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# **COMMON RESPIRATORY VIRUS INFECTIONS IN CYSTIC FIBROSIS - FROM IMMUNITY TO VACCINE**

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# Common respiratory virus infections in cystic fibrosis – from immunity to vaccine

## THESIS FOR LICENTIATE DEGREE

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**CYSTIC FIBROSIS  
IS A SUPERPOWER**

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*Dedicated to all people with CF in the world*



## ABSTRACT

Cystic fibrosis is the most common lethal monogenetic disease in Caucasians. It is caused by mutations in the cystic fibrosis transmembrane conductance regulator protein which is responsible for appropriate ion and water transport across epithelial cell membranes. The main clinical problems in cystic fibrosis are lung disease, pancreatic insufficiency and cystic fibrosis-related diabetes.

Respiratory virus infections predispose individuals with cystic fibrosis to bacterial infections and chronic colonization of the airway which exacerbate lung disease. The mechanisms behind this are poorly understood but the immune system is evidently involved.

In this thesis, we studied common cold-causing enteroviruses, the Coxsackieviruses, in cystic fibrosis. In Paper I, we showed that a part of adaptive immune response towards Coxsackieviruses, namely production of neutralizing antibodies, is impaired in an experimental mouse model for cystic fibrosis (carrying the delF508 mutation). In Paper II, we elaborated on this finding and studied whether the delF508 mice could be protected from Coxsackievirus infection by vaccination and showed that vaccination was safe and efficient. We found that the production of virus-neutralizing antibodies upon vaccination in the delF508 mice was initially weak but improved upon a booster dose. We studied the frequency of Coxsackievirus infections in individuals with cystic fibrosis and found that they are common in this patient group. We conclude that common respiratory virus infections in cystic fibrosis can be successfully prevented by vaccination, which could potentially contribute to better lung function.

Disease mortality is increased six-fold in individuals with cystic fibrosis-related diabetes, the pathogenesis of which is largely unknown. An autopsy study, where pancreatic tissue from cystic fibrosis patients was used as control, discovered presence of enterovirus in islets of cystic fibrosis patients with diabetes. In Paper III, we studied pancreas autopsy material from another cohort of cystic fibrosis patients with diabetes and found that 80% were positive for enterovirus in the islets compared to 40% in non-diabetic controls without cystic fibrosis. We also searched for serological evidence of a link between previous enterovirus infections and the development of cystic fibrosis-related diabetes but found no such relationship. A low-grade infection which does not induce antibody response, or a long-term persistent infection might be an explanation to this. We conclude that the role for enteroviruses in development of cystic fibrosis-related diabetes should not be excluded.

In conclusion, this thesis contributes to the field of cystic fibrosis by revealing a potential immune defect in response to viral infections. It also demonstrates that common respiratory virus infections can potentially be targets for preventive treatments in cystic fibrosis. In addition, the potential role of enterovirus involvement in the pathogenesis of cystic fibrosis-related diabetes has been presented, motivating for further studies.

## LIST OF SCIENTIFIC PAPERS

- I. Svedin E, **Utorova R**, Huhn MH, Larsson PG, Stone VM, Garimella M, Lind K, Hägglöf T, Pincikova T, Laitinen OH, McInerney GM, Scholte B, Hjelte L, Karlsson MCI, Flodström-Tullberg M. A Link Between a Common Mutation in CFTR and Impaired Innate and Adaptive Viral Defense. *The Journal of Infectious Diseases*, 2017, 216(10):1308-1317.
- II. Virginia Stone, **Renata Utorova**, Marta Butrym, Amirbabak Sioofy-Khojine, Minna M. Hankaniemi, Emma Ringqvist, Anirudra Parajuli, Terezia Pincikova, Björn Fischler, Ferenc Karpati, Vesa P. Hytönen, Heikki Hyöty, Lena Hjelte, Malin Flodström-Tullberg. Coxsackie B virus infections are common in cystic fibrosis and can be prevented by vaccination. *Manuscript*.
- III. **Renata Utorova**, Amirbabak Sioofy Khojine, Terezia Pincikova, Jussi Lehtonen, Sami Oikarinen, Lena Hjelte, Noel G. Morgan, Sara J. Richardson, Heikki Hyöty, Malin Flodström-Tullberg. Investigating the role for enterovirus infections in cystic fibrosis-related diabetes. *Manuscript*.

## LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

- SI. Lind K, Svedin E, **Utorova R**, Stone VM, Flodström-Tullberg M. Type III interferons are expressed by Coxsackievirus-infected human primary hepatocytes and regulate hepatocyte permissiveness to infection. *Clinical Experimental Immunology*. 2014, 177(3):687-95.
- SII. Larsson PG, Lakshmikanth T, Laitinen OH, **Utorova R**, Jacobson S, Oikarinen M, Domsgen E, Koivunen MR, Chaux P, Devard N, Lecouturier V, Almond J, Knip M, Hyöty H, Flodström-Tullberg M. A preclinical study on the efficacy and safety of a new vaccine against Coxsackievirus B1 reveals no risk for accelerated diabetes development in mouse models. *Diabetologia*. 2015, 58(2):346-54.



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## LIST OF ABBREVIATIONS

CF	Cystic fibrosis
CFALD	Cystic fibrosis-associated liver disease
CFRD	Cystic fibrosis-related diabetes
CFTR	Cystic fibrosis transmembrane regulator
CVB	Coxsackievirus B
DC	Dendritic cell
DIOS	Distal intestinal obstruction syndrome
EV	Enterovirus
HAV	Hepatitis A virus
HbA1c	Hemoglobin A1c
IFN	Interferon
iNKT	Invariant NK T (cell)
IL-1 $\beta$	Interleukin-1 $\beta$
IL-6	Interleukin-6
IL-8	Interleukin-8
ILC	Innate lymphoid cell
MHC	Major histocompatibility complex
NK	Natural killer (cell)
OGTT	Oral glucose tolerance test
PI	Pancreatic insufficiency
RSV	Respiratory syncytial virus
RV	Rhinovirus
T reg	Regulatory T cells
T1D	Type 1 diabetes
T2D	Type 2 diabetes
Th	T-helper cell

Th17

T helper 17 cell

TLR

Toll-like receptor

TNF

Tumor necrosis factor

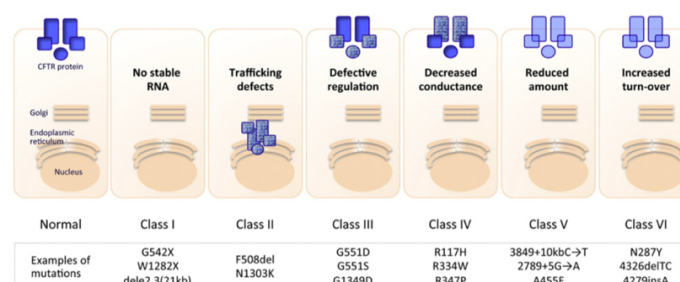


# 1. CYSTIC FIBROSIS

## 1.1 Etiology

Cystic fibrosis (CF), previously known as mucoviscidosis, is the most common monogenetic disease that causes preterm mortality in Caucasian populations (1). It is a complex multi-organ disease that affects the airways, the pancreas, the digestive tract, the hepatobiliary system, and the reproductive organs as well as metabolism and the immune system. The incidence of CF varies across the world and is approximately 1:3.000 – 1:4.000 amongst white Europeans while it is significantly less common in other ethnic groups (2). It is estimated that around one in 25-30 Caucasians carries a disease-causing mutation in the affected gene (2).

CF is caused by mutations in the transmembrane conductance regulator (*cftr*) gene which encodes a chloride and bicarbonate ion channel that is predominantly expressed in epithelial cell membranes. By regulating chloride and bicarbonate currents across the cell membrane and through interactions with sodium channels, the CFTR protein contributes to the balancing of ion concentrations in the liquid at apical cell surfaces (3, 4). CFTR mutations cause an imbalance in ion concentrations which leads to accumulation of a thick viscous mucus in many organs, hence the original name of the disease - mucoviscidosis. Over 2.000 CFTR mutations have been identified to date but not all result in disease (5). The known mutations are now classified into seven different classes/types depending on the effect the mutation has on CFTR protein production (Figure 1) (6, 7). Most mutations result in non-functional or absent CFTR protein leading to the accumulation of chloride ions outside the cell which is pathognomonic for classic CF. One of diagnostic criteria is increased sweat chloride concentration which is measured by a sweat test. Some mutation classes are associated with more severe disease, where the most common mutation, and also the one with the worst prognosis, is the deletion of a phenylalanine at position 508 (denoted as  $\Delta F508$ ) which accounts for nearly 70% of CF worldwide. Interestingly, the *cftr* genotype does not always correlate with the severity of disease which has recently introduced the role of environment and the so-called ‘modifier genes’ into pathogenesis of CF (8).



**Figure 1. Classes of CFTR mutations.** Adopted from *I. Fajac, K. De Boeck / Pharmacology & Therapeutics 170 (2017) 205–211.*

## 1.2 Clinical manifestations of cystic fibrosis

CF is a complex disease affecting the whole body. Individuals with CF are managed by multidisciplinary teams of clinicians and paramedical specialists with appropriate experience, that are guided by national and international standards of care. The major cause of morbidity and mortality in CF is lung disease characterized by gradual decline in lung function due to bacterial infections and lung tissue destruction. Eventually, for many individuals with CF lung transplantation becomes inevitable. Pancreatic insufficiency (PI) is another major manifestation present in the majority of individuals with CF and is the result of chronic obstructive pancreatitis. It results in the replacement of pancreatic tissue with fibrotic tissue and cysts, hence the name cystic fibrosis. The hepatobiliary system is also often affected in CF where it manifests as cystic fibrosis-associated liver disease (CFALD). Many individuals with CF struggle with malnutrition, vitamin deficiencies and osteopenia which in turn are the consequence of pancreatic insufficiency and intestinal dysfunction. Infertility is very common in men and can be the only symptom of CF. The second major co-morbidity after lung disease in over 40% of adults with CF is cystic fibrosis-related diabetes (CFRD) which contributes to a worsened overall prognosis.

### 1.2.1 Lung disease

The central pathological mechanisms of lung function decline in CF are chronic bacterial infections and a pro-inflammatory environment (9, 10). Lung disease starts early in life when the widening of the airway, or bronchiectasis, occurs and can be radiologically visualized (3). Bronchiectasis is believed to result from protease hypersecretion by neutrophils, a cell subset which is significantly increased in the airways of individuals with CF (11). As bronchiectasis develops, the airways become more susceptible to infections with common pathogenic bacteria such as *Haemophilus influenzae* and *Staphylococcus aureus* prevalent in children and as they age, *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Stenotrophomonas maltophilia*. These recurrent bacterial infections lead to a decline in lung function and therefore require aggressive treatment.

The mechanisms behind increased susceptibility to bacterial infections are complex. Airway dehydration due to electrolyte imbalance, mucociliary dysfunction and inflammation are believed to be the consequences of faulty CFTR function (10, 12, 13). There is evidence suggesting that CFTR mutations lead to mucus volume depletion at epithelial surfaces which in turn negatively affects the airway cilia movement, thereby inhibiting mucociliary clearance of pathogens (13). The resulting milieu is beneficial for bacterial infections and colonization which is associated with and driven by high levels of pro-inflammatory markers such as IL-1 $\beta$ , IL-6, IL-8 and TNF (reviewed in (14)).

Respiratory viruses are also involved in exacerbating CF lung disease (15-18). Viral infections in individuals with CF are as common as in general population but are longer in duration and cause more severe symptoms (17, 19). The most common viruses recovered from the

symptomatic CF airway are rhinoviruses/enteroviruses (RV/EV), respiratory syncytial virus (RSV) and influenza viruses (19-22). Interestingly, one study has shown that individuals with CF have higher viral loads both in the upper and in lower airways during a symptomatic infection compared to healthy controls (23). This could partly explain why respiratory viral infections cause worse symptoms in CF. Moreover, there is significant amount of evidence indicating that respiratory virus infections predispose the CF lung to colonization by common bacterial pathogens (e.g. *Haemophilus* species, *Moraxella* species and *Streptococcus pneumoniae*) that cause serious exacerbations (24, 25).

It has been proposed that there is a link between anti-viral defense and bacterial infection/colonization in the CF airway. The immune mechanisms behind this have been studied with varying results and still remain largely unknown. Some studies report exaggerated immune responses, whilst others report that the effects of the immune system are dampened (26, 27). There is evidence that viral clearance is impaired in epithelial cells in CF (15, 27, 28). *In vitro* studies have shown that these cells secrete lower amounts of type I interferon (IFN) in response to *Pseudomonas aeruginosa* which makes them less efficient in the initiation of adaptive immune responses (29). In summary, a large gap still exists in our understanding of the mechanisms behind the susceptibility of the CF airways to and interactions between viral and bacterial infections.

### **1.2.2 Gastrointestinal manifestations**

Gastrointestinal organs are severely affected in CF and account for a large part of CF-related morbidity. The main extrapulmonary complications are PI and CFALD. Individuals with CF are also at significantly higher risk for gastrointestinal cancers such as colon cancer, cancer of the biliary tree and pancreatic cancer, which has recently introduced a view that CF should be seen as a gastrointestinal cancer syndrome (30, 31).

PI is the most common gastrointestinal feature of CF. In the majority of individuals with CF (80-90%) PI manifests already from birth, or even stages before and is usually associated with higher disease burden and worse prognosis (32). The pathological mechanism behind PI is chronic inflammation driven by premature activation of digestive proenzymes due to imbalance in the constitution of pancreatic secretions which are thick and lack bicarbonate (33). Digestive enzymes in the intestine are therefore deficient which leads to poor absorption of macronutrients, steatorrhea and growth retardation. Per year, a few newborns are diagnosed by presentation with meconium ileus which is an obstruction of the distal ileum. It is caused by a thick protein-rich plug and is a strong predictor of CF with PI. In older children and adults with PI, the distal intestinal obstruction syndrome (DIOS) is common but it also happens in pancreas sufficient patients indicating the complexity of the disease (34).

CFALD is the third most common comorbidity in CF and up to 70% of individuals with CF are found to have liver damage upon autopsy (35). The most common pathological findings are focal and multilobular biliary cirrhosis which occur due to obstruction and plugging of bile

ducts with the thick and harmful bile salt-rich bile (36). Hepatic steatosis is even more common but its origin is largely unknown since the CFTR is not expressed in hepatocytes (37). In some individuals, CFALD develops into liver failure leading to liver transplantation.

With increasing life expectancy and rapid evolution of medical care, other CF complications have become important. Vitamin D and K deficiencies are common in children with CF and are largely the result of the above-mentioned gastrointestinal complications and malnutrition (38). As a result of vitamin deficiencies, together with chronic inflammation and frequent infections, intermittent corticosteroid therapies and physical inactivity, osteopenia is common (39).

### **1.2.3 Cystic fibrosis-related diabetes**

CFRD is the most common comorbidity after lung disease in CF. With increasing life expectancy CFRD is becoming more prevalent, affecting about 50% of people with CF above the age of 30 (40). CFRD is associated with severe *cftr* genotypes (such as the  $\Delta F508$  mutation) and is more common in females (41). Diagnosis is uncommon in children under the age of 10 but has been reported in infants (42, 43). Association with type 1 diabetes (T1D) is absent and the presence of autoantibodies and genetic predisposition to T1D in CF patients with CFRD is more similar to that seen in the normal population rather than T1D cohorts (44).

Morbidity and mortality risks are both increased when CFRD is present. Mortality risk is six times higher in patients with CFRD than in those without CFRD (41). Diabetes is also associated with a significant decline in lung function because of an increased frequency of exacerbations and a greater reduction in lung function (40, 41, 45). Moreover, such deterioration of lung function can be seen years before diabetes diagnosis is made (46, 47). Early insulin therapy in CFRD is crucial since it not only improves lung function and reduces exacerbation frequency but also improves nutritional state (48).

CFRD is classified by WHO as a disease of the exocrine pancreas and the diagnostic criteria are identical to the other diabetes types. Screening for CFRD is done by random testing of blood glucose or at the annual screening with an oral glucose tolerance test (OGTT) which has the highest specificity for detecting CFRD. Measurements of hemoglobin A1c (HbA1c) are not recommended for screening purposes but are used as a follow-up tool for guidance regarding insulin treatment. Just as with other diabetes patients, individuals with CFRD are recommended to undergo surveillance for diabetic complications.

The pathophysiological mechanisms behind CFRD development are not fully understood. Although insulin resistance can be present due to frequent corticosteroid use, the main feature of CFRD is insulin deficiency. Historically, it has been accepted that insulin deficiency is the result of destruction and fibrosis of pancreatic exocrine tissue with the consequent loss of pancreatic islets and insulin-producing  $\beta$ -cells. However, CF patients without PI are still at higher risk for developing diabetes than the general population (41). This and the fact that diabetes development occurs over decades (although PI is established during the first year of



life), indicates that the mechanisms behind diabetes development in CF are more complex. Moreover, autopsy studies show that the degree of  $\beta$ -cell loss seen in individuals with CFRD is similar to that seen in those without CFRD and furthermore, that islet architecture is generally altered in CF with a decrease in both  $\alpha$ - and  $\beta$ -cells and an increase in  $\sigma$ -cells (reviewed in (49)). These findings imply that intrinsic islet-cell mechanisms involving CFTR could contribute to the development of CFRD. As a matter of fact, two recent independent findings regarding CFTR involvement in insulin secretion by the insulin-producing  $\beta$ -cells point towards such intrinsic pathophysiological mechanisms in CFRD (50, 51). Furthermore, improvement of first-phase insulin secretion upon treatment with one of the CFTR modulator drugs is consistent with the hypothesis that there is an intrinsic  $\beta$ -cell defect in CF (52, 53).

CFRD also shares common features with type 2 diabetes (T2D) such as the correlation with the presence of amyloid in the islets of CF patients with diabetes but not in the islets of those without diabetes (54). Interestingly, family history of T2D is a risk factor for developing CFRD (55). Moreover, the finding that there is an impaired first-phase insulin secretion is also shared between T2D and CFRD, making the pathogenesis puzzle of CFRD even more complex (56). In summary, there is evidence that both structural and functional mechanisms are involved in the development of CFRD.

Insulin deficiency is the main feature of both T1D and CFRD, whilst there are otherwise no strong associations between the two diabetes types. Environmental factors, such as viruses, have been associated with T1D development. One of the strongest associations is with a group of EVs, the Coxsackieviruses (CVBs). There is both direct and indirect evidence of the CVBs' role in pathogenesis of T1D, where the most impressive is the significantly more common detection of virus in the pancreatic islets of T1D patients than in non-diabetic controls (57). Interestingly, a cohort of individuals with CF was used as control in the aforementioned study, where enterovirus was detected in the islets of patients with CFRD but not in patients without CFRD (57). The authors suggest that enteroviruses could be involved in the pathogenesis of CFRD in a similar manner to T1D. To current knowledge, potential associations between CFRD and enteroviruses have not been studied elsewhere.

### **1.3 Treatment of cystic fibrosis**

CF is a multi-organ disease and clinical presentation may vary greatly from patient to patient. There has been an enormous breakthrough in the treatment of the underlying genetic defects during the past year. Nevertheless, the golden standard of CF treatment originates from early 1960s with the main principle being intensive symptomatic treatment.

The main clinical presentations of CF are respiratory and gastrointestinal symptoms. Treatment of lung symptoms is directed towards airway clearance by physiotherapy and infection control. Aggressive management of bacterial lung infections is utilized from early childhood with the

use of oral, intravenous and inhaled antibiotics aimed at eradicating infection and restoring lung function. When pulmonary exacerbations occur, either bacterial or viral, they should be treated with antibiotics and airway clearance therapy should be intensified. Chronic bacterial infections lead to progressive lung damage and lung function decline which, if terminal and irreversible, results in lung transplantation. The gastrointestinal symptoms, most commonly steatorrhea and poor weight/weight loss, are managed with enzyme replacement therapy, vitamin supplements, calorie-rich nutrition and insulin treatment when diabetes is present.

During the past decade, new types of treatment, the so-called CFTR modulators, have become available. Studies are ongoing all over the world to show their short- and long-term effects in people with CF. CFTR modulators are taken orally and aim to correct the underlying CFTR defect. Ivacaftor, which targets the G551A mutation, was the first drug developed for individualized CF therapy. Its effects on disease improvement have been significant, although there is the major drawback that it can only be used in a small fraction (5%) of people with CF (58, 59). When Lumacaftor, which is specific for the most common delF508 mutation, became available it was combined with Ivacaftor and showed enhanced efficiency in patients homozygous for the delF508 mutation, meaning that an individualized treatment became available for about 40-50% of the CF patients worldwide (58). In 2018, another combination treatment with Ivacaftor was approved in the US for use in patients with homozygous delF508 mutation which represents almost 90% of CF population. Recently, a triple combination drug that is even more superior became available for individuals with homozygous delF508 mutation (60). In general, these novel drugs improve lung function and lower the amount of pulmonary exacerbations but other effects such as improved glucose control are also anticipated (58, 61). Furthermore, there are several drug candidates in clinical phase 1 and 2 trials as well as in the preclinical pipeline which should lead to dramatic improvement of CF management (60).

Implementation of aggressive multidisciplinary treatments in specialized centers around the world has increased the life expectancy of people with CF to almost 50 years (62). Most European countries have also introduced newborn screening which will benefit the societies at both individual and socioeconomical level. Additionally, it is apparent that a new era of evolving gene-guided therapy will further increase life quality and survival in CF.

## **2. THE IMMUNE SYSTEM**

The collection of cells and mechanisms that protects an organism against infectious disease-causing pathogens and non-infectious substances is referred to as the immune system. The main role of the immune system is to protect from and eradicate microbial invaders without causing harm to the host. Moreover, the immune system is able to discriminate between self and non-self, protect against tumors and cancer and repair damaged tissues. These various elements of the immune system have been successfully utilized in treatment of and protection against many diseases.

### **2.1 Innate and adaptive immunity**

The immune system consists of two arms, a natural/native immunity (innate) and acquired immunity (adaptive). Innate immunity is present in utero (e.g. before birth) and is the first line of defense against pathogens. It consists of outer and inner epithelial barriers, the specialized cells that reside in or near these barriers (monocytes, macrophages, neutrophils, dendritic cells (DCs), mast cells, innate lymphoid cells (ILCs) and natural killer (NK) cells) and the molecules produced by these cells. The innate responses are rapid and universal but do not develop any memory, and as such, they need to be repeated every time a pathogen is encountered. The cells of the innate immune system recognize patterns present on microbes by specialized receptors (e.g. Toll-like receptors, TLRs) and react by producing factors (such as complement proteins, cytokines and chemokines) which amplify defense mechanisms in order to prevent microbial spread. Inflammation and tissue repair are also initiated by the innate immune system. Upon encountering a microbe, the innate immune responses are activated and relay signals (antigens) to the adaptive immunity compartment. The adaptive immune system consists of T and B lymphocytes which are initially naïve but upon activation, they proliferate and differentiate into T-helper (Th) cells, cytotoxic killer T-cells or antigen specific T-effector cells or in the case of B lymphocytes, antibody-producing cells. Due to the vast variation of antigens that the adaptive immunity encounters, long-lived and specific memory (both cellular and humoral) is developed to allow for rapid response after repeated exposure and invasion of a particular pathogen.

### **2.2 Evidence of an impaired immunity in cystic fibrosis**

There is substantial amount of evidence showing that CFTR mutations play a role in the function of both innate and adaptive immune system in CF. For a long time, the CF airways have been recognized as having a pro-inflammatory milieu with favorable conditions for bacterial colonization. Airway epithelium plays an important role in the initial defense against microbes and CFTR mutations have been associated with exaggerated inflammatory responses. These responses start early in childhood and in the long run damage the tissue (10, 27, 63). Although

required for tissue repair, inflammation in the CF airways becomes persistent which does not favor efficient bacterial clearance and results in chronic infections.

In order to protect the body from infection, the airway epithelium functions as an innate barrier which 1) removes pathogens/particles by mucociliary transport; 2) activates innate immune mechanisms to try and limit the spread of the pathogen; and 3) enables activation of the adaptive immunity for an efficient elimination of the pathogen. It has been shown that CF lung epithelial cells have dysregulated production of mucins making airway clearance from mucus, and thereby foreign particles and pathogens, difficult (13, 64). Such alternations in mucus composition are associated with infections caused by more aggressive antibiotic-resistance bacteria (65). The cell composition of the CF airway is also altered and dominated by overactive neutrophils and inefficient macrophages (66). Neutrophil elastase, TNF and IL-8, all strong proinflammatory mediators, are increased at a young age in the CF airways, even in the absence of infection (67, 68). Moreover, there is also evidence of impaired IFN production in CF airway epithelial cells which can result in dysfunctional defense against bacteria and viruses (26, 29, 69). In summary, there is quite a bit of evidence documenting dysregulated innate immune mechanisms in the CF epithelium that promote inflammation and favor bacterial growth.

As with many other chronic diseases, the severity of CF is associated with certain gene clusters which are responsible for the adaptive immune responses (70, 71), implying that adaptive immunity is involved in the pathogenesis of CF. Indeed, there is sufficient amount of evidence of dysfunctional adaptive immunity in CF. First, expression of the major histocompatibility complex (MHC) class II molecule, a key molecule on peripheral DCs that links innate and adaptive immune responses, is lower in individuals with CF compared to healthy controls (72). In addition, it has been observed that T cells have impaired functions. For instance, the function and numbers of the anti-inflammatory regulatory T cells (T regs) tend to decrease with age and with the acquisition of chronic bacterial infections that drive disease progression (73). Second, the proinflammatory Th17 cell responses are stronger in the airway of CF patients, supporting the concept that CF is a chronic inflammatory disease (74). Third, CFTR seems to be involved in the enhancement of B cell responses, notably in the lung, which is in line with the persistent increase in levels of total IgG and IgA in CF patients (75). Another cell type that is possibly dysregulated in CF is the invariant NKT (iNKT) cells which are upregulated in the absence of CFTR, causing excessive cell death and consequently inflammation (76). Clearly, quantitative and qualitative defects in leukocytes are involved in pathogenesis of the CF lung disease.

A defective anti-viral response has also been suggested to contribute to pulmonary exacerbations in the CF lung, but the evidence is insufficient. One of the anti-viral mechanism studied is the IFN signaling, and the evidence of its dysregulation is contradictory and some of it could be explained by the viral evasion mechanism and not by a CF-specific dysfunction (23, 26, 69, 77, 78). Studies examining pro-inflammatory cytokine levels after viral infection in either cell culture or in patients have been indefinite but conclusions are difficult to draw due to the variability of viruses and cell cultures used in such studies (reviewed in (22)). Altogether, existing evidence suggests that mutations in CFTR contribute to defects in both innate and adaptive immune system but significant gaps in our understanding exist. In addition to these

defects, the complex bacteria-virus interactions play a part, further magnifying the need for more research.

### **2.3 Vaccine use in cystic fibrosis**

Morbidity and mortality rates caused by infectious diseases can be decreased by vaccination. The detrimental effects of bacterial and viral respiratory infections on lung function in CF could also be prevented by efficient vaccines. There are currently no uniform European immunization schedules for individuals with CF and they are recommended to follow national programs in order to obtain general protection. Immunization coverage amongst both children and adults is generally high and continues to rise but they are still not optimal considering the number of vaccine-preventable diseases that continue to occur. Moreover, there is evidence showing that children with chronic diseases are at risk for delayed completion of vaccination schedules and that adults are at risk for low vaccination coverage because of lack of access to vaccinations (79, 80). It is also well-known that vaccine-induced immunity to some pathogens wanes over time (81, 82). Given these facts and the rising problem of opponents to vaccines, there is a risk that the rates of vaccine-preventable diseases continue to increase.

Vaccines are a safe method to provide immunity to large populations. They rely on a strong humoral and memory response by B cells. The most efficient vaccines available are those against viruses and are composed of live-attenuated or inactivated viruses. Another type of vaccines produced, the subunit vaccines, are developed mostly against bacteria. Both vaccine types induce highly pathogen-specific neutralizing antibody responses which are rapidly initiated upon pathogen encounter. Additionally, there is evidence of so-called heterologous vaccine effects which implies that some vaccines can further benefit the immune system by additional stimulation of both innate and adaptive immunity inducing partial protection against other infections, which for instance is observed with the measles vaccine (83). In conclusion, there is no doubt that the use of vaccines is fundamental for both individual and herd immunity.

There is little to no data regarding vaccine coverage among individuals with CF. Besides the routine vaccines against measles, mumps, rubella, polio, diphtheria, tetanus and pertussis, individuals with CF should also be immunized against influenza, hepatitis, varicella and RSV as most of these infections can be severe in individuals with CF and some can also be detrimental for transplantation. It is therefore especially important to prevent these potentially life-threatening infections.

Individuals with CF are particularly at risk for pulmonary deterioration upon infections with respiratory viruses as well as with pathogenic bacteria. *Pseudomonas aeruginosa* causes a significant increase in morbidity and mortality due to its high virulence but an efficient vaccine has yet to be identified in clinical trials (84). Efficacy of pneumococcal vaccines' use in CF is lacking evidence which has unfortunately broadly halted its use (85). Development of new and potent vaccines, such as for instance the vaccine against *Haemophilus influenzae*, is therefore

crucial for preventive care in CF. Several efficacious vaccines against respiratory viruses, such as influenza and varicella, and soon RSV, are available, and it seems reasonable that they are employed in CF because these viruses can cause serious pulmonary disease. In summary, vaccine use is a potentially powerful strategy for preventive therapy in CF.

Immunogenicity of common vaccines in CF has been scarcely studied. There is evidence that individuals with CF have a lower immune response to intramuscular pandemic influenza vaccination which can be bettered by addition of an adjuvant (86). On the contrary, children with CF generally respond well to oral polio vaccine despite their gastrointestinal disease status (87). Moreover, there are reports describing reduced vaccine responses in other chronic diseases. As such, children with chronic liver disease, who are at risk for worse outcomes if infected with hepatitis A virus (HAV), show an inadequate protection many years after initial immunization (88). In conclusion, immunogenicity of vaccines seems to depend on the type and administration route. Although individuals with CF are not considered as immunodeficient, there is extensive evidence of specific immune dysfunctions in CF. In the light of this fact, a concern should be raised regarding whether vaccine responses in individuals with CF are sufficient.

### 3 AIMS OF THE THESIS

The overall aim of this thesis was to explore the possibility that there is a defect in the immune defense against common cold viruses in CF and if one such common virus infection in CF can be prevented by vaccination.

Specific aims:

- to study the antiviral immune response towards a common cold virus, an enterovirus, in an experimental mouse model for CF (**Paper I**);
- to examine how common infections with one family of enteroviruses are in individuals with CF (**Paper II**);
- to study whether a vaccine against these enteroviruses is safe and can protect against an acute infection in a mouse model for CF (**Paper II**);
- to examine the state of immunity towards a common enterovirus vaccine in individuals with CF (**Paper II**);
- to investigate whether there is a temporal relationship between enterovirus infections and the onset of CFRD (**Paper III**).

## 4 METHODOLOGICAL CONSIDERATIONS

The following section is an overview of the main methodology utilized in this thesis. A detailed description of materials and methods may be found in the respective papers included (**Paper I**, **II** and **III**).

### 4.1 Virus strains and virus infections

In **Paper I** and **II**, the CVB3 (Nancy strain) was used for all infection experiments in mice. The virus was propagated in HeLa cells. Mice were injected with CVB3 intraperitoneally in a total volume of 200  $\mu$ l PBS or RPMI-1640 medium. Animal health state was monitored and mice that showed signs of illbeing were sacrificed.

In **Paper II** and **III**, CVB1-6 as well as polioviruses 1 and 3 (the Sabin strains) were used for determination of neutralizing antibody titers against these viruses. We did not study immunity towards polio 2 because the eradication of this virus was announced a few years ago.

### 4.2 Vaccine

In **Paper II**, mice were vaccinated by interscapular injection of 1.8  $\mu$ g of a monovalent CVB3 vaccine. It was produced by our collaborators at the University of Tampere, Finland. CVB3 was propagated in Vero cells and purified by sucrose pelleting and gelatin affinity chromatography resin (89). The virus was inactivated in 0.01% (v/v) formalin for 5 days at 37°C and diluted in vaccine buffer (M199 medium with 0.1% Tween 80, 1.8 $\mu$ g dose).

### 4.3 Cell lines

In **Paper I** and **Paper II**, HeLa cell monolayers were used for measuring CVB3 titers in the blood and organs of CVB3 infected mice and CVB3 neutralizing antibody titers in serum of vaccinated animals. Neutralizing antibody titers in human serum towards CVB1-6 and polio 1 and 3 were studied using Green monkey kidney (GMK) cells monolayers.

### 4.4 Animals

Two strains of mice were used in studies of immune response towards CVB3:

The **delF508** mouse model, which is homozygous for the *Cftr*<sup>*tm1EUR*</sup> mutation (90), the most common CF mutation in humans, was used to study the immune response to either CVB3 infection in **Paper I** or to CVB3 vaccination followed by CVB3 infection in **Paper II**.



The **TCR $\alpha$ -knock out** mouse model was used in **Paper II** to study the role of T cells in the neutralizing antibody response to CVB3 vaccination. These mice are homozygous for the *Tcr $\alpha$ <sup>tm1Mom</sup>* mutation which causes a loss of the alpha beta T-cell receptor and results in the absence of functional T-cells.

#### **4.5 Patients**

The serological patient data presented in **Paper II** and **II** comes from a retrospective analysis of a cohort of individuals with CF (n=65), monitored monthly/yearly at the Stockholm CF Center, Karolinska University Hospital Huddinge.

We performed a nested case-control study where we looked at whether any new CVB infections during the year preceding CFRD diagnosis were correlated with CFRD onset. The nested case-control study consisted of nine case-control groups of CF patients from our initial CF patient cohort. We collected serum samples from cases at the time of diabetes diagnosis and one year prior to their diagnosis. Sampling was performed in an identical manner for the controls that were matched by age, gender and genotype.

In **Paper II**, we present results of histopathological studies of autopsy material from a cohort of deceased CF patients with CFRD. These studies were performed by our collaborators in Exeter, England.

The serological studies on serum from Swedish CF patients were approved by the regional ethical board in Stockholm, Sweden. The study of autopsy material from individuals with CFRD were approved by The West of Scotland Research Ethics Committee. Both studies complied with the Declaration of Helsinki.

#### **4.6 Enterovirus PCR**

In **Paper III** we analyzed presence of entero- and rhinovirus RNA as indicator of an acute infection by using real time PCR.

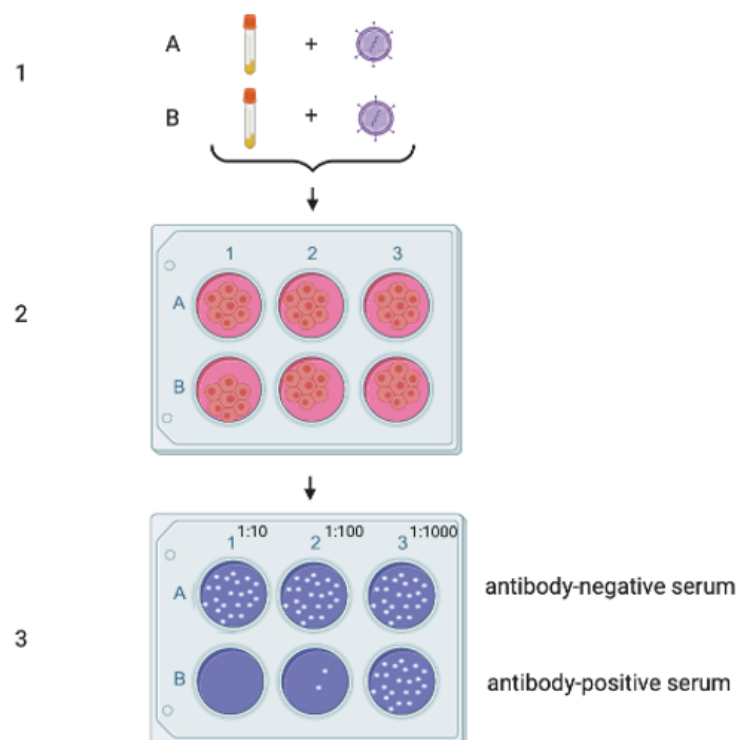
#### **4.7 Plaque assay and plaque neutralization (reduction) assay**

In **Paper I** and **II**, virus titers were measured by standard plaque assay and quantified as PFU/ml or PFU/g tissue.

Both in **Paper I, II** and **III**, titers of neutralizing antibodies against CVB3, CVB1-6 and polio 1 and 3 respectively were measured by plaque neutralization (reduction) assay (Figure 2). This

method is commonly used for quantification of highly specific neutralizing antibodies against a virus of interest and it enables to look directly at the ability of a sample to inhibit viral infection. In short, (1) a sample is incubated with virus in order for receptor binding of the neutralizing antibodies to occur, thereafter (2) the sample-virus mixtures are applied to cell monolayers and incubated allowing for infection of the cells. If the sample is neutralizing antibody negative (Figure 2, 3A) the virus is capable of infecting the cells and plaques (a small round area of killed by virus cells) are formed. If the sample is neutralizing antibody positive (Figure 2, 3B) no plaques are formed because the neutralizing antibodies bind to the virus and inhibit infection of the cells.

Neutralizing antibody titer measurements of serum from mice were performed at Center for Infectious Medicine, Karolinska University Hospital Huddinge, Sweden. Measurements of neutralizing antibody titers towards CVB1-6 and polioviruses in human serum were performed at the Department of Virology at Tampere University, Finland.



**Figure 2. Plaque neutralization (reduction) assay for determination of neutralizing antibody titers against a virus.**

#### 4.8 ELISA

In **Paper I**, anti-CVB3 IgM and IgG antibodies were measured by ELISA that was established using CVB3 virus-like particles (VLPs) obtained from our collaborators in Tampere, Finland (91). This method enabled comparison of anti-CVB3 IgM and IgG antibody titers to the virus-

specific neutralizing antibody titers. The total IgM and IgG in mouse serum was measured using a commercial ELISA (Mabtech).

#### **4.9 Histology**

In **Paper I** and **II** mouse tissue sections was routinely processed and embedded in paraffin for histological evaluation of signs of infection or disease.

#### **4.10 Immunohistochemistry**

In **Paper I** and **II**, paraffin-embedded mouse tissue was stained with hematoxylin and eosin for histological examination.

In **Paper III**, pancreatic tissue from diseased individuals with CF obtained from Network for Pancreatic Organ Donors with Diabetes (nPOD) program was studied for the presence of enterovirus-specific protein. These analyses were performed by our collaborators' lab in University of Exeter Medical School, England. In short, paraffin-embedded section of pancreas tissue was stained for the enterovirus-specific viral protein 1 (VP-1).

#### **4.11 FACS**

In **Paper II**, FACS analysis was performed on purified mouse splenocytes in order to confirm the absence of functional T cell in the TCRA-knock out mouse model. The cells were stained for anti-CD4, anti-CD8a, anti-TCR $\beta$  or anti-B220 antibodies (Biolegend).



## 5 RESULTS AND DISCUSSION

### 5.1 PAPER I

#### ***A Link Between a Common Mutation in CFTR and Impaired Innate and Adaptive Viral Defense***

There is evidence that respiratory virus infections predispose individuals with CF to chronic bacterial colonization of the airways thereby contributing to greater morbidity (17, 19, 20, 22). Moreover, the duration of upper respiratory illness is prolonged while the frequencies of infections do not seem to differ from the healthy population (17-19). To investigate why the above is observed, in **Paper I** we studied the immune response to a common respiratory virus, CVB3, in an experimental mouse model for CF. In this paper we present new evidence of an immune defect in these animals.

The mouse model used in **Paper I** harbors the most common homozygotic *cfr* mutation in humans, the delF508 mutation. Our first striking finding in the delF508 mice was that they have significantly lower survival rates upon CVB3 infection with both a high and a low virus dose compared to wt mice when exposed both a higher and a lower virus dose (**Paper I**, Figure 1A and 1B respectively). The amount of virus found in blood on days 3 to 5 after infection in the delF508 mice was however similar to that detected in wt mice (**Paper I**, Figure 2A), indicating that the virus initially spread at the same speed in both wt and the delF508 mice. However, measurements of the viral titers in the different organs showed that the delF508 mice had significantly higher viral titers, compared to wt mice, and they also had a delay in virus clearance on day 7 post infection (**Paper I**, Figure 2B and 2C). At the same time, histological evaluation of the infected delF508 mice organs showed no differences in the severity of infection in the affected organs compared to wt mice (**Paper I**, Supplementary Figure 1).

To further investigate the finding that there was a delay in viral clearance in the delF508 mice, we studied the key innate immune factors that are important for an effective antiviral defense. IFNs are crucial signaling molecules in the induction of immune responses against enteroviruses (92). We therefore started by looking at IFN production in mice. To address if the delF508 mice had a generalized defect in producing IFNs upon virus infection, we first exposed the animals to stimulation with the double stranded RNA homopolymer polyI:C. PolyI:C is known to mimic viral induced IFN production in mice (93, 94) and is recognized by the same pattern recognition receptors as enteroviruses (TLR3 and MDA5) (95, 96). Upon polyI:C injection, the delF508 mice had a slower induction of IFN $\alpha$  than wt mice (**Paper I**, figure 3B), while production of IFN $\beta$  and IFN $\lambda$  did not differ between between the delF508 and wt animals (**Paper I**, Supplementary Figure 3 and 4). We also looked at the expression of two other genes important for antiviral defense, namely iNOS and OAS, and found that they were both induced upon stimulation with polyI:C but their expression did not differ between the delF508 and wt mice (**Paper I**, Supplementary Figure 5A and 5B).

The finding that the delF508 mice had an impairment in their ability to clear virus led us to consider whether any impairments existed in a crucial part of the adaptive immune response involved in the control of infections, namely the production of virus neutralizing antibodies. We first measured the levels of serum IgM and IgG antibodies after CVB3 infection and discovered that the delF508 mice had detectable levels of both antibody isotypes, but these were significantly lower than those found in wt mice (**Paper I**, Figure 4A). To further evaluate whether these antibodies had neutralizing capacity, we performed neutralization assays and found that the delF508 mice had a delayed neutralizing antibody response to CVB3 infection and, moreover, significantly lower titers of neutralizing antibodies against the virus compared to wt mice (**Paper I**, Figure 4B and 4C respectively). These striking findings demonstrated that the delF508 mice have a defect in their antibody production against CVB3.

Passive immunization studies were performed in order to see whether the delF508 mice could be protected from CVB3 infection by the transfer of antibodies (serum) generated in infected wt mice. The results showed that passive immunization is a potentially efficient strategy to prevent such infections in our CF mouse model (**Paper I**, Figure 5).

We next moved on to study the different immune cell populations and mechanisms that could be involved in the defective antibody production in the delF508 mice. We found no major differences in cell counts of B-, T- and NK-cells between the delF508 and wt mice (**Paper I**, Supplementary Figure 6A-I). Using TNP-Ficoll stimulation, which induces T cell-independent antibody production (T cell-independent antigens account mostly for bacterial products), we found no differences in IgM and IgG levels (**Paper I**, Figure 6A). In contrast, injection of a T cell-dependent antigen (T cell-dependent antigens are generally proteins), using rSFV-βGal, resulted in significantly lower levels of both IgM and IgG in the delF508 mice on day 7 post injection (**Paper I**, Figure 6B). However, the antibody levels in the delF508 mice successfully rose to the levels seen in the wt mice on day 12 post injection (**Paper I**, Figure 6B), suggesting that only the initial antibody production stage is impaired in the delF508 mice.

In conclusion, the main finding in **Paper I** is that mice harboring the most common *cftr* mutation have a delayed in their initial production of neutralizing antibodies towards a common cold virus.

It has been shown that CFTR is expressed in lymphocytes including B cells and there is increasing evidence of CFTR dysfunction in immune cells in CF (75, 97, 98). Production of neutralizing antibodies is a result of B cell activation, clonal expansion and differentiation into plasma cells. One activated B cell can produce several thousands of plasma cells, which enables rapid amplification of an antibody response towards a proliferating microbe. Both T cell-dependent and T cell-independent B cell responses are driven by the same activation processes involving BCR signaling and activation of transcription factors needed for proliferation, maturation and antibody secretion. Our findings in **Paper I** indicate that there is a problem in the T cell-dependent antibody production in mice homozygous for the delF508 mutation, whereas the T cell-independent response seems to function properly. This suggests that the

problem is unlikely to lie within the B cells' activation, maturation or antibody secretion. We hypothesize that the defect could lie somewhere prior to the involvement of the B cells: for instance, from antigen recognition by innate cells up to the contact between T and B cells.

T cells in particular along with antigen presenting cells (APCs) are essential for T cell-dependent B cell responses (as the name implies). It has been reported that both cell counts and the function of T helper cells, cytotoxic T cells and DCs are decreased in individuals with CF and in the delF508 mouse model (99-102). One of the crucial factors required for the induction of an efficient anti-viral defense by these cell types are IFNs. Interestingly, one of the main antigen-presenting cell types, the plasmacytoid DCs (pDCs) are also the major source of IFN $\alpha$  (103). The initial delay in IFN $\alpha$  production that we saw in the delF508 mice could possibly be explained by decreased counts or functions of DCs, which in turn could be the link to the defect T cell-dependent antibody response we observed. Given the above, a defect in innate immunity could lead to a defect in the adaptive immunity in CF. It can be concluded that studies examining the mechanisms and kinetics of T cell-dependent antibody production in CF against both viruses and bacteria are highly warranted.

## **5.2 PAPER II**

### ***Coxsackie B Virus Infections Are Common in Cystic Fibrosis and Can Be Prevented by Vaccination***

In **Paper I**, we discovered that the delF508 mouse model had an impaired antibody response to infection with an enterovirus, which made them more susceptible to infection than the wt mice. This finding was in line with our hypothesis and previous indications that individuals with CF could also have a defect in their immune system that results in them suffering from prolonged and more severe viral infections (17, 19). As a result, these infections could predispose the CF lung to bacterial colonization (15, 20, 104-106). Ultimately, viral infections could be potential preventive therapy targets by vaccination strategies. Given our finding regarding the poor antibody response after viral infection in the delF508 mouse model, we wanted to examine whether the response to vaccines was also impaired in CF. In **Paper II**, we therefore focused on examining the immune response towards enterovirus vaccines in CF.

We utilized our existing CF mouse model and an experimental monovalent vaccine against CVB3 in order to study neutralizing antibody development after immunization. Being one of the most common respiratory pathogens found in CF, the enteroviral immune response including immunizations are highly interesting to investigate (20, 21, 106). In addition, our finding that the delF508 mice have an impaired T cell-dependent antibody response raised the question of whether the response to CVB3 vaccination was T cell-dependent or -independent.

First, a knock-out mouse model lacking T cells (TCR $\alpha$  knock-out mice) was established in order to study whether antibody production towards the CVB3 vaccine was dependent on T cell help. Using the T cell-deficient mice we found that the second phase of antibody response (the

IgG response) that occurs after vaccination was T cell-dependent, while the early antibody response (the IgM) was not affected by a lack of T cells (**Paper II**, Figure 2).

Having found that the immune response to the CVB3 vaccine is T cell-dependent, we went on to study antibody development in the delF508 mouse model after vaccination. The delF508 mice showed a good initial antibody response to the vaccine which however, in a different manner to the wt mice, waned by the expected time of antibody class-switch (**Paper II**, Figure 3, day 14). Nevertheless, after a booster vaccination the neutralizing antibody levels rose to a comparable level to that seen in the wt mice (**Paper II**, Figure 3). Moreover, we confirmed the protective effect of the CVB3 vaccine in the delF508 mice by challenging them with the CVB3 virus after vaccination and the delF508 mice were protected from acute infection (**Paper II**, Figure 4).

In summary, the results of **Paper II** give a strong indication that immunization against common respiratory viruses could be a potent preventive therapy option for individuals with CF. In order to stress the importance of our observations, we studied the prevalence of CVB infections in individuals with CF. We presented completely novel data showing that these infections are frequent but not more common than in healthy individuals. Every third individual with CF had experienced at least one infection with at least one of the six known CVB serotypes (**Paper II**, Table 2). Moreover, we analyzed immunity to a common and well-studied enterovirus vaccine, namely the highly immunogenic inactivated polio vaccine, and found that all individuals with CF had good protective levels of polio-specific neutralizing antibodies (**Paper II**, Figure 1). This indicates that individuals with CF have a good and long-lived immunologic memory after vaccination against an enterovirus.

There is scarce but significant evidence regarding the poor response to virus vaccines in individuals with CF. Interestingly, the waning of the initial antibody response seen after the first immunization in our CF mouse model is in line with the data reported in humans: a significant proportion of individuals with CF have a poor response to one dose of adjuvant-free influenza vaccine (86). These findings support the existing idea that special immunization strategies (for example the use of booster doses or adjuvants) are needed for good protection mediated by vaccines in individuals with CF. Collectively, our findings in **Paper I** and **II** suggest that common respiratory virus infections could be future targets for preventive therapy in CF and that vaccination of this patient group might require special immunization strategies.

### 5.3 PAPER III

#### ***Investigating the Role for Enterovirus Infections in Cystic Fibrosis-Related Diabetes***

There is a lot of evidence supporting the role of EV in T1D. It is known that the risk for developing T1D is increased when EVs, and particularly CVBs, are found in blood (107-113). Further strongly convincing evidence is that EV proteins are often found in pancreatic islets of



T1D patients to a greater extent than in those without the disease (112, 113). Interestingly, in one such study, where a small group of individuals with CF was used as a control group, the EV protein was detected in the islets of individuals with CF who also had CFRD but not in those without diabetes (57).

In **Paper III**, we elaborated on the above-mentioned finding and, in collaboration with the authors of the original study, performed histological studies examining another cohort of individuals with CFRD histologically. We were also interested in whether there could be a similar temporal association to that seen in T1D between CVB infections and CFRD onset.

The presence of EV in pancreatic tissue was assessed by immunohistochemistry which enabled direct visualization and localization of virus. Four out of five tissue donors with CFRD were positive for the virus in the pancreatic islets while most of the non-diabetic control donors did not show any positivity for the viral protein. Although no statistical significance was reached, the findings strengthen the case for possible EV involvement in the pathogenesis of CFRD (in at least a subgroup of individuals). Increasing the number of donors would be a natural progression for this study. Moreover, in a similar manner to the recently introduced concept of “endotypes” in T1D (114, 115), CFRD could also have a heterogenic etiology which emphasizes the need for an even larger study cohort.

The second aim of **Paper III** was to investigate whether there was a temporal association between CFRD onset and a previous CVB infection. For this, we performed a nested case-control study where we analyzed serum samples for the appearance of new CVB infections preceding diagnosis of CFRD. No such relationship was noted, although the small cohort size was the major limiting factor of the study. Another important aspect is that EV infection could be of a low-grade persistent character and present exclusively in the pancreatic islets thereby evading the general immune response, which could explain the absence of neutralizing antibodies in serum. By looking at each study subject individually, we identified one patient who lost neutralizing antibodies towards two CVB serotypes in one year – a striking finding given the fact that CVB immunity, like for other enteroviruses, is generally long-lasting (116). One could speculate, that more individuals with CFRD might have lost immunity towards the hypothesized diabetogenic CVB serotype years before diabetes diagnosis and looking at earlier historical serum samples from these patients could possibly reveal that.

To the best of our knowledge, there is no previously published data to compare with that examines the potential involvement of viruses in the pathogenesis in CFRD. Further investigations in a larger study cohort would therefore be intriguing.

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In the middle of the covid-19 pandemic I am ending my PhD journey halfway by writing this licentiate thesis. I cannot hide I am both sad and happy about it and the decision to stop halfway was as overwhelming as the change the covid-19 brought into our lives. Nevertheless, I am very proud of and grateful for everything that I have accomplished. Hereby, I would like to thank those who have supported me along the way:

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