies of free will and not to diagnose a disease. They receive, however, monetary compensation.

Another point to consider is the samples collected contain a lot of biological information (DNA information etc.) which usually is investigated together with clinical information from the patient's journal. This makes blinding/anonymizing imperative.

8.2 STUDY SUBJECTS

In the clinic, we routinely screen patients to be eligible for research and after an informed consent is obtained, samples (both blood and BAL fluid) are collected and stored in our biobank. **All of the papers** rely on the existence of sample material from patients and healthy controls.

Paper I - The same cohorts as in project II was used in the discovery phase for project I. In the verification phase, an additional 216 sarcoidosis BAL samples were added together with 33 samples from healthy controls. In total, 251 BAL samples from sarcoidosis patients and 49 BAL samples from healthy controls were included in this project. All sarcoidosis patients had an active disease, defined as either progression on chest radiograph, physical symptoms or worsened lung function in accordance with a sarcoidosis disease. All patients and healthy controls gave written and informed consent.

Paper II - This paper included 345 BAL and 141 serum samples from LS, nLS, asthma and healthy controls in the screening stage. An additional 22 BAL samples from fibrosis patients and 269 serum samples from patients with myositis were added in a subsequent verification stage. The same criteria for active disease as in paper I for sarcoidosis patients were applied. The sarcoidosis patients were diagnosed according to the American Thoracic Society (ATS)/European Respiratory Society (ERS)/ World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) criteria (109) and subgrouped into LS and nLS cohorts. Healthy controls were neversmokers and did not take any prescription drugs. The asthma patients were mild allergic asthma patients, currently out of season and with access to bronchodilators but not under corticosteroid treatment. All patients and healthy controls gave written and informed consent.

Paper III - BAL and serum samples from both LS (n=12) and nLS (n=12) were initially analyzed using LC-MS/MS after melongel purification. As controls, a cohort of severe asthmatics (n=20) and a cohort of RA-patients (n=14) were added together with healthy subjects (n=12). The severe asthmatic patients stood on a regime of continuous high dose corticosteroid inhalations. Clinical characteristics and biomarkers measured as part of the BIOAIR study included BMI, sputum and blood cell counts, exhaled NO, lung function, serum periostin, YKL-40, total IgE and CRP. For the RA patients the Fc-glycan information were taken from published data using the mean values between anti-citrullinated peptide

antibodies (ACPAs) and the remaining paired polyclonal IgGs from respective patient. For more details see Lundstrom et al (185).

Paper IV - Through international collaborations in France and Netherlands, two sets of IPF cohorts were provided. In total a 154 serum samples (France) and 99 matched serum and BAL samples (Netherlands) were provided. These samples were shipped on dry ice and then treated exactly the same as the rest of the samples from the other cohorts. The original cohorts from paper I and II functioned as controls.

8.3 BRONCHOALVEOLAR LAVAGE AND PROCESSING OF SAMPLES

Bronchoscopy is a crucial procedure for medical doctors to assess a patient's airway, and essential for the ability to take samples from the mucosa or pulmonary tissue.

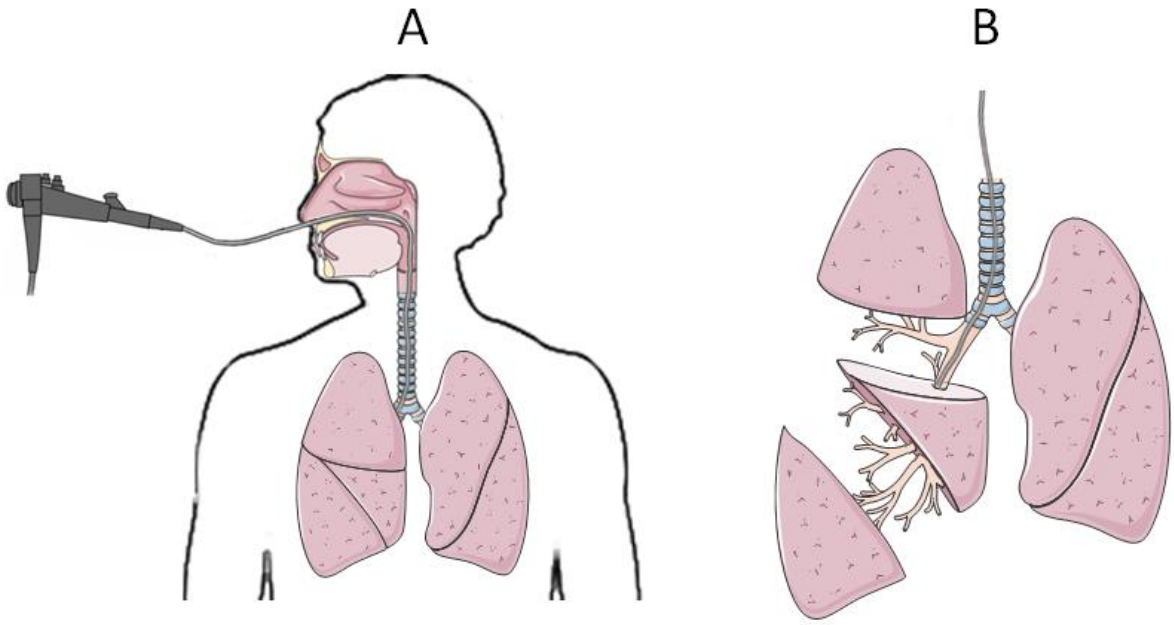

Figure 7. **Bronchoscopic procedure and collection of BAL**. A) Via inserting a flexible bronchoscope into the patients' airways, the physician can observe the epithelium and take samples, such as BAL. B) Sterile saline solution is instilled in the right middle lobe and then aspirated in order to produce bronchoalveolar lavage fluid (BAL).

For bronchoalveolar lavage a flexible bronchoscope is inserted into the airways via one of the nostrils, then 50 ml of saline (0.9%) solution is instilled in the middle lobe, this instillation process is repeated 5 times for a total of 250ml of saline solution, though not all fluid is recovered. The samples are then without delay put on ice and transported to the laboratory, then processed, aliquoted and stored in -80 or -150 until further use. BAL cell count is performed by separating the cells from the supernatant, then fixating the cells on cytospin slides with cells subsequently stained before determining the differential count by microscope. For **all projects** the samples were processed in the same way, with minimal freeze thaw cycles. At processing, the samples were thawed in cold water and then centrifugated at 4000/g using ultrafiltration tubes (Amicon Ultra-4, 3kDa, Cork, Ireland)

8.4 MULTIPLEX BEAD ARRAY

The method primarily used in this thesis is an immunoassay based on multiplex array techniques; these techniques emerged in the beginning of the 2000s, initially only being able to analyze a few samples at a time (186, 187). Since then the capacity has greatly increased and it is now theoretically possible to run the entire proteome (188).

The major benefit of these microarray technologies is the opportunity to screen large ranges of antigens on planar arrays, using an unbiased selection of antigens, in combination of extended analysis with the bead-based assays.

In our work we used multiplex bead array analyses in **project I, II and IV**, in which color-coded beads are coupled with either antigens or antibodies and then incubated with biotinylated patient samples. The samples can be analyzed by a reader to generate semi-quantitative results for statistical analysis (189). A schematic overview of the procedure can be viewed below (Figure 8). In this set-up antibodies are coupled with the beads in order to detect antigens in patient samples (as done in paper II) (190), the method can be turned around and the beads can be coupled to proteins in order to detect autoantibodies in the sample (as done in project I) (189).

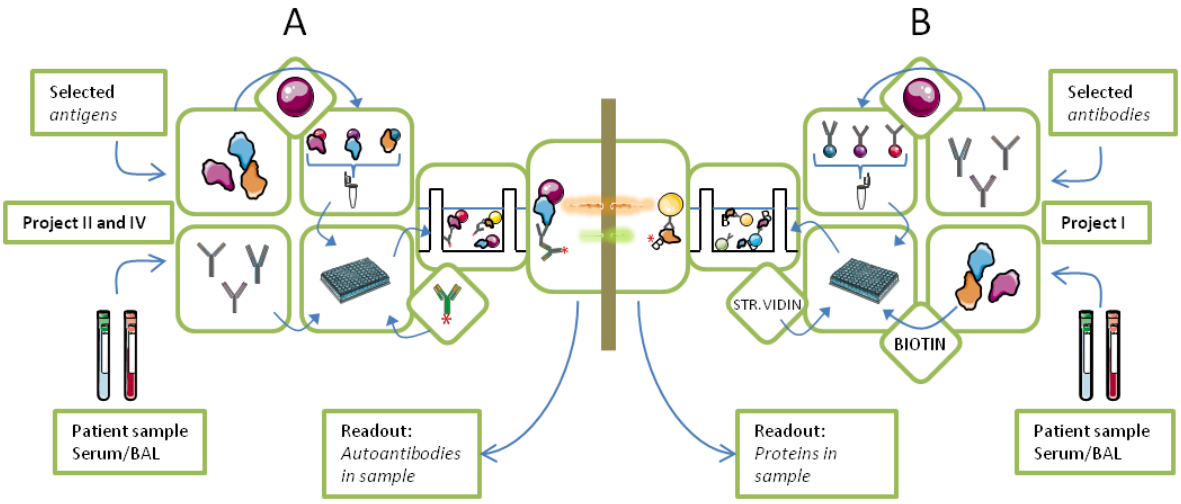

Figure 8. Schematic of the mSBA method. Autoantibody detection: A) Patient samples are aliquoted into plates; simultaneously selected antigens are coupled to microscopic color coded magnetic beads with an identifiable ID; bead-coupled antigens are pooled; the pooled antigen stock is added to each sample on the plate separately; a phycoerythine labeled anti-IgG is added to each well on the plate; following incubation each sample is fed through a reader detecting the simultaneous presence of bead/antigen connected to sample antibody and the anti-IgG antibody. Thus, *each patient is tested for presence of the autoantibodies of interest*. **Protein detection:** B) Each patient sample is aliquoted to a well; all patient samples undergo biotinylation; simultaneously; antibodies (ab) towards proteins of interest are aliquoted to individual wells and coupled to magnetic beads with an identifiable ID; aliquoted ab are pooled; together with streptavidin, patient samples are incubated individually with pooled ab; each sample is run separately. Detection similar to (A), subsequently, *each patient sample is tested for presence of the proteins of interest*. STR.VIDIN - Streptavidin

8.5 PULMONARY SPIROMETRY

Patients in all papers go through lung function tests, spirometry, to assess their pulmonary status. Certain parts of a spirometry are more relevant to certain patient groups. For example forced expiratory volume under 1 second (FEV1) is more relevant to asthmatic patients since it measures obstructivity in the small airways, whereas the diffusing capacity for carbon monoxide (DLco) is more relevant to IPF patients, since excess pulmonary fibrosis impairs gas exchange

8.6 LC-MS/MS

In mass spectrometry the protein/peptide samples are analyzed through the ratio between mass and charge. This method can be used both for wide detection of many proteins as well as analysis of specific protein targets. In **project III,** liquid chromatography tandem mass spectrometry (LC-MS/MS) was applied in a shot-gun approach in order to characterize the Fc-glycan part of the IgG in serum and BAL fluid of sarcoidosis patients and healthy controls(182).

8.7 ELISA

Enzyme-linked Immuno-sorbent Assay (ELISA) belongs to the category of immunoassays, similar to the bead array method. In contrast to mSBA, ELISA is not a high throughput method, however, several commercial kit towards virtually all proteins are readily available. In **Project I** we utilize a sandwich ELISA-kit, as per manufacturers protocol (DY279, R&D), to detect presence of autoantibodies towards CCL2 and for validation of previous results from the mSBA method.

8.8 STATISTICAL ANALYSES

For **all papers,** univariate analyses were performed using Student's t-test, with equal or unequal variance conditioned by F-test.

P-values were corrected using FDR concordant with total number of comparisons. Linear regression analysis was performed for the correlation between BAL and serum. Correlation between variables and clinical factors were obtained using two-tailed Pearson or Spearman correlation (depending on normality test). Statistical software from Graph Pad Prism (Graph Pad Prism, GraphPad Software Inc., LaJolla, USA) was used to do analyses for **paper I-IV**. In **paper III**, Multivariate modelling using Principal Component Analysis (PCA) and Orthogonal projections to latent structures discrimination analysis (OPLS-DA) was done using SIMCA 14.0 with mean centring, UV scaling, and log transformation. Performance of the model was described through cumulative correlation coefficients (R^2), and the predictive ability (Q^2) through seven-fold cross validation calculations and *p*[CV-ANOVA]). In **paper III** the data was normalized by GlcoAge index.

For **paper I and IV,** in order to compare the sample groups, the readout intensities from the planar and bead-array analyses were converted into binary variable based on reactivity frequencies for each sample, i.e. the sample was determined to be reactive or non-reactive. Correction for multiple testing was applied through false discovery rate (FDR) in accordance with the Benjamini-Hochberg method (also in **paper III**). In **paper I and II**, receiver operating characteristic (ROC) was used to discern sarcoidosis patients from healthy individuals by relative antibody intensities.

Different methods were applied for **paper II** and **paper IV** when it came to determine reactivity, and the use of reactivity cut-offs. **Paper II** was initiated by screening from planar arrays, and a sample median was determined for each batch. After which a high cut-off value was set to sample median + 20x median absolute deviation (MAD) and a low threshold of sample median + 10x MAD. Antigens were selected for further analysis if two or more sample met the high threshold and/or if four or more samples met the low threshold. In **paper IV** an antigen specific cutoff was assigned using the distribution of resulting reactivities across a selection of possible MAD cutoffs by determining where the slope is nearing a local minimum beside the highest peak (i.e. where the curve flattens out after the highest point). Figure 9 illustrates both methods; the implications of the two strategies are discussed in greater detail in "Result and discussion" section of **paper IV.**

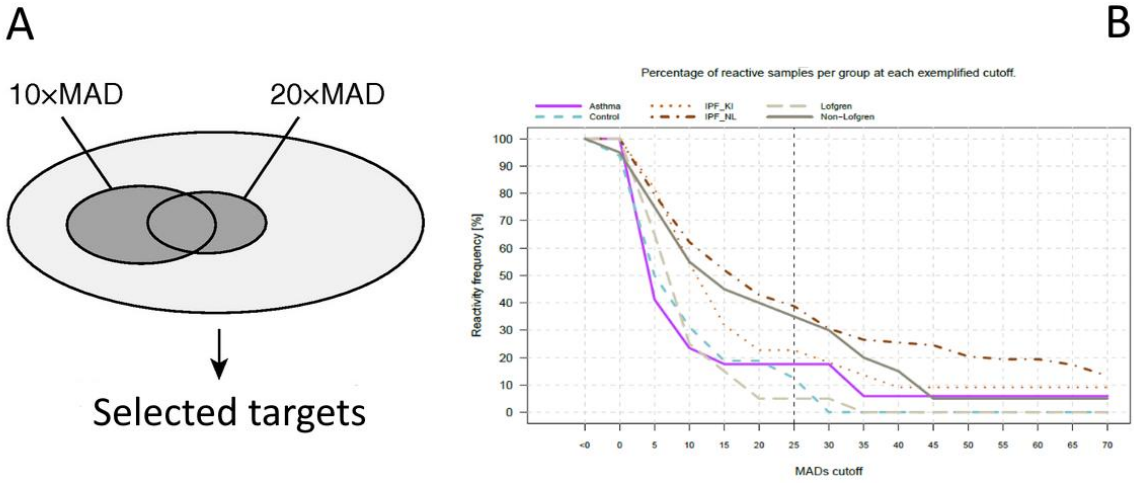

Figure 9. Overview of the methods to determine reactivity. A) From paper II, after screening two thresholds were determined. The higher threshold was set to sample median + 20xMAD and a lower threshold of sample median + 10xMAD. For an antigen to be reactive, either two samples had to meet the higher criteria, or four samples had to meet the lower criteria. B) An antigen specific cutoff was assigned using the distribution of resulting reactivities across a selection of possible MAD cutoffs by determining where the slope is nearing a local minimum beside the highest peak (Representative example). MAD- median absolute deviation.

9 RESEARCH AIMS

In this thesis we were investigated the role of autoantigens, autoimmune antibodies and immunopeptides in two interstitial diseases, sarcoidosis and idiopathic pulmonary fibrosis (IPF). The diseases do not have a completely known pathophysiology or aetiology. We hypothesize that identification of specific antigens can help elucidate of the pathogenesis of sarcoidosis as well as of IPF, and in the long perspective this could lead to therapeutic targets.

The aims are therefore to:

- Identify key markers of inflammatory responses in sarcoidosis and IPF
- Identify new immune targets (cells, cytokines) for possible treatment.
- Identify peptides (externally derived or auto-antigens) implicated in sarcoidosis or IPF.
- Generate targets for functional test/antigens capable of stimulating T-cells derived from patients with interstital disase

10 RESULTS AND DISCUSSION

10.1 PROJECT I

Sarcoidosis Patients Display Elevated Levels of FN1 and CCL2 in BAL Fluid

In this project we set out to investigate the protein profile of sarcoidosis patients in order to find proteomic targets associated with disease and/or inflammation.

We used the mSBA platform in BAL samples to analyze antibodies targeting selected proteins from the literature. In addition to the two sarcoidosis groups (LS and nLS), BAL fluid from asthmatics and healthy individuals were included for extended reference.

Initial protein concentration analyses showed a significantly higher concentration for the processed BAL samples of sarcoidosis patients compared to both healthy controls and asthma patients ($p<0.0001$). In addition, there was also a prominent diversity in concentration throughout all samples ranging from 0.2 to 9.8 mg/ml.

Following analysis of 68+ 249 BAL samples from sarcoidosis, asthma and healthy controls by multiplex bead array, fibronectin 1 and C-C motif chemokine 2 (CCL2) were the two proteins with the highest significance. These proteins had significant elevated presence in the sarcoidosis cohort compared with controls ($p<0.001$).

In order to verify the results for FN1 and CCL2 from the first assay, the protein levels were explored using the original bead array with an additional independent antibody, together with a sandwich immunoassay on the bead array. The results from the three assays confirmed the original observation and can be viewed in figure 10.

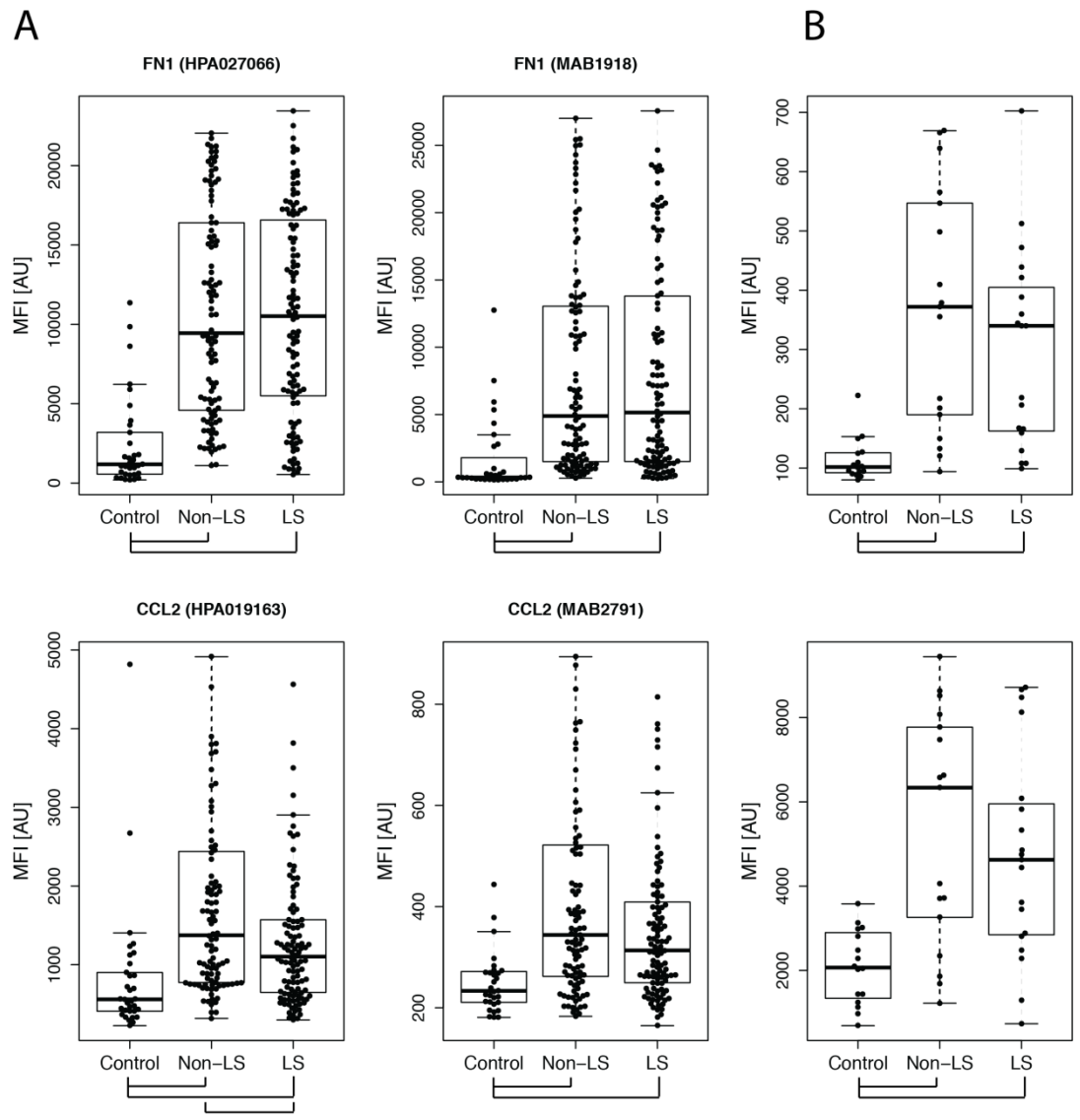

Figure 10. FN1 and CLL2 levels in BAL. A) Elevated concentration of FN1 and CCL2 were discovered in BAL from sarcoidosis patients compared to controls. B) Sandwich immunoassay showing verified results from figure (A) Outliers were omitted from the figure for illustration purpose, significant differences between groups indicated ($p < 0.05$). Adapted from (191)

While the quantity of FN1 and CCL2 in BAL fluid was elevated in sarcoidosis patients in contrast to controls and asthma patients, the measured protein levels were unable to separate the LS subgroup against the nLS subgroup. Unlike the BAL samples, analysis of serum samples yielded no statistical significant difference between the cohorts with regard to protein levels.

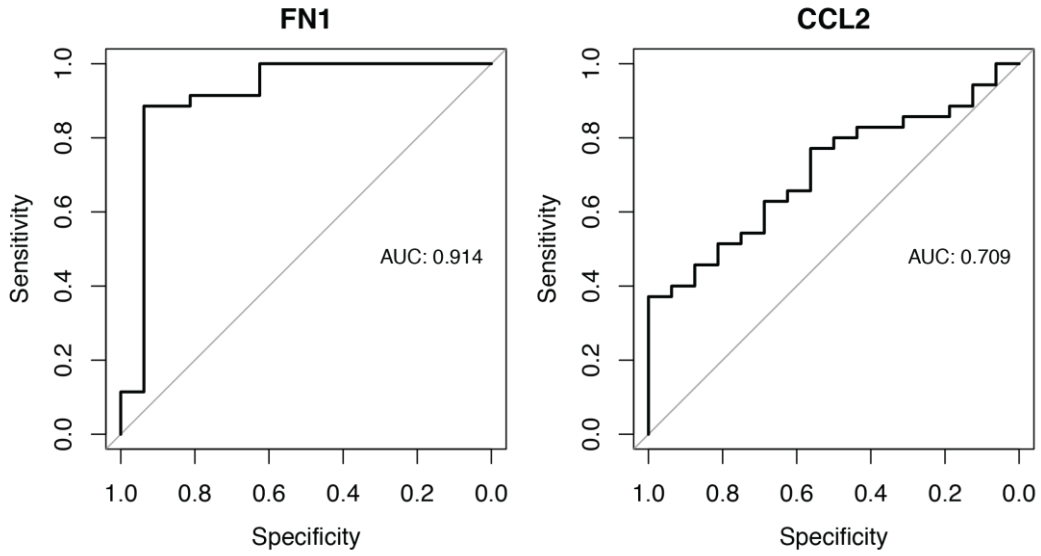

Figure 11. **ROC analysis of FN1 and CCL2.** In order to establish if the levels of FN1 and CCL2 would separate sarcoidosis from controls, ROC analysis was carried out. Results indicated area under the curve (AUC) of 0.91 for FN1 and 0.71 for CCL2. (190).

In order to evaluate if the levels of FN1 and CCL2 would separate patients with sarcoidosis from healthy controls, a ROC analysis was executed. Results showed an area under the curve (AUC) of 0.91 for FN1 and 0.71 for CCL2 (Figure 11).

In addition to the proteins found to differ between sarcoidosis and controls, the protein cadherin (CDH5) correlated with BAL findings. The CDH5 protein had a positive correlation with the percentage, as well as cell count, of lymphocytes in BAL (Spearman's ρ 0.55 and 0.71)

Discussion of project I

Here we set out to investigate the prevalence of proteins in serum and BAL from sarcoidosis patients in order to find sarcoidosis associated proteins. Even though sarcoidosis is viewed as systemic disease, over 90% of patients have manifestations in the lung; therefore, using BAL is likely the most suitable approach for identifying proteins in this condition. Several proteins were also discovered with elevated levels in BAL of sarcoidosis patients compared to controls. Though these differences were not represented in serum samples, indicating that even though sarcoidosis is a systemic disease, the lung is the primary site for the active disease.

Two proteins (FN1 and CCL2) stood out with the most significant differences between BAL and controls. Elevated levels of fibronectin have previously been demonstrated in BAL fluid from interstitial lung diseases including sarcoidosis (192-195). The previous studies used ELISA, and the overlapping results between these studies and our data suggest that the bead array assay is appropriate for analyses in BAL. Moreover, the similar levels of FN1 in serum between sarcoidosis patients and controls corresponds to earlier findings in plasma (194). The current opinion considers increased levels of fibronectin in BAL as an indicator for inflammation in the lung, more so than a disease specific response of sarcoidosis patients (192, 196, 197).

We discovered elevated levels of CLL2 in BAL of sarcoidosis patients compare to controls, these findings are concordant with previous studies where CCL2 as well as CCL5 was in BAL in all sarcoidosis stages (198).

We were unable to detect differences in serum levels of CCL2 between cohorts, this finding is consistent with earlier papers (199). Neither could the prevalence of any protein in the study distinguish the LS and nLS subgroups. This is fairly unexpected since LS and nLS have distinct clinical phenotypes and genetic variation (2). Nevertheless, this project presents possible protein targets for further investigations in pulmonary disease.

10.2 PROJECT II

Profiling of the Autoantibody Repertoire in Sarcoidosis

In this project we set out to characterize and relate the antibody repertoire between sarcoidosis patients and healthy controls.

Results from the planar arrays demonstrated a high heterogeneity between individuals when it came to antigen reactivity, where some samples were only reactive towards a few antigens and others demonstrated reactivity to several. Even after converting the sample intensities into binary variable by applying cut-off criteria (see method section), i.e. each sample was determined to be reactive or non-reactive for each antigen, a major individual variation persisted, although a majority of the reactivities were confined to single individuals (Figure 12A). A noticeable variation between individuals was detected when compiling the total sum of reactivities, ranging between 0 to 200 antigens per sample. A trend was observed where the sarcoidosis patients displayed a higher total reactivity frequency compared to controls. This was particularly evident for the nLS group vs controls, where a significant difference could be observed (p=0.05) (Figure 12B).

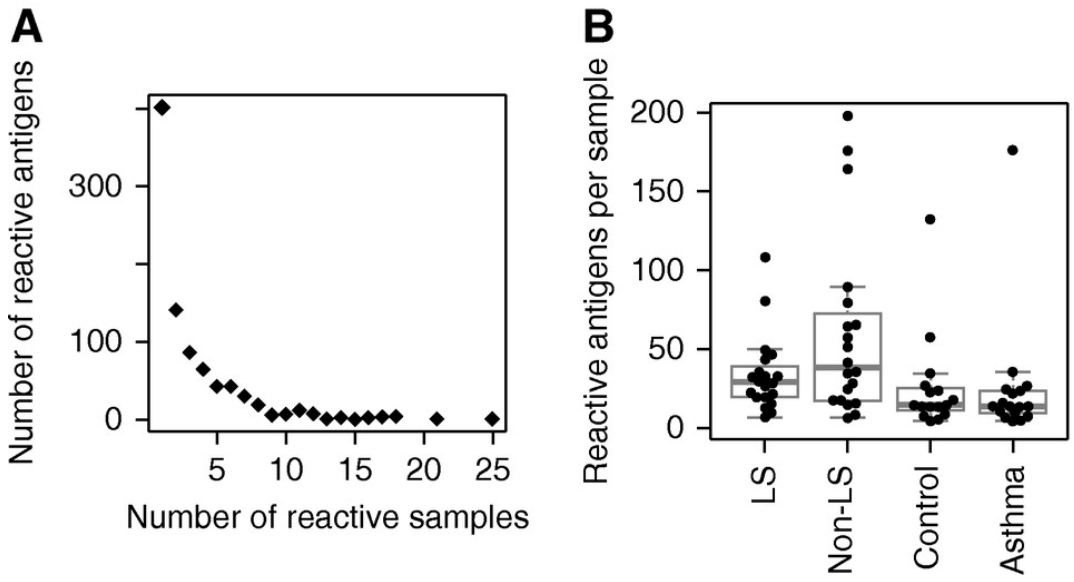

Figure 12. Screening and antigen selection from planar arrays. (A) Reactivity profiles for all samples groups revealed a major individual variation, although a majority of the reactivities were confined to single individuals. (B) A plot displaying total number of reactive antigens per group. Sarcoidosis patients demonstrate a higher total reactivity frequency compared to controls, particularly non-Löfgren patients, which compared to controls had a significantly higher total reactivity frequency. ($P < 0.05$). Adapted with permission of the American Thoracic Society. (189)

By calculating and comparing reactivity frequencies between cohorts, a set of four antigens were of specific interest. The set consisted of Zinc finger 688 (ZNF688), Mitochondrial ribosomal protein L43 (MRPL43), Nuclear receptor coactivator 2 (NCOA), Adenosine diphosphate–ribosylation factor GTPase activating protein 1 (ARFGAP1).

ZNF688 and MRLP43 displayed the highest difference between sarcoidosis patients and healthy controls. ZNF688 demonstrated reactivity in 71% of the sarcoidosis patients, 45% in healthy controls and 53% in patients with asthma. The ZNF 688 antigen was also the target with the highest total reactivity throughout all samples with 56% reactivity frequency. For MRLP43 the reactivity in sarcoidosis patients were 12% compared with 2% in the healthy controls and no reactivity in the asthma cohort. A difference could be observed when comparing LS and nLS where 14% of LS patients demonstrated reactivity towards MRLP43 while only 2% of the nLS did (Figure 13).

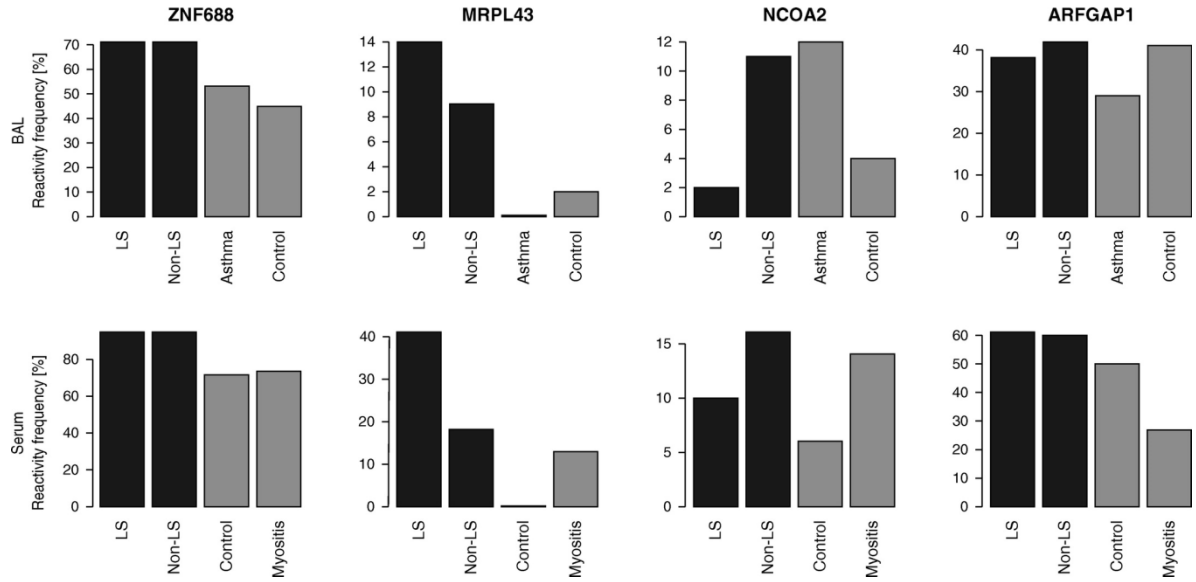

Figure 13. **Summary of reactivity frequencies**. Zinc finger protein 688 (ZNF 688), Adenosine diphosphate–ribosylation factor GTPase activating protein 1 (ARFGAP1), Nuclear receptor coactivator 2 (NCOA2), Mitochondrial ribosomal protein L43 (MRLP43), Löfgren syndrome (LS). non-Löfgren syndrome (Non-LS). Adapted with permission of the American Thoracic Society. Copyright © 2021 American Thoracic Society. All rights reserved.

Conversely, a stronger difference between reactivity frequency of LS and nLS was observed for the protein fragment of NCOA2. For NCOA2 only 2% of the LS patients displayed reactivity compared to 11% in nLS patients. In addition to the other findings, ARFGAP1 also displayed high reactivity throughout all cohorts.

Receiver operating characteristic (ROC) was performed for reactive samples from ZNF688 and ARFGAP, where ARFGAP was the antigen with high reactivity across all cohorts. Area under the curve (AUC) analysis showed values of 0.79 for ZNF688 and 0.76 for ARFGAP1 signifying that ARFGAP, despite similar reactivity between cohorts, could distinguish patients from healthy controls comparable to ZNF688 based on the relative intensity levels (Figure 14). In addition, both antigens showed significant difference in a two-group comparison between controls and sarcoidosis patients where both displayed a significant p-value of < 0.01.

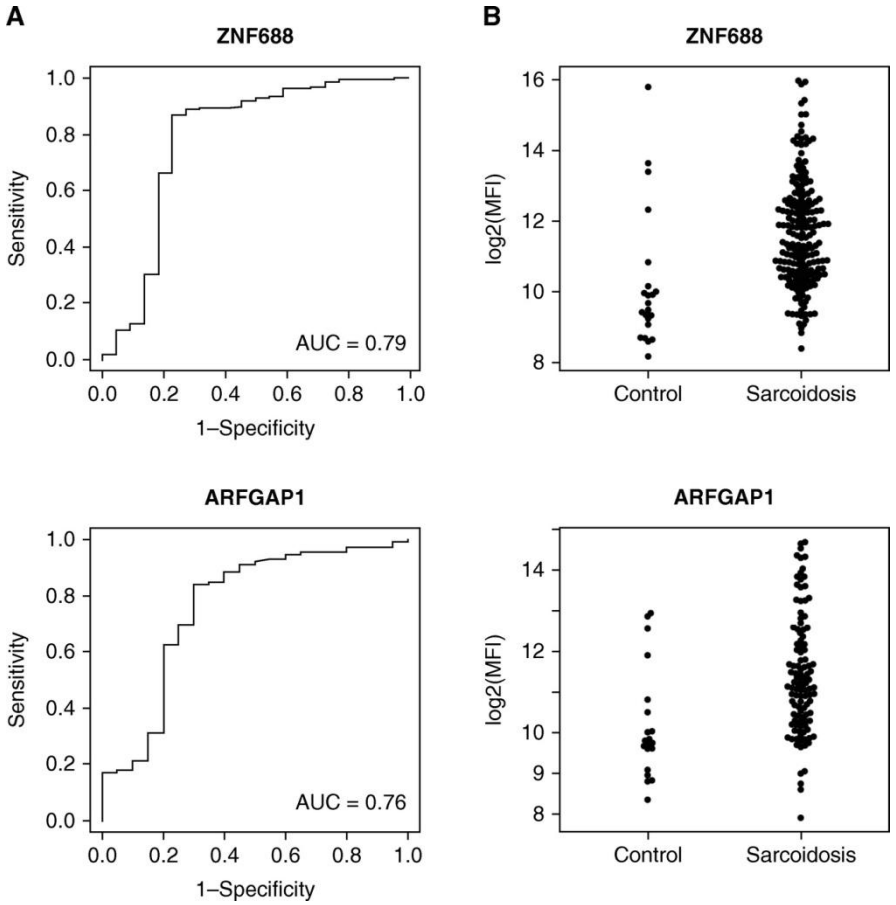

Figure 14 .**Sample classifications.** (A) ROC analysis using only reactive samples demonstrating relative intensity levels of antibodies toward zinc finger protein688 (ZNF688) and adenosine diphosphate–ribosylation factor GTPase activating protein 1 (ARFGAP1). (B) Levels of reactive antibodies towards ZNF 688 and ARFGAP1 in samples from patients with sarcoidosis compared to healthy controls. Both antigens with P < 0.001. Area under the curve (AUC), Median fluorescent intensity (MFI). Adapted with permission of the American Thoracic Society. Copyright © 2021 American Thoracic Society. All rights reserved. (189)

Discussion of project II

Despite that the pathophysiology suggest a cellular mediated immune response through CD4+ helper cells, there are indications of autoimmunity, or autoimmune components in sarcoidosis. One is the proposal of Vimentin as an autoimmune target (200), to add to the discovery of the four targets in our paper. Another is the concurrence of sarcoidosis with other autoimmune disorders (201), where there is one autoimmune disease there can be more (202). Another, slightly contradicting, but interesting fact is that most if not all people demonstrate the ability to produce autoantibodies, however, far from all subjects have a disease. It highlights the importance to separate autoimmunity with the presence of autoantibodies; presence is not equal to function.

When comparing the antibody reactivity profiles in our study there was a considerable variability, most of the antigens only exhibited reactivity in single individuals. This is coherent with previous findings which has stated strong individual variation patterns (12, 21).

In this type of hypothesis generating set-ups with a high throughput system there is a higher likelihood of discovering differences between cohorts, than compared to a hypothesis driven one. When performing multiple testing, statistically, you increase the chance of finding significant differences. However, the differences might a product of repeated testing and thus not accurate. Hence it is important to correct for the increased probability of finding significant differences. The trade off is that true differences could be masked if a too harsh method is applied; in our investigation of these antigens the results were not statistically significant after correcting the P-values for multiple comparisons using false discovery rate (FDR).

The sarcoidosis group, and nLS cohort in particular, demonstrated a higher total number of reactivities compared to controls and asthma patients, potentially reflecting a more prominent inflammatory state, though this group has previously been described with hypergammagloublinemia (203). Furthermore, increased levels of IgG and IgM secreting cells in BAL has been observed in sarcoidosis patients compared to healthy controls, however there was no difference in serum (203). Patients with asthma were more similar to healthy controls, with regard to total number of reactivities, than to sarcoidosis patients. It is important to note that these patients were mild allergic asthmatics out of pollen season;

therefore it is likely that the asthmatics were in a "steady state". It also important to note that, in this paper, the reactivity definition is based on a cut-off values that can be varied and thus yield altered frequency levels and outcomes. The method in this paper differs from that of project IV which uses a statistical model to determine the cut-off levels for each sample.

This paper set out to profile the autoimmune repertoire in patients with sarcoidosis with regard to both BAL and serum. The paper indentified four potential autoimmune targets that were present in both BAL and serum. Particularly ZNF688 and ARFGAP1 demonstrated significantly higher reactivities compared to controls.

10.3 PROJECT III

Altered Fc Galactosylation in IgG4 in Serum for Chronic Lung Diseases

We explored the possibility of Fc glycans from BAL and serum to associate with pulmonary inflammation, as well as the correlation of glycosylation status between BAL and serum.

Multivariate analyses were carried out in order to explore the general trend, as well as the correlation within each individual, between serum and BAL profiles. A principal component analysis (PCA) demonstrated a separation (R^2=0.46, Q^2=0.35), where the two main proponents behind the separation could be attributed to differences in BAL versus serum in addition to individual-specific differences when it came to cohort with or without the combination of age.

For further study of which Fc glycans were propelling the separation between BAL and serum, an orthogonal projection to latent structures- discrimination analysis (OPLS-DA) was performed. The OPLS-DA analysis included all sample groups, and the successfully separated BAL from serum with a robust model (R^2=0.90, Q^2=0.85, p=1.33×10−26 by cross-validated (CV)-ANOVA)). In the glycan screening, 31 Fc glycans variants out of a 64 total correlated with 95% confidence in serum while only 5 variants correlated with the same confidence in serum. This would suggest a more variable IgG composition in serum compared to BAL with regard to Fc glycan expression.

When investigating Fc glycan differences between patient subgroups and controls, OPLS-DA analysis was able to discern between healthy controls, LS and nLS (R^2=0.28, Q^2=0.24, p=4.4×10^{-12}). In this analysis, controls and LS patients had the biggest difference, while nLS was positioned in between (Results shown as: mean±SD tcv[1]; Controls=2.9±2.4, LS=−0.9±2.1 and nLS=−2.1±2.1). Here nLS seems to distance itself further from healthy controls than LS, interestingly since LS is usually viewed as the more active disease compared to nLS which is of a more chronic character.

We used the major agalactosylated form (more proinflammatory, FA2) and the major galactosylated (less proinflammatory, FA2G2), to investigate the correlation between serum and BAL for the ratio of log(FA2/FA2G2) for each sample (Figure 15). The log(FA2/FA2G2) ratio correlated particularly well for IgG4 in all groups (R^2all=0.95), while the other IgG subclasses had inferior scores; IgG_1(R^2all=0.87) IgG_2/$_{(3)}$ (R^2all=0.76).

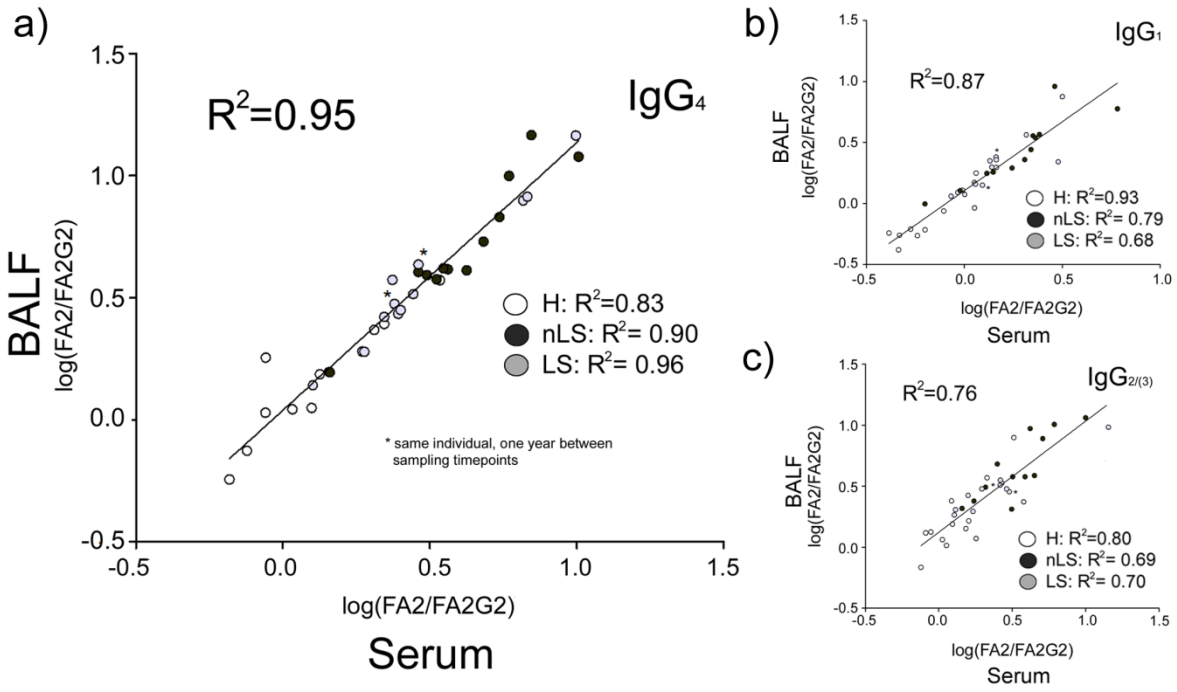

Figure 15. Intra-individual correlations between BAL and serum. Graphs showing the correlation between BAL and serum using the logarithmic ratio of the agalactosylated form (FA2) and the galactosylated form (FA2G2) for each sample. a) IgG4 (R^2=0.95), b) IgG1(R^2=0.87) and c) $IgG_{2/(3)}$) (R^2=0.76). H - Healthy controls; nLS - non-Löfgren's syndrome; LS - Löfgren's syndrome. #: Dual data-points for individual who underwent bronchoscopy twice with a year in between BAL samplings.

The levels of truncated or agalactosylated Fc glycans increases with age, additionally, the increase is also not linear. So in order to examine how much the difference in galactosylation is due to disease and not age, and to correct for the age difference between cohorts, we normalized our data with the help of GlycoAge index (204). After GlycoAge index normalization, log(FA2/FA2G2) of IgG_4 stayed significant both in BAL ($p=1.1\times10^{-2}$) and serum($p=1.6\times10^{-2}$).

To examine whether log(FA2/FA2G2) of IgG_4 is disease specific to sarcoidosis or not, we added serum samples from severe asthmatic (SA) patients and previously published Fc glycan data from RA patients to the analyses. The results from comparisons to the aforementioned groups (LS and nLS) evidently demonstrated that increased age-corrected IgG_4 log(FA2/FA2G2) ratio is not specific to sarcoidosis. The analyses showed that most of the SA patients had very high log(FA2/FA2G2) IgG_4 ratios in serum (0.73±32). The RA group scored lower log(FA2/FA2G2) ratio (0.37±26) than sarcoidosis and asthma patients, but still slightly higher than healthy controls.

A significant difference in log (FA2/FA2G2) ratio in serum was observed between healthy with RA patients and the three cohorts with pulmonary inflammatory disorders (LS, nLS and SA) ($p=9.2x10^{-4}$) (Figure 16). Based off these results we propose that age-corrected log(FA2/FA2G2) ratio in serum can be separated in low, medium and high-range values (⩽0.3, 0.3–0.6, ⩾0.6), where the high-ranged values suggest strong inflammatory activity in the lung. (Figure 16a and 16b).

In addition, using ROC analysis, the GlycoAge corrected log(FA2/FA2G2) ratio of IgG_4 in serum could separate sarcoidosis and severe asthmatics (SA) vs. healthy and RA subjects with a mean ± standard error (SE) AUC of 78±6%. When clustering the more chronic lung disorders (nLS and SA) and comparing them to healthy and RA, the AUC increased to 83±6%. Furthermore, when only comparing against healthy controls the AUC increased to 90±5% for the nLS + SA group (Figure 16).

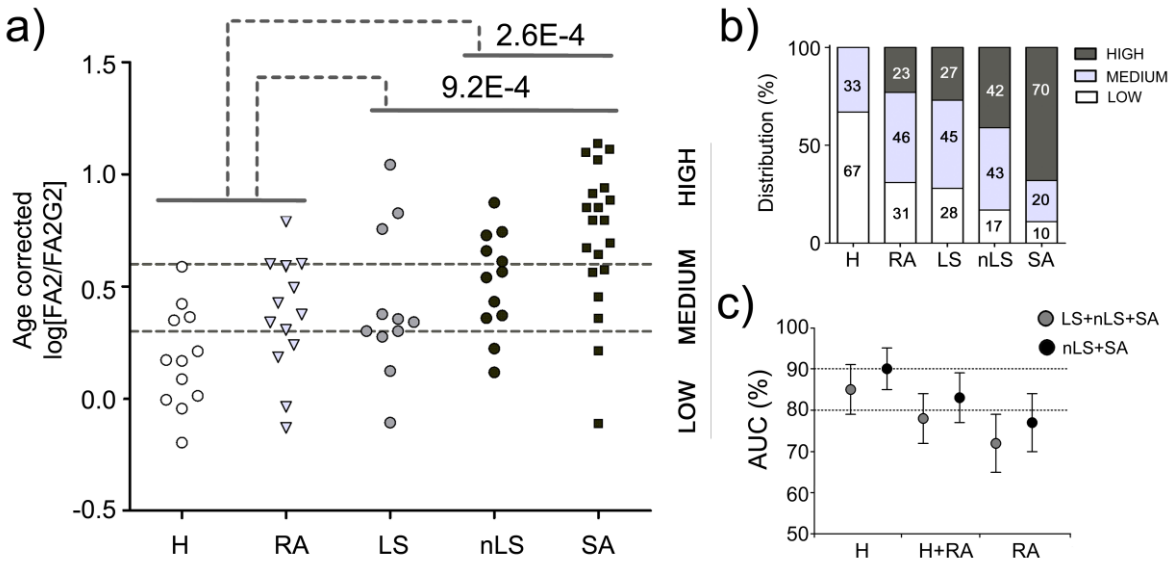

Figure 16. Ratios for log(FA2/FA2G2) across all groups. a) Age-corrected serum log(FA2/FA2G2) ratio of IgG4 for healthy controls (H), rheumatoid arthritis (RA), Löfgren's syndrome (LS), non-LS(nLS) sarcoidosis and severe asthma (SA). Dotted lines indicate thresholds for low- (⩽0.3), medium- (0.3–0.6) and high-range values (⩾0.6). b) The disposition (%) for each sample group according to the low-, medium-, high-value ranges. c) AUC values from ROC analyses demonstrating comparisons between sample groups with and without pulmonary diseases. Adapted from (46).

Discussion of project III

At times it can be challenging to differ between sarcoidosis and IPF, as stated previously, there are even cases of concurrent disease (168). Furthermore, neither sarcoidosis nor IPF have any good markers for prognosis that is universal for all patients. For sarcoidosis patients there is a connection to better prognosis if you express the DRB1*03 haplotype, or if you have high levels of AV2.3+ T-cells in BAL, though this is not applicable for everyone. Usually, invasive investigations are needed to be performed in order to acquire samples for analysis, though particularly bronchoscopy is generally viewed as an uncomfortable procedure by patients. A marker of inflammatory status that can be captured via a blood test would be of great benefit in clinical practice. By using the information of glycosylation status of different IgG subsets from our study, it would be possible to acquire information of inflammatory status in the lung from a standard blood sample.

In this paper we demonstrated that Fc galactosylation status of IgG in BAL and serum correlates closely, and remains significant even after correcting for age differences. The correlation was particularly strong for IgG_4 in patients with pulmonary disorders compared to controls and RA patients. Alterations in IgG Fc glycan domains has been reported in several studies, most notably regarding RA patients where the IgG_1 Fc glycans are more prominent but less galactosylated (35, 185, 205).

In this paper we successfully indentified IgG_4 galactosylation status as a candidate marker for chronic respiratory inflammation. This marker also demonstrate a high correlation between BAL and serum (R^2=0.95) and could be readily available through blood sampling. As it was designed as a pilot study the study cohort was drastically skewed when it came to age and sex differences between cohorts, though the sex matched RA patients exhibited no difference in galactosylation between men or women. With regard to age differences the study results were adjusted accordingly. Overall the project presents an interesting pathway for future biomarker investigations in Sarcoidosis.

Based on the results of project III we conclude that the Fc galactosylation status of IgG_4 could potentially be used as a serum marker for severity in chronic pulmonary inflammation.

10.4 **PROJECT IV**

High Throughput Screening and Comparison of IPF and Sarcoidosis Samples

The mSBA approach is used for both sarcoidosis and IPF samples in order to evaluate similarities and differences between fibrosis associated diseases.

When observing the reactivities across the entire set of samples, Zinc finger 688 (ZNF688), Collagen 5A1 (COL5A1), displayed the highest reactivity frequencies in BAL (84.7% and 73.3% respectively) and blood (26.2% and 15.1% respectively) Both heat shock protein A4 (HSPA4) (BAL 13.1% and serum 33.2%) and ADP-ribosylation factor GDPase-activating protein 1 (ARFGAP1) (BAL 15.0% Serum 40.7%) demonstrated high reactivity frequencies in both BAL and serum, though not as distinct as COL5A1 and ZNF 688 (Figure 17).

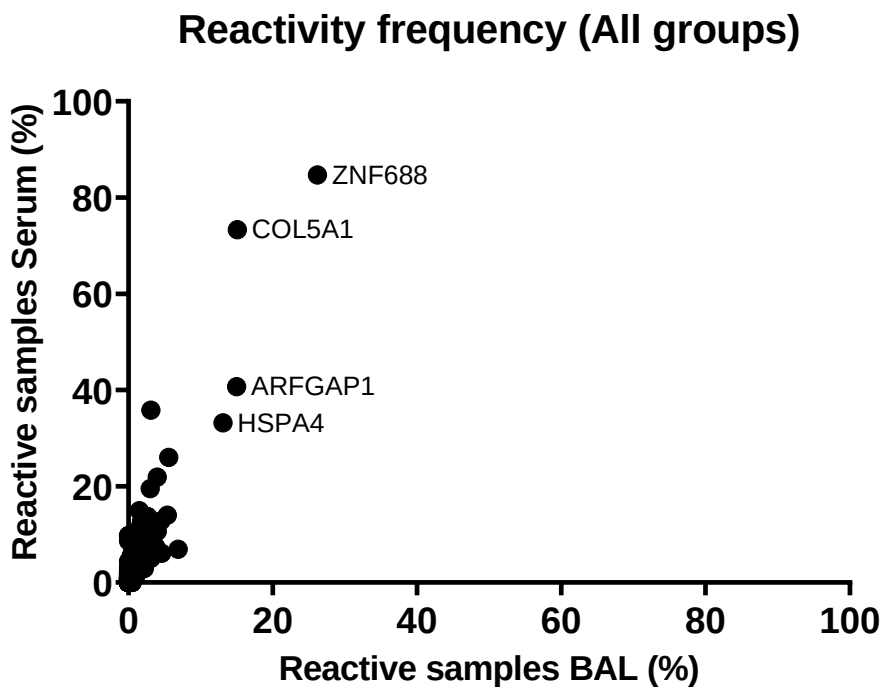

Figure 17. Overall reactivity frequency. Initial screening. Reactivity frequency (Percentage of reactive samples) by all groups towards all. Serum on the y-axis and BAL on x-axis.

When evaluating IPF and sarcoidosis patients, the COL5A1 target showed a statistically significant higher frequency of reactivity between both IPF and nLS compared to LS in BAL ($p<0.005$ and $p<0.05$ respectively, (Fig 18a).There were high reactivity in BAL for ZNF688, ARFGAP and HSPA4 for both LS and nLS, though no statistical difference could be distinguished between the groups (Fig 18b). For IPF the difference was also significant in serum ($p=0.007$), however, no difference could be observed in serum between LS and nLS ($p=0.49$). Also, no statistical difference could be discerned comparing IPF to nLS patients ($p=0.3$) in either BAL or serum (Figure 18).

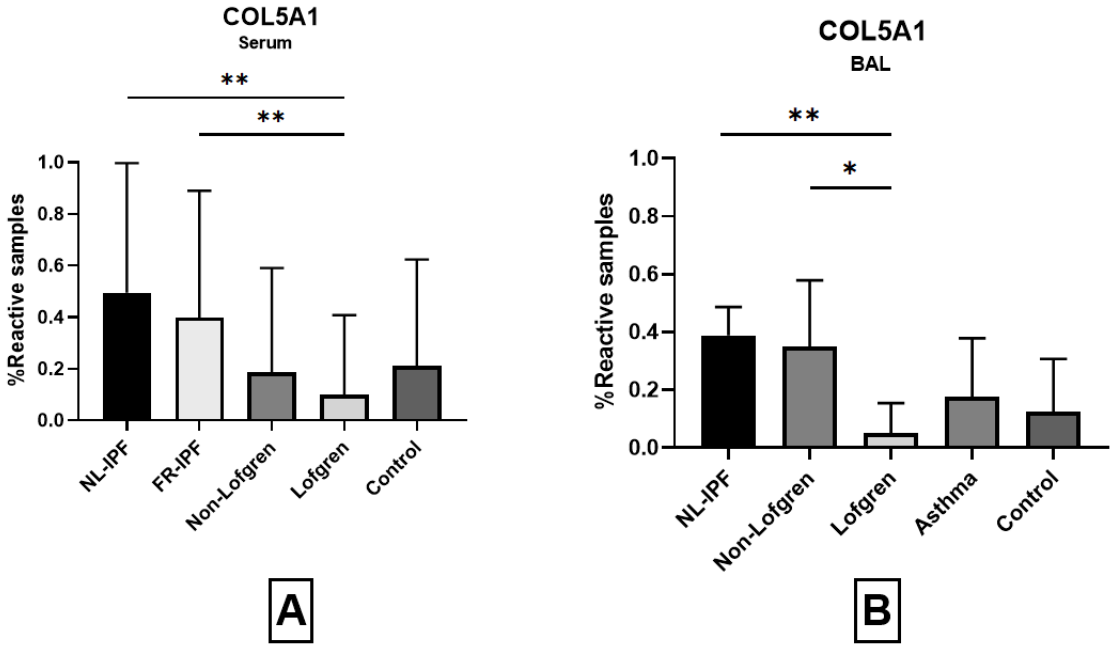

Figure 18. Reactivity frequency against COL5A1 in BAL and Serum. Comparison of the different cohorts. Line with** indicates significant difference between groups at $p<0.005$, a * indicates significant difference between groups at $p<0.05$

In addition to the IgG reactivity assays, an epitope mapping analysis of the COL5A1 protein fragment (Table 1 supplementary) was performed by means of peptide array. Results displayed clear heterogeneity in the reactivity between the compared groups, though peptide number 8 (Y Y T E G D G E G E T Y Y Y E) & 10 (G E G E T Y Y Y E Y P Y Y E D) (Supplementary Table 2) yielded high signals across all diseases, as well as healthy controls, in both serum and BAL (Fig 19 A-F). Despite only performed in serum, (Figure 19 A) results seem to indicate a preference of reactivity towards peptide number 1-3 in the French IPF cohort. For the matched BAL and serum samples of the Dutch cohort a clear overlap of preferred reactive peptides can be seen in Figure 19 B. The same can be observed for the unmatched cohorts of LS and nLS as seen in Figure 19 C and D.

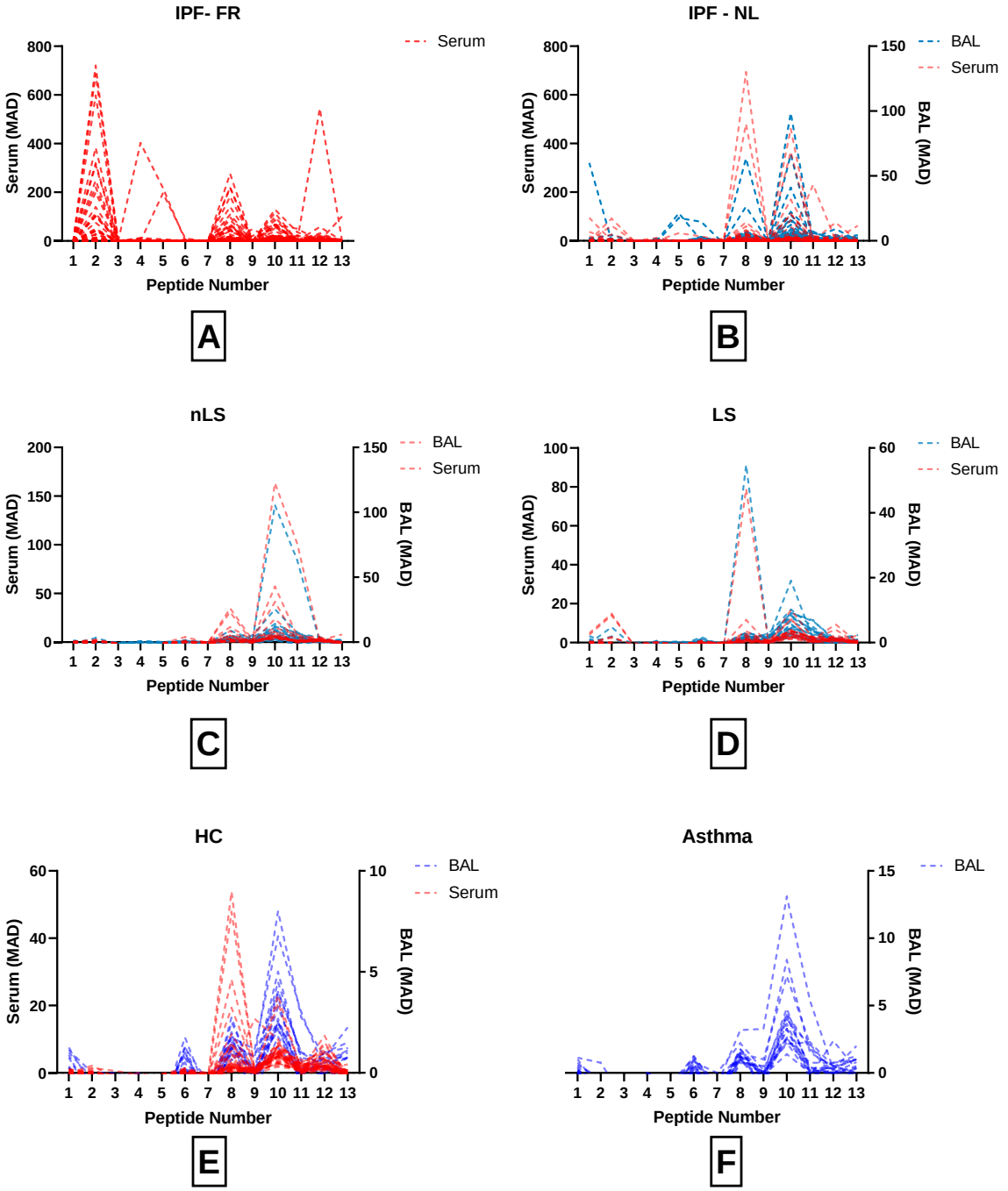

Figure 19A-F. COL5A1 epitope mapping analysis in Serum and BAL. Each graph represents a cohort with its corresponding MFI value (y-axis) for Serum (Left) and BAL (right) for each peptide (1-13) (x-axis). Figure F displays the asthma cohort for BAL only.

Discussion of project IV

In this study we investigated potential autoreactivity in serum and BAL samples from IPF and sarcoidosis patients by high-throughput microarray analyses. One of our main findings was that nLS sarcoidosis showed similarities to IPF regarding prevalence of autoantibodies in general and COL5A1 in particular. In fact, nLS were more similar to the IPF cohorts than to LS. This is interesting considering the tendency to develop fibrosis in both IPF and nLS, but not in LS nor the asthma or healthy controls. For the protein fragment of collagen 5A1 (COL5A1), a high reactivity frequency was found in both IPF and nLS but not in LS, further emphasizing the similarities between the two cohorts able to develop fibrosis.

When it comes to determining whether a sample is reactive or not, a different method was applied in this project compared to project II. In this project we assigned an antigen specific cutoff using the distribution of resulting reactivities across a selection of possible MAD cutoffs by determining where the slope is nearing a local minimum beside the highest peak (i.e. background peak)(Fig. 9). The advantage to use this method compared to setting subjective thresholds is that this method is unbiased, therefore you run less risk of inadvertently setting threshold that benefit your result, it is also adjusted for each individual antigen. The downside is that some antigens display a slow decline in reactivity distribution, which makes the initial background peak drawn out. This leads to very high cutoffs which run the risk of cutoffs being too strict and loose valid data point. Nonetheless, the method used in project IV appears to be more robust and works reasonably well.

IPF and nLS displayed higher levels and frequencies of auto-reactivity compared to all other diseases and controls, both in BAL and in serum. Of specific interest are the protein fragments representing ZNF688 and COL5A1 with significantly higher reactivity frequencies.

Anti-ZNF688 reactivity has been implicated in both SLE and sarcoidosis, as noted by previously published papers (189, 206). Furthermore, in lung tissue it is expressed solely by ciliated epithelial cells that are crucial for airway homeostasis and protection from extrinsic pollutants (189). Our results demonstrate a higher reactivity in IPF and nLS patients as compared to healthy controls and LS patients. This coincides with previous findings of increased anti-ZNF688 in sarcoidosis patients, though to our knowledge, ZNF688 has not previously been associated with IPF.

In contrast to ZNF688, COL5A1 has been implicated in numerous diseases and conditions, most notably Ehlers-Danlos syndrome (207) and adenocarcinoma metastasis (208). Furthermore, autoimmunity towards COL5A1 is suspected to play an important role in pulmonary transplant rejection and the occurrence of bronchiolitis obliterans (BOS) (162, 209). The HLA-DRB1*15 allele was overrepresented in a cohort of IPF patients who had undergone transplant, in addition, an association between HLA-DRB1*15 and COL5A1 autoantibodies was detected in pre-transplant IPF patients (162, 210, 211). This supports our findings where IPF patients demonstrated a significant higher frequency of autoantibodies against COL5A1. The connection to HLA-DRB1*15 is interesting since it links to sarcoidosis where the HLA variant corresponds to a worse prognosis and thus higher likelihood of fibrosis (1). With this in regard, it provides the possibility that early detection of autoantibodies against COL5A1 in patients with HLA-DRB1*15 could foreshadow future fibrosis development in IPF and/or sarcoidosis.

In our peptide analysis of the COL5A1 protein fragments, some heterogeneity was apparent; however there were clear overlaps where several peptides were present across all cohorts in both BAL and serum (Figure 19A-F). Potential cross-reactivity and genetic/social variability could explain the variations, however all IPF and nLS cohorts displayed reactivity to peptides within the COL5A1 fragment, further highlighting the potential of autoreactivity against COL5A1 in fibrosis development.

Autoantigens were found in a majority of patient samples, including healthy controls, although that cohort displayed a lower frequency of autoreactivity and also relatively lower titers. Altogether, autoreactivity was higher in IPF and in nLS patients compared to all other groups; both groups include a higher fibrosis rate than the other diseases potentially linking autoreactivity to fibrosis development. The protein epitope of COL5A1 is proposed as an autoimmune target and/or marker of fibrosis, and is of interest for further investigation.

11 CONCLUSIONS

In this thesis we have investigated the autoreactive range of antigens and antibodies in patients with sarcoidosis and IPF as well as control subjects, as well as various different control diseases. We discovered several autoreactive targets with potential autoimmune effects. Many of the targets have been present in both BAL and serum which provide a possibility of finding a marker that can monitor its disease from a blood sample, which would be beneficial and more comfortable for the patient.

Project I, II and IV can be categorized as semi-targeted, since the main feature is to generate interesting targets for further studies, but utilize a top-down approach where we select the proteins and antibodies that we are investigating. This generates a vast amount of data and the most difficult part is to decipher which target or what result actually matter and is not just an epiphenomenon. This is similar to a game of Tetris, where one has to figure out where all the pieces fit.

The main conclusions from this thesis are therefore;

- The immunopeptidomic profiles in BAL differs between sarcoidosis patients and control subjects.
- Non-Löfgren patients generally have a higher titer of autoantibodies than patients with löfgren syndrome.
- In comparison to controls, ZNF688 and ARFGAP1 displayed with significantly higher frequency of reactivity in sarcoidosis patients.
- Based on the results of project III we suggest that the Fc galactosylation status of IgG_4 could potentially be used a serum marker for level of inflammation in lung disease.
- With regard to autoantibody profiles, Non-Löfgren patients are more similar to IPF patients, rather than Löfgren syndrome patients.
- The protein epitope of COL5A1 is proposed as an autoreactive target and/or marker of fibrosis in IPF and non-Löfgren sarcoidosis, and is of interest for further investigation.

12 POINTS OF PERSPECTIVE

By investigating samples from IPF, sarcoidosis and controls we will gather a repertoire of autoantibodies displaying reactivity for each disease as well for healthy subjects. Both the mSBA analyses as well as the IgG-OMICS approach has revealed potential antigens/autoantibodies, however to test their function further studies need to be conducted. We collaborate with Professor Andrew Fontenot (University of Colorado) to generate hybridomas expressing TCRs of interest, i.e. identical TCRs of lung T cells of different patients. These hybridomas are produced to recognize peptides presented on the DRB1*03 molecule. T cell hybridomas will be used for screening a peptide library. Our studies may result in the identification of disease markers that could be suitable targets for immunotherapy and/or serve as disease markers.

It is also possible to use T-cell hybridomas as well as BAL cells expressing VA2.3+ peptides on TCRs, in stimulate by the candidate antigens as identified in our previous studies. Cytokine production and proliferative capacity will be analyzed by Fluorospot/Flow cytometry.

We intend to test if the identified protein targets are able to stimulate T-cells from the disease groups. One approach is to take antigens such as vimentin, ZNF688 or Col5A1 from patients with IPF or sarcoidosis and analyze on ELISpot in order to understand if generated autoantibodies and their corresponding autoantigen can stimulate production of, for example, interferon gamma as an indication that the antigen can elicit a function. Particularly Vimentin since such autoantibodies has been described for both IPF and sarcoidosis, in addition to COL5A1 which could benefit both disease groups if properly associated to fibrosis.

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