Prognostic factors in cystic fibrosis - the impact of antibody response and platelet activation

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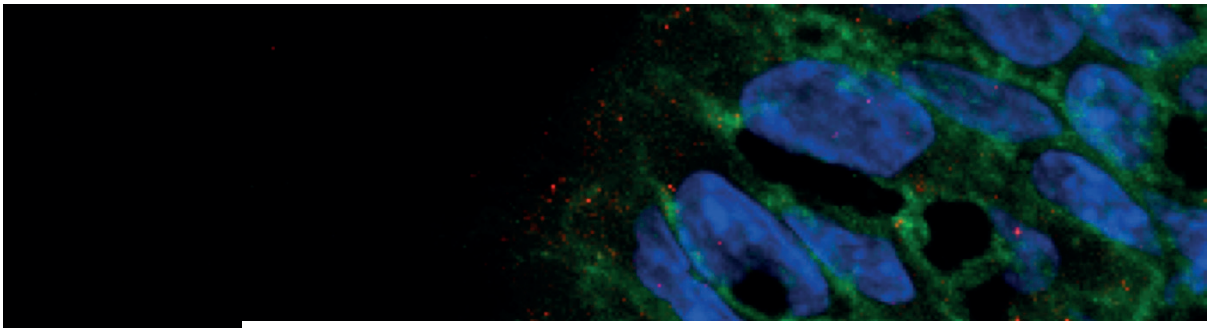

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ULRIKA LINDBERG
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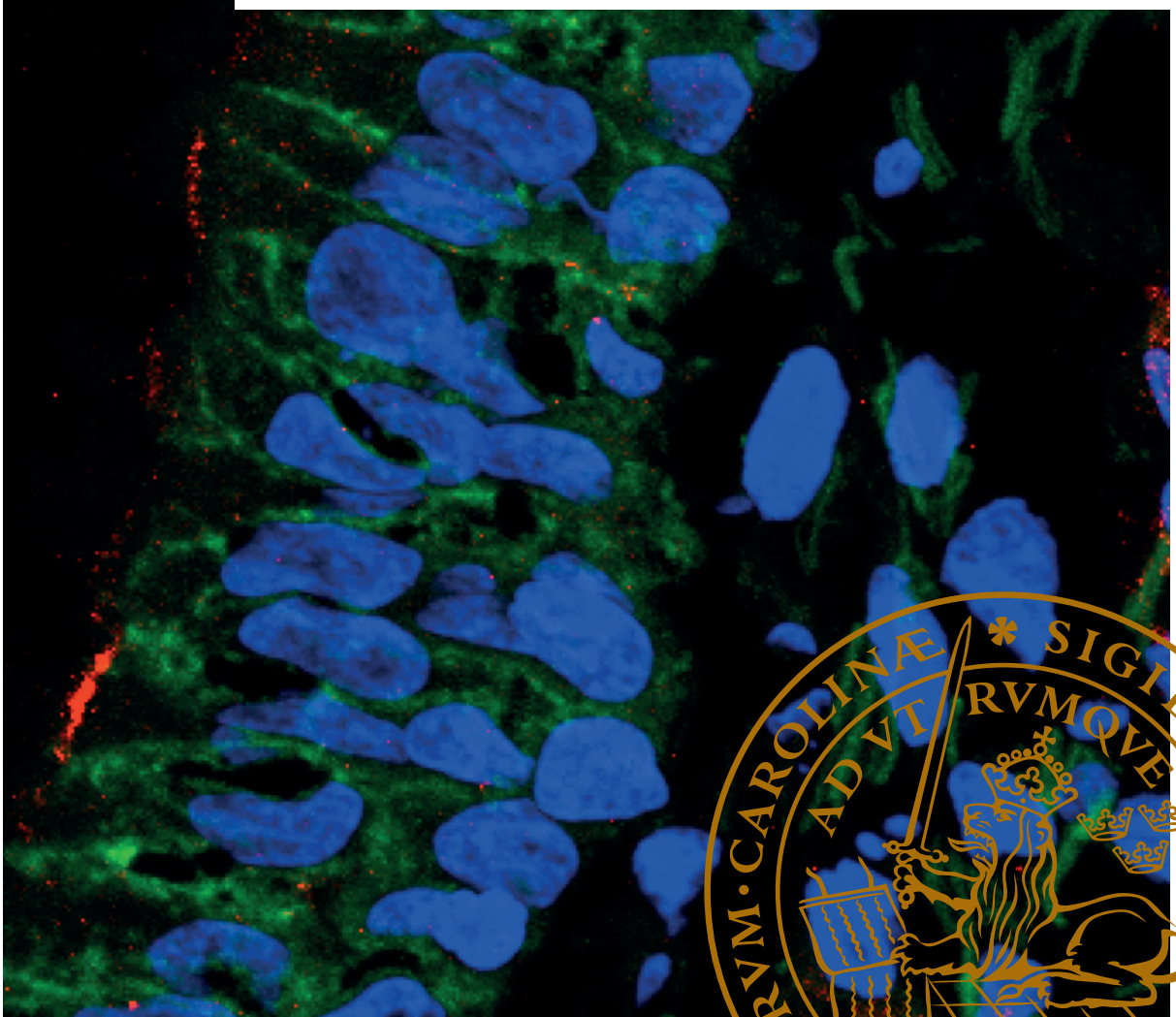

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Abstract

The prognosis in cystic fibrosis (CF) has improved dramatically over the last decades, but the clinical course is variable. Many factors influence prognosis, and colonisation with Pseudomonas aeruginosa is one of them. Earlier studies have shown that patients with P. aeruginosa who have developed anti-neutrophil cytoplasmic antibodies specific for BPI, bactericidal/permeability-increasing protein (BPI-ANCA), have worse prognosis. Platelets are involved in the immune response in acute and chronic inflammation, but their role in CF is not well studied. The overall aim of this thesis is to evaluate BPI-ANCA as a prognostic factor, investigate why BPI-ANCA is formed and investigate platelet activation in CF in general and in association with BPI-ANCA.

(I) Long time prognosis in 46 adult CF patients was studied. BPI-ANCA was measured at inclusion and evaluated, over a ten year period, together with lung function and P. aeruginosa colonisation. BPI-ANCA correlated significantly to outcome, stronger than colonisation status. Lung function at inclusion was also a very important predictor.

II): The relation between BPI-ANCA and three P. aeruginosa serology tests was investigated in 117 CF patients. There was only a week correlation between serologies and BPI-ANCA and BPI-ANCA had a higher capability of predicting end-points. All tests identified chronic colonisation but none of them predicted future colonization.

(III): In order to study if molecular charachteristics of different P. aeruginosa strains determine if BPI-ANCA develops, two screening methods were employed. Strains from BPI-ANCA negative and BPI-ANCA positive patients were compared using mRNA microarray and 2D gel electrophoresis on outer membrane fractions. Both methods suggested that expression of flagellin A could be related to the development of BPI-ANCA. However, analysis on the gene level using PCR in a cohort of 37 CF patients did not confirm this assumption.

(IV): Platelet activation occurs during infection and inflammation. Activated platelets bind to and modulate monocytes and neutrophils and influence the inflammatory processes. Increased platelet activation has been seen in CF. Platelet function in plasma from 22 CF patients was compared with healthy controls. Platelet aggregation, platelet activation, platelet-leukocyte complex formation, and leukocyte activation were analysed. We confirm that platelet activation is increased in CF patients. Increased platelet aggregation and platelet-monocyte activation was observed but clearly activated isolated platelets were not detected in ex-vivo samples. Levels of platelet/monocyte complex formation correlate with lung function decline, CRP and BPI-ANCA (IgG and IgA). The increased reactivity measured by AUC collagen indicates in-vivo priming of the platelets and it also correlates with BPI-ANCA.

Conclusions: The results from study I and II both show that BPI-ANCA is a stronger prognostic factor than P. aeruginosa colonisation on its own and we suggest that it is a marker of a negative host-pathogen interaction. Flagellin expression is possibly involved in the generation of BPI-ANCA, but ist is not only the expression of flagellin A that matters as there are many differences between bacteria in BPI-ANCA positive and negative patients. Platelets are activated in CF plasma, and this correlates with clinical findings in the patients.

Key words: Cystic fibrosis, prognosis, antineutrophil cytoplasmic antibodies, ANCA, bactericidal/permeability-increasing protein, BPI, platelet activation

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Coverphoto: CFTR expression in CFTR-null pigs treated with AAV2H22-CFTR gene transfer by Joseph Zabner et al, The University of Iowa

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To all CF patients

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Publications

The thesis is based on the following papers, which will be referred to by their Roman numerals I-IV.

I. **BPI-ANCA and Long-Term Prognosis among 46 Adult CF Patients: A Prospective 10-Year Follow-Up Study**. Clinical and Developmental Immunology Volume 2012, Article ID 370107, 8 pages doi:10.1155/2012/370107. Ulrika Lindberg, Malin Carlsson, Claes-Göran Löfdahl and Mårten Segelmark

II. **BPI-ANCA provides additional clinical information to anti- pseudomonas serology - results from a cohort of 117 Swedish cystic fibrosis patients**. J Immunol Res. 2015;2015:947934. doi: 10.1155/2015/947934. Epub 2015 Jul 26. Ulrika Lindberg, Malin Carlsson, Thomas Hellmark, and Mårten Segelmark.

III. **The role of bacterial characteristics for the development of BPI-ANCA in cystic fibrosis patients.** Ulrika Lindberg, Kristian Riesbeck, Yu-Ching Su, Thomas Hellmark, Mårten Segelmark. Manuscript.

IV. **Platelet activation in cystic fibrosis patients correlates to clinical status and BPI-ANCA**. Ulrika Lindberg, Lisbeth Svensson, Thomas Hellmark, Mårten Segelmark, Oonagh Shannon. Manuscript

Publication not included in the thesis

i. **Extensive endoscopic image-guided sinus surgery decreases BPI-ANCA in patients with cystic fibrosis**. Scand J Immunol. 2012 Dec;76(6):573-9. doi: 10.1111/j.1365-3083.2012.02775.x. Aanaes K, Rasmussen N, Pressler T, Segelmark M, Johansen H K, Lindberg U, Hoiby N, Carlsson M, Wieslander J, Buchwald C.

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Abbreviations

ABPA	allergic bronchopulmonary aspergillosis
ALI	acute lung injury
AMP	antimicrobial protein
AP	alkaline protease
ASL	airway surface liquid
BAL	bronchoalveolar lavage
BMI	body mass index
BPI	bactericidal/permeability-increasing protein
CF	cystic fibrosis
CFF	Cystic Fibrosis Foundation
CFLD	CF liver disease
CFRD	CF related diabetes
CFTR	cystic fibrosis transmembrane conductance regulator
COPD	chronic obstructive pulmonary disease
CT	computed tomography
DC	dendritic cells
DIOS	distal intestinal obstruction syndrome
ECFS	European Cystic Fibrosis Society
ELA	elastase
ESS	endoscopic sinus surgery
ExoA	exotoxin A
FEV1	forced expiratory volume in one second
FEV1%pred	forced expiratory volume in one second, % of predicted

G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage stimulating factor
GNB	Gram-negative bacteria
GERD	gastroesophageal reflux
HLA	human leukocyte antigen
HRCT	high-resolution computed tomography
HFCWO	high frequency chest wall oscillation
HMGB1	high mobility group box 1 protein
HS	hypertonic saline
IBD	inflammatory bowel disease
IFRD1	interferon-related developmental regulator 1
IL	interleukin
ILD	interstitial lung disease
IIE	indirect immunofluorescence
IRT	immunoreactive trypsinogen
LB	lysogene broth
LBP	LPS binding protein
LPS	lipopolysacharid
LXA_4	lipoxin A4
LTB4	leukotriene B4
MALDI-TOF-MS	matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry
MBL	mannose-binding lectin
MI	meconium ileus
MMP	matrix metalloprotease
MPA	microscopic polyangitis
MPO	myeloperoxidase
NADPH	nicotinamide adenine dinucleotide phosphate
NBS	newborn screening

NE	neutrophil elastase
NETs	neutrophil extracellular traps
NO	nitric oxide
NSAID	nonsteroidal anti-inflammatory drug
NTM	non-tuberculosis mycobacteria
OGTT	oral glucose tolerance test
OM	outer membrane
OMP	outer membrane protein
PCR	polymerase chain reaction
PEP	positive expiratory pressure
PERT	pancreatic enzyme replacement therapy
PI	pancreatic insufficiency
PS	pancreatic sufficiency
RA	rheumatoid arthritis
PR3	proteinase 3
ROS	reactive oxygen species
SLE	systemic lupus erythematosis
TGF-β1	transforming growth factor β1
TIS	tobramycin inhaled solution
TNFα	tumour necrosis factor alpha
TLR	toll-like receptors

Abstract

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Cystic fibrosis

Cystic fibrosis – an inheritable disease

As early as in the 18th century the symptoms of CF were described in texts from Germany and Switzerland, with the message that if your baby´s skin tasted salty, the child would soon die (1). It was, however, not until 1936 that the characteristic changes in the pancreas and the lungs were described by Fanconi and two year later by Andersson (2, 3). The genetic and molecular background to this lethal disease was first described in 1989 when the gene coding for the cystic fibrosis transmembrane regulator (CFTR) was identified ((4, 5). Since then more than 2000 different mutations in the CFTR gene have been identified (The Clinical and Functional TRanslation of CFTR (CFTR2); available at http://cftr2.org.).

The genetic background to CF

Individuals affected by CF have mutations on both copies of the gene coding for CFTR, resulting in reduction or absence of CFTR function. The gene is located on the long arm of chromosome 7, encoding a 1480 amino acids long protein (4, 5). Although tremendous improvements in treatment have been made, CF remains one of the most common fatal hereditary diseases in the world, and worldwide, more than 80 000 people are suffering from CF (6). Life expectancy is increasing and for patients born after year 2000, it is predicted to be over 50 years (7).

Mutations causing CF are divided into six different groups, according to the CFTR defect and the changes the defect causes in the production, circulation or function of CFTR on the cell membrane ((8). In class I mutations CFTR is absent because of deficient protein translation. Class II mutations show defect processing of CFTR, resulting in less functioning CFTR on the cell membrane. Class III mutations produce CFTR that does not open properly (gating mutations) and class IV mutations have defect protein conductance. In class V mutations CFTR has reduced synthesis or stability. Class VI mutations, the last group to be added to this list, have an increased turnover of CFTR protein at the cell surface (6). Lately, a class VII has been suggested, including mutations where no CFTR is produced at all, because no mRNA transcription occurs. These mutations were earlier included in class I together

with stop-mutations (6) and others suggest that a class IA and IB should be created in stead (9) as they share clinical outcome. Mutations in class I-III (including the newly suggested class VII/IA) are generally referred to as severe mutations, usually associated with pancreatic insufficiency (PI), whereas class IV-VI are less severe (10).

Table 1. Classification of CFTR mutation classes (adapted from De Boeck and Amaral 2016)

Class	CFTR defect
I	Defective protein production/No protein
II	Defective protein processing/ No traffic
III	Defective protein regulation/Impaired gating
IV	Defective protein conductance
V	Reduced amount of functioning CFTR protein
VI	Increased turnover of CFTR at the cell surface,
VII or IA	No mRNA transcription/No protein

The physiological function of CFTR

CFTR is an ion channel, found in many cell types in the body, regulating ion traffic over the cell membrane. The defect CFTR influences many systems in the patient, causing a multi-organ disease ((11).

CFTR is a membrane protein with multiple domains, belonging to the large family of adenosine nucleotide-binding cassette transporters. It consists of two transmembrane domains, two nucleotide-binding domains and a regulatory domain, unique for CFTR (12). The CFTR is expressed in epithelial organs, for instance airway surface epithelium and submucosal glands, but there is also evidence for CFTR presence on other cells in the body, for instance in platelets (13) and other cells involved in innate and adaptive immune response, such as neutrophils and macrophages (14).

CFTR dysfunction causes CF

CFTR works as an anion channel conducting Cl^- and bicarbonate. Via the amiloride-sensitive epithelial Na^+ channel, ENac, in the airways, CFTR also functions on the Na^+ absorption (15-17) causing an increased Na^+ absorption. The dysfunctioning regulation of ENac by CFTR is proposed to play an important role in CF lung disease, but exactly how important that role is, is under debate (18, 19).

Mutations in CFTR cause basic ion transport defects that in turn change the environment in the airways, leading to inflammation, infection and lung function impairment. The leading theories about how CFTR dysfunction causes CF are, in short: 1) *the airway surface dehydration theory*, where lack of Cl^- secretion in epithelial cells results in low hydration of the airway surface liquid and 2) *the decreased HCO_3^- transport theory*, where secreted mucins are maintained in an aggregated and poorly

soluble form (20-23) and pH is lower than in normal airways (24). Both these defects affect the airway surface liquid (ASL), decrease mucociliary clearance of inhaled pathogens and influence the response to infection.

Studies on CFTR-deficient mice, generated to mimic CF lung disease (βENaC-Tg-mice) and CFTR-deficient pigs and ferrets have proven the dehydration concept. In these animals, hyper concentrated mucus decreases mucociliary clearance and mucus plugging appears, leading to inflamed airways and bacterial infection (25-27). These animal mo dels have become important tools to further study and understand CF disease in humans.

Lately it has also been acknowledged that the decreased HCO_3- secretion in CF hampers the expansion of mucins, the giant glycoprotein molecules that compose mucus (28) and that the reduced pH leads to impaired bacterial killing in CF pigs (29). The link between CFTR dysfunction and mucus phenotype has taken long to understand, probably because of the difficulties in studying the big and highly glycosylated mucin molecules (23). So, both the dehydration theory and the bicarbonate theory have experimental support and both mechanisms contribute to explaining the pathogenesis of CF airway disease.

CF diagnosis

CF investigation is traditionally started because of typical symptoms of CF (table 2). The child presenting with meconium ileus (MI), an acute bowel obstruction seen in newborns, failure to thrive or steatorrhea alerts the clinician to suspect CF and ask for a sweat test.

Newborn blood spot screening (NBS) has been introduced as it has become evident, that an early diagnosis prevents symptoms and improves prognosis (30). Long-term follow-up of children in Sydney, Australia, diagnosed with NBS compared to children diagnosed based on symptoms show that an early diagnose improves survival (31). More and more countries now perform screening of newborns to diagnose CF as early as possible. These early tests are usually based on a blood test, measuring immunoreactive trypsinogen (IRT) in a blood spot (32). If high levels are found, the test is completed with a DNA test, but different strategies exist, including a confirming IRT test and in some countries extended genetic analysis by sequencing (33).

Children with a positive screening test are referred to a CF centre for further diagnostic evaluation with a sweat chloride test. NBS has taught CF caretakers much about different mutations and corresponding phenotypes, as also patients with mild mutations and atypical symptom are identified earlier. The logarithm for each country´s screening procedure is essential, and has to be adjusted to the mutations

prevalent in the population. NBS programmes are constantly evaluated, as new mutations are discovered and incorporated in existing programmes, and other mutations are in some cases excluded from the programmes (34) as they are considered not disease causing. Bioethical implications such as detection of carriers of the CF gene and problems with inconclusive diagnoses are important aspects to study further (33).

Table 2. Symptoms characteristic for CF diagnosis (adapted from Amaral 2016)

Organs involved	Symptoms
Lower airways	Respiratory symptoms of acute or persistent charachter
	Sputum production and chronic cough
	Obstructive lung disease
	Lower airway infections
	Colonization with pathogens typical for CF
	Bronchiectasis on chest radiograph
Upper airways	Nasal polyps
	Chronic sinusitis
Gastrointestinal tract	Pancreatic insufficiency, malabsorption
	Failure to thrive
	Meconium ileus
	Rectal prolapse
	Hepatobiliary disease
	Recurrent pancreatitis
	Distal intestinal obstruction syndrome (DIOS)
Sweat glands	High Cl^- concentration in sweat
Male reproductive system	Congenital absence of vas deferens
Metabolism	Fat-soluble vitamin deficiency
	Salt-loss syndrome with salt depletion

In Sweden, and other countries without NBS, the diagnosis starts with the identification of symptoms, consistent with CF.

The diagnosis of CF should be based on the presence of one or more characteristic clinical symptoms and laboratory evidence of an abnormality in the CFTR gene or protein. Still, it is sometimes difficult to classify some patients (35). New mutations are identified and we do not know in which phenotype the mutation will result. Patients with mild disease may carry two disease causing mutations but have normal or borderline sweat chloride levels; these patients are referred to as atypical CF or non-classic CF (36).

If CF is suspected clinically, the first test to perform is the sweat test. This test, established by Gibson and Cooke in 1959 (37) is still the gold standard. Appropriate performance of the test is very important and it should be done in a standardized way.

The test involves transdermal administration of pilocarpine by iontophoresis to stimulate sweat gland secretion, followed by collection and quantification of sweat and analysis of chloride concentration.

Nasal potential difference measurement (nasal PD) is another way to investigate CFTR function in patients. In CF patients the nasal PD is more negative than in healthy persons, and the rise after application of amiloride is greater (38, 39). Nasal PD can be used if the sweat test is inconclusive in a patient where CF is suspected and the DNA test does not identify more than one CF causing gene (36).

Intestinal current measurement (ICM) is another way to demonstrate CFTR dysfynction. This test is done on intestinal epithelium from rectal or jejunal mucosal biopsies, but is mainly used in research settings (40).

CF symptoms and treatment

The treatment of CF should be multidisciplinary; team based, individualized and start at diagnosis. The most important aspects of treating CF are nutrition, physical activity, antibiotics and airway clearance treatments (41). In the future, not too far away hopefully, CF treatment will include mutation specific CFTR modulation or gene therapy and more effective anti-inflammatory treatment.

Starting with the earliest symptoms: treating meconium ileus (MI)

In patients with uncomplicated MI, conservative treatment with diluted Gastrografin^{R} enema is an effective initial treatment. Various surgical methods are used to treat MI, including resection with enterostomy, primary anastomosis, and purse-string enterotomy with intra-operative lavage (42, 43).

Nutrition

Infants with CF commonly have symptoms of malabsorption, due to PI. Patients with PI need treatment with pancreatic enzyme replacement therapy (PERT) and fat-soluble vitamins (D and E) to obtain normal growth and nutritional status. CF patients also have an increased risk of malnutrition because of increased energy demands, caused by infections and increased metabolism. Careful nutritional support must start early, and patients should be monitored closely (44, 45, 46).

Airway clearance to prevent mucus plugging and infections

Chest physiotherapy for airway clearance is an important part of CF treatment. There are different treatment traditions in different countries and only few studies comparing these have been published (47). It is very important that airway clearance treatment is individualized and adjusted to the clinical situation. Positive expiratory

pressure (PEP) is a commonly used method of airway clearance, for instance in Sweden, whereas, in some countries, high frequency chest wall oscillation (HFCWO) using a vest is popular. In a recent study where the vest was compared to PEP, investigators actually found a difference in disadvantage of the vest (48). Aerobic exercise is recommended for airway clearance and for its additional positive effects on overall health (49) and is an important part of CF treatment (50). In a study, performed during two months, investigators found that increases in exercise capacity resulted in significantly improved lung function and self-reported habitual activity (51).

Mucolytic and airway hydrating treatment

Airway clearance and mucolytic treatment are usually combined in the daily treatment performed by the patient. Dornase alfa, recombinant human DNase, degrades the excess DNA left by dying neutrophils in the inflamed airways. Dornase alfa has been shown to reduce markers of inflammation in CF (52), influence lung function decline and decrease pulmonary exacerbations (53).

Hypertonic saline (HS) and mannitol are hyperosmolar substances, rehydrating CF airways by means of osmosis, in this way improving airway clearance. The use of HS can reduce pulmonary exacerbations and improve lung function (54). Mannitol has shown sustained, clinically meaningful benefit in airway function in CF (55, 56) but no benefit is seen in the combination with dornase alfa (57). Dornase alfa and hydrating substances influence the ASL in two different ways and there is not enough evidence to conclude which treatment is superior in improving lung function (57).

Preventing Staphylococcus aureus infections with prophylactic antibiotics

S. aureus bacteria often infect small children and start causing lung damage early, and the use of prophylactic antibiotics to prevent such infection is standard treatment in Europe (41), whereas children in the US do not receive such treatment. The reason for this difference is that a trend to higher number of *P. aeruginosa* infections in children age 4-6 years on prophylactic treatment was observed (58) and the interpretations of this study differ between countries. In Sweden, many children are treated with flucloxacillin to prevent *S. aureus* infection, but there are differences between Swedish CF centres.

Treating infections: exacerbations and chronic infections

Pulmonary infections are responsible for most of the morbidity and mortality in CF. Pathogens colonizing CF airways tend to develop in a, for CF, typical way, starting with *S. aureus* and *Hemophilus influenzae*, followed by *P. aeruginosa*, *Burkholderia cepacia* and other opportunistic pathogens including mycobacterial, fungal, anaerobic and viral infections (picture 1).

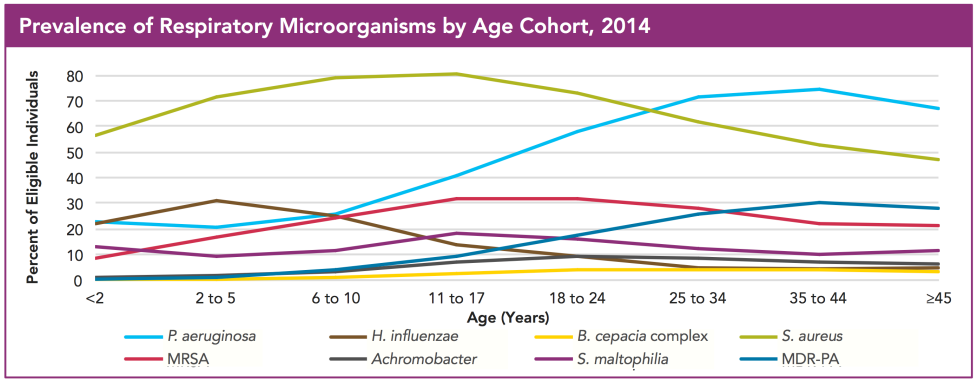

Figure 1. From CFF annual report 2015.

Treating pulmonary exacerbations is important, as it is common that patients never recover their earlier lung function level after an exacerbation (59). The definition of an exacerbation has not been agreed on. Patients experiencing a clinical deterioration should be treated promptly, in most cases with antibiotics, administered orally or intravenously, depending on clinical status and pathogen (41). In some patients inhaled antibiotics may be the choice when treating a clinical deterioration.

Detection and eradication of a new *P. aeruginosa* infection are very important tasks in CF care but the optimal combination of antibiotics to use in this situation is not known (60-62). In many patients the infection eventually becomes chronic. Chronic *P. aeruginosa* is in most countries, and according to international guidelines, treated with long-time inhaled antibiotics as they improve lung function and reduce exacerbations (63). The treatment tradition in Sweden has for many years been based on intravenous antibiotics more than inhaled and results have been similar to other countries (64, 65). So far, studies of long-time inhaled antibiotics against *P. aeruginosa* are in favour of tobramycin inhaled solution (TIS), but the differences are small (63, 66, 67, 68, 69). The arsenal of available inhaled antibiotics is growing, making it easier to find a treatment optional for the individual patient (70, 71).

Non-tuberculous mycobacteria (NTM) may cause chronic pulmonary infection, and have been identified as a major threat to CF patents´ health. These bacteria are difficult to diagnose and treat. The US Cystic Fibrosis Foundation (CFF) and the European Cystic Fibrosis Society (ECFS) recently published a consensus document for the management of NTM pulmonary disease in CF (72).

Aspergillus fumigatus is a fungus species commonly found in CF. Allergic bronchopulmonary aspergillosis (ABPA) is an immunological/allergic reaction, caused by *A. fumigatus*; this diagnosis should be considered when patients do not respond to antibiotics as expected. Standard treatment of ABPA includes oral corticosteroids. At

present, there are no randomised controlled trials to evaluate the use of antifungal therapies for the treatment of ABPA in CF (73). The relevance of *A. fumigatus* in patients with CF not affected by ABPA is unclear but persistent *A. fumigatus* infection is an independent risk factor for hospital admissions (74).

Immune-modulation with macrolides

Macrolides are part of standard treatment in CF, as these antibiotics effect both infection and inflammation through different mechanisms (75). There is evidence that macrolides affect biofilm-producing organisms, of which *P. aeruginosa* is the most important. Macrolides also influence neutrophilic inflammation in several ways (75). Macrolide treatment improves respiratory function and reduces pulmonary exacerbations (76) in patients chronically infected with *P. aeruginosa*. In children and teenagers with CF, not infected with *P. aeruginosa*, treatment with azithromycin did not improve pulmonary function (77).

Treating CF-related diabetes (CFRD)

CFRD is a common complication in CF, more common in severe mutations and with increasing age. In US publications, CFRD is present in about 20% of adolescents and in 40-50% of adults (78). In 2014, CFRD was present in 27% of adult CF patients in Sweden (65). Treating and diagnosing CFRD is an important task for the CF team. Unlike type 1 diabetes, patients with CFRD never develop total absence of insulin secretion. Few CF patients have normal glucose metabolism and patients with impaired glucose tolerance should be carefully monitored as they have a high risk of developing CFRD (78).

CFRD is treated similarly to insulin-dependant diabetes in other patients, but often require lower doses of insulin. Nutrition in CFRD differs from diabetes in general, due to the high calorie demand in CF and calories should almost never be restricted. A multi-disciplinary management is important. Monitoring for diabetes related complications is done in the same way as in other diabetes patients (41, 79, 80).

Distal intestinal obstructive syndrome (DIOS)

Distal intestinal obstructive syndrome is a specific clinical entity, different from constipation, affecting many CF patients. DIOS is defined as an acute complete or incomplete faecal obstruction in the ileocaecum. In a European multi-centre study, the incidence was approximately six episodes per 1000 patient-years in patients less than 18 years of age (81). Most CF patients affected by DIOS are pancreatic insufficient, have a severe genotype, and many have a history of meconium ileus at birth (81). The treatment is based on rehydration and stool softening laxatives and often involves lavage of some kind. In most cases a conservative regime is successful and only few cases have to undergo surgery. To prevent relapse it is important to

avoid dehydration and to optimize pancreatic enzyme treatment. Laxatives are commonly used (82).

Sinusitis

Nasal and sinus mucosal disease is very common in patients with CF as the defective CFTR-channels also affect the sinonasal mucosa (83). Common problems are nasal congestion, polyposis, mucopurulent material and aplasia of the paranasal sinuses, most often the frontal sinus (84). CF patients with these symptoms are usually treated with topical corticosteroids and those with extensive sinus symptoms are offered endoscopic sinus surgery (ESS), with good but often short-lasting results, as the problems tend to recur. Lately, the interest in sinus infections has increased dramatically as the sinuses are considered a bacterial reservoir for pulmonary infections, and in some centres patients without symptoms are prophylactically operated with ESS to help eradicate the bacterial reservoir and thus improve pulmonary outcome (85).

Liver disease

CF patients often develop liver diseases; this is due to the CFTR defect in cholangiocytes. The most common entity is focal biliary cirrhosis, which results from biliary obstruction and periportal fibrosis (86). According to earlier studies approximately 5-10% of CF patients develop multilobular liver cirrhosis before the age of ten (86). Many of these patients develop portal hypertension and complications as variceal bleeding. Liver failure usually may develop in adulthood and accounted for 3.3% of deaths in 2014 (Cystic Fibrosis Foundation Patient Registry 2015 Annual Data Report). Treatment with ursodeoxycholic acid is often initiated if signs of liver disease are detected, and might halt disease progression, although there is substantial disagreement about how effective the treatment is (86-88). The use of ursodeoxycholic acid could be used as an indicator of the total prevalence of liver disease of all categories and in Sweden approximately 20% of all CF patients are treated with this drug (65). Liver transplantation is possible, but the optimal timing is difficult to establish. Indications differ from other liver diseases where chronic hepatic failure is the main indication. In CF, other extra-hepatic parameters are important to consider, including worse nutritional status and the influence on pulmonary function (86).

Complications to CF: Pancreatitis, haemoptysis, pneumothorax

Pancreatitis occurs in 20% of CF patients with pancreatic sufficiency (PS) (89, 90) and there is a clear association between CFTR mutation and risk of pancreatitis. Pancreatitis can be a very problematic symptom in PS CF patients as it in some patients causes chronic pain, difficult to treat.

Haemoptysis is common in CF and can become life threatening, although this is very uncommon. Approximately 4% of all CF patients will experience a massive haemoptysis at some time (91), the incidence increasing with age. Haemoptysis is often a sign of pulmonary infection and in many cases treatment with antibiotics is indicated (92). In Sweden, minor haemoptysis is usually treated with tranexamic acid. If a major bleeding would occur, access to interventional radiology for bronchial artery embolism can be life saving. In 2014, no major haemoptysis was registered in the Swedish CF population (65).

Pneumothorax is another complication, although not so common, that increases with age and severity of lung disease. The incidence is approximately 0.64% (93) similar to the Swedish incidence reported in 2014 (0.48%) (65). CF patients with a large pneumothorax or if clinically instable should be treated with a chest tube. Pleurodesis can be considered in recurrent pneumothorax (92).

Malignancies in CF

As patients live longer an increased incidence of gastrointestinal malignancies in CF patients has been noted and there is a growing awareness of the association (94-96). It has been suggested that screening for colon cancer in CF patients should be implemented and it is important that persistent or unexplained symptoms from the gastrointestinal tract are promptly investigated.

Preventing psychosocial problems and depression

It is clear that living with a chronic disease like CF can be emotionally challenging, both for the patient and for their relatives. Diagnosis of a small child, via NBS or due to clinical symptoms, is traumatic for the parents, and it is essential to support parents in this situation. Anxiety and depression is problematic in parents and patients. Within the CF team, interdisciplinary work and the use of cognitive-behavioural theories for psychological problems are essential. A well-planned transition from paediatric to adult care is also very important (97).

Lung transplantation

Lung transplantation has become an established therapy in CF patients with end-stage lung disease. Lung transplantation is potentially life saving and the survival seen after lung transplantation is more favourable than seen in patients with COPD and pulmonary fibrosis (98). It is essential to select candidates correctly and the awareness of CF-specific issues both before and after transplantation is important as CF disease influences many aspects on the transplantation (99). In patients who do not want to undergo lung transplantation or have contraindications for such operation we have to plan for palliative care. Palliative care in CF should improve quality of life for patients and their relatives, facing the end the patients´ life (100).

Treating CF by treating inflammation

Inflammation is an important target for treatment in CF, regardless of when it starts (101). Dornase alfa, is an enzyme which selectively cleaves DNA. Dornase alfa has been shown to reduce markers of inflammation and neutrophil-associated metalloproteinases (52) in some studies, but others have found increased levels of elastase activity in sputum (102) indicating that a combination of dornase alfa with a protease inhibitor would be a potentially successful way to decrease inflammation in CF airways.

Inhalation of HS improves airway hydration and airway clearance, and studies have shown that it also increases antioxidants and reduces IL-8 in the airways of CF patients (103). Macrolides have immune-modulatory effects and reduce the inflammatory response (see above) (104). The anti-inflammatory effect of ibuprofen in CF is well studied, but the clinical use of ibuprofen remains limited, mainly due to side effects caused by required doses (105). An anti-inflammatory drug that reduces neutrophil influx and inhibits substances that degrade lung tissue, for instance neutrophil elastase (NE) would be a very important treatment in CF (101).

Physical exercise can also have an immune-modulating and anti-inflammatory effect (101, 106).

New treatments for CF

The Cystic Fibrosis Foundation (CFF) provides an excellent ”drug development pipeline”. Most important are treatments where a cure could be achieved, such as gene therapy or CFTR correction on the protein level. Other potential pharmacological targets are airway dehydration, inflammation and infection, and several interesting substances are under clinical and preclinical investigation.

Gene therapy

The optimal way to cure CF would be to correct the defect gene with gene therapy but so far, this approach has been unsuccessful. In a recent Cochrane review (107) the authors analysed four randomized controlled studies of in total 302 patients. One study of liposome-based CFTR gene transfer therapy, where patients were treated with a monthly dose of nebulized, non-viral gene therapy, demonstrated some improvements in respiratory function, (108) but so far the efficacy is too low to support routine use.

Very recently, two new studies were published. In a study by Cooney et al, CF pigs received aerosolized CFTR by a lentiviral vector delivered to the nose and lungs. Evidence for increased functional CFTR was found in excised tissue after two weeks, and an increase in airway surface pH and bacterial killing was observed (109). From

the same institution, another group published a study where an adeno-associated viral capsid was used to transfer CFTR to CF pigs and also in this study, positive results were seen in tissue from the treated animals. Partially corrected anion transport, increased ASL pH and bacterial killing were found (110).

Ivacaftor – the first drug to rescue mutant CFTR

Ivacaftor, a small molecule drug, is a drug that potentiates the defect CFTR by improving deficient gating and/or condactance of Cl^- channels, was detected by molecular screening. This drug works on gating mutations (class III mutations), where the best-known mutation is G551D, by improving gating and thereby increasing the open probability of the CFTR channel (111). Ivacaftor was approved in 2012 and has shown to be a very effective treatment for patients with a gating mutation (112, 113). Unfortunately, class III mutations account for less than 10% of mutations in the CF population worldwide. The cost for this treatment is very high, but the treatment is effective and available in Sweden and many other countries.

Ivacaftor plus lumacaftor

Clinical trials of a combination of lumacaftor (a corrector of CFTR) and ivacaftor (potentiator) have shown effect in patients homozygous for ΔF508, improving lung function with 2.6-4% and reducing exacerbations (114). This combination therapy has been approved and is currently used in some countries, but the cost is very high (US $ 250 000 per year) and so far, this treatment is not available in Sweden.

Prognostic factors in cystic fibrosis

Although life expectancy in CF has increased substantially in later years (7, 115, 116), CF is still a life-shortening disease. In a Canadian CF registry based study, the median expected survival age had increased from 31.9 years in 1990 to 49.7 years in 2012 (116). The improving survival is probably pointing at a median survival of >50 years of age for individuals born in 2000 even without therapy correcting defect CFTR (7).

Adult CF patients now outnumber children in most developed countries. Patients surviving beyond the age of 40 represent many different genotypes, from homozygous ΔF508 to milder mutations and single organ disease. Improved survival gives us new challenges, including co-morbidities, for instance CFRD (117).

As progressive pulmonary disease is the main cause of morbidity and mortality in CF, factors influencing lung function become factors influencing prognosis. These factors can be divided into two main groups: non-modifiable risk factors and modifiable risk factors: In short, sex, CFTR mutation, MI and pancreatic status are the most common non-modifiable risk factors, and nutrition, respiratory infection, pulmonary

exacerbation represent the most common modifiable factors influencing lung function decline in different studies (118).

Genetic factors: mutation class, pancreatic status and modifier genes

CFTR genotype as prognostic factor

There are many descriptions of phenotypic variability in CF, according to CFTR genotype (119). Mortality rates and clinical phenotype have been compared between genotypes according to the six classes on the basis of their functional effect on CFTR production. McKone et al reported that compared with class II, classes IV and V have a significantly lower mortality rate and milder clinical phenotype. There are distinct genetic subgroups associated with milder clinical manifestations and low mortality. Patients with mutations class I to III, with almost no residual function of CFTR, have similar phenotypes and higher mortality than patients with mutation type IV to V, where CFTR has some residual function (10). In another study by McKone, a big cohort was divided in two groups; high-risk mutations (class I-III) and low risk (class IV-V) and patients with high-risk mutations had a much greater risk of dying (relative risk 2.25) than low-risk mutations (class IV-V), during the 5-8 years follow-up in the study. The association was partly depending on lung function, pancreatic insufficiency, *P. aeruginosa* colonisation and nutritional factors (120). Lung function and BMI were associated with worse survival on multivariate analysis, and CFTR genotype remained an independent predictor of survival. Class VI mutations have been characterized after these studies were made and belong to the low risk mutations.

Pancreatic exocrine function

Pancreatic sufficiency (PS) has been associated with improved survival and milder disease and studies have suggested that PS is the mechanism by which milder CFTR mutations influences phenotype (119, 121, 122). PS is more common in CF patients with milder mutations and is associated with better survival (122). The impact of pancreatic status on lung function decline has been investigated and it is a well-established notion that PI is a risk factor for rapid lung function decline and worse prognosis (123-126). But as PI is associated with high-risk mutations it is not an independent risk factor after adjusting for CFTR genotype (120).

Genetic variations beyond CFTR genotype: the role of modifier genes

Even between patients with the same genotype, there is a wide variability in disease, and in pulmonary phenotype (119). Other genetic mechanisms influencing the course of disease must exist as monozygous twins have a significantly higher concordance of disease, than dizygous twins, suggesting that there are other genetic

factors involved than CFTR (127). The notion that patients surviving for over 40 years did not have residual CFTR function, when tested with nasal PD and compared to young patients with corresponding mutation types, also indicates that other genes than CFTR influence disease course (128). These genes are called modifier genes.

Systematic studies to identify modifier genes in CF have been performed by the European CF Twin and Sibling Study (129) and the CF gene modifier study (GMS). Mekus et al concluded that CF disease severity is modulated by other inherited components than the CFTR gene itself. To quantify the contribution of modifier genes to variation in CF lung disease severity another study on monozygous twins compared to siblings was performed and it was estimated that as much as 50-80% of the pulmonary phenotypic variability could be accounted for by non-CFTR genetic variation (130).

Mannose-binding lectin

Mannose-binding lectin (MBL), is involved in innate immune response, and the gene coding for MBL was one of the first candidate genes to be investigated as a modifier gene. Patients with low expression of MBL were found to have more severe pulmonary disease. The presence of MBL variant alleles was associated with poor prognosis and early death in patients with CF (131, 132). However, these findings have not been detected in all studies regarding MBL as a modifier gene (133).

In a recent review and meta-analysis (134), authors concluded that genotypes associated with MBL insufficiency were associated with earlier acquisition of *P. aeruginosa*, reduced pulmonary function in adults, and an increased risk of death or lung transplantation

Liver disease in CF has also been suggested to be significantly associated with a mutated mannose binding lectin (135) but other studies have found conflicting data (136).

Other modifier genes

A number of genes have been investigated as potential modifier genes in CF, for instance:

- TNFα -238 G/A (as well as MBL2 O/O) genotypes appear to be genetic modifiers of survival of cystic fibrosis.(137).
- HLA haplotypes have been investigated and certain haplotypes have more *P. aeruginosa* infections (138).
- Cytokines and inflammatory mediators like TNFα and IL-10 have been evaluated for polymorphism, and found to influence CF lung disease (139).
- Variants in the gene coding for TGFβ1, a protein secreted by neutrophils in response to infection, have been suggested as modifier genes, but findings are contradicting (137, 140, 141).

- IFRD1 protein, involved in regulating gene expression, has been suggested to influence CF through regulation of neutrophils (142, 143).

Modifier genes most probably contribute to the establishment of CFRD (144). New techniques have made it possible to perform large studies such as genome-wide associations studies. Wright at al, from the Gene Modifier Study, studying ΔF508 patients, reported that one locus on chromosome 11p13 and one on 20q13 (145) both influence CF disease.

Identifying and understanding these disease-modifying genes can generate new treatments and in the future the patients´ genetic profile could help identifying the best possible treatment (139).

Early childhood factors

CF lung disease starts early in life and delayed diagnosis influences our patients. Children who had early pulmonary symptoms at the age of three had lower pulmonary function when they were six, compared to patients with fewer symptoms in early childhood. Treatment early in life aiming for improved growth, nutrition and preventing lung disease affects pulmonary function (146).

Meconium ileus occurs very early in life and its possible effect on lung function decline has been evaluated. Investigators have found diverging results. Some find a steeper lung function decline (147) and others find opposing results (148, 149). Lai found that patients who had had MI were more likely to be malnourished later, thus influencing morbidity and mortality (150).

Through the different NBS programmes adopted in many European countries, Australia and most of the US and Canada, we now know, that an early diagnosis is beneficial and that it influences long term growth, prevents malnutrition and increases survival (30, 151, 152, 153).

In Canada not all parts of the country have NBS and a comparative study between screened and not-screened children with CF was performed. It was shown that NBS influences early indicators of long-term health. The NBS patients are diagnosed earlier, have their first clinic visit at a younger age and have a lower incidence of *P. aeruginosa* and *S. aureus*. After adjusting for age at clinic visit, gender, pancreatic status, and *P. aeruginosa* infection status, mean z-scores for weight-for-age and height-for-age were higher in NBS patients, with no differences in BMI-for-age. A twice as high incidence of first occurrence of *P. aeruginosa* in non-screened children, as compared to NBS children was found. The prevalence of *P. aeruginosa* was 28% in NBS and 61.8% in non-screened CF children (151).

Lung function and nutritional status, two factors influencing survival, are improved by NBS and thus these programmes improve long-term health for CF patients.

Rate of lung function decline

A fast decline in lung function, especially in young patients, has been reported as a negative prognostic factor (154, 155). Young patients with a *high* baseline FEV1 actually have a higher risk of faster lung function decline, compared with patients who had a lower baseline FEV1 at the same age (156, 157). In adults, baseline lung function has not been seen as predictive of rate of lung function decline (124).

Exacerbations per year correlated with increased mortality in a study performed by Harness-Brumley (158) and pulmonary exacerbations correlate with increased lung function decline, especially in young children (159). CFRD is also a risk factor for increased rate of lung function decline (125, 126).

In a recent review, *P. aeruginosa* infection and pancreatic status were found to be the most common factors seen to influence rate of lung function decline (118).

Physical capacity

It has been known for many years that a higher level of physical capacity in patients with CF is associated with a better prognosis. In 1992, Nixon concluded that although aerobic fitness might just be a marker for less severe illness, measurement of peak VO_2 appeared to be valuable for predicting prognosis (160).

This was evaluated again by Pianosi who found that higher final peak VO_2 (peak oxygen uptake) is a marker for longer survival in CF patients (children age 8-17) as patients with peak VO_2 of less than 32 ml/min/kg had a dramatic increase in mortality, in contrast to those whose peak VO_2 exceeded 45 ml/min/kg. Rate of decline in physical capacity and lung function were also significant predictors (161).

Combining BMI, lung function and peak VE/VO_2 (peak ventilatory equivalent ratio for oxygen) in a multivariate model to predict mortality in young CF patients, age 11-14 years, was by Hulzebo et al found to be the best predictive model (162).

Pseudomonas aeruginosa infection

The important impact of *P. aeruginosa* infection in CF has been acknowledged for many years and is well studied (163-165). Chronic *P. aeruginosa* infection influences patients´ survival (166) and rate of lung function decline (64, 124, 126, 157, 167,

168) and therefore the eradication of new *P. aeruginosa* infections and treatment of chronic *P. aeruginosa* are essential parts of CF care (41).

Other important pathogens influencing prognosis

Not only *P. aeruginosa* infection contributes to lung function decline and prognosis, but many other infections as well.

- *Burkholderia cepacia*: epidemic *B. cepacia* colonisation is associated with excess mortality (158, 169).
- MRSA infection has been found to accelerate lung function decline (159, 170, 171).
- Chronic *Aspergillus fumigatus* (found in two cultures) was associated with increased lung functions decline and hospital admissions in children in one study (74), although this notion is not generally accepted.
- Chronic infection with *Mycobacterium abscessus* has a negative influence on lung functions decline, to a higher level than other mycobacteria (172-174).
- *Achromobacter xylosoxidans*, an emerging CF pathogen, can cause high levels of inflammation and influence lung function decline (175).

Why is it worse to be a female CF patient?

It was observed long ago that females have a three to five years shorter median survival than males (176-178) but the reasons for this are unknown. Demko et al did not see worse nutritional status in females, and the difference in survival was noticed already before puberty. In the Demko study, median age for initial positive mucoid *P. aeruginosa* culture was 7.4 years in females, 8.4 years in males. The notion that females acquire *P. aeruginosa* and other pathogens at an earlier age, and have worse prognosis with all pathogens expect *Hemophilus influenzae* was reported also by Harness-Brumley (158).

The gender gap has been questioned, as life expectancy increased and some investigators did not find it. Stephenson (116) suggested that the explanation for the worse prognosis was the fact that females developing CFRD had a worse prognosis. In a recent study (158) the difference in survival between male and female CF patients still existed, 2.8 years, a level similar to 1995.

Different explanations have been suggested, for instance:

- Estrogen and progesterone have been shown to have impact on CFTR gene expression (179) and data suggest that estrogen can interact directly with CFTR to alter anion transport (180-182).

- Levels of exhaled nitric oxide are influenced by sex hormones (183).
- Lung function changes in female CF patients have been found during menstrual cycles (184).
- 17-β-estradiol inhibits IL-8 release by the estrogen receptor in CF bronchial epithelial cells which makes females more vulnerable to infection and colonization (185).
- In vivo levels of estradiol correlated with infective exacerbations in women with CF, with the majority occurring during the follicular phase (186).
- Estrogen inhibits Ca^{2+} signalling and ASL volume homeostasis in non-CF and CF airway epithelia by attenuating Ca^{2+} influx. Estrogen antagonists as tamoxifen prevented the effect (187).
- Estrogen acts directly on *P. aeruginosa* conversion to mucoid form and biofilm production through a mutation of *mucA*, a gene involved in biosynthesis of alginate, in vitro (186, 188).
- Estradiol effects mucus expression in bronchial epithelia via post-tranlational modification of mucins thereby increasing mucus amount (189).

In a recent review (190) authors conclude that the exact mechanisms are not fully identified yet, but gender definitely affects lung infection, decline in pulmonary function and nutritional status.

Nutritional status

BMI, used as index of nutritional status, is known to be a factor influencing survival. Patients with CF referred for lung transplantation with a BMI less than 18 kg/m^2 are at high risk of death over the next year (191). Similar findings have been reported in other studies (192).

In the aging CF population, a higher BMI is associated with increased probability to live longer (128). Hulzebos also found BMI to be a significant predictor of survival in both univariate and multivariate models (162).

Cystic fibrosis related diabetes, CFRD

The prevalence of CFRD is 60% in adult patients with severe mutations, but only 14% in patients with mild mutations. Mortality for CFRD patients older than 30 years is higher than for CF patients without diabetes and both mortality and CFRD prevalence are higher at every age in females than males (193). CFRD has a direct influence on mortality, as it is associated with increased risk of death within each genotype group (193).

Inflammatory markers of lung disease

Anti-body responses against *P. aeruginosa* have been analysed since the 1970ies and used as markers of lung inflammation and tissue damage (163, 164). A relation between high titres against *P. aeruginosa* and poor prognosis has been shown (163).

Chronic infection is followed by chronic inflammation and inflammatory mediators released from the airway epithelium, neutrophils and macrophages. NE, IL-8 and TNF-α in BAL and sputum correlate with disease severity and can be used as markers of inflammation and lung tissue damage (14, 194-197). The lysosomal cysteine proteases cathepsin 6 and 8 influence CF lung pathophysiology (198, 199).

In a recent study, CF patients were followed for more than ten years, end-point being death or lung transplantation. Serum levels of anti-Pseudomonas IgG, IgA, plasma IL-6, urine TNFr1 (tumor necrosis factor receptor 1), sputum IL-8, TNF-α, NE, cathepsin S and cathepsin B were measured at inclusion. Anti-Pseudomonas IgG and IL-6 positively correlated with mortality. However, multivariate analysis demonstrated that after adjusting for lung function, anti-Pseudomonas IgG was not independently related to mortality. An association between IL-6 and mortality was observed, but none of the sputum markers showed any clear association with survival. The authors conclude that biomarkers of inflammation cannot be used as independent prognostic factors in CF (200).

Statistical models for predicting mortality in CF

Why are statistical models for predicting risk of death in CF needed? Expected survival time is critical for deciding when a CF patient should be referred for lung transplantation. Lung transplantation does not cure CF and survival for adults varies. Median survival, internationally, in adults was only 6.4 years in 2008 (201). A recent long-term follow-up of lung transplantation results from Lund, Sweden, reports that survival rates for CF patients are indeed much better, showing that CF patients had a median survival of 16.2 years in this centre (202). However, we also see that the median survival of patients with an FEV1 of less than 30% has increased from 1.2 years in 1990 to 5.3 years in 2002 (203) which makes optimal timing of transplantation even more important.

The first multivariate models for predicting survival were created when waiting time for lung transplantation could be up to two years, and were thus created to identify patients at risk of dying within this time. Most models include: lung function (FEV1%pred), nutritional status, blood gas measurement, age and sex, as these were factors identified in a retrospective study in 1992 (192). In the study by Kerem et al, 28% of patients studied died during the study period of ten years, a number very different from what we see in later years.

FEV1 below 30% of predicted was the most significant predictor of mortality as 50% of these patients died within two years. Females had a worse prognosis and young patients deteriorating fast had a higher risk of dying. Patients with a weight-for-height below 70% also had a mortality rate above 50% within two years, in patients over 18 years. The conclusions drawn from this study were that patients with an FEV1 below 30% of predicted should be considered candidates for lung transplantation and females and young patients (<18) should be referred earlier than that. The Kerem study became a reference when evaluating patients for referral to lung transplantation, but later studies have completed the picture, as there is a wide variability in rate of decline of lung function and some patients can be stable at a low level for longer time. Rapidly declining FEV1 values, especially in patients <15 years of age, should be factors to consider when referring to lung transplantation, as these predict mortality within two years (154, 155).

In 2001 Liou (204) created multivariate logistic regression models for 5-year survivorship, based on age, FEV1, gender, weight-for-age z-score, pancreatic status, CFRD, *S. aureus* infection, *Burkholderia cepacia* infection, and annual number of acute pulmonary exacerbations.

In a multivariate model from 2014, mortality in adolescents was best predicted by including FEV1%pred, peak VE/VO2 and BMI (162). Aaron et al (205) predicted one-year survival in a Canadian cohort combining factors associated with chronic disease severity and factors associated with exacerbations and decline in FEV1 preceding year to calculate the 1-year survival probability.

What distinguishes patients who survive longer? Pancreatic sufficiency, male gender, and some milder mutations are associated with a slower rate of pulmonary function decline (123). We also know, from a study where nasal PD was measured in long-term survivors, that long survival couldn't be explained by residual CFTR (128).

In the clinical every-day setting, predicting life expectancy and identifying prognostic factors may help both health professionals and patients to prioritize treatment at the right time, in the right patient (116).

The inflammatory process

Neutrophil function in infection and inflammation

Polymorphonuclear leukocytes (PMN), here referred to as neutrophils, are the most abundant leukocyte subset in the blood stream and essential for the acute and chronic inflammatory response. They are usually the first leukocytes at the site of inflammation and help eliminate pathogens by different mechanisms. Neutrophils are generated from myeloid precursors in the bone marrow and up to $2x10^{11}$ cells are produced daily. The circulatory life span of a neutrophil is generally considered to be about 8 hours (206) but this is under debate (207) as some investigators have found evidence that neutrophils can live as long as 5.4 days (208). During inflammation, it is clear that the life span of neutrophils increases several times (209). The production process is regulated by colony stimulating factor (G-CSF), which in its turn is regulated by IL-17 from T cells. IL-17 release is controlled by IL-23, originating from macrophages and dendritic cells.

Neutrophils are normally most abundant in bone marrow, spleen, liver and lung, where they wait, ready to be activated and sent to sites of inflammation (209). Lung vasculature is especially rich in mature neutrophils (210). Neutrophils are recruited to the site of infection via different mechanisms, involving interactions between cytokines, chemoattractants and molecules responsible for cell adhesion. This process leads to migration over the endothelium, extracellular matrix and finally through the alveolar epithelium to the site of infection.

Neutrophilic killing of pathogens

Neutrophils carry granules that are formed during maturation (211). Granules are filled with pro-inflammatory proteins (211, 212) and are of three main types. Azurophilic (primary) granules contain myeoloperoxidase (MPO), NE, BPI, defensins and cathepsin G. Specific (secondary) granules contain lactoferrin, lysozyme, NADPH oxidase and collagenase, among others. Tertiary granules contain MMP9 and collagenase (211). When released, the granule content can act in both intra- and extracellular environments. These three types of granules can be further divided in subtypes.

Neutrophils also carry secretory vesicles, which easily can be mobilized. Upon stimulation, the secretory vesicles transport molecules essential for cell adhesion (β2-integrin), to the cell surface, where they are integrated in the cell membrane (211).

Neutrophils kill pathogens by three main mechanisms:

- Phagycytosis, in which the neutrophil engulfs the pathogen, then encapsulates it in phagosomes, killing it with antibacterial proteins from granules and reactive oxygen species (ROS) generated by the neutrophil.
- Degranulation, where the antibacterial proteins from granules are released extracellular to kill the pathogen together with ROS.
- By releasing neutrophil extracellular traps (NETs) (213, 214).

When neutrophils die, they have to be removed. Under normal conditions, this can happen in the liver, spleen and bone marrow. Apoptotic neutrophils are removed by macrophages and dendritic cells (DCs), a process regulated mainly by the cytokine axis, consisting of IL-23, IL17 and G-CSF. Monocytes are also able to phagocytise apoptotic neutrophils (215).

Once the pathogen is eliminated the anti-inflammatory phase starts. This involves different mediators, for instance IL-10 and cytokine and chemokine receptor antagonists. Pro-inflammatory mediators such as prostaglandin I2 and LTB4 are produced during the start of inflammation, but later a lipid mediator switch occurs and cells in the inflamed area start producing resolving mediators such as lipoxin A4 (LXA_4), protectins and resolvins and thus stop neutrophil migration (216).

NETs in lung disease

Activated neutrophils can also kill pathogens by generating NETs. NETs are composed of granular contents released into the extracellular space together with core DNA elements to which antimicrobial proteins and enzymes are attached (213). NE and MPO are involved in regulating the creation of NETs (217). NADPH oxidase-derived ROS is usually also required in the generation of NETs (218). By its structure and because of its high concentrations of antibacterial molecules, NETs facilitate phagocytosis and prevent pathogens from spreading (219). Antimicrobial substances as histones and proteases situated in the NETs can act directly to kill trapped pathogens. NETs can also be negative for the patient, as they disturb microcirculation, fill the alveoli with debris and act in an immunogenic way, damaging host tissue. Regulatory substances are altered in different lung diseases resulting in the accumulation of DNA in the lungs (220).

The inflammatory process in CF lung disease

A dysregulated inflammatory process

It was long believed, that the inflammatory process seen in CF lung disease predominantly was caused by bacterial infections, but in later years, is has become evident, that the inflammation process starts even before there are any bacteria present. Already in 1995 proof of very early inflammation was found in infants with CF with BAL in form of increased neutrophil count, NE and IL-8 levels (221, 222). Neutrophils are considered crucial for this early and uncontrolled inflammatory response (221). But there were also investigators who found normal inflammatory levels in infants, as long as they were not infected (223). Abnormalities in tracheal development, plugging of bronchioles and a vigorous inflammatory response are also seen in young CF children, indicating early inflammatory effects without infection (224). Airway obstruction and bacterial infection cause bronchiectasis and eventually destruct normal bronchial structure (225). The presence of NE early in life predicts the development of these irreversible lung damages (226).

When bacteria enter the airway lumen they attract neutrophils, which initiate an inflammatory response, release proteases and oxidants that damage the airways. CF lung disease is characterized by an excessive neutrophil inflammation and traditionally, neutrophils were believed to express little or no CFTR (227). Later studies have shown that CFTR is present in neutrophil phagolysosomes (228). This influences neutrophil function and impairs bacterial killing as CFTR channel expression affects neutrophil chlorination of phagocytised *P. aeruginosa* bacteria. BAL fluid from CF patients alkalinized cytosolic pH and suppressed apoptosis, a process that influences neutrophil cell death (229). The CFTR defect thereby influences the neutrophil ability to clear infection.

CF airways attract neutrophils, through attractants like IL-8, HMGB1, IL-17 and others, and show a decreased neutrophil clearance (221, 230, 231). A multitude of pro-inflammatory mediators have been identified as up regulated; TNF-α, IL-1β, IL-6, IL-8, IL-17, IL-33, GM-CSF, G-CSF and HMGB-1, which all play different inflammatory roles in the host (230, 232, 233).

The excess neutrophil accumulation in the airways creates abundant mucus, which has to be cleared, either by coughing, or degradation of apoptotic neutrophils by macrophages. Neutrophils in CF tend to undergo necrosis as alveolar macrophages are abnormal (101) a process that also increases the production of NETs and debris in the airways. In this way the inflammatory process continues, as necrotic neutrophils release chemoattractants and more neutrophils are recruited.

Neutrophils release proteases, for instance matrix MMP8 and MMP9, NE, cathepsin G and proteinase-3 (PR3), all which degrade connective tissue and damage lung structure. NE is long considered to be the most important protease (234), affecting the airways directly by digesting elastin and other proteins (235). NE levels have been shown to correlate with lung function decline (194, 236) and have been suggested as a biological marker in CF (226). The high levels of proteases in CF patients, measured by BAL, exceed the natural protective defence systems, for instance protease inhibitors such as α1-protease inhibitor. Although levels of α1-protease inhibitor are increased many-fold in CF, it is not enough to balance the protease effect (231, 237).

Neutrophil function is also influenced by mucins, as MUC5AC, MUC5B and MUC2. Mucins are important in regulating the airway surface liquid, as they are tethered to the epithelial cell cilia and attract water to maintain hydration of the epithelium. CFTR dysfunction causes decreased pH, which leads to impaired mucin function and influences the airway epithelium. The mucus layer becomes denser and more tightly adhered to the epithelium (23, 238). The dehydrated airway mucus decreases neutrophil capturing and killing of bacteria on epithelial surface in the lungs by influencing neutrophil motility (239). It has also been suggested that mucus stasis on its own could start the inflammatory process, which is seen in CF-mice (240).

NETs in CF lung disease

More recently, the presence of NETs and proteins associated with NETs has been demonstrated (220, 241) and it is now believed that the excess DNA in CF sputum and BAL is mainly derived from NETs. A correlation between lung function and levels of extracellular DNA indicates that the accumulation of NET-DNA in the airways contributes to lung function decline in CF (242). Pro-inflammatory cytokines and neutrophil chemokines can also initiate NET generation (241). The chronic infections and decreased capacity of clearing these infections perpetuates the inflammation and the excessive NET formation actually helps *P. aeruginosa* create biofilms and acts as pro-inflammatory parts in biofilms (243). NE and MPO on NETs further increase tissue destruction via degradation of connective tissue and endothelial cell matrix (244, 245). However, the excess formation of NETs in CF is not efficient in killing pathogens (242, 246).

Lymphocytes influence neutrophils in CF

Lymphocytes are present in large amounts in CF lung tissue and both B cells and T cells express CFTR. CF T cells produce less IL-10, an anti-inflammatory substance, than normal T cells (247). Different subsets of lymphocytes exist in CF lungs, and especially Th17 cells produce IL-8 and IL-17, promoting neutrophil influx (248).

CF airways are unable to resolve inflammation

There is also an inability to resolve the on-going inflammation that perpetuates the inflammatory process, as NE increases IL-8, which in its turn increases NE (249). CF airways are deficient in molecules regulating inflammation, such as IL-10, nitric oxide (NO) and LXA_4 (230, 250, 251). IL-10 helps terminate the inflammatory response, down-regulates production of pro-inflammatory cytokines and chemokines and induces neutrophil apoptosis. There have been attempts to treat CF with IL-10, and it showed effect in mice, where it improved survival and decreased inflammation (252). The decreased NO level may contribute to airway smooth muscle contraction, bronchial obstruction and inflammation (253, 254).

Lipid mediators in lung inflammation

The lipid metabolism in CF has been considered important for long time, as many lipid mediators are involved in the regulation of inflammation. There is an imbalance of membrane composition of lipids in CF, which further increases the pro-inflammatory effects (101).

In summary, the excessive neutrophil inflammation in CF airways causes a perpetuating inflammation. Killing of bacteria is impaired due to CFTR dysfunction and mucus alteration. The inflammation causes degradation of lung tissue, which leads to bronchiectasis and lung function decline. The inflammatory process is dysregulated in many ways and there is an imbalance between pro-inflammatory and resolving factors where the pro-inflammatory side wins the battle.

ANCA

ANCA antigens

Autoantibodies directed against granule proteins of PMNs are called ANCA (anti-neutrophil cytoplasmic antibodies). ANCA was initially detected with indirect immunofluorescence (IIE), where different patterns, p-ANCA (perinuclear) and c-ANCA (cytoplasmic) were seen. Nowadays antibodies are usually detected by immunochemical assays such as ELISA and purified proteins are used as antigens (255). Most ANCA antigens are found in the azurophilic, primary, granules, for example the serine proteases neutrophil elastase, cathepsin G, PR3 and azurocidin. These granule proteins are important mediators in the elimination of pathogens by PMNs. BPI, described in more detail below, and MPO are other antimicrobial proteins found in primary granules and known to be target for ANCA.

ANCA in vasculitis and other inflammatory diseases

The strongest correlation to ANCA is found in pauci-immune small vessel vasculitis, such as microscopic polyangitis (MPA) and granulomatosis with polyangiitis (GPA). Typical clinical findings in MPA and GPA are necrotizing inflammation in the smallest blood vessels and in GPA also granuloma formation. Kidneys, respiratory tract and the skin are the organs most often involved. ANCA with specificity for MPO and PR3 are present in the majority of untreated patients, and as the disease often comes in flares, ANCA levels increase with a flare of disease and decrease with treatment and during remission (255, 256). ANCA is considered to be pathogenic in vasculitis.

ANCA is frequently found in inflammatory bowel disease (IBD) but no relation to disease severity has been reported (257-260). ANCA have been described in many other diseases as well, for instance systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), endocarditis, other chronic infections, and malignancies of the bone marrow (255).

Bactericidal/permeability-increasing protein (BPI) and its relatives

BPI is a cationic, antimicrobial peptide (AMP) of 55-60 kDa with a boomerang-shaped structure. BPI is a neutralizing peptide acting against bacterial endotoxins (LPS) and able to damage the permeability barrier of Gram-negative bacteria (261, 262).

BPI has two functionally important domains; the amino-terminal (N-terminal) domain binds to the lipid A/inner core of LPS from many Gram-negative bacteria, while the carboxy-terminal (C-terminal) domain promotes bacterial attachment to neutrophils and monocytes, which eventually leads to phagocytosis (263).

AMPs are secreted by cells exposed to bacteria, and by different blood cells (264) and have many roles in the defence against pathogens. They kill bacteria directly or act as chemoattractants, and some AMPs are able to bind to LPS to detoxify or to introduce it to immune cells, initiating an immune response. BPI is one of two major AMPs able to start such immune response (264) and LPS binding protein (LBP) the other.

When BPI was discovered, it was mainly believed to origin from neutrophil granules, but has later been found in many other cells and tissues, such as eosinophils, monocytes, epithelia of the alimentary tract, nasolacrimal ducts, lachrymal glands and tears. Dermal fibroblasts and epithelia of the genital tracts also express BPI (265).

BPI belongs to a family of proteins called lipid transfer (LT)/LBP family, considered evolutionary related. Substances of this family, for instance Palate, Lung and Nasal epithelium Clone (PLUNC) and LBP are important in the innate immune system (266). By transferring LPS from one binding site to another on the cell surface, LBP acts pro-inflammatory, whereas BPI has an anti-inflammatory effect. PLUNC also exhibit anti-inflammatory and anti-bacterial effects by reducing airway surface tension and disrupting *P. aeruginosa* biofilm (267).

BPI plays an important role in opsonization as it enhances the delivery of antigenic substances from bacteria to DCs (268) and mediates the reaction between bacteria and phagocyte (263). BPI acts in an antibacterial, anti-inflammatory and anti-angiogenic way. BPI reduces effects started by LPS, such as neutrophil stimulation, TNF-α production and pyrogenicity, by binding to LPS (269) and has been investigated as a potential treatment against infections. It has been shown that BPI has anti-angiogenic properties (270), by inhibiting growth, inducing detachment and apoptosis of endothelial cells involved in angio-genesis. As such, BPI could be a possible treatment for disorders where angio-genesis is important. BPI has a chemical similarity to platelet factor 4 (PAF4), which could be the reason why it has these anti-angiogenic effects (270).

Using recombinant BPI it has been shown possible to overcome the pro-inflammatory effects of LBP (271). Recombinant amino terminal fragment of BPI ($rBPI_{23}$) was found to reduce circulating endotoxin, release of TNF-α, IL-6, IL-8 and IL-10 among other effects in humans (272). The recombinant fragment $rBPI_{21}$ was used as adjunctive treatment of meningococcal sepsis in children and showed effect on morbidity, although it did not improve mortality rate in a small clinical study (273). Unfortunately, big doses of recombinant BPI are very expensive and difficult to produce. Other methods of production and delivery of BPI have been demonstrated, for instance based on an adenoviral vector (274), the use of a chimeric protein (275) or murine BPI expressed on gas vesicle nanoparticles (276).

The regulation of BPI production is complex and cell-type dependant (265).

Diseases associated with BPI are either related to mutations in BPI or to the presence of ANCA against BPI. A BPI polymorphism in intron 5 resulting in decreased plasma level of BPI was associated with an increased risk of developing COPD (277). Lower levels of BPI in synovial fluids have been observed in patients with arthritis related to psoriasis (278).

ANCA against BPI

The first report about ANCA against BPI came in 1995 and reported that BPI-ANCA was present in serum from vasculitis patients (279).

BPI-ANCA has been reported in many different diseases, such as CF, IBD, reactive arthritis, and transporter associated with antigen processing (TAP) deficiency (280-282). BPI-ANCA has also been found in patients with chronic pulmonary infections (283) and found to correlate with the severity of clinical symptoms. The serum BPI-ANCA titre decreased with the improvement of the clinical picture, a pattern seen also in CF (284).

The mechanisms causing these autoantibodies are not known, but different explanation have been suggested in the CF context.

BPI-ANCA in CF

The first publication about BPI-ANCA in CF was published in 1996 (285). BPI-ANCA, of both IgA and IgG type, has been associated with *P. aeruginosa* colonization, lung function decline and worse prognosis in CF (286-289). BPI-ANCA in CF is also significantly associated with several other negative factors, such

as low BMI and low FEV1%pred (290). BPI-ANCA inhibits BPI-mediated phagocytosis, an effect that has been demonstrated in several studies. Antibodies incubated with leukocytes, BPI and *E. coli* show decreased BPI-induced phagocytosis. Purified BPI-ANCA from patients with CF, vasculitits or IBD was able to inhibit the effect of BPI (289). Sera from BPI-ANCA positive CF patients inhibited PMN-mediated killing of *P. aeruginosa* in vitro in a dose-dependant way (291). It has also been demonstrated that monoclonal BPI-ANCA can activate PMN by release of ROS from granulocytes, a process increasing inflammation, and thus perpetuating chronic inflammation, as seen in CF (292). Together, these findings suggest why the presence of BPI-ANCA is negative in CF, as it impairs anti-bacterial effects necessary for the host.

Two different types of BPI-ANCA have been identified; ANCA directed against the C-terminal domain or the N-terminal domain, but no clinical or biological differences have been reported regarding the two sub-types (289, 293, 294).

It is not known how BPI-ANCA develops, but different mechanisms have been suggested. Diseases where BPI-ANCA is common have a very long and profuse exposure to Gram-negative bacteria (GNB) and endotoxin in common, a situation in which neutrophils are excessively activated and accumulated, leading to extensive mobilization of BPI (295).

The generation of BPI-ANCA

Certain epitopes on *P. aeruginosa* may trigger ANCA production by acting as a molecular mimic of BPI as certain BPI epitopes show strong similarity to outer membrane (OM) proteins from *P. aeruginosa* and *E. coli* (289) (293, 294). Helper T cells specific for *P. aeruginosa* would then provoke BPI-dedicated B cells into autoantibody production. Alternatively, the chronic infection with prolonged delivery of BPI-coated bacterial membrane particles to DCs, may initiate processes that induce auto-immunity (295, 296). Only one study has described the presence of BPI-ANCA in very young children, even before the development of clinical disease in CF (297) a finding that could indicate that the inflammation in CF starts very early, a theory now accepted, or that BPI-ANCA can be established without extensive bacterial load.

Skopelja et al recently presented a new theory suggesting that BPI is cleaved by elastase from *P. aeruginosa*-mediated NETs (298). This process created two molecules introduced to the immune system by DCs, in this way breaking tolerance to self. *P. aeruginosa* strains without bacterial elastase were not able to cleave BPI. This theory is in line with earlier findings regarding NETs as autoimmune initiators (220)

Other autoantibodies in CF

Autoantibodies are common in CF patients. Lachenal et al performed a screening in sera from 144 CF patients for a wide range of antibodies and found autoantibodies in 40% of the patients, of which 59% were specific against BPI (290). In this study no other ANCA were found, but ANA was found in 25%. IgM rheumatoid factor was found in 2.7%, anti-CCP in 7.6%, and anti-gliadin IgA in 12.5% of the patients.

Bactericidal/permeability-increasing protein fold containing family A (BPIFA) 1, is a protein secreted in the upper airways similar to BPI exhibiting antimicrobial effects of the same kind. The protein was formerly named SPLUNC-1, but was renamed due to its similarity with BPI. Budding et al found anti-BPIFA1-antibodies to be elevated in 48% of investigated CF patients compared to healthy controls. BPI-ANCA was elevated in over 90% of patients in this study. Levels of anti-BPIFA1 did not decrease after lung transplantation, which was seen with BPI-ANCA levels, in this and other studies (284, 299).

In systemic sclerosis with pulmonary complications autoantibodies against endothelin-1 type A receptor (ETAR) are present. In a recent study, Budding et al analysed the presence of anti-ETAR in patients undergoing lung transplantation and found overall higher anti-ETAR autoantibody titres in sera taken before lung transplantation in the CF patient group as compared to COPD but not in CF compared to ILD. The antibody titres were not influenced by lung transplantation and no association between autoantibody levels and clinical data was found (300).

Pseudomonas aeruginosa in CF

The role of *P.aeruginosa* infection in CF lung disease is a huge field of research, and the aim of this chapter is merely to clarify a few aspects regarding *P. aeruginosa*; this includes the detection of infection, the role of anti- *P. aeruginosa serology* in eradication and classification of infection and the attempts to develop a vaccine. *P. aeruginosa* diversification, mutagenicity and quorum-sensing will also be briefly described.

P. aeruginosa has been identified as the most significant pathogen in CF for a very long time (163) (301) (165). Chronic infection with *P. aeruginosa* is common in CF patients and in many CF clinics up to 80% of the adult population used to be affected (168) although in more recent reports this number is decreasing. In Sweden, 42% of all CF patients were considered chronically colonized with *P. aeruginosa* in 2014 (65). In a majority of these patients, chronic infection is preceded by intermittent colonization.

Detection of P. aeruginosa infection in CF

Bacterial cultures

In CF, early detection of infection is essential. Early and aggressive treatment of new *P. aeruginosa* colonisation can successfully eradicate the pathogen in many cases. To achieve this, regular culture of expectorated sputum is strongly recommended, not only when patients have symptoms as more than 50% of children diagnosed with *P. aeruginosa* for the first time can be asymptomatic (302). Culturing sputum is usually un-complicated in adult patients, as they produce enough sputum, but it is often more complicated in children.

Cultures from lower airways can be obtained in different ways: expectorated sputum, cough swab, induced sputum or via bronchoscopy with bronchoalveolar lavage (BAL). Other techniques are also available. BAL is the “gold standard” for examining pathogens and inflammation in young children (303) but has its practical limitations

as it requires anaesthesia and is costly. Another limitation is that BAL usually examines only one or two segments of the lung (304).

Cough swabs means that a swab is placed in the posterior pharynx and the patient is asked to cough, a procedure that can be challenging in young children. It is debated if performing this test after physiotherapy or nebulised hypertonic saline can increase sensitivity. In a recent study, induced sputum from eleven young (3-7.4 years old) non-expectorating CF patients was compared to BAL (304) and a good bacteriologic correlation was found. Collins et al found that induced sputum procedure reduced the need for BAL when 7% hypertonic saline was nebulised for 15 min and sputum was collected at 5 min intervals, and finally physiotherapy was performed for collection of more sputum (305). In Sweden, the use of cough swabs in children is limited. Instead a technique is used where the nasopharynx is subjected to suction with a plastic catheter (personal communication).

When searching for *P. aeruginosa*, the result from culturing sputum is comparable with BAL culture (306) and high negative and positive predictive values are achieved, while cultures from the oropharynx are not useful (307).

Molecular testing

Techniques based on molecular testing instead of culture, are also used and show promising results. PCR for early detection of *P. aeruginosa* has been evaluated (308) and studies indicate that PCR is useful as a complement for early detection of *P. aeruginosa* in CF patients. 16S rRNA sequencing is another technique that can add information about present bacteria.

P. aeruginosa serology in CF

In the 1970ies and 1980ies, different antibodies against *P. aeruginosa*, in form of precipitins, elastase and proteinase antibodies, were found to correlate with the clinical status of CF patients (309, 310) and it was initially suggested that patients with a stronger immune response, represented by high levels of antibodies, against *P. aeruginosa* infection had a more severe lung disease (311). The analysis of antibodies against *P. aeruginosa* started, with Hoibys work on precipitins (163). Different commercial test are now available, most often as ELISA-tests measuring different *P. aeruginosa* antigens as elastase (ELA), alkaline protease (AP) or exotoxin A (ExoA). Measuring antibodies against *P. aeruginosa* has been shown to be useful in characterizing patients with different infection status; elevated titres found early in the course of infection constitute a risk factor for developing chronic infection (312, 313). Serology may also be useful to monitor response to therapy (314) as patients

who clear an early infection exhibit a significant decrease in antibody titres. In patients in whom antibiotic therapy fails the titres increase instead. There is considerable variation between patients concerning early antibody responses and treatment decisions should not be based only on serology (314).

Serum antibodies may be detected before the organism is detected, especially in children who do not produce sputum and have few symptoms. In BAL samples from CF children diagnosed through neonatal screening, serology for *P. aeruginosa* became positive six to 12 months before the organism was isolated. A longitudinal monitoring of antibody titres, in combination with pulmonary X-ray findings, could facilitate diagnosing early *P. aeruginosa* infection in young children (315, 316) although there is still some controversy about this. Daines found a positive predictive value of serology for first positive culture within the next six months of only 76.2% and maximum negative predictive value of 72.1% and concluded that serology did not appear to be useful for predicting first culture of *P. aeruginosa* (317).

Mauch (313) found in his review that studies showed a good correlation between anti-*Pseudomonas* antibody titres and microbiological culture, and that *Pseudomonas* serology can be useful to evaluate the colonization/infection status of the patient. The author concluded that there is support for the use of *Pseudomonas* serology in the follow-up of CF patients, something that is already done in the Nordic countries, but is not as a role in the US.

In patients already chronically colonized with *P aeruginosa* serology is nowadays not considered to add any relevant information (315).

Classification of P. aeruginosa infection in CF

Until 2003 there was no universally accepted definition of chronic *P. aeruginosa* infection. Different definitions were used in different countries, some of them involving the use of serology, some not. Lee (318) et al suggested and validated a new set of definitions, the "Leeds criteria". In the Leeds criteria patients are categorized into four different groups, based on standard cultures obtained during the preceding twelve-month period:

- Chronic: More than 50% of sputum samples are positive for *P. aeruginosa*.
- Intermittent: 50% or less of samples are positive.
- Free of infection: No growth of *P. aeruginosa* during previous twelve months, however, previously cultures have been positive.
- Never: *P. aeruginosa* has never been cultured from sputum or cough swab.

If cultures are taken too seldom, the accuracy of the classification will decrease and thus the Leeds criteria requires frequent sampling; at least four sputum cultures per year in adults, and four to six in children. Antibodies against *P. aeruginosa* were not included in these criteria, as only few centres have access to prompt testing (318). High association between infection category and antibody levels, after adjustment for age, was found.

In 2006, an evaluation of the Leeds criteria was published. A good agreement was found between colonisation category and clinical status as well as with levels of antibodies (319).

The European Cystic Fibrosis Foundation uses the following pragmatic definition of chronic *P. aeruginosa* colonization in their annual report (65):

Patient should be defined as chronically infected if he/she fulfils the criteria now or has done in recent years and the physician has no reason to think the status has changed:

a. modified Leeds criteria, chronic infection: >50% of the sputum samples positive, collected during the last 12 months. At least 4 sputum samples during that period;

b. and/or significantly raised bacteria-specific antibodies according to local laboratories.

Vaccination against P. aeruginosa in CF

Treating established *P. aeruginosa* infection in CF lungs is difficult and a vaccine would be very useful, also in other patient groups. There have been many attempts to create such vaccine, based on different antigenic components from the bacteria, such as OM products, surface polysaccharides and flagellar proteins (320). Antibodies against the whole bacteria or OM proteins can be found in serum or BAL after infection, indicating that a vaccine could be based on these components (321). Surface polysaccharides, such as alginate, have been investigated for vaccine purposes, but these substances are poorly immunogenic and protective antibodies do not develop. Polysaccharides have been combined with toxins or flagellar proteins to increase immunogenicity (322-324) and several studies have been performed with such vaccine candidates. Repeated immunization with a vaccine based on a conjugate of O-polysaccharide-toxin A, an octavalent vaccine, showed low but sustained levels of antibodies in patients (324) and in a long-time follow-up of vaccinated patients, a lower rate of chronic *P. aeruginosa* infection was seen (325). A bigger study was started, with more than 400 patients included, by the industry, (Aerugen Berna Vaccine) but this study was terminated early and no results have been published.

P. aeruginosa is able to move because of a single polar flagellum built of polymerized flagellin proteins of two major serotypes: A and B (322). Flagellar proteins contribute to the invasive capacity of *P. aeruginosa* and are also involved in adhesion to host cells and mucins (326). Flagella induce inflammation by binding to toll-like-receptor 5 (TLR5) (327). *P. aeruginosa* strains that initially colonise CF patients are generally flagella positive and composed of A or B or both flagella subtypes (328). Vaccines based on flagellar proteins, instead of polysaccharide antigens, show high and persisting antibody titres against flagella antigens and several studies in animals and humans have been performed. Doring et al included almost 500 CF patients in a phase III study and found high and long-lasting IgG antiflagellar titers. They also saw a lower risk of chronic *P. aeruginosa infection,* the primary end-point, (RR 0.66), compared to placebo, finding a protective effect of 34%. The vaccine seemed to protect from certain strains as *P. aeruginosa* strains with flagella subtypes included in the vaccine were less frequently isolated from vaccinated patients than in the placebo group (329). As antibiotic treatment against *P. aeruginosa* has become more efficacious, early eradication therapy may decrease the interest in future vaccine studies as it is very difficult to prove that the vaccine protects from chronic infection (328). Based on available studies, Cochrane reviews conclude that vaccines against *P. aeruginosa* cannot be recommended (330) and there is no vaccine available.

Gurgling with anti-*Pseudomonas* antibodies from egg yolk, IgY, has been investigated as a treatment to prevent *P. aeruginosa* colonization for a long time, and the treatment has shown positive results in a small group of CF patients, but so far no double-blind randomized study has been performed. A phase III study is underway and a new study evaluating IgY treatment in mice showed promising results (331).

Another option in order to prevent, and also treat, *P. aeruginosa* infection, is passive immunization with monoclonal antibodies against *P. aeruginosa* able to neutralize different toxins (332, 333). Studies on mice treated with multifunctional bispecific antibodies against the serotype-independent type III secretion system virulence factor PcrV and persistence factor Psl (exopolysaccharide) showed positive results. A similar antibody has been tested in a phase I study in humans. A trend towards reduced inflammation in the airways was seen in this short study, but no differences in clinical outcome or *P. aeruginosa density* (334).

P. aeruginosa adaptation and diversification in CF

P. aeruginosa undergoes different changes to adapt to the environment in the CF lung. The CF lung is a stressful place, and to persist there, the bacteria must overcome different challenges induced by host response, antibiotics and co-existence with other pathogens. *P. aeruginosa* is ecologically flexible, due to its large genome, with many

regulatory genes and genes involved in catabolism and transport of organic compounds (335, 336).

The best-known adaptation is the emergence of mucoid colonies. This is caused by overproduction of the polysaccharide alginate. The change to a mucoid appearance is acknowledged as a marker for chronic infection and is, although seen also in other chronic lung infections, almost pathognomonic for CF lung disease (337). Alginate is involved in the emergence of biofilm, which protects the pathogen from antibiotics and host responses (338). The bacteria also lose their motility and develop hypermutators (339). Increased antibiotic resistance is another common adaptation (340). Toxins like Exotoxin S, U, T and Y secreted via the type III secretion system, and quorum sensing (QS) system, control other important virulence factors, such as pyocyanin and elastase. QS is a cell-density dependent regulatory system, involved in the control of gene expression of multiple genes. Gradually, the bacteria lose acute virulence factors and instead develop chronic virulence factors. Much is known about the acute virulence factors, less about chronic, apart from bio-film formation (341).

Pyocyanin is a well-described virulence factor, which together with pyoverdine results in the characteristic green colour of wild type *P. aeruginosa* grown on agar plate. Pyocyanin is an important virulence factor that interacts with PMNs and epithelial cells accelerating apoptosis. It also impairs phagocytosis of apoptotic cells and induces IL-8 expression in vitro (342-345). In a study by our group (346) pyocyanin producing strains from CF patients were associated with BPI-ANCA negative patients. BPI-ANCA positive strains were less actively harmful in their interaction with epithelial cells and induced less IL-8 and less cell death than strains from BPI-ANCA negative patients. This finding indicates that strains from BPI-ANCA positive patients have adapted to the CF airways.

Platelets

Platelet function in haemostasis and the immune system

Platelets are small cytoplasmic cell bodies without a nucleus, present in large numbers in the circulation. Platelets are derived from megakaryocytes, which are resident in the bone marrow (347). Platelets express a number of receptors on their surface and different effectors in their granules. GPIIb/IIIa, $P2Y_1$, $P2Y_{12}$ and GPIa/IIa are examples of important receptors that all contribute to aspects of platelet activation (348). Activation of the platelet occurs when a receptor binds an activation ligand and a downstream signalling follows. Well-known activators are collagen, adenosine diphosphate (ADP), thromboxane, and thrombin (349). In haemostasis, these receptors attach to collagen or von Willebrand factor, exposed by vascular damage or endothelial activation, and bond with platelet-adhesion receptors. This induces the activation of platelets and release of granule contents including pro-coagulatory mediators such as thrombin and prostaglandins. This process leads the formation of a thrombus, involving leucocytes and red blood cells as well.

The role of platelet aggregation in response to vascular damage and maintaining haemostasis is well known, but platelets are also involved in the innate immune response. The role of platelets in immunity is becoming more and more acknowledged. Platelets play multiple roles in the inflammatory and immune response. Platelets possess many different receptors and adhesions molecules that help them interact with both immune cells and pathogens, most notably CD62P mobilised from granules to the surface of activated platelets (350). Platelet granules also contain different modulatory mediators, including cytokines and chemokines such as TGF β and PF4 (351). When activated, intracellular signalling results in a rearrangement of the cytoskeleton that changes the shape of the platelet, after which surface-adhesion molecules are activated and granules released.

Intergrins are cell surface-adhesion molecules found on many different cell types. They help cells interact with extracellular matrix and other cells and are abundantly expressed on platelets (352). Integrin functions on platelets include interaction with one another, leucocytes, endothelial cells and extracellular matrix. GPIIb/IIIa, the predominant platelet integrin, is recognized by the monoclonal antibody PAC-1 that

binds to the active conformation of the GPIIb/IIIa complex on activated platelets (353).

Platelets bind to leukocytes, activate them, and influence neutrophil functions, such as degranulation. By releasing chemoattractants, platelets further promote leukocyte recruitment. Platelets release mediators able to bind to and activate neutrophils, for instance CD40L. CD40L is an important activator of macrophages and increases their killing of microbes (354). Mediators such as CD40L also activate the endothelium and induce adhesion molecule expression, a process that further supports leukocyte adhesion.

Platelets are also able to recognize and directly bind, and via sequestering, kill pathogens (350). Platelets, interacting with Kupffer cells in the liver, scan the vasculature for pathogens, and upon detection they may capture and isolate the pathogen in a platelet aggregate (355). Platelets recognize pathogens via the expression of pattern-recognition receptors, such as TLR (355) and are able to recognize LPS via TLR4. Upon recognition, platelets bind to neutrophils and NET formation can be induced (356). The platelet-derived activation of leukocytes is not restricted to the blood vessels, but can also take place in tissues (357).

Platelets have the ability to bridge leukocytes to the endothelium, giving them access to the vessel wall via important adhesion molecules, such as P-selectin (CD62P) (353). Platelets have also been shown to bind to lymphocytes and enhance lymphocyte adhesion in lymph nodes (358, 359).

Platelet activation occurs during inflammation and infection, and assays of platelet activation may provide diagnostic or prognostic information during inflammatory conditions.

Platelet activation in disease

Platelet activation plays an important role in the inflammatory process in different lung diseases ((360, 361) and in many vascular diseases, such as coronary heart disease, thrombotic disease and ischaemic stroke, but also in diabetes, sickle cell disease and HIV ((362-366). Increased platelet activation is also seen in renal failure patients, psoriasis and Crohn´s disease (367-369) and many other diseases. The treatment with anti-platelet therapy in cardiovascular disease is well established.

Platelets have also been implicated in the development of autoimmune disease, for instance systemic lupus erythematosis (SLE), via the soluble marker of vascular inflammation, CD40L, for which platelets are the main source (370). Inhibition of CD40L decreases inflammation in several models (371) and depletion of platelets increase survival in a mouse model for SLE (370).

It is well known that the pro-thrombotic platelet activity in vascular disease can be inhibited by anti-platelet therapy, but treatment of the pro-inflammatory platelet-derived effect in infection and inflammation is more controversial (372). In an animal model of acute lung injury (ALI) platelets play an important role in the recruitment of neutrophils to the lung and platelet depletion diminished PMN accumulation in the intravascular, interstitial and alveolar compartments (373).

Platelet activation in CF

Platelet activation in CF has been reported to be increased by different investigators (374-377), although McGivern (378) found normal levels of platelet activation.

The platelet activation seen in CF patients may be both a direct and indirect effect of the primary defect in CF, the dysfunctional CFTR. CFTR is found on human platelets and CFTR blockade has been demonstrated to influence platelet release of mediators involved in the inflammatory response (13). In particular, Mattoscio et al found that CFTR blockade of platelets reduced LXA_4 formation. Lipoxins are anti-inflammatory lipid mediators that modulate neutrophil inflammation (13). LXA_4, is formed during platelet-monocyte interaction and is important in the resolution of inflammation (250). The early and continuous inflammation seen in CF most probably also influences platelet activation indirectly, as part of the vicious circle of infection and inflammation.

Zhao et al (379) found that platelets play an important role in lung inflammation in CFTR-deficient mice. Inhibition of platelet aggregation or depletion of neutrophils diminished LPS-induced lung inflammation in these mice. Zhao also found that anti-platelet aggregation treatment with acetylsalicylic acid decreased LPS-induced thrombocytopenia and lung inflammation in CF mice.

O´Sullivan, on the other hand (377) found increased platelet activation in CF in form of monocyte-platelet aggregation, neutrophil-platelet aggregation and increased platelet surface P-selectin (CD62P), but did not identify neither CFTR nor CFTR-specific mRNA in normal, human, platelets.

Present investigations

Aims of the present studies

The overall aim of this thesis was to compare the impact of different prognostic factors in CF, with emphasis on BPI-ANCA, increase our knowledge about BPI-ANCA and platelet function in CF and investigate how and why BPI-ANCA is established in some, but not all, CF patients. Specific aims of the present studies were

1. To evaluate BPI-ANCA as a long-term prognostic factor in a cohort of adult CF patients (study I) and in children and adult CF patients (study II).
2. To compare the prognostic value of BPI-ANCA with clinical data and serological findings related to *Pseudomonas aeruginosa* in a cohort of children and adult CF patients (study II).
3. To investigate molecular characteristics of *Pseudomonas aeruginosa* isolates from BPI-ANCA negative and BPI-ANCA positive patients to understand why BPI-ANCA develops only in some patients (study III).
4. To investigate platelet activation in CF patients compared to healthy controls and to correlate these results to clinical findings (study IV)

Patients and methods

Patients

Patients were recruited from the CF centre in Lund: children from the Department of Paediatrics (study II and III) and adults from the Department of Respiratory medicine and Allergology, Skåne University Hospital, Lund, Sweden (study I-IV). The cohorts in study I and II had been established during earlier studies of BPI-ANCA, as both were long-term follow up studies (286, 287) The cohort in study III was partly established during earlier investigations (346), but extended during the course of this work, and new patients were recruited from the adult CF centre.

Patients for study IV were recruited from the CF centre in Lund in 2015 and only adult patients were included.

CF diagnosis was confirmed in all patients, and information about CFTR mutations and clinical data was retrieved from patient records.

Ethics

The Regional Ethical Review Board in Lund approved the studies and written, informed consent was signed by all patients, or, when children were included by their parents.

Statistical analysis

Statistical calculations in study I were performed using SPSS for Windows version 19. Survival curves were estimated using Kaplan-Meier method. Log rank tests were used to compare survival between sub-groups. Cox proportional hazard regression was used to estimate hazard ratios.

In study II Pearson correlation coefficient (r) was used to examine and compare serology and BPI-ANCA in relation to bacterial colonization, lung function, future colonization and long time outcome. Receiver operator curves (ROC) were generated to graphically illustrate sensitivity and specificity for these assays. Area under curve (AUC) with 95% confidence interval (ci) was calculated. Analysis was performed with GraphPad PRISM 6, version 6.0a, 2012.

Statistics in study III was performed using the Mann-Whitney U-test. In study IV Pearson correlation coefficient (r) was used for correlations (GraphPad, PRISM 6, version 6.0a, 2012).

Lung function

Lung function was measured by spirometry and calculated according to age, sex and height. In study I, II and III, FEV1%pred, was calculated according to Solymar (380) and Quanjer (381). In study IV results from the annual review, performed at the Department of Clinical Physiology in Lund, were used, and the Swedish reference table created by Berglund was used to calculate FEV1%pred (382).

Microbiology

In all four studies, sampling, transport, and culture were performed according to routine procedures. History of bacterial colonization was obtained from patient records as far back as possible, and *P. aeruginosa* colonization was defined according to the Leeds criteria (318) where Leeds class I (chronic) consists of patients with more than 50% positive cultures during the last year, class II (intermittent) have *P. aeruginosa* in 50% or less of the sputum cultures, class III have had the pathogen before, but not during the last year, and class IV have never had *P. aeruginosa* growing in their sputum cultures. In study III *P. aeruginosa* strains were longitudinally isolated at Clinical Microbiology, Laboratory Medicine Skåne, Lund, Sweden, and all clinical isolates were stored at -80°C until analyzed.

BPI-ANCA

BPI-ANCA was analysed with ELISA and measured at time of inclusion in each study. Purified BPI was obtained from Wieslab AB (Lund, Sweden) or Euro Diagnostica (Malmö, Sweden) and direct binding was performed (383). Purified BPI were coated onto microtiter plates at a concentration of 1µg/ml. Serum samples were diluted 1/80 and incubated for one hour. Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-human IgA. BPI-ANCA was quantified from a calibrator curve that was serially diluted and the results expressed as arbitrary units (U). The cut off level for IgA BPI-ANCA was determined to be ≥ 67 arbitrary units per litre (U/L) from the mean absorbance value of 42 normal paediatric sera + 3S. The cut-off level for IgG BPI-ANCA was set to 50 AU (287). In study I and II, BPI-ANCA of IgA subclass was evaluated, as earlier studies had shown that IgA correlates slightly better with lung function than IgG (287).

Anti-Pseudomonas serology

P. aeruginosa serologies (study II) were analysed using anti-Pseudomonas IgG EIA E15, a commercially available test from Mediagnost, Reutlingen, Germany. Antibodies against three exoproteins, AP, ExoA and ELA were measured at the time of inclusion. Serum or plasma samples were diluted and added to wells of microtitre plates, coated with AP, ExoA or ELA. After washing, the conjugate (anti-human IgG peroxidase labelled immunoglobulin) was added and incubated again for 2 hours at 37°C. After a final washing step, substrate was added and further incubated for 30 minutes at room temperature. The reaction was terminated on addition of stop solution accompanied by a change from blue to yellow. The absorbance of the colour

reaction product was measured on a microtitre plate reader. Kappler et al have investigated specificity and sensitivity of this analysis (315).

mRNA microarray

In study III an mRNA microarray was performed on six clinical isolates of *P. aeruginosa*, three from BPI-ANCA-positive and three from BPI-ANCA-negative patients, and the reference strain PAO-1. Strains were cultured three times at three different occasions, in 50 ml LB medium at 37°C until reaching early log phase (OD600=0.5). Total RNA was harvested by RNeasy kits form Qiagen (Copenhagen, Denmark), and the quantity and quality were analysed by Nanodrop and Agilent 2100 Bioanalyzer. The global mRNA expression patterns were analysed in all 21 RNA samples using Affymetrix gene chips for *P. aeruginosa*. Probe summarization and data normalisation were performed using the robust multi-array analysis (RMA). A SAM (significance analysis of microarray) analysis was performed to identify significantly differentially expressed genes between BPI-ANCA-positive and BPI-ANCA-negative patients. A heat map of the microarray was generated. Differentially regulated genes were interpreted by using the Pseudomonas genome project website (384) or the UniProt database (385).

Extraction of outer membrane fraction and analysis on two-dimensional (2D) gel electrophoresis

In study III outer membrane proteins (OMPs) were isolated from cultures of five BPI-ANCA-positive and five negative strains of *P. aeruginosa* based upon the method of Lecoutere et al (386). OMPs were separated on 2D-gel electrophoresis as previously described (387). After centrifugation at 8,000×g for 10 min to remove any precipitates supernatant was removed and applied to a precast 7 cm pH 3-10 and 4-7 immobilized pH gradient (IPG) gel strip by in-gel sample rehydration method. The strips were covered with mineral oil and allowed to rehydrate overnight. Isoelectric focusing was performed using IPGphor II electrophoresis unit (GE Healthcare Biosciences). The focused strips were equilibrated in equilibration buffer for 15 min followed by incubation in the same buffer. After the equilibration, strips were run in a second dimension of SDS-polyacrylamide gel. Electrophoresis was conducted in a PROTEAN II MINI GEL cell electrophoresis unit. Protein spots on gels were visualized with Coomassie blue staining for protein sequence identification with mass spectrometry. Targeted stained protein spots were excised and subjected to protein identification by MALDI-TOF-MS. Protein identification was performed by Protein Analysis Service available at Alphalyse (Odense, Denmark).

Flagellin A and B genotyping by PCR

Clinical *P. aeruginosa* isolates were cultured on LB agar overnight and harvested. Bacterial DNA was isolated using innuPREP Bacteria DNA Kit (Analytik; Jena, Germany). DNA concentrations were measured using Nanodrop, and all samples were diluted to 100ng/µl. Flagellin A and B specific primer pairs were designed by alignment of 23 published flagellin A genes and 18 flagellin B genes. A common forward primer binding to both genes was used together with a flagellin A specific reverse primer or a flagellin B specific reverse The flagellin A primers generated a PCR product of 793-800 base pairs (bp) and the flaggelin B primers a 719 bp amplicon. The PCR was performed by 30 cycles (30 sec at 95°C, 30 sec at 56°C and 30 sec at 72°C). The PCR products were analysed on a 1.2% Agarose gel.

Results and discussion

BPI-ANCA and prognosis in CF patients

The aim of **study I**, a prospective study, was to follow the progress of lung disease in 46 adult CF patients to elucidate the significance of a positive IgA-BPI-ANCA as a prognostic factor, in relationship to level of lung function and *P. aeruginosa* colonization. The patients were included between 1995 and 1998 and the cohort followed until December 31st 2009. Death and lung transplantation were end-points. Patients are described in table 3.

In total seven patients reached an end-point within five years after inclusion and 15 within ten years. At the end of the study 19 patients were either transplanted or dead.

The well-known association between *P. aeruginosa* colonization in CF patients and adverse clinical outcome can be seen also in this study, but bacterial colonisation categorized by the Leeds classification was not a significant determinant of outcome (p= 0.113).

After ten years, eleven (42 %) out of the 26 patients with chronic or intermittent *P. aeruginosa* colonization at inclusion had experienced an end-point, and on December 31st 2009 14, 54%, of patients in this group were either dead or had received a lung transplant. Compared to this, the patients who were free from earlier *P. aeruginosa* (Leeds III) or who had never been infected with *P. aeruginosa* (Leeds IV), did better as at time of final follow-up only five of these patients (20%) had reached end-point.

Table 3 Description of patients in paper I at baseline

Number of patients	Total	Males	Females	
(n)	46	26	20	
Age:	Mean	Range		
(years)	26.2	18.4-44.6		
CFTR mutation:	ΔF508/ΔF508	Others		
(n)	24	22		
FEV1.0 % predicted:	> 80 %	50-80%	<50%	
(n)	16	17	13	
IgA BPI-ANCA:	Negative (≤67 U)	Positive (>67-200 U)	High (>200 U)	
(n)	17	18	11	
(mean age, years)	27.5	26.3	24.6	
Leeds classification of P. aeruginosa colonization:	I (chronic)	II (intermittent)	III (free)	IV (never)
(n)	24	2	8	12
Diabetes mellitus:	yes	no	NA	
(n)	5	34	1	

In contrast to Leeds groups, IgA-BPI-ANCA level was significantly correlated to outcome. The hazard ratio for one standard deviation of BPI-ANCA, used as a continuous variable, was calculated to 1.76 (95% CI: 1.25-2.48 p=<0.001). After ten years 15 patients had reached an endpoint, out of these only two (13 %) were IgA-BPI-ANCA negative at inclusion. The median IgA-BPI-ANCA level of all patients reaching an end-point within ten years was 251 ELISA units as compared to 69 for the 31 patients who did not experience such an event.

As expected, lung function at inclusion was a very important predictor for the long-term prognosis. None of the patients with a normal FEV1%pred at inclusion reached end-point during the follow-up. Patients with a severe lung damage at inclusion reached end-point to a very high degree, 11 out of 13 patients. The hazard ratio for reaching an end-point was, for each standard deviation of better FEV1%pred at baseline, 0.334 (0.18-0.60; p=<0.001).

A positive IgA-BPI-ANCA was associated with low lung function at inclusion. The moderate sample size and the association between low lung function and adverse outcome in this cohort makes it difficult to analyse whether IgA-BPI-ANCA provides any additional information when FEV1%pred is known. But it is interesting to note that among patients with severe lung damage all patients with high ANCA levels (>200 AU) reached end point within ten years as compared to three out seven with lower values.

In **study II** we evaluated the relation between BPI-ANCA and different *P. aeruginosa* serologies to investigate if BPI-ANCA gives the same information as *Pseudomonas* serology tests. We compared BPI-ANCA with serology with respect to lung function impairment, prediction of outcome, detection of chronic *P. aeruginosa* colonization and prediction of future colonization. The cohort is described in table 4.

Table 4. Description of patients in study II at baseline

P. aeruginosa infection status	Chronic Leeds I	Intermittent Leeds II	Free from Leeds III	Never Leeds IV	Total
n	48	11	16	42	117
Median age (IQR) ***years***	21 (17-27)	17 (10-23)	15 (7-22)	16 (7-25)	19 (11-25)
Sex m/f *n*	27/21	4/7	5/10	24/18	60/57
Mutation **ΔF508/ΔF508** **ΔF508/other** **other/other** **ΔF508/unknown *n***	28/17/2/1	7/4/0/0	10/5/1/0	20/16/3/3	65/42/6/4
Pancreatic insufficiency ***n (%)***	47 (98%)	8 (73%)	14 (87,5%)	31 (74%)	100 (85%)
Median lung function ***FEV1% pred*** **(IQR)**	62 (41-85)	95 (90-101)	90 (74-102)	89 (80-104)	84 (60-96) (n=112)

In the whole cohort, 25 patients (18%) died or were lung transplanted during the 10-year follow-up. In the Leeds I group, 20 patients (42%) either died or were lung transplanted. In the Leeds group II, III and IV only five patients (1,1 and 3 respectively) died or were lung transplanted. One of the patients in Leeds group IV died from an accident, not related to CF. At follow-up the remaining 28 patients in the chronically colonized group had a lung function of 61% of predicted FEV1 (IQR 50-76). In Leeds group II, III and IV the follow up lung functions were 86%, 69% and 88% respectively.

BPI-ANCA had a higher capability of predicting the end-points in the chronically colonized group (AUC= 0.77, p=0.002) compared with serology tests; AUC for AP 0.7 P (p = 0.02), ELA 0.65 (p = 0.09) and ExoA 0.54 (p=0.6). This finding is in line with the results from study I, where BPI-ANCA level, in contrast to Leeds class, was significantly correlated to outcome.

The results from study I and II both show that BPI-ANCA is a stronger prognostic factor than *P. aeruginosa* colonization on its own, and a probable explanation for this

is that the presence of ANCA shows that an unfavourable host-pathogen interaction has occurred.

BPI-ANCA and anti-Pseudomonas serology

It is known that BPI-ANCA correlates with *P. aeruginosa* colonization and it has been discussed if BPI-ANCA is just another anti-*Pseudomonas* serology. In **study II** we compared BPI-ANCA and three different serology tests and found that they were all useful for identifying patients with chronic *P. aeruginosa* (AUC between 0.822 and 0.929). There were no statistical differences between the tests. Among the chronically colonized (Leeds I) patients, the values obtained with the three *Pseudomonas* serology tests (AP, ELA and ExoA) correlated better to each other (r values: 0.37, 0.46 and 0.58) than they did with the levels of IgA-BPI-ANCA (r values: 0.12, 0.21, 0.02).

To examine the ability of the different tests to detect subclinical colonization, and in that way detect future permanent colonization, we compared BPI-ANCA and serology tests among those who during a follow-up of three years changed their colonization status from Leeds II, III and IV to Leeds I. Twelve patients underwent such a change. None one of the tests were able to identify such patients.

BPI-ANCA is associated with lung function impairment and in study II correlation between lung function impairment (100-FEV1.0%pred) and IgA-BPI-ANCA in the chronically colonized group gave an r-value of 0.44. A value in the same range was achieved with the anti-AP test (r=0.35), while a lesser degree of correlation was seen for the anti-ELA test (r=0.20) and hardly any with the anti-ExoA test (r=0.06). ROC curves were also created to evaluate the ability to detect lung function impairment (FEV1.0<80%pred). We found that IgA-BPI-ANCA exhibited the highest value (AUC 0.799) while the corresponding values for the three bacterial serology tests ranged from 0.516 to 0.689.

Thus, BPI-ANCA shows better correlation with *P. aeruginosa* induced lung function impairment and negative prognosis and has a similar capacity to detect chronic colonization as standard anti-Pseudomonas serology. The interpretation of these result, as in study I, is that BPI-ANCA shows that something more than *P. aeruginosa* colonization has occurred in the patient, influencing lung function decline and long time prognosis.

Why does BPI-ANCA develop?

The finding that some CF patients who have been chronically colonized with *P. aeruginosa* for many years don´t develop BPI-ANCA, and that these patients seem to have a better prognosis (study I), could be related to differences in the strains of *P.*

aeruginosa in these patients. Different strains could generate differences in the immunological response in the host, thereby influencing the inflammatory process, prevalent in CF patients.

In **study III**, three different methods were used to look for such differences between *P. aeruginosa* strains. First, six clinical isolates of *P. aeruginosa*, three from BPI-ANCA-positive and three BPI-ANCA-negative patients, and the reference strain PAO-1 were submitted to mRNA analysis. Differentially regulated genes were interpreted by using the Pseudomonas genome project website (384) or the UniProt database (385). A large number of genes were differentially expressed in the six isolates studied. A higher expression of genes associated with polyamine metabolism and lipid A biosynthesis was seen among isolates from ANCA-positive patients, whereas genes related to quorum sensing, phenazine metabolism and flagellin assembly were found to have a lower expression.

Next, extraction of outer membrane fraction and analysis on two-dimensional (2D) gel electrophoresis was performed on *P. aeruginosa* cultures from five BPI-ANCA-positive and five negative patients. The 2D gel electrophoresis showed a distinct pattern suggesting that flagellin A is a factor involved in bacteria related to the development of BPI-ANCA. We found 10 and 6 protein spots that were distinct in ANCA-negative and ANCA-positive *P. aeruginosa* strains, respectively. Five out of the 10 spots found in ANCA-positive strains were related to flagellin A.

Table 5. Patients recruited for the PCR analysis in study III

BPI-ANCA status	Number of patients	Sex (f/m)	Age (years) mean	Mutation type (PI/PS)	Lung function (FEV1%pred) mean	Pseudomonas colonization Leeds class I/II
IgA positive	21	8/13	31.5	20/1	69	20/1
IgA negative	16	9/7	31.1	15/1	70	14/2
IgG positive	19	9/10	34.2	18/1	62	17/2
IgG negative	18	8/10	28.3	17/1	77	17/1
Both IgA-and IgG positive	11	4/7	32.2	11/0	66	10/1
Both IgA and IgG negative	8	4/4	25.3	8/0	84	7/1
All patients	37	17/20	31.3	35/2	69	34/3

This finding was very interesting, as it suggested that different types of flagellin from *P. aeruginosa* bacteria would induce different pathogen-host interactions, influencing the prognosis for the patient. To try this hypothesis, a bigger cohort of 37 CF patients, with well-defined colonization data and BPI-ANCA status, was created. These patients are described in table 5. All strains, *i.e* also those analysed with mRNA and 2D-gel-eletrophoresis, were subjected to PCR analysis, but to avoid bias, statistics was based only on strains from the 37 new patients. However, the pattern was not persistent when analysed in a larger number of patients, and there were several patients with flagellin B-carrying isolates who had developed BPI-ANCA. There was a tendency towards a difference between flagellin A and B in the BPI-ANCA IgG-positive group, but it was not statistically significant (p=0.18).

When we analysed the initial 10 strains we found partly diverging PCR results, compared to the 2D-gel and mass-spectrometry. This result raises the question of which method is more efficient in identifying differences between bacterial strains.

Platelet activation in cystic fibrosis patients

It is well known that platelet activation occurs during inflammation and that activated platelets release pro-inflammatory mediators, such as lipid metabolites and chemokines. Activated platelets bind to and modulate the function of immune cells, such as monocytes and neutrophils and may thereby influence the inflammatory processes and sustained lung tissue damage seen in CF.

In **study IV** we investigated platelet function in blood from 22 CF patients compared with healthy controls. Platelet aggregation, platelet activation, platelet-leukocyte complex formation, and leukocyte activation were analysed. We also correlated platelet function to clinical data in CF patients, including BPI-ANCA. The cohort is described in table 6.

In **study IV** we confirm that platelet activation is increased in CF patients, however, the results are assay dependent. Increased platelet aggregation and platelet-monocyte activation was observed but significantly activated isolated platelets were not detected in ex-vivo samples when measuring CD62P or PAC-1 on the platelet surface. The inability to detect an activated platelet population in ex-vivo samples may reflect a preferential association of activated platelets with monocytes or shedding of CD62P from the surface of activated platelets. It is known that activated platelets shed their CD62P to plasma, but continue to circulate and are active. We detected significantly increased levels of platelet-monocyte complexes, which is a more robust marker of in-vivo platelet-activation.

Table 6. Patients participating in study IV

Patients (n)	22		
Sex (n)	f=9	m=13	
Age (years)	34.0(mean)	SD: 9.45	Range: 20.7-54.4
Pancreatic function (n)	PI 19	PS 3	
Lung function FEV1%pred (n=20)	72.0 (mean)	SD: 21.5	Range: 32-110
Bacterial colonization (n)	Chronic P. aeruginosa 11	Chronic S. aureus 11	Other bacteria 4
Platelet count	292 (mean)	SD: 99.7	Range: 179-620
CRP	6.1 (mean)	SD: 8.4	Range: 0.65-38
Azithromycin (n)	Yes 12	No 10	
IgG	13.4 (mean)	SD: 3.1	Range: 9.5-22
IgA BPI-ANCA	282.3 (mean)	SD: 603.8	Range: 2-2251
IgG BPI-ANCA	56.9(mean)	SD: 92.6	Range 0-372
CF related diabetes	Yes 5	No 17	

Platelet activation observed in CF patients may be a direct or indirect effect of the primary defect in CF, *i.e.* the dysfunctional CFTR. CFTR is found on human platelets and CFTR blockade has been demonstrated to have impact on platelet release of mediators involved in the inflammatory response (13).

We demonstrate here that levels of PMC formation correlate with lung function decline, CRP and BPI-ANCA (IgG and IgA). The increased reactivity measured by AUC collagen indicates in-vivo priming of the platelets and correlates also with BPI-ANCA. The strong correlation between BPI-ANCA and *P. aeruginosa* colonization together with the positive correlation between BPI-ANCA and platelet activation suggest that the platelet activation in this cohort is secondary to pulmonary inflammation rather than a direct consequence of the CFTR mutation.

Conclusions and future perspectives

BPI-ANCA as prognostic factor

The studies in this thesis show that BPI-ANCA can tell us something about the prognosis for the CF patient. Patient chronically colonised with *P. aeruginosa*, who develop a positive BPI-ANCA should be monitored closer and receive more intense treatment as the presence of BPI-ANCA indicates a negative host-pathogen interaction and a worse long-term prognosis than for a patient without BPI-ANCA.

Generation of BPI-ANCA

Lack of a functional BPI has been associated with decreased clearance of the mucoid strain of *P. aeruginosa* (388). BPI-ANCA has been shown to decrease the anti-bacterial effect of BPI ((389) which makes it realistic that BPI-ANCA itself influences airway disease in CF, not only by representing a more adapted and durable *P. aeruginosa* infection, with excess NET formation, but also via a negative and direct impact on the innate immune system. There are patients, who develop BPI-ANCA although they are not colonized with *P. aeruginosa*, indicating that the process may not be depending exclusively on *P. aeruginosa*.

We still do not know what causes the immune system to react against "self" and produce antibodies against BPI. Recently, a new study about BPI-ANCA and other auto-antibodies in CF and RA was published (390). Skopelja et al found NE, citrullinated and carbamylated proteins in NETs from CF patients. They identified BPI localized on the neutrophil cell membranes and on NETs. BPI was often co-localized with NE, which could be expected, as both substances are released from the azurophilic granules. Next, they found that neutrophil BPI was cleaved by *P. aeruginosa* elastase, creating two molecules that can be introduced to the immune system by DCs. *P. aeruginosa* strains without elastase were not able to cleave BPI.

Our findings in study III are partly in line with these findings, as the level of expression of elastase probably can vary in different strains. However, in study III we found no correlation at all between BPI-ANCA and anti-elastase antibodies (anti-ELA), and a very low correlation between anti-ELA and lung function impairment.

The length of *P. aeruginosa* colonization has not been studied in connection with the establishment of BPI-ANCA, but BPI-ANCA is associated with pyocyanin negative *P. aeruginosa* strains (346), a finding associated with bacterial adaptation in chronic infection. A possible influence in this process is that the longer the patient harbours strains producing NETs, the more BPI-ANCA antibodies develop. Mucoid *P.*

aeruginosa strains adapted to CF airways are known to secrete less exotoxins than nonmucoid strains (341) and in the mRNA analysis performed in study III, none of the *P. aeruginosa* genes, related to elastase, las A or las B, were found among up regulated genes, a finding that contradicts the explanation suggested by Skopelja et al.

Platelet function

Platelet function in CF has not been studied in detail and, to my knowledge; we are the first to correlate platelet activation with lung function impairment and other clinical findings as BPI-ANCA. Platelets participate in the chronic inflammation in CF, and it is possible that the increased activation is correlated to inflammation level in the patient. Our cohort is small, but we still find these indications of clinical relevance. These findings indicate that further studies of platelet activation in relation to clinical findings and prognostic factors in CF are warranted.

Further studies

The results of these studies inspire to further studies. The results from study III, where we found flagellin A to be associated with BPI-ANCA positive strains, were very interesting. We interpreted flagellin expression as either flagellin A or B, and designed our PCR primers on published sequences. The 5' end of the flagellin A and B DNA are very homologous. The middle part of the gene has several insertions and deletions that differ between the genes and this part was used for our analysis. The flagellin A gene is shorter and lacking about 450bp in the 3' end. However, a deeper analysis of the middle part of the gene shows there might not be such clear cut between flagellin A and B and it is possible that our PCR is not the optimal way to answer our hypothesis. To further analyse if flagellins are involved in BPI-ANCA generation, it would be necessary to sequence flagellar genes comparing *P. aeruginosa* strains from BPI-ANCA positive and negative patients.

NET formation in relation to BPI-ANCA is another interesting subject, especially after the new publication from Skopelja et al. It would be interesting to study if NET formation and/or *P. aeruginosa* elastase production in the strains associated with BPI-ANCA differ from BPI-ANCA negative strains.

We know that BPI-ANCA levels decrease after lung transplantation, but BPI-ANCA has not been evaluated as an indicator of treatment effect. Measuring BPI-ANCA before and after treatment of a pulmonary exacerbation would be a way to broaden the clinical use of BPI-ANCA. As new treatments are becoming available to CF

patients, BPI-ANCA level could be one way to evaluate efficacy. Cooperation with my clinical CF colleagues in Sweden has been discussed, where BPI-ANCA would be included in the follow up panel in patients treated with new CFTR correctors and/or potentiators.

A mechanism suggested in earlier studies about BPI-ANCA, is that epitopes on *P. aeruginosa* and other bacteria may trigger ANCA production by acting as a molecular mimic of BPI as certain BPI epitopes show strong similarity to outer membrane (OM) proteins from *P. aeruginosa* and *E. coli* (289) (293, 294). In study III we identify OM proteins, using 2D-gel electrophoresis, which are associated with BPI-ANCA positive strains. It could be of interest, to compare these proteins with BPI.

A logical development of our findings in study IV would be to investigate platelet activation and platelet-neutrophil complex formation on site for the actual inflammation in CF, *i.e.* in sputum. The formation of NETs in connection with platelet activation in CF is another interesting aspect. Anti-platelet therapy is effective in many diseases and it would be interesting to study how platelets in CF patients react to treatment with Ibuprofen or Azithromycin. Platelet activation level in response to treatment of an exacerbation in CF patients is another interesting aspect.

The knowledge about different aspects of CF disease increases every day. We see the light in the tunnel and hope that treatments able to cure the underlying genetic defect or the different protein defects will be available to all CF patients in the future. We also expect better treatment against infections and inflammation in CF lung disease. My findings in this thesis will not cure CF, but prognostic factors help us identify patients in need of closer monitoring. Investigating the underlying processes of prognostic factors increases our knowledge about this disease. I am proud to be part of this process and hope to continue adding knowledge in this inspiring field of research.

Populärvetenskaplig sammanfattning

Den ärftliga sjukdomen cystisk fibros (CF) drabbar dem som har två anlag för sjukdomen, d v s ett från varje förälder. Det är en relativt ovanlig sjukdom, men anlaget för sjukdomen bärs av ca var 30:e person i Sverige. Anlagsbärare är inte sjuka. I Sverige finns det i nuläget ca 700 personer som har CF, i världen som helhet ca 85 000.

Sjukdomen beror på att ett protein, CFTR, nödvändigt för att cellernas saltbalans och pH ska fungera normalt, produceras i för liten mängd eller på ett felaktigt sätt. Olika fel i genen påverkar proteinet på olika sätt och detta gör att patienternas sjukdom kan variera utifrån det genetiska felet.

De flesta CF patienter får sin diagnos som litet barn, många gånger p.g.a. dålig tillväxt. Den dåliga tillväxten beror på att bukspottskörteln (pankreas) inte fungerar normalt, vilket gör att man inte kan ta upp fett och fettlösliga vitaminer ur kosten. CFTR-defekten gör att man utsöndrar saltare svett än normalt och svettestet är därför, i kombination med gentester, det vanligaste sättet att komma fram till diagnosen CF. I många länder har man infört screening av nyfödda för att hitta barn med CF så tidigt som möjligt, dock inte i Sverige. Vissa typer av CF är mildare och ger inte så tydliga symtom vilket gör att dessa patienter ofta får sin diagnos betydligt senare i livet.

Det felaktiga CFTR sitter i cellernas yta (membran) och påverkar transporten av salter. Vid CF blir vätskan utanför cellens yta, framför allt i luftvägarna, seg och trög vilket leder till att bakterier lättare kan få fäste och ge infektioner. Infektionerna blir svåra att bekämpa, p.g.a. att CFTR inte fungerar. På så sätt blir det en ond cirkel av infektion (bakterier/virus/svamp) och inflammation (vita blodkroppar m.m.) som på sikt förstör lungvävnaden och sänker patientens lungfunktion.

CF behandlas med många olika terapier, varav de viktigaste är pancreasenzymer, antibiotika, slemlösande och luftrörsvidgande inhalationer respektive olika typer av fysioterapi för att hosta upp slemmet. Fysisk aktivitet är en viktig del av behandlingen, eftersom den gör att patienterna får upp slem, öppnar upp luftvägar och mår bättre generellt.

Modern behandling har gjort att överlevnaden vid CF har ökat dramatiskt sedan 1960 och 70-talen. Man menar nu att barn med CF som fötts efter år 2000 har stor chans att bli 50 år. Men även med modern behandling så utvecklar CF-patienter med

åren, vissa redan i tonåren, en allvarlig lungfunktionspåverkan, som innebär att lungtransplantation behöver övervägas. Nya läkemedel för att behandla CF genom att rätta till CFTR har utvecklats på senare år, och ett av dessa är så effektivt att patienterna blir i princip helt friska. Tyvärr finns sådan medicin idag bara för det fåtalet patienter som har en särskild typ av genetiska mutationer, men fler är under utveckling. Även genterapi är på väg, men den typen av behandling har visat sig vara väldigt svår att lyckas med. Nya mediciner som behandlar infektioner och påverkar den kroniska inflammationen är också viktiga att få fram.

Patienter med CF drabbas i väldigt varierande grad av sin sjukdom, och det är viktigt att känna till de faktorer som påverkar prognosen, så att man i görligaste mån kan påverka dem. Behandlingen vid CF är omfattande och tidskrävande och om man visste vilka som har mest nytta, respektive mindre behov, av behandling, så skulle det kunna hjälpa oss och patienterna att göra behandlingen optimal.

Infektion med bakterien *Pseudomonas aeruginosa* är en sådan faktor, där vi vet att kronisk infektion innebär sämre prognos för patienterna, och behandlingen av denna bakterie blir därför extra viktig. Hos vissa patienter med CF, framför allt hos dem med *Pseudomonas*-infektion, kan man uppmäta så kallade auto-antikroppar mot ett kroppseget protein, BPI, ett fynd som visat sig vara kopplat till sämre prognos. Auto-antikroppar innebär att kroppens immunförsvar har reagerat mot sig själv, något som egentligen inte ska hända. BPI är involverat i kroppens försvar mot bakterier och antikropparna gör att denna funktion påverkas negativt.

Det övergripande syftet med den här avhandlingen har varit att utvärdera nyttan av att mäta denna antikropp, BPI-ANCA, och att försöka förstå varför den uppkommer, eftersom vi då kanske skulle kunna påverka förloppet vid sjukdomen. Vi har också tittat på hur blodplättar, trombocyter, påverkas vid CF. Trombocyter har många viktiga roller i kroppen, bl.a. i immunförsvaret mot bakterier.

I **arbete I**, följdes 46 CF-patienter under mer än tio år, för att se om ett enstaka blodprov avseende BPI-ANCA kunde förutsäga hur det skulle gå för patienterna. Det visade sig att om BPI-ANCA låg högt, så var det en faktor som talade för att patienten löpte stor risk för att vara antingen avliden eller lungtransplanterad vid studiens slut. BPI-ANCA jämfördes med andra faktorer och var inte bättre på att förutsäga framtiden än den lungfunktion som patienten hade, d.v.s. en låg lungfunktion sa också mycket om prognosen. Däremot var BPI-ANCA bättre som prognostisk faktor än enbart information om vilken typ av bakterieinfektion patienten var drabbad av.

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CF är en svår sjukdom som drabbar många organ i kroppen. CFTR defekten finns i många olika typer av celler och påverkar oändligt många system på cellnivå. Detta gör CF till en otroligt svår, men samtidigt spännande, sjukdom att studera. Denna avhandling är en liten pusselbit i arbetet med att förstå CF. Jag hoppas få förmånen att fortsätta arbeta och forska inom området cystisk fibros.

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