From DEPARTMENT OF CLINICAL SCIENCE, INTERVENTION AND TECHNOLOGY, DIVISION OF PEDIATRICS
Karolinska Institutet, Stockholm, Sweden

VITAMIN D INSUFFICIENCY IN CYSTIC FIBROSIS: PREVALENCE, CONSEQUENCES AND INTERVENTION

Terezia Pincikova

Karolinska Institutet

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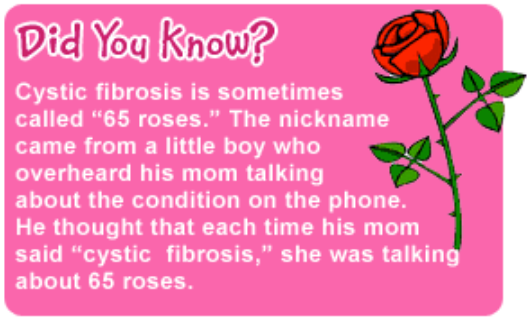

Dedicated to all the CF patients in the whole world

ABSTRACT

Cystic fibrosis (CF) is the most common life-shortening autosomal recessive disease in Caucasians. The major cause of morbidity and mortality is lung disease, characterized by a vicious circle of infection and inflammation. Management of CF requires a multifaceted approach, where intensive chest physiotherapy is combined with aggressive antibiotic treatment, and tight nutritional follow-up. Vitamin D insufficiency has high prevalence among CF patients worldwide. Vitamin D is crucial for maintaining healthy skeleton. Recently, extraskeletal functions of vitamin D have been described. These include immunomodulatory and glucose-lowering properties. Due to lack of relevant studies, vitamin D supplementation in CF is recommended only as one of the means of maintaining bone health. The aim of this thesis was to increase our current understanding of the impact of vitamin D supplementation in CF patients, and provide data needed for designing an efficient vitamin D supplementation policy.

First of all, we showed that a majority of Scandinavian CF patients had a suboptimal serum 25-hydroxyvitamin D (s25OHD) level (**Paper I**), and that the vitamin D doses needed to increase it were high. Cholecalciferol was more efficient at increasing s25OHD than ergocalciferol, and s25OHD monitoring was needed (**Paper III**).

Secondly, we propose that vitamin D induces a broad spectrum of immuno-modulatory actions in CF. In the well-defined Scandinavian CF population (n=898), s25OHD was associated negatively with serum total IgG, and positively with lung function (FEV1) in a robust multiple linear regression (MLR) model (**Paper I**). In line with that, three months long ergocalciferol supplementation in Stockholm CF patients decreased serum total IgG and IgM, whereas cholecalciferol decreased expression of the costimulatory molecule CD40 on dendritic cells. Patients receiving any form of vitamin D decreased T cell activation and IL-8 levels at the end of the supplementation. On the other hand, certain mechanisms of innate immunity were enhanced, such as MCP-1 and sTREM-1. Soluble CD14 increased only in patients reaching 92 < s25OHD < 97 nmol/L, which suggests bell-shaped relationship between s25OHD and soluble CD14. Notably, increase in s25OHD levels was associated with positive changes in lung function and in respiratory quality of life scores (**Paper III**).

Moreover, we demonstrated that s25OHD < 30 nmol/L, s25OHD < 50 nmol/L, and vitamin D insufficiency degree are independent determinants of HbA1c values in Scandinavian CF patients in a MLR model. This indicates that vitamin D may have glucose-lowering properties in CF. In addition, s25OHD < 30 nmol/L and vitamin D insufficiency degree determined the risk of CF-related diabetes (**Paper II**).

In **Paper IV** we aimed to assess the ability of CF bronchial epithelial (CFBE) cells to convert the inactive 25OHD to the active $1,25(OH)_2D$, which is an important mechanism ensuring adequate local concentrations of the biologically active $1,25(OH)_2D$ *in vivo*. Upon addition of 25OHD (100 nmol/L), the amplitude of the increase in $1,25(OH)_2D$ was smaller for the CFBE cells than the non-CF human bronchial epithelial cells (12.0 vs. 33.2 pmol/L). These results indicate that cells harbouring mutations in *cftr* may have impaired ability to activate vitamin D.

In conclusion, this thesis contributes to the understanding of the multifunctional importance of vitamin D in CF. It creates hypotheses about role of vitamin D in chronic inflammation, diabetes and lung function, which need to be studied further.

LIST OF PUBLICATIONS

This thesis is based on the following papers:

I. PINCIKOVA T, Nilsson K, Moen IE, Karpati F, Fluge G, Hollsing A, Knudsen PK, Lindblad A, Mared L, Pressler T, Hjelte L; Scandinavian Cystic Fibrosis Study Consortium. Inverse relation between vitamin D and serum total immunoglobulin G in the Scandinavian Cystic Fibrosis Nutritional Study. *Eur J Clin Nutr, 2011, 65(1): 102-109.*

II. PINCIKOVA T, Nilsson K, Moen IE, Fluge G, Hollsing A, Knudsen PK, Lindblad A, Mared L, Pressler T, Hjelte L; Scandinavian Cystic Fibrosis Study Consortium. Vitamin D deficiency as a risk factor for cystic fibrosis-related diabetes in the Scandinavian Cystic Fibrosis Nutritional Study. *Diabetologia, 2011, 54(12): 3007-3015.*

III. PINCIKOVA T, Paquin-Proulx D, Sandberg JK, Flodström-Tullberg M, Hjelte L. Clinical and immunological impact of vitamin D treatment in cystic fibrosis: A randomized, controlled pilot trial. *Manuscript.*

IV. PINCIKOVA T, Flodström-Tullberg M, Hjelte L. Cystic fibrosis bronchial epithelial cells have impaired ability to activate vitamin D. *Manuscript.*

The papers will be referred to by their numerials I-IV.

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LIST OF ABBREVIATIONS

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25OHD	25-hydroxyvitamin D
ABPA	Allergic bronchopulmonary aspergillosis
AUC	Area under curve
BMD	Bone mineral density
BMI	Body mass index
BPI-ANCA	Anti-neutrophil cytoplasmic antibodies specific for bactericidal/permeability-increasing protein
BW	Bodyweight
CF	Cystic fibrosis
CFALD	Cystic fibrosis associated liver disease
CFBE	Cystic fibrosis bronchial epithelial cells (CFBE41o- cell line)
CFQ-R	Cystic Fibrosis Questionnaire Revised
CFRD	Cystic fibrosis related diabetes
cftr	Cystic Fibrosis Transmembrane conductance Regulator (gene)
CFTR	Cystic Fibrosis Transmembrane conductance Regulator (protein)
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
D2	D2 vitamin (ergocalciferol)
D3	D3 vitamin (cholecalciferol)
DBP	Vitamin D binding protein
DCs	Dendritic cells
ELISA	Enzyme-linked immunosorbent assay
FEF25%	Forced expiratory flow rate 25%
FEF50%	Forced expiratory flow rate 50%
FEF75%	Forced expiratory flow rate 75%
FEV1	Forced expiratory volume in one second
FVC	Forced vital capacity
Free-s25OHD	Free seum 25-hydroxyvitamin D
HBE	Human bronchial epithelial cells (16HBE14o- cell line)
HMGB-1	High mobility group box protein-1
HOMA-%B	Homeostatic Model Assessment-Beta cell function

Ig	Immunoglobulin
IL	Interleukin
iNKT cells	Invariant natural killer T cells
IP-10	Interferon gamma-induced protein-10
ISI	Insulin sensitivity index
LL-37	The antimicrobial peptide cathelicidin
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
MLR	Multiple linear regression
NETs	Neutrophil extracellular traps
NFκB	Nuclear factor kappa B
OR	Odds ratio
PBMCs	Peripheral blood mononuclear cells
PEF	Peak expiratory flow
PMA	Phorbol 12-myristate 13-acetate
PRRs	Patter recognition receptors
PTH	Parathyroid hormone
QLP	Parent-reported quality of life
QoL	Patient-reported quality of life
RCT	Randomized controlled trial
s25OHD	Serum 25-hydroxyvitamin D
sCD14	Soluble CD14
SD	Standard deviation
SR	Sedimentation rate
sTREM-1	Soluble triggering receptor expressed on myeloid cells-1
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
Th	T helper
TNF-α	Tumour necrosis factor-alpha
Tot-s25OHD	Total seum 25-hydroxyvitamin D
Tregs	T-regulatory cells

1 CYSTIC FIBROSIS

Cystic fibrosis (CF) is the most common life-shortening autosomal recessive disorder in populations of European origin (1). The reported incidence in Caucasians varies considerably, depending on the population sampled and the method of detection (2). The incidence of CF in Finland is one of the lowest among all Caucasian populations, 1:25000 newborn, (3), whereas the highest incidence at birth is in Ireland, 1:1014 (4). Also the occurrence of CF in Brittany in western France (1:2000) ranks as one of the highest (5). In Sweden, the incidence of CF is 1:5600 live births (6).

CF is a multi-organ disease with very complex underlying molecular mechanisms. They include dysregulated ion transport, epithelial functioning, immunity, absorption of nutrients and vitamins, and dysregulated glucose and calcium homeostasis. This translates into a complicated clinical picture with a broad range of phenotypic heterogeneity.

1.1 ETIOLOGY OF CYSTIC FIBROSIS

CF is caused by mutations in the Cystic Fibrosis Transmembrane conductance Regulator (*cftr*) gene on chromosome 7. The commonest mutation is deltaF508, which accounts for 70% of CF-causing mutations in most Caucasian populations (7). It has been hypothesized that a selective pressure could have led to the high prevalence of this mutation. Indeed, it is possible that there exists an unknown positive function for deltaF508 *cftr* in the heterozygote form, offering a biological advantage for CF heterozygotes. For example, *in vitro* and mouse studies have well defined resistance to typhus and cholera as potential advantages of the mutated *cftr* gene (8, 9).

Frequencies of mutations in the *cftr* gene vary a great deal between and within individual countries. DeltaF508 shows a northwest-to-southeast gradient, with a maximum in Denmark (87% of all identified mutations in the *cftr* gene) and a minimum (21%) in Turkey (10). Interestingly, in a sample of Iranian CF patients, the deltaF508 allele frequency was 21%, and 60% of the patients had none of the 29 common *cftr* mutations they tested for (11). It is currently being debated how the geographical distribution may help to identify potential origins of *cftr* mutations. Moreover, investigation of the age of the *cftr* mutation variants helps to provide historical insights in the context of understanding population migrations (5).

The *cftr* gene encodes for a chloride channel located in the apical side of the cell membrane of epithelial cells (12). However, the pathophysiological picture found in CF cannot be explained by the loss of CFTR function as a chloride channel only. The CFTR modifies the properties of ion channels; for example, it controls the activity of the epithelial Na+ channel, ENaC. It is unclear what is more important for the development of CF lung disease, the decreased secretion of Cl- or the increased reabsorption of Na+ (13). Both of these mechanisms influence water transport, which results in dehydration of mucosal secretions. Important to note, there is certainly much left to discover about the CFTR function, as the CFTR seems to influence a number of proteins, a list of which is still growing.

The major cause of morbidity and mortality in CF is progressive pulmonary disease (14). In the CF lung, abnormal ion transport causes inadequate hydration of luminal secretions, which results in accumulation of thick ("viscous") mucus in CF airways. This is where the old term for CF, 'mucoviscidosis', comes from. The dehydrated mucus leads to impaired mucociliary clearance, which is one of the key components of innate immunity. Therefore, the hallmark of CF lung disease is the vicious circle of infection and inflammation, ultimately leading to parenchymal destruction.

In general, classes I, II and III of *cftr* mutations are associated with more severe CF phenotype, as they result in CFTR that is absent or nonfunctional at the cell surface. Classes IV and V are associated with less severe phenotypic consequences, because they give rise to partially functional CFTR at the cell surface. As regards the gastrointestinal tract, pancreas and sweat ducts, the *cftr* mutations are a reliable predictor of the CF disease. This is not the case for the CF lung disease, where numerous 'modifier genes' play a substantial role in determining disease severity and CF-related morbidities (15-19). However, even here *cftr* gene mutations may have some predictive value. It has been for example shown that CF patients carrying two class I mutations risk developing more severe lung disease compared to patients with at least one class II mutation (20). In addition, environmental influences are important in this regard as well (21-27).

1.2 ABERRANT INFLAMMATORY RESPONSES IN CYSTIC FIBROSIS

Airway epithelial cells function as the first line of defence against pathogens and CFTR is expressed in their apical membrane, as well as in the submucosal glands in the airways (28, 29). Therefore, the link between CFTR and epithelial function has been extensively studied. Given its role in ion transport, CFTR contributes to regulate the airway surface liquid homeostasis (30, 31). Studies using primary airway cultures and mice over-expressing the beta subunit of the ENaC channel consistently demonstrated that the ion imbalance leads to airway surface dehydration (32, 33), which results in decreased beating of the cilia and stagnation of mucus that in the long run manifests as a lung disease resembling human CF (34).

The impaired mucociliary clearance reduces bacterial clearance; thus, initiating and sustaining inflammation (35). The pulmonary inflammation is also driven by ceramide accumulation (36-39), reduced ability to clear *Pseudomonas aeruginosa* and *Burkholderia cepacia* infection (40-44), intrinsic proinflammatory properties of CF fibroblasts and epithelial cells (45-47), as well as by prolonged NFκB activation and sustained cytokine secretion upon bacterial challenge in CF (48). The inability of deltaF508-CFTR to reach cell surface leads to inherently high levels of NFκB, which has a broad spectrum of consequences, as reviewed previously (49). CF airway epithelial cells have also decreased surface expression of the pattern recognition receptor (PRR) TLR-4 (50, 51), which contributes to the CF-specific airway epithelial dysfunction. The decreased functioning of the epithelial barrier in CF has been recognized as the major factor driving the airway inflammation in CF, and in 2012, Prince and Cohen have named CF as "a mucosal immunodeficiency syndrome" (52).

The CF lung is environment rich in pathogen- and danger- associated molecular patterns, which activate PRRs (53). Moreover, substances like neutrophil elastase and haemoglobin activate PRRs indirectly, contributing to the vicious circle of inflammation in CF (54, 55). Also, proline-glycine-proline and high mobility group box protein-1 (HMGB1), recently discovered potent proinflammatory substances, are elevated in the sputa of CF patients (56, 57). In addition, reduced levels of the anti-inflammatory cytokine IL-10 have been described in CF airways (58, 59). Collectively, this leads to a proinflammatory shift and to high levels of cytokines such as tumour necrosis factor-alpha (TNF-α), IL-6, IL-1β in the bronchoalveolar lavage fluid and sputum of CF patients (60).

It is currently agreed that the PRR overstimulation contributes to the CF lung pathology, but there are also reports saying that inadequate PRR stimulation has negative clinical impact in CF. For example, female CF patients have poorer lung function compared to well-matched male patients. One possible explanation of this gender gap might be impaired PRR-induced NFκB activation and IL-8 expression (61).

The recruitment of leucocytes into airways is orchestrated by chemokines (62). A role in CF lung pathology has been mainly described for IL-8 (CXCL8), but also for CCL2, CCL3, CCL4, CCL20, CCL17 and CCL22. In CF, several defects in chemokine signalling have been reported. For example, the epithelial expression of the chemokine CCL5 is dependent on CFTR, and thus, defective in CF cells (63). Moreover, IL-8 acts on both CXCR1 and CXCR2, and CXCR1 is cleaved by proteolysis in CF (64). The biological relevance of these defects remains to be determined.

As explained above, leucocytes are constantly recruited into the CF lungs. In fact, CF lung disease is dominated by a neutrophilic airway inflammation (65). Generally, neutrophils act via phagocytosis, granule release and formation of neutrophil extracellular traps (NETs). Neutrophils in CF are a double-sided sword: On one hand they are needed for the defence against pathogens, on the other hand they are causing lung damage. Importantly, neutrophils from CF patients display a spectrum of functional abnormalities, such as blunted phagocytic activity and reduced apoptosis rate (66-68). CFTR is expressed in neutrophil phagolysosomes and plays a role for the microbicidal action of neutrophils (43). The currently accepted notion is that neutrophils are recruited in excessive quantities to CF lung and sustain there the vicious circle of inflammation due to frustrated phagocytosis and inefficient killing.

A considerable part of the immune surveillance of the lungs is covered by alveolar macrophages. The numbers of alveolar macrophages in non-infected CF children are higher than in matched controls without CF, and their quantity is correlated with the monocyte chemoattractant protein-1 (MCP-1) concentration (69). Albeit recruited in high quantities, CF macrophages are functionally defective; for example, human macrophages with absent functional CFTR were shown to display intrinsically decreased bactericidal ability (70).

Additionally, CF monocytes were described to be defective in a few more aspects. Triggering receptor expressed on myeloid cells-1 (TREM-1) is normally induced on monocytes and neutrophils by lipopolysaccharide (LPS) and its activation results in secretion of proinflammatory cytokines (71). TREM family contribute to fine-regulate the innate immune response (72). In CF, the expression of TREM-1 on monocytes in the circulation is low. On top of that, when stimulated *ex vivo* with LPS, CF monocytes do not upregulate TREM-1, which is abnormal. This hyporesponsiveness was attributed to the very high plasma LPS levels in CF, and it was demonstrated that these LPS levels are able to induce an endotoxin tolerance state in non-CF monocytes *in vitro* (73). It was concluded that CF monocytes are locked in an endotoxin tolerance state (74).

The cross-talk between the innate and adaptive immunity is regulated by dendritic cells (DCs). The function of DCs in the CF lung is impaired due to low sphingosine-1-phosphate (75), and they display a delay in early differentiation phase (76). This suggests that activation of the adaptive immune response may be delayed in CF, which could be one of the reasons for the increased infection susceptibility in the CF lung.

The immune system of the CF lung is compartmentalized, with neutrophils dominating the alveolar and bronchial space, and T lymphocytes in the subepithelial layer. Currently, we recognize four CD4+ T cell subsets: Th1, Th2, T-regulatory (Treg) and Th17 cells. Th17 lymphocytes produce cytokines IL-17 and IL-22, and regulate both granulopoiesis and recruitment of neutrophils, thereby linking innate and adaptive immunity (53). The primary function of the Th17 subset is clearance of certain microorganisms via stimulating mucosal innate immunity and IL-17 has pleiotropic effects including recruitment and activation of neutrophils (77). However, Th17 pathway has also been implicated in autoimmunity (78, 79). In CF, IL-17 levels were observed to be increased both in bronchoalveolar lavage fluid (80) and in induced sputum (81). It is being debated whether this is beneficial or harmful (53, 77). Except for Th17 cells, there are other important sources of IL-17, and one of these are NKT cells. NKT cells belong to “innate-like” T lymphocytes that can promptly produce a wide array of cytokines and chemokines after endogenous or exogenous TCR-mediated activation. Through this property, NKT cells are viewed as important cells to conduct early effector and regulatory functions and cover the gap before adaptive immunity gets fully activated. In CF patients, there is no inherent defect in glycolipid antigen presentation to NKT cells (82), and as these cells can improve the course of lung inflammation caused by *Pseudomonas aeruginosa* or *Mycobacterium tuberculosis* (83, 84), it is being hypothesized that therapies augmenting NKT cell activation could be a novel approach to treat antibiotic-resistant bacterial infections (82). However, this has not been tested yet. On the contrary, the requirement of NKT cells in altered lung function and in development of emphysema in chronic obstructive pulmonary disease (COPD) has recently been demonstrated (85, 86). In this setting, NKT cells seem to be critical for the development of lung pathology in COPD. Collectively, further studies are clearly necessary to establish the relevance of NKT cells in the human system, including the context of CF pathology.

Beyond the excessive recruitment of Th17 cells, previous research showed that T cells from CF patients display an intrinsic shift towards Th2-related cytokines upon activation (87, 88). This was supported by experiments with CFTR -/- mice, which mounted an exaggerated IgE response towards *Aspergillus fumigatus*, with higher levels of IL-13 and IL-4 (88). Indeed, Th2–biased immune responses are observed in patients with CF (89). CFTR-deficient lymphocytes revealed an enhanced flux of intracellular Ca^{2+} in response to TCR activation. This was accompanied by an increase in nuclear localization of the nuclear factor of activated T cell, which could drive the IL-13 response (88). In summary, T lymphocytes with dysfunctional CFTR display some abnormalities, which is due to the intrinsic CFTR defect.

Abnormalities of B cells in CF have not been described. However, CF patients were found to have higher IgG and IgA serum concentrations compared with healthy controls, even when they are stable (90). In addition, elevated levels of specific anti-pseudomonas antibodies are risk factor for developing chronic pseudomonas infection (odds ratio (OR) 4.9 for IgG, and OR 2.7 for exotoxin A). There are several diagnostic tests for measurement of these antibodies available. These tests are highly sensitive and specific and antibiotic treatment based on serology may be advisable in selected cases, in order to eradicate the initial pseudomonas infection and prevent/delay the development of chronic pseudomonas infection/colonization (91).

1.3 CLINICAL MANIFESTATIONS AND TREATMENT

1.3.1 Lung disease in cystic fibrosis

In CF, multiple epithelial organs are affected, such as pancreas, liver, intestine, reproductive system, sweat glands, and, particularly, the respiratory tract. The decreased chloride secretion into the airways and increased sodium absorption from the airways lead to relative dehydration of the airway mucus, impaired mucociliary clearance, accumulation of purulent secretions and chronic infection (92).

A common bacteria infecting children with CF is *Staphylococcus aureus*, which was the major cause of death in children during the pre-antibiotic era (93). Today, the most common bacteria associated with CF respiratory tract infection and the main pathogen leading to chronic lung disease is *Pseudomonas aeruginosa*, with 75% prevalence in adult CF patients in Canada (94), and 70% prevalence in Swedish adult CF patients (95). Recently, non-tuberculous mycobacteria such as *Mycobacterium avium complex* and *Mycobacterium abscessus* have revived increased attention in the field of CF, as bacteria leading to chronic infection associated with drop in lung function (96). Moreover, CF patients are susceptible to contracting lung colonization by the *aspergillus species*, most commonly *Aspergillus fumigatus*, and often respond with hypersensitivity reactions, such as allergic bronchopulmonary aspergillosis (ABPA) (97).

The prominent cause of morbidity in CF are intermittent episodes of increased respiratory symptoms (“exacerbations”), such as cough and increased volume of

sputum (98), which are often associated with increase in acute phase reactants and decrease in quality of life (99). Treatment of the exacerbations consists of antibiotics (100) with a multidisciplinary approach, including intensified chest physiotherapy and management of poor nutritional status, depression and diabetes (101).

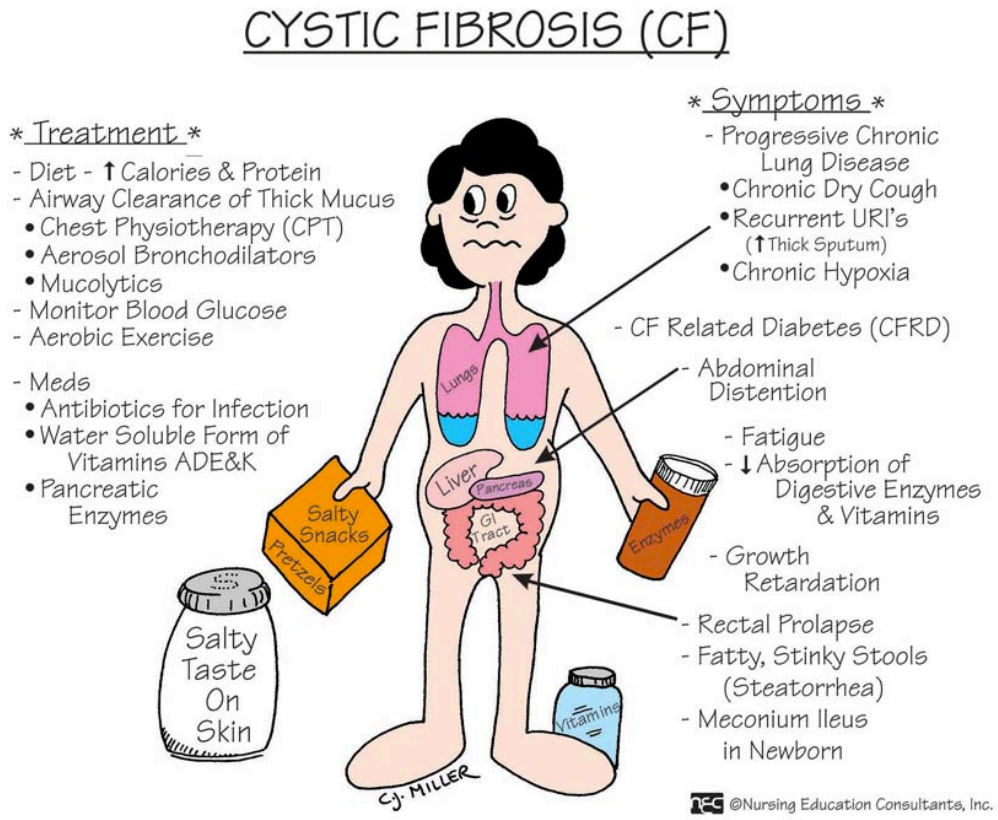

Figure 1. Symptoms and treatment of cystic fibrosis. Reprinted with permission from Zerwekh, JoAnn, Nursing Education Consultants, Inc., Chandler, AZ, 2013.

1.3.2 Gastrointestinal tract disorders in cystic fibrosis

The gastrointestinal tract is a significant source of morbidity in CF, as CFTR dysfunction affects the intestinal tract and the pancreatic and hepatobiliary ducts in a similar fashion as the lungs (102). Distal intestinal obstruction syndrome (DIOS) and meconium ileus are essentially specific for CF, while other gastrointestinal clinical entities are more common or behave differently in CF (103).

The majority of CF patients (around 85%) suffer from pancreatic insufficiency, resulting in malabsorption and insufficiency of fat-soluble vitamins. Pancreatic insufficient patients need to take pancreatic enzyme preparations with every meal (104), and fat-soluble vitamin supplements are recommended for all CF patients (105). Genotype strongly predicts pancreatic functional status: Pancreatic insufficient patients tend to have class I–III mutations and those with pancreatic sufficiency most often have class IV–V mutations (106).

The earliest gastrointestinal manifestation of CF is meconium ileus, a neonatal bowel obstruction. It occurs in 6-20% of CF neonates. It is rarely fatal with prompt treatment, and long-term outcome is not worse than that of other CF patients (107). If the abnormal meconium produces colonic instead of distal ileal obstruction, the entity is called meconium plug syndrome, which is not pathognomonic for CF (108). "Meconium ileus equivalent" occurring after the neonatal period is termed distal intestinal obstruction syndrome, as it seems to be a separate clinical entity with pathogenesis different from that of meconium ileus. It occurs most commonly in adolescent and adult CF patients, and should be differentiated from simple constipation, which is also very common in CF (109). Other gastrointestinal disorders related to CF include gastro-esophageal reflux disease, intussusception, appendiceal disease, volvulus, rectal prolapse and enteric infections (103).

Fibrosing colonopathy used to receive attention in the past, as it was associated with use of high-strength pancreatic enzyme preparations coated with methacrylic acid copolymer (110). We do not see this complication any more, since the cessation of use of the methacrylic acid copolymer in the enzyme preparations. Instead, gastrointestinal malignancies in CF patients are increasingly getting into focus. Even if the overall burden of cancer in CF patients remains low, CF patients have a considerably increased risk of digestive tract cancer, which increases further after lung transplantation (111).

It is widely recognized that liver is one of the organs affected by CF. Occasionally, the CF-associated liver disease (CFALD) may be a dominant manifestation of CF disease. In CFALD, the primary defect is in the bile ducts and not in hepatocytes. The common observation found is focal biliary cirrhosis consistent with that seen in partial biliary obstruction and can be seen in 70% of CF adults > 20 years of age (112). The vast majority of patients with CFALD will present already in childhood or adolescence (113, 114), and in most of them, the CFALD will have very little if any impact on their clinical wellbeing. However, the trickiest about CFALD is the fact that the rate of progression is extremely individual and unpredictable, and can sometimes become very rapid, progressing to liver decompensation if left untreated (103). In Scandinavia, around 4% of all CF patients develop clinical CFALD, which may eventually lead to liver transplantation.

In general, gastrointestinal conditions in CF respond well to medical management or prompt surgery when indicated. Indeed, modern treatment seems to positively influence liver disease because CFALD today is less common, less progressive, and less serious than previously reported (115).

1.3.3 Vitamin D insufficiency in cystic fibrosis

Due to lack of attention to vitamin D in CF in the past, patients used to be given multi-vitamin supplements in uniform dose and serum 25-hydroxyvitamin D (s25OHD) level would not be monitored (116). Later it was shown that the use of specific vitamin D supplements provides protection against vitamin D insufficiency whereas use of multivitamins does not (117, 118). As a consequence, systemic levels of vitamin D

and/or its metabolites studied in various CF patient populations were lower than in control non-CF subjects during the past few decades (116, 119-126). Vitamin D insufficiency has continued to be very prevalent among both the paediatric and adult CF patients despite routine specific vitamin D supplementation given today (117, 127, 128), even if there is a trend towards higher s25OHD levels in CF over recent years (129).

One third of CF infants detected by newborn screening have low s25OHD levels at initial evaluation (130), which suggests that some of the risk factors for vitamin D insufficiency are inherent to CF. The underlying reasons for vitamin D insufficiency in CF include pancreatic insufficiency with malabsorption, little body fat, low circulating vitamin D binding protein (DBP) concentrations (131), impaired absorption of ergocalciferol, accelerated excretion of vitamin D before hepatic hydroxylation of the vitamin (132) and CFALD causing impaired hepatic hydroxylation (133). Moreover, low adherence with vitamin D supplement prescriptions has also been highlighted as a reason contributing to the high prevalence of vitamin D insufficiency in CF (134).

Sun exposure has been confirmed as an important determinant of s25OHD levels in CF patients (135). In line with that, s25OHD values were shown to exhibit a clear season-dependent pattern (135, 136) and the US CF Foundation recommends a yearly screening for vitamin D status that should be done preferably at the end of winter (137).

1.3.4 Long-term complications of cystic fibrosis

With improving medical care, survival age increases and long-term complications of cystic fibrosis become more prevalent. For example, with increased life expectancy of CF patients, bone disease has become a common complication (138). Specifically, CF-related low bone mineral density (BMD) and its complications, such as osteoporosis and osteopenia, have high prevalence worldwide (139). Low BMD is present already at young age, in CF adolescents (140) and children (141), and its prevalence increases with age.

Untreated CF-related low BMD may result in fractures (139). One of the consequences may also be severe kyphosis (142). In the long run, this can lead to morbidity with deformity (143) and diminished lung function (144, 145), which may accelerate the progression of CF disease. Therefore, the maintenance of bone health in CF patients is crucial. Among the Swedish CF patients we currently do not see these severe complications of low BMD, which may probably be attributed to the advanced multidisciplinary medical care available today.

Current prevention and treatment recommendations for CF-related low BMD are primarily based on targeting the known risk factors for CF-related low BMD (138, 146). These include pancreatic insufficiency leading to malabsorption and low BMI, delayed puberty, glucocorticoid use (147), immunosuppression after lung transplantation (148), CFTR dysfunction (149), systemic inflammation (150), vitamin K insufficiency (151), compromised calcium balance (152), cystic fibrosis related

diabetes (CFRD) (153), reduced daily physical activity (154) and vitamin D insufficiency (120, 124).

Despite the increasing prevalence of CF-related low BMD, the most common complication of CF still remains CFRD. Currently, the prevalence of CFRD is 33% in some CF populations (155). On top of that, CFRD has a major impact on the clinical status of patients with CF - it is uniformly recognized that CFRD diagnosis is associated with poor lung function and earlier death (155-158).

Due to its clinical significance, CFRD has been studied extensively. In spite of that, its etiology remains puzzling. What we know for sure is that CFRD shares certain pathological features both with type 1 and type 2 diabetes (159). One of the principal causes of CFRD is probably the loss of pancreatic beta cells as a result of pancreatic fibrosis, but it is clearly not the only contributing factor in CFRD development. In a multivariate model of 377 cases of CFRD in 3275 CF patients in the UK, the following risk factors were independently associated with CFRD diagnosis: CFTR mutations class I or II (versus classes III-V), increasing age, female gender, worse pulmonary function, liver dysfunction, pancreatic insufficiency, and corticosteroid use (160). It remains to be determined which of these factors play a significant causative role in the etiology of CFRD, and which of them are only by-standers or consequences of CFRD. In general, the CF clinical and research community has preliminarily agreed that CFRD primarily results from an insulin-insufficient state, which is aggravated by worsening insulin resistance (161).

1.3.5 Treatment of cystic fibrosis

The therapy available for CF patients today is symptomatic and requires a multifaceted approach given that multiple organs are affected. In the current practice, the main focus of the therapy is strict infection control combined with tight nutritional follow-up.

The infection control includes aggressive use of oral or intravenous antibiotics directed against CF-related pathogens (101) (162), such as aminoglycosides (100), macrolides (163), cephalosporines and penicillins (164), fluoroquinolones and others (165, 166). Given in a prophylactic or therapeutic manner, inhalation antibiotics also find their place in the CF treatment (167, 168). In addition, the growing problem of multi-drug resistance and slow development of newer antibiotics has led to the re-emergence of colistin. In 1970s, its use in some countries decreased due to high incidence of nephrotoxicity and neurotoxicity. Recently, however, colistin has been increasingly used and newer studies have shown lesser toxicity and good efficacy (169).

Except for the prominent use of antibiotics, therapies directed at dampening the excessive inflammation in CF have been a part of the multifaceted CF treatment since a long time ago, and are now receiving increased attention (170). It is currently recognized that ibuprofen is safe (171) and can slow the progression of lung disease in CF children (172, 173). Other immunomodulatory agents that appear to have beneficial effect on the disease progression in CF are oral corticosteroids and azithromycin (174).

Taken together, this indicates that implementing strategies that would modulate lung inflammation could theoretically be beneficial for CF patients (172).

Treatment of the CF-related lung disease also includes mucolytic medications, such as bromhexin, acetylcystein, and hypertonic saline inhalations. Intensive chest physiotherapy is irreplaceable. In some cases, recombinant human DNase inhalations are prescribed as well (175).

Inferior nutritional status negatively affects survival of both pre- and post- transplant CF patients (176), as well as their quality of life (177). Thus, a crucial part of the symptomatic management of CF patients is close nutritional status monitoring and provision of individually adjusted nutritional advice and intervention (178). Moreover, CF patients have disturbances in lipid metabolism, including low status of linoleic and docosahexaenoic acids (179, 180), and their supplementation is believed to have beneficial effects with promising preliminary results (181-183). Therefore, food rich in energy and essential fatty acids is an important part of management of CF patients (175).

Advances in the symptomatic CF disease management have been increasing the life span of the CF patients worldwide. It has been calculated that 95% of the CF patients born in Scandinavia today will live longer than 25 years (6). This has led to increased prevalence of long-term complications of CF, such as CFRD and CF-related low BMD, and the focus of the therapy has been shifting towards these complications. Another consequence of the increased life span and the tight symptomatic management of CF patients, is a significant healthcare burden associated with treatment of pulmonary exacerbations and with long-term prophylaxis in CF (184), as well as with relevant healthcare costs (185). Finally, the longevity and increased prevalence of comorbidities, have led to the need for highly complicated and time-consuming spectrum of treatments. This extremely high treatment burden often results in non-adherence, which may have detrimental impact on the outcomes (186).

There are several novel treatments for CF emerging that utilize two different approaches in order to try to correct the basic defect: (1) gene therapy, aimed at correcting the defect in the *cftr* gene, and (2) therapy aimed at correcting the alteration in the CFTR protein (187). Both remain at research level, but especially the treatments focused on repairing the defective protein have shown promising preliminary results, with several molecules under development. These include Ataluren (PTC124), which is a molecule making the ribosomes become less sensitive to the premature stop codons in *cftr*; Lumacaftor (VX-809), which is corrector molecule directed at class II mutations; Ivacaftor (VX-770), which is a CFTR potentiator with efficacy for Gly551Asp mutation in CF patients. Ivacaftor has now become a licenced medication called Kalydeco, and is indicated for patients with the Gly551Asp mutation > 6 years of age. These treatments are currently undergoing clinical trials for other genetic mutations; for example, Kalydeco in combination with Lumacaftor are being tested in patients with deltaF508 mutation in homozygous form. Promisingly, Ivacaftor had potentiating effect on all CFTR forms with gating defects when tested *in vitro* (188). Taken together, treatments correcting the CFTR protein damaged by the most common mutations are

still missing (187), but the development proceeds very quickly and has showed promising results so far.

Similarly, there is currently no evidence to support the use of *cftr* gene therapy as a treatment for CF lung disease (189). A few RCTs have evaluated topical *cftr* gene delivery to the lung, using either viral or non-viral delivery systems. Promisingly, ion transport in the lower airways was significantly changed towards normal values upon administration of the *cftr* gene transfer agent (190). Initially, even improvement in FEV1 was reported (191); however, this was later not confirmed in a larger-scale study (192). One study found "influenza-like" symptoms as an adverse event of the *cftr* gene transfer agent, with risk ratio 7.00 (190). In summary, the results indicate that the novel treatments targeting the *cftr* gene or protein have potential to work, but future studies need to investigate clinically relevant outcome measures and safety in more detail. There is currently a gene therapy study ongoing in UK, where 124 patients have been randomized to receive placebo or gene therapy in form of liposome-mediated gene transfer. Recruitment of the patients has been completed and the trial will be finished by the end of this year (193).

As the potential causative therapy for the majority of the CF patients still mainly remains at research level, the only currently available treatment of the end-stage pulmonary disease is lung transplantation. CF patients have increasingly improving outcomes with transplantation and the post-transplant median survival increases in excess of 10 years. Common issues in the lung transplanted CF patients remain *diabetes mellitus*, renal dysfunction, bone disease and hypertension (194).

2 VITAMIN D

2.1 VITAMIN D: FROM DISCOVERY OF A VITAMIN TO RE-CLASSIFICATION AS A HORMONE

Vitamin D deficiency in the past was defined by the clinical recognition of rickets. Rickets is a childhood disease of impaired skeletal development, which is marked by bending and distortion of the bones under muscular action, by the formation of nodular enlargements on the ends and sides of the bones, and by delayed closure of the fontanelles (195). The term is said to have derived from the ancient English word “wricken” (means "to bend"). It was also in England where the term “rickets” first appeared: in 1634, “rickets” was listed as one of the causes of death in London’s Annual Bill of Mortality (196). In Europe, rickets was also called “English disease”, because it was endemic in larger British cities at the turn of the 19th century.

Figure 2. Children suffering from rickets. Adapted from http://www.talkorigins.org/ with permission; original source not found.

At the beginning of 19th century, rumours began circulating in Europe, claiming that cod liver oil could cure rickets (197-199). Moreover, a relationship between rickets and lack of sunlight was observed. For example, in 1822, rickets was more common among children in Warsaw as compared with those living in the rural surroundings, and Sniadecki pointed out inferior sun exposure as the underlying reason (197, 200). Additionally, in 1878 in Wales, children with rickets were treated by sun exposure on hospital balconies (201). It was also noted that areas of temperate latitude or heavy air pollution had greater prevalence of rickets; which again pointed to the sunlight as a protective factor (200, 201). After World War I, children with rickets at a hospital in Vienna were treated with UVB lamp, sunlight, cod liver oil, nothing or with combination of UVB lamp and cod liver oil. Children who received the combination of cod liver oil and UVB lamp were cured from rickets most rapidly. Another interesting observation was that partial UVB body exposure induced full body healing (202, 203).

Elmer McCollum and colleagues induced rickets in rats, healed them with cod liver oil, and then oxidized the cod liver oil in order to isolate the substance with anti-rachitic properties. They saw that the factor with anti-rachitic properties was distinct from the substance with anti-xerophtalmic properties (which had been discovered earlier and named “fat-soluble A”). They claimed it was a vitamin, and because it was the fourth vitamin discovered, it got the name “vitamin D” (197, 198, 204).

During 1960s, the metabolite of vitamin D which we currently use as a measure of vitamin D status (25-hydroxyvitamin D; 25OHD) was discovered, and it was

determined that it is produced in the liver (205-208). The biologically active vitamin D metabolite (1,25-dihydroxyvitamin D; 1,25$(OH)_2$D) was found at the end of 1960s and its production was originally described to occur in the kidney (209-211). The structure of this metabolite was confirmed in 1971 (212-214). In 1980, synthesis of vitamin D in the skin exposed to UV radiation was described (215). As a consequence of these discoveries, the scientific community has gradually re-classified vitamin D as a hormone (197, 215, 216).

Today the definition of vitamin D deficiency has changed from the clinical diagnosis of rickets to a definition based on s25OHD concentration (217). The s25OHD concentration is currently universally accepted as measure of vitamin D status, because it represents the amount of circulating vitamin D supply that is available to the peripheral tissues throughout the body. On the other hand, no universal definition of vitamin D deficiency exists at present. There are several various definitions based on s25OHD concentration. The Institute of Medicine (2011) defines s25OHD < 30 nmol/L as deficiency, 30 nmol/L < s25OHD < 50 nmol/L as insufficiency, and s25OHD > 50 nmol/L as sufficiency. If basing the definition of vitamin D insufficiency on s25OHD levels where parathyroid suppression and/or calcium absorption is observed to begin to plateau, then deficiency is s25OHD < 50 nmol/L, insufficiency 50 nmol/L < s25OHD < 75 nmol/L and sufficiency s25OHD > 75 nmol/L (218, 219). However, even this definition is imperfect, because the studies evaluating parathyroid suppression and/or calcium absorption were relatively small, excluded children and demonstrated large individual variation in the endpoints (218, 220-222). Another problem is that there are several different methods for s25OHD measurement available, and there is significant variability between laboratories even when using the same method (223, 224).

Interestingly, vitamin D intoxication usually doesn't occur until s25OHD > 450 nmol/L (225). The question is, why the physiological concentration window of s25OHD is so wide, and whether there is no extra use of concentrations that are higher than the currently recommended 50 or 75 nmol/L.

2.2 VITAMIN D METABOLISM

As the rumours during the 19th century indicated, today we know that vitamin D is synthesized in the skin by sun/UVB exposure (215), as well as it is ingested in diet. It occurs naturally in egg yolks, mushrooms and fatty fish, such as salmon, sardines, tuna, cod and mackerel (217). Moreover, some food items in Sweden are fortified with vitamin D, for example milk with ≤ 1.5% fat, unsweetened light yoghurt, unsweetened light sour ("fil") milk, and margarines (226). The major source of vitamin D, however, is the dermal synthesis. In the skin, exposure of human skin to solar UVB radiation (wavelengths: 290–315 nm) leads to conversion of 7-dehydro-cholesterol to pre-vitamin D3. Pre-vitamin D3 is then rapidly converted to vitamin D3 by temperature- and membrane-dependent processes (227). The amount of vitamin D production in the skin depends on the incident angle of the sun and thus on latitude, season and time of the day (228). With each deviation from the equator by 10 degrees, there is a progressive decrease in solar UVB radiation exposure (229).

There are two basic forms of vitamin D: Ergocalciferol (vitamin D2) is produced via irradiation of yeasts and mushrooms, and cholecalciferol (vitamin D3) is in food of animal origin and produced by dermal synthesis under UV light exposure (230). Both of these basic forms are metabolized in the liver to 25OHD (also called calcidiol). The circulating s25OHD is then further metabolized in the kidney to $1,25(OH)_2D$ (also called calcitriol) by the enzyme 1α-hydroxylase (Cyp27B1). $1,25(OH)_2D$ represents the biologically active form of vitamin D and binds to the vitamin D receptor (VDR). The VDR belongs to the nuclear receptor family and is expressed in the majority of the tissues in human body, including immune cells, skin, placenta and pancreas (231, 232). Similarly, not so long ago it has been discovered that the vitamin D activating enzyme, the 1α-hydroxylase (Cyp27B1), is expressed not only in the kidney, but also in other tissues (233, 234), including bone, placenta, prostate, keratinocytes, macrophages, T-lymphocytes, dendritic cells and several cancer cells (235). Taken together, the wide distribution of VDR and 1α-hydroxylase indicates that vitamin D might have other functions except for the classical, well described, role in rickets prevention and calcium homeostasis maintenance. Indeed, the locally produced active form of vitamin D exerts a broad spectrum of auto- or paracrine effects.

Currently it is clear that the second vitamin D activation step is carried out by a single enzyme, the 1α-hydroxylase (CYP27B1). However, it is not certain what enzyme(s) produce 25OHD. It has recently been confirmed that CYP2R1 is the major enzyme responsible for that, but there clearly must be also other, yet unknown, enzyme(s) that are contributing (236).

The biologically active $1,25(OH)_2D$ induces its own destruction by rapidly inducing the enzyme 25OHD-24-hydroxylase (CYP24A1), which in turn leads to multistep catabolism of both 25OHD and $1,25(OH)_2D$ (237).

2.3 CLASSICAL FUNCTIONS OF VITAMIN D

It is fascinating how old vitamin D is from evolutionary perspective. Since more than 500 million years ago, vitamin D has been synthesized by phytoplankton (238). Here, its function initially could have been the protection of UV-sensitive macromolecules when they were exposed to sunlight for photosynthesis (228). Later on, as animals with vertebral skeletons living in the calcium-rich ocean evolved and moved onto land, the maintenance of calcium homeostasis became a major problem. It was vitamin D that ensured the efficient intestinal calcium absorption from dietary sources and ultimately was essential for the development and maintenance of the calcified skeleton of mammals (239). Vitamin D provided by sunlight or diet is still critical for most vertebrates for maintaining their skeletal integrity. This is the case for humans as well (238, 240).

The primary function of the active form of vitamin D is regulation of calcium and phosphorus homeostasis. It binds to the VDR, which promotes the expression of an epithelial calcium channel and a calcium-binding protein, thereby increasing the efficiency of intestinal calcium absorption (218, 241, 242). In a similar way, it

enhances the intestinal phosphorus absorption (243). Moreover, vitamin D regulates the calcium and phosphorus homeostasis indirectly via regulation of parathyroid hormone (PTH) levels. The parathyroid glands locally convert the circulating 25OHD into $1,25(OH)_2D$, which inhibits the synthesis of PTH (244). High PTH in turn stimulates the production of $1,25(OH)_2D$. Therefore, vitamin D deficiency leads to secondary hyperparathyroidism with $1,25(OH)_2D$ production stimulated with the high PTH levels. This is the reason why, paradoxically, vitamin D deficiency is often associated with normal to high $1,25(OH)_2D$ levels. $1,25(OH)_2D$ also binds to VDR in osteoblasts, which induces the expression of receptor activator of NFκB ligand, and that stimulates pre-osteoclast to turn into a mature osteoclast. The mature osteoclast mobilizes calcium and phosphorus from the bone in order to maintain calcium and phosphorus homeostasis. In that way, vitamin D contributes to maintain adequate calcium and phosphorus levels in the circulation, which promotes the mineralization of the skeleton (218).

It is generally agreed that long-term vitamin D deficiency leads to persistently increased PTH levels in blood and increased bone turnover, as indicated by the biochemical markers for bone formation and resorption. Eventually, this leads to decrease in the rate of bone mass accumulation (245). If it happens in very young age, it may result in the disease called rickets. Clinically, deficiency of vitamin D is usually more common than either isolated calcium or phosphorus deficiency and continues to be the commonest cause of rickets (246), as it was suspected during the 19^{th} century. Rickets is characterized as a failure of mineralization of the organic matrix of bone, particularly at the epiphyseal growth plate, with deformities of the skeleton. However, rickets also includes muscle weakness, atrophy and spasms (247). For a very long time, the importance of vitamin D for muscle function was not recognized. Only recently, the association between muscle strength and vitamin D status has been demonstrated by several independent studies (245, 248). The underlying mechanisms of vitamin D action have not been clearly elucidated yet. VDR-active-vitamin-D complex seems to play a role in muscle development by regulating calcium content inside muscle cells and stimulating their growth and proliferation (249, 250). Moreover, research has elucidated several pathways of the molecular action of vitamin D in muscle, which include both genomic and non-genomic actions (251).

At later stages in life, persistent severe vitamin D deficiency results in the clinical entity called osteoporosis. Osteoporosis used to be defined by low bone mass and micro-architectural deterioration of bone tissue, giving rise to increased bone fragility and osteoporotic fractures. According to this definition, the diagnosis of osteoporosis requires the presence of a fracture. Today, the World Health Organization defines osteoporosis by BMD measurement, so that osteoporosis can be diagnosed and treated prior to incident fracture. Practically, the diagnosis of osteoporosis can be made when BMD at any site < 2.5 standard deviations (SD) below the young adult standard (a T-score of < -2.5). A precursor of osteoporosis is osteopenia, where the BMD T-score is ≥ -2.5 but < -1 SD (252). A growing bulk of data suggests that osteoporosis has its origins in childhood and adolescence when, at the end of skeletal maturation, optimal peak bone mass is not achieved (253, 254). Therefore, research has mainly been focused on potential factors that could influence the accrual of bone mass and vitamin D status was determined to be one of them. A large randomized controlled trial (RCT) was done in Finland, where girls aged 11-12 years were

randomized to serve as controls or receive vitamin D supplements for 1 year. This trial showed that vitamin D supplementation significantly increased bone mineral augmentation of the proximal femur (255). Similar results were achieved in a population of adolescent Lebanese girls (256), and further supported by a meta-analysis of six RCTs, which showed that vitamin D supplementation lasting at least three months had a positive effect on BMD of the lumbar spine and total bone mineral content (257).

Osteomalacia is defined as impairment of bone mineralization. It differs from osteoporosis, where bone mass is decreased with a normal ratio of mineral to matrix. In osteomalacia, the ratio of mineral to matrix is abnormally decreased. Osteomalacia is much less common than osteoporosis and can be definitively diagnosed only by bone biopsy. Typically, osteomalacia is associated with pain, myopathy, and fracture. The most common cause of osteomalacia in older adults is vitamin D deficiency (252, 258).

2.4 EXTRAMUSCULOSKELETAL EFFECTS OF VITAMIN D

The wide distribution of VDR and 1α-hydroxylase indicates that vitamin D might have other functions besides the classical, well described, role in rickets prevention and calcium homeostasis maintenance. Indeed, the locally produced active form of vitamin D exerts a broad spectrum of auto- or paracrine effects (228). For example, vitamin D has a major impact on the immune system, which has been covered by many outstanding review articles (for example (259-261)). In summary, vitamin D seems to stimulate the innate and dampen the adaptive immunity in a very complex manner. For example, it induces the production of tight junction proteins, and thus contributes to the intactness of epithelial barriers covering the skin, gut, and both urinary and respiratory tracts (262-264). In the epithelial cells, vitamin D also directly up-regulates the expression of antimicrobial peptides (265). In the lung, VDR is expressed in murine and human respiratory epithelial cells (266-268) and human lung epithelial cells can convert the inactive 25OHD to the active $1,25(OH)_2D$ (269). The active vitamin D directly induces the expression of VDR-regulated genes including the antimicrobial peptide cathelicidin (LL-37) in the respiratory epithelial cells (269). Collectively, this suggests that vitamin D is produced locally in the lung and that the epithelial cell derived active vitamin D contributes to the host innate immune defense.

Moreover, vitamin D stimulates the actions of cells associated with innate immunity such as monocytes and macrophages. Triggering receptor expressed on myeloid cells (TREM) family seem to fine-tune the innate immune response (72). TREM-1 is a receptor induced by LPS on monocytes and neutrophils and its activation results in secretion of proinflammatory cytokines (71). It was originally described on neutrophils and monocytes, but was later identified on epithelial cells as well (270). Importantly, the active $1,25(OH)_2D$ induced TREM-1 both in airway epithelial cells and monocytes/macrophages (271, 272), which is one of the mechanisms how vitamin D contributes to boost the innate immune response. Another mechanism is the induction of antimicrobial peptides in monocytes/macrophages. For example, the $1,25(OH)_2D$-VDR pathway upregulates LL-37 production in human monocytes, which accelerates the antimicrobial function of autophagolysosome in *Mycobacterium marinum* infection (273). Moreover, $1,25(OH)_2D$ strongly stimulated the production of IL-1β in human monocyte-derived macrophages treated with LPS

or PMA (274) and enhanced IL-1β secretion from infected macrophages *in vitro* (275). Other effects of the active vitamin D on macrophages may in contrast include down-regulation of the production of proinflammatory cytokines (276) and inducible NO-synthase as well as cyklooxygenase-2 (277). However, the prevailing effect of vitamin D on macrophages seems to be stimulatory, as vitamin D deficiency impairs macrophage maturation (278) and addition of the active vitamin D to macrophages *in vitro* increases chemotaxis and phagocytosis (279, 280).

Should the immunomodulatory effect of vitamin D be clinically relevant, then there should be a causative relationship between vitamin D supplementation and decreased risk of infection. A typical infectious disease that has been clearly associated with low vitamin D status, and responded to treatment with vitamin D, is tuberculosis. The positive effect of sunlight has been noted throughout the history of tuberculosis treatment. Herman Brehmer was a Silesian student who suffered from tuberculosis. In 1854 he travelled to the Himalayan mountains due to his botanical interests, and as a coincidence, he cured there his tuberculosis (281). Moreover, vitamin D in high doses was used to treat active tuberculosis in the pre-antibiotic era. For the tuberculosis of the bone, vitamin D supplementation caused some improvement (282).

Children suffering from tuberculosis taking additional vitamin D had more pronounced clinical and radiological improvements upon tuberculosis treatment compared with controls not taking extra vitamin D, but in another trial, additional vitamin D treatment did not have any effect on mortality of patients with tuberculosis (283, 284). *In vitro* data clearly show that the active form of vitamin D has potential to decrease mycobacterial burden (275, 285) and very recently, data gathered by RCTs consistently confirm the previously suggested role of vitamin D in the treatment of tuberculosis (286, 287).

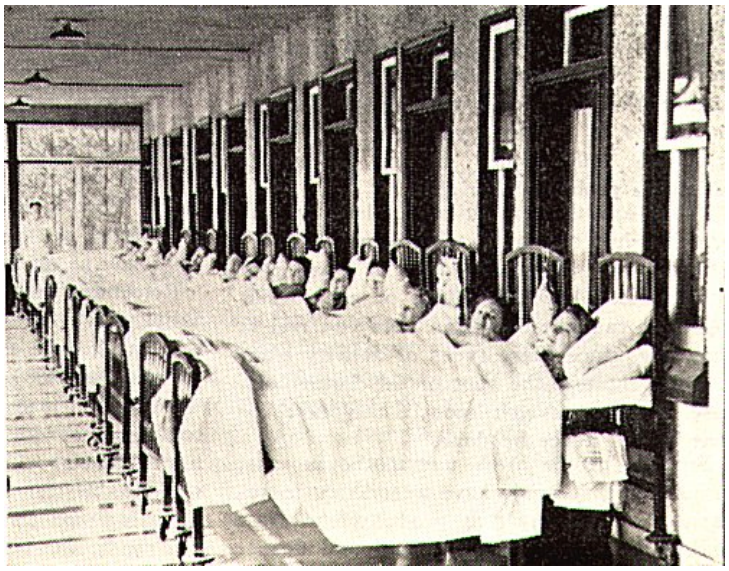

Figure 3. Tuberculosis patients were placed in sanatoria on balconies for sun exposure. Adapted from (288). Reprinted with permission of the Lung Association of Saskatchewan, Canada.

It has been hard to demonstrate causative relationship between vitamin D insufficiency and respiratory tract infections in children. There are many positive descriptive association studies available. For example, low serum 25OHD levels were associated with increased risk of laboratory-confirmed viral respiratory tract infections in children from Canadian Hutterite communities (289). However, published data from RCTs show mixed results, due to heterogenous study designs, poor compliance, and lack of s25OHD monitoring. Promising results have been shown in a placebo-controlled RCT,

where 1200 IU vitamin D per day during four winter months reduced number of children with influenza A in Japanese schoolchildren (290).

In the adult population, several studies have indicated a potential role for vitamin D supplementation as means of enhancing the control over respiratory tract infections (291-293). In patients with increased infection susceptibility, vitamin D supplementation significantly reduced the overall infectious burden score, compared with placebo (294). A meta-analysis of 11 placebo-controlled RCTs suggests that vitamin D has a protective effect against respiratory tract infections, and once-daily dosing pattern seems to be most effective (295).

While enhancing innate immunity, vitamin D dampens the adaptive immune response by reducing the production of Th1 and Th17-associated cytokines (e.g. IL-2, IFN-γ, IL-17 and IL-21) and decreasing plasma cell differentiation and the production of IgM and IgG, as reviewed previously (296). In line with this, low vitamin D levels have been associated with autoimmune and allergic diseases (261, 297). The suppressive effect of vitamin D on adaptive immune response is mediated via pleiotropic actions of vitamin D, which have been revealed during the past decade by immense amount of experimental studies. The active form of vitamin D inhibits proliferation, maturation, survival and differentiation of myeloid DCs (298, 299). This is done via several various mechanisms, which include inhibition of IL-12 production (300), increased IL-10 production (301), and down-regulation of co-stimulatory molecules (302). Eventually, this leads to decreased T cell activation (303). Moreover, vitamin D exerts direct effects on T and B cells, altering their response to activation. CD4+ T cells increase their VDR expression upon activation and vitamin D regulates > 100 genes in these cells (304). The emerging picture is a switch from a Th1/Th17 to Th2/Treg profile (305, 306). The direct effect on B cells involves inhibition of their differentiation into memory B cells and plasma cells, but also decreasing their proliferation and immunoglobulin production (307).

Not only immunomodulatory effects but also glucose-lowering properties of vitamin D have been reported. Several excellent reviews have covered this topic (308, 309). In brief, vitamin D insufficiency may very well play a role as a contributing factor in the etiology of *diabetes mellitus,* as vitamin D supplementation in childhood could decrease the incidence of type 1 diabetes (T1D) (310). Moreover, vitamin D may have a potential to improve the clinical course of both T1D and type 2 diabetes (T2D), since the s25OHD concentration is positively associated with insulin secretion and peripheral insulin sensitivity in T2D (311). Intervention studies have shown contradictory results, some showing positive effect of vitamin D supplementation on insulin sensitivity when given to vitamin D deficient populations (312). Some studies demonstrated even a positive effect on insulin secretion (313). Simultaneously, there are numerous RCTs studying the effect of vitamin D supplementation on the same outcomes, which delivered negative results (314-316). Also the findings of the largest intervention study studying the effect of vitamin D-plus-calcium supplementation on the risk of developing *diabetes mellitus* in postmenopausal women are disappointing (317). It may well be the case that *diabetes mellitus* is a group of clinical entities with various pathophysiology leading to the same clinical manifestation, and that vitamin D has preventive or therapeutic effect only in a few of these entities. Studying all cases of

T1D or T2D may then dilute the effect that would otherwise be detectable in the specific pathophysiologically defined subgroups.

Low s25OHD levels were described to be associated with increased risk of various forms of cancer (318-320), and there is inverse relation between s25OHD and total cancer incidence and mortality (321). In addition, low s25OHD is associated with worse prognosis in cancer (322). On top of that, there are numerous *in vitro* studies supporting the epidemiological data, which have helped to clarify the anticancer effect of vitamin D on molecular level. As a consequence, vitamin D is being debated as a potential preventive or therapeutic agent in cancer (323). Disturbances in the vitamin D pathway were also associated with cardiovascular and metabolic diseases, as well as with reproduction and neurocognitive disorders (324, 325).

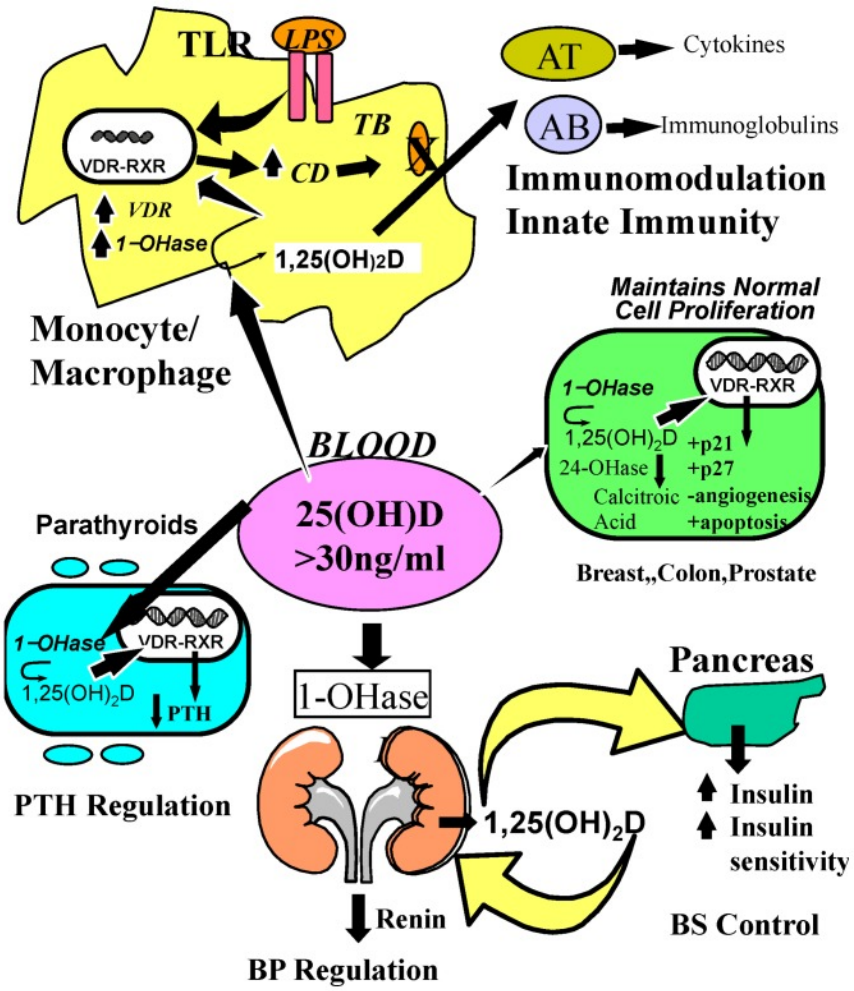

Figure 4. Extramusculoskeletal effects of vitamin D. Adapted from (228). Reproduced with permission from Michael F. Holick.

2.5 VITAMIN D IN CYSTIC FIBROSIS: WHERE ARE WE?

Despite routine vitamin supplementation, vitamin D and/or its metabolites were lower in various CF patient populations than in control non-CF subjects (116, 125-127). Importantly, one third of CF infants detected by newborn screening were reported to have low s25OHD level at the initial evaluation (130). Taken together, vitamin D insufficiency has been a very common observation among CF patients, and is present already very early in their life.

Today, vitamin D insufficiency is recognized as one of the observed risk factors associated with poor bone health in CF (120, 124). This is the reason why the UK CF Trust, the European CF guidelines and the US CF Foundation Consensus Panel consistently recommend routine vitamin D supplementation for CF patients. As a second-hand choice, bisphosphonates are endorsed for patients with very low BMD (T/Z score $\leq$ -2.0), with history of fragility fractures or after lung transplantation, but owing to several potential safety concerns and common adverse events their broader use cannot be recommended (137, 138, 146, 326). Interestingly, while oral and intravenous bisphosphonates have been proven to increase BMD in CF patients (327), no relevant studies have managed to prove that vitamin D supplementation improves BMD in CF (328).

CF is characterized by the vicious circle of infection and inflammation, with impaired ability to clear lung infections, as discussed in detail above. In this context, vitamin D supplementation might be beneficial in CF patients. However, so far there are very few studies available that have addressed this question. For example, it has been demonstrated that human bronchial and tracheal cell lines with mutated CFTR respond to the active form of vitamin D by increasing the expression of cathelicidin mRNA levels (267, 329), which indicates that vitamin D *in vivo* maybe could decrease the infection susceptibility in CF. Moreover, pretreatment of respiratory epithelial cells with active vitamin D prior to challenge with LPS or conditioned medium from cultures with *Pseudomonas aeruginosa* decreased production of the proinflammatory cytokines IL-6 and IL-8 (329). These results suggest that vitamin D may have a beneficial immunomodulatory effect in CF, and so far only one RCT has tested this hypothesis. In a double-blinded, placebo-controlled RCT, Grossmann et al. tested the effect of a single dose of 250000 IU of cholecalciferol on antimicrobial peptide concentrations and markers of inflammation. They randomized 30 adult CF patients hospitalized with a pulmonary exacerbation. Three months after the vitamin D administration they observed a major reduction in TNF-α and a trend towards reduction in IL-6, with no changes in IL-1β, IL-8, IL-10, IL-18BP and neutrophil gelatinase-associated lipocalin (330). The effect seemed to be clinically meaningful, because the unadjusted one-year survival and number of hospital-free days were increased in the group randomized to receive cholecalciferol, as compared with the placebo arm (331). This was further supported by a recent observational study, where higher s25OHD levels in CF children were associated with lower rates of pulmonary exacerbations, and in adolescents, better lung function measured by FEV1 (332). The data produced by these studies are very promising, and more clinical trials are clearly warranted to investigate the potentially

beneficial effects of vitamin D supplementation on antimicrobial defense and inflammation in the CF lung.

In a cohort of CF patients with ABPA, heightened Th2 reactivity correlated with lower mean s25OHD, and *in vitro* addition of $1,25(OH)_2D$ reduced DC expression of the costimulatory molecule OX40L, increased DC expression of the transforming growth factor beta (TGF-β), increased TGF-β expression by Tregs and reduced Th2 responses by CD4+ T cells from CF patients with ABPA (333). These *in vitro* data were later supported by *in vivo* mice experiments, which showed that vitamin D-deficient mice had increased expression of OX40L on lung CD11c+ cells, and OX40L was critical for exaggerated Th2 responses to *Aspergillus fumigatus in vivo* (334). Collectively, these results indicate that vitamin D may have a therapeutical potential in CF patients suffering from ABPA.

Based upon the bulk of data above, vitamin D may promote antibacterial defense in the lung by stimulating the production of antimicrobial peptides. At the same time, it may dampen excessive inflammatory responses and prevent/treat ABPA. In addition, several studies suggest that vitamin D insufficiency may contribute to the high prevalence of depression in CF (335, 336). Theoretically, CF patients could benefit from vitamin D supplementation in other aspects as well. For example, the suggested glucose-lowering or anti-cancer properties of vitamin D could be beneficial in CF population, as high prevalence of *diabetes mellitus* and gastrointestinal cancer is reported in CF. In summary, more studies on the impact of vitamin D insufficiency on various clinical outcomes in CF are needed.

As regards the spectrum of potential extra-skeletal benefits of vitamin D supplementation in CF, too few studies have addressed these questions, and there is definitely no evidence available for CF yet. Therefore, the current vitamin D recommendations were designed with focus on bone mineralization only. However, there are no directly applicable studies of good quality, which would clearly prove the benefit of vitamin D for bone health in CF. Therefore, British recommendations for vitamin D supplementation in CF are ranked as "C" (*UK CF Trust 2007*), and the latest European (146) and US (137) guidelines are mainly consensus based due to lack of evidence and low number of relevant studies available. Thus, vitamin D in CF seems to be a research area with great potential that has been explored very little so far, and any new piece of information could make a big change for the management and wellbeing of CF patients worldwide.

3 AIMS

Vitamin D insufficiency is common in CF patients despite vitamin D supplementation. Vitamin D has recently been shown to have immunomodulatory and glucose-lowering properties, besides its well-known benefits for the bone health. CF patients have chronic inflammation and locally increased infection susceptibility in the lung. Moreover, they suffer from long-term complications such as bone disease and CFRD.

Based on current knowledge, it is reasonable to hypothesize that vitamin D supplementation providing an improved vitamin D status will have a beneficial effect in CF. Evidence supporting this is however lacking and it is still debated what are optimal serum levels of vitamin D in CF.

3.1 OVERALL AIM

The overall aim of this thesis was to explore the effect of vitamin D in CF hoping that the extended knowledge could help to provide relevant information needed for revision of the current recommendations for vitamin D supplementation in CF, and generate new hypotheses that can later be tested in larger intervention studies.

In this thesis, I have tested the hypothesis that low vitamin D status is a contributing risk factor for the exaggerated inflammatory response, the impaired glucose tolerance, the low lung function and the decreased quality of life in CF, by using epidemiological descriptive methods, intervention with vitamin D supplementation and cell culture experiments.

3.2 SPECIFIC AIMS

To determine whether low vitamin D status or intake is associated with increased chronic inflammation and lower lung function in CF, by studying the relationship between s25OHD or vitamin D intake parameters, and serum total IgG or FEV1 in the Scandinavian CF population (**Paper I**).

To evaluate whether low vitamin D status or intake is associated with impaired glucose tolerance in CF, by studying the relationship between s25OHD or vitamin D intake parameters, and HbA1c, OGTT result and CFRD in the Scandinavian CF population (**Paper II**).

To test causality of the associations of vitamin D with chronic inflammation and lung function in CF, in a pilot RCT (**Paper III**).

To determine optimal dosing strategy and optimal s25OHD levels in CF, in a pilot RCT (**Paper III**).

To test the hypothesis that free-s25OHD concentration is a more precise measure of vitamin D status than total s25OHD (tot-s25OHD), as assessed by its correlation with the biological responses to vitamin D supplementation (**Paper III**).

To compare effectiveness of ergocalciferol versus cholecalciferol at increasing s25OHD levels in CF, and investigate other potential differences between them with respect to a wide spectrum of studied outcomes, in a pilot RCT (**Paper III**).

To explore in more detail the immunological effects of vitamin D supplementation or the immunological changes accompanying increasing s25OHD levels in CF, in a pilot RCT (**Paper III**).

To analyse the ability of CF bronchial epithelial cells to activate vitamin D and to investigate how vitamin D influences these cells, by performing *in vitro* experiments with bronchial epithelial CF and non-CF cell lines (**Paper IV**).

4 MATERIALS AND METHODS

All materials and methods applied in this thesis have been described in detail in the respective papers (**Paper I - IV**). This section provides a brief overview of the methodology.

4.1 PATIENTS

The Scandinavian CF Nutritional Study is a cross-sectional study designed to explore the nutritional status of the Scandinavian CF patients. CF patients from Sweden, Norway and Denmark (n=898) were enrolled when in clinically stable condition. Data collected in this study were used to analyse the relationship between vitamin D and serum total IgG, lung function and glucose homeostasis (**Paper I** and **II**).

In the pilot RCT in this thesis, 16 Stockholm CF patients were randomized to receive cholecalciferol, ergocalciferol or to serve as controls (**Paper III, Figure 1**). There were no significant differences in the baseline characteristics between the three intention-to-treat arms (**Paper III, Table 1**). One patient in each study arm left the trial shortly after completing the baseline visit; the remaining 13 patients completed the study and were included in the analyses. These per-protocol groups did not differ in their baseline characteristics (**Paper III, Table E1).**

The patient studies were approved by the regional ethics review boards in the respective countries and complied with the Declaration of Helsinki. All randomized patients or their parents gave informed written consent (**Paper I - III**). The pilot RCT was registered at ClinicalTrials.gov before enrolment of the first patient (**Paper III**).

4.2 CELL LINES AND CULTURE

CFBE41o- (CFBE) and 16HBE14o- (HBE) are well-characterized continuous airway epithelial cell lines that have been compared in detail previously (337). The cell lines were a gift from Dr. Dieter C. Gruenert (University of California, San Francisco, CA) and were used in this thesis as *in vitro* model for CF and non-CF airway, respectively (**Paper IV**).

The cells were cultured in MEM medium with 10% fetal bovine serum, 1% glutamine and 1% antibiotics (penicillin/streptomycin) and were maintained at 37° C in a humidified incubator in the presence of 5 % CO2. They were grown adherent on flasks/plates pre-coated with LHC medium supplemented with 0.03 mg/mL bovine collagen I, 0.1 mg/mL bovine serum albumin and 0.01 mg/mL human fibronectin (**Paper IV**).

4.3 VITAMIN D TREATMENT

In the pilot RCT, 16 CF patients were randomized to receive ergocalciferol (D2 vitamin), cholecalciferol (D3 vitamin) or to serve as controls. Patients < 16 years of age received a starting dose of 35000 IU ergocalciferol or cholecalciferol per week, and those ≥ 16 years old received a starting dose of 50000 IU ergocalciferol or cholecalciferol per week. The weekly dose was given as seven once-daily doses. Three months of supplementation were followed by two months of washout. One of the inclusion criteria was s25OHD < 75 nmol/L at the most recent visit, and the initial vitamin D dose was further individually adjusted, aiming to reach s25OHD of 100 – 125 nmol/L at the end of intervention. The trial was open-labelled and all control patients as well as all patients allocated to the vitamin D arms continued their regular vitamin supplementation (**Paper III**).

In the *in vitro* experiments, HBE and CFBE cells were cultured for 22 or 48 hours prior to addition of $25OHD_3$. $25OHD_3$ was dissolved in ethanol and added at concentration of 100 nmol/L in the media. Adding media alone or adding ethanol served as controls. After 24 hours stimulation with $25OHD_3$, the media was harvested (**Paper IV**).

4.4 VITAMIN D INTAKE CALCULATION

In the cross-sectional studies, food vitamin D intake was assessed using a seven-day food record. Three nationally designed and pre-coded forms were used, and patients and/or parents were instructed by a CF dietician how to complete them. The records were coded and analysed for energy and nutrients including vitamin D. Supplemented vitamin D intake was calculated from the data on the mean daily intake of vitamin and mineral supplements, taking into account forgotten or skipped doses. Total daily vitamin D intake was calculated as the sum of food and supplemented daily vitamin D intake, and further adjusted per kg bodyweight (BW) (**Paper I** and **II**).

In the pilot RCT, the patients were instructed to ingest the vitamin D together with food and pancreatic enzymes. Patients allocated to the intervention arms filled in an adherence questionnaire at each study visit throughout the intervention period. The total ingested vitamin D dose was then counted combining the information on the ordered dose with the patient reported adherence (**Paper III**).

4.5 SEASON AND SUN EXPOSURE ASSESSMENT

Fluctuation of s25OHD levels was previously described in healthy Scandinavian population (338). Therefore, season was a potential confounder to consider in the patient studies included in this thesis. We defined the summer season as May–October and the winter season as November–April (**Paper I**). Dark part of the year was defined as 23rd September to 19th March and light part of the year as 20th March to 22nd September (**Paper I and II**).

In the pilot RCT, the sum of hours spent in the sun was divided by the total number of hours between the visit and the previous visit, and multiplied by 1000. This was further multiplied by a coefficient based on the reported skin reaction (1 if no skin reaction, 5 if lightly sunburnt, 10 if considerably sunburnt). "All day in the sun" was assessed as 6 hours, "in a sunny location, considerably in the sun but not all the time" was assessed as 4 hours and one solarium visit as 0.5 hours (**Paper III**).

4.6 CIRCULATING VITAMIN D MEASUREMENT

In the cross-sectional studies, tot-s25OHD (the sum of tot-s25OHD$_2$ and tot-s25OHD$_3$) was analysed at inclusion at the Research Laboratory, Department of Paediatrics, Haukeland University Hospital in Bergen. A modified version of high performance liquid chromatography with a UV detection method, described by Aksnes, was used (339) (**Paper I** and **II**).

In the pilot RCT, tot-s25OHD was analyzed by DiaSorin immunochemical method, which is the standard method at the Karolinska University Hospital Huddinge clinical chemistry laboratory. Free-s25OHD was calculated from tot-s25OHD, albumin and vitamin D binding protein (DBP) as previously described (340, 341). 1,25(OH)$_2$D (the circulating "active vitamin D") was immunoextracted from plasma and quantified by plate-bound enzyme-linked immunosorbent assay (ELISA) (**Paper III**). Similarly, 1,25(OH)$_2$D was measured by ELISA following immunoextraction from the harvested cell culture media in **Paper IV**.

4.7 VITAMIN D STATUS CLASSIFICATION

In this thesis, vitamin D status was primarily assessed by measuring the circulating tot-s25OHD levels, as it is currently internationally agreed. In line with that, in **Paper II**, four degrees of vitamin D insufficiency were defined as follows: tot-s25OHD $\geq$ 75 nmol/L (degree 0); 50 nmol/L $\leq$ tot-s25OHD < 75 nmol/L (degree 1); 30 nmol/L $\leq$ tot-s25OHD < 50 nmol/L (degree 2); tot-s25OHD < 30 nmol/L (degree 3). In addition, all patients were classified either as '0' (being above) or as '1' (being below) with respect to the following cut-offs for tot-s25OHD: 30, 50, and 75 nmol/L.

Of note, s25OHD binds to DBP (with high affinity) and to albumin (with lower affinity), which affects levels of the fraction of s25OHD that is available to be converted to the active vitamin D locally by various cells expressing the enzyme 1-α hydroxylase (341, 342). Indeed, free-s25OHD was superior compared with tot-s25OHD when analyzing the effect of vitamin D on plasma lipids (343). Therefore, study outcomes in **Paper III** were analyzed with respect to free-s25OHD levels as well as to tot-s25OHD concentrations. In this trial, all patients allocated to the intervention arms increased their tot-s25OHD concentration, and reached peak tot-s25OHD at 8 or 12 weeks of intervention. To explore the effect of various tot-s25OHD levels, we grouped the patients primarily according to their peak tot-s25OHD as follows: Peak 1 group 58 - 82 nmol/L; Peak 2 group 92 – 97 nmol/L; Peak 3 group 111 - 129 nmol/L.

In a similar manner, patients were grouped according to the highest free-s25OHD at 8 or 12 weeks into Peak 1, Peak 2 and Peak 3 groups and according to tot-s25OHD below or above 90 nmol/L.

4.8 GLUCOSE HOMEOSTASIS ASSESSMENT

A deviant oral glucose-tolerance test (OGTT) curve in CF patients is considered to be a precursor of CFRD. Therefore, in **Paper II**, a 75 g OGTT was performed at inclusion to assess glucose tolerance. It was regarded as pathological if 2-hour plasma glucose was ≥ 7.8 mmol/L (included both impaired glucose tolerance and *diabetes mellitus*).

In **Paper II**, diagnosis of CFRD was made at or before inclusion into the study, when one of the following criteria was met: fasting plasma glucose ≥ 7.0 mmol/L at two occasions or 2 hour plasma glucose during OGTT was ≥ 11.1 mmol/L. In patients < 9 years of age, OGTT was carried out only in cases where anamnesis raised suspicion of CFRD. Another criterion for the diagnosis of CFRD was HbA1c > 6.5% (> 47.5 mmol/mol) at inclusion.

HbA1c mirrors the long-term glycaemic exposure in patients with or without *diabetes mellitus*. In **Paper II** it was used as a proxy for glucose homeostasis. It was measured as per cent of total haemoglobin by ion exchange chromatography with high-performance liquid chromatography at the respective hospital laboratories. The only centre using a different method was Oslo, where HbA1c was measured at the Department for Medical Biochemistry, Oslo University Hospital, by an immunological method (Tina-quant, Roche Diagnostics, Basel, Switzerland).

Patients participating in the pilot RCT have done a 3-hour 75 g OGTT. So far, several glucose homeostasis markers based both on measurements done in fasting state and during the OGTT were preliminarily analysed (**Preliminary data**). Approximate blood glucose area under curve (AUC) was calculated as (G_0+G_15+G_30+G_45) x 15 + (G_60+G_90) x 30 + (G_120+G_180) x 60. Insulin sensitivity index (ISI) measured by Stumvoll was calculated as follows: 0.222-0.00333 x BMI - 0.0000779 x ins_120 - 0.000422 x age. Beta cell function was quantified approximately as HOMA-%B = 20 x (ins_0/6.945) / (G_0-3.5).

Abbreviations used in the formulas:

G_0	fasting blood glucose
G_yz	blood glucose at yz minutes of OGTT
ins_0	fasting blood insulin
ins_yz	insulin at yz minutes of OGTT
BMI	body mass index
HOMA-%B	the homeostatic model assessment used to quantify beta cell function

4.9 ANALYSES OF SOLUBLE INFLAMMATORY MARKERS

4.9.1 Enzyme-linked immunosorbent assay

In **Paper I**, high sensitivity ELISA was performed to measure IL-10 in the sera from 124 randomly chosen patients followed at Stockholm CF Center. At each visit during the pilot RCT (**Paper III**), plasma was collected by two subsequent centrifugations and cryopreserved at -80 C until ELISAs were used to measure levels of TGF-β, immunoglobulin E (IgE), LL-37, soluble CD14 (sCD14), DBP, soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) and lipopolysaccharide (LPS).

4.9.2 Luminex assay

IL-10, IL-17A, monocyte chemoattractant protein-1 (MCP-1) and interferon gamma-induced protein-10 (IP-10) were measured by Luminex xMAP technology, using fluorescent-coded beads (**Paper III**).

4.9.3 Standard methods at the Karolinska University Hospital Huddinge clinical laboratories

Standard methods at the Karolinska University Hospital Huddinge clinical laboratories were utilized to measure serum calcium, albumin, PTH, C-reactive protein (CRP), sedimentation rate (SR), antitrypsin, orosomucoid, haptoglobin, IgG, IgM, IgA, IL-1β, IL-8, IL-6, TNF-α, leucocytes, neutrophils, monocytes, basophils, eosinophils and lymphocytes in **Paper III**.

4.10 FLOW CYTOMETRY

Flow cytometric analyses were done in **Paper III**, in order to phenotype peripheral blood mononuclear cells (PBMCs) and evaluate various PBMC subpopulations quantitatively and qualitatively. For this purpose, liquid nitrogen-cryopreserved PBMC specimens were thawed and washed, and counts and viability were assessed. Cells were washed, stained with monoclonal antibodies and fixed before flow cytometry data acquisition.

For staining of FoxP3, samples were first stained for extracellular markers as described above, and then washed, fixed, permeabilized and stained with anti-FoxP3 APC.

BD LSR II instrument was used to aquire data. Data was analysed using FlowJo version 9.6 by both TP and DPP independently, and concordant findings only were reported.

4.11 LUNG FUNCTION TEST

To assess lung function, dynamic spirometry volumes in liters were measured (Forced Vital Capacity, FVC; Forced Expiratory Volume in the first second, FEV1; FEV1 expressed as percentage FVC, FEV%; Peak Expiratory Flow, PEF; Forced Expiratory Flow rates, FEF25%, FEF50%, FEF75%). Percentage of predicted (% predicted) values were counted using the Solymar and Quanjer reference equations for patients < 19 years and ≥ 19 years of age, respectively (344, 345).

4.12 QUALITY OF LIFE QUANTIFICATION

The need for clinically meaningful health-related quality of life measures as endpoints in clinical trials has been highlighted by The US Food and Drug Administration consensus report on patient-reported outcomes (346). Therefore, in **Paper III**, Cystic Fibrosis Questionnaire Revised (CFQ-R) was used to measure patient-reported and parent-reported quality of life (QoL and QLP, respectively).

CFQ-R consistently discriminates among stages of disease severity based on lung function (347), is well validated (348, 349) and has been used and described previously (350, 351). It uses a 4-point Likert scale and takes approximately 20 minutes to complete. The CFQ-R raw scores are converted into standardized scores (0–100) for each domain, with higher scores indicating better quality of life. Minimal clinically important difference score for the Respiratory domain is 5.0 points (352).

In **Paper III** we used two self-report versions of the CFQ-R: the CFQ-R Child Version for 6 to 13 years old children and the CFQ-R Teen/Adult Version for patients 14 years of age and older. Moreover, one parent-report version was used: CFQ-R Parent Version as a measure of the parent's report of their child's quality of life for children aged 6 to 13.

4.13 CONFOUNDING FACTORS

A widely accepted surrogate outcome measure in CF is the lung function parameter FEV1 (353, 354). It is correlated inversely with serum total IgG, which is a marker of the chronic inflammation in CF and negative long-term prognosis indicator (14, 355). **Paper I** aimed to determine whether vitamin D parameters could partially explain the variation in serum total IgG and/or FEV1 in Scandinavian CF patients. In order to get an indication of whether s25OHD determines these outcome variables in an independent manner, correction for other known IgG/FEV1 determinants in CF had to be made. This was done by building a multiple linear regression (MLR) model with IgG/FEV1 as dependent variables. FEV1 decreases and IgG increases with factors contributing to lung morbidity and overall mortality in CF. Therefore, age, gender, pancreatic insufficient versus sufficient phenotype, infection/colonization status, genotype, additional diagnosis of CFRD, z-BMI, oral steroid and chronic macrolide use were put into the model as independent variables together with vitamin D variables.

In **Paper II** we aimed to investigate whether vitamin D parameters are independent determinants of the glucose homeostasis approximated by measuring HbA1c and evaluating CFRD. To do this, the MLR model corrected for the 'country' variable (to account for the potential differences between the participating countries), as well as for factors that were previously shown to be independently associated with increased risk of impaired glucose homeostasis and additional diagnosis of *diabetes mellitus* in CF. These were genotype, age, female gender, inferior pulmonary function, liver dysfunction, pancreatic insufficiency, and corticosteroid use (160).

4.14 STATISTICAL ANALYSES

All statistical analyses in this thesis were performed using STATISTICA (Versions 7 and 10), SPSS (Versions 17 and 21) or Prism (Version 6). Where normal distribution of values was needed, values were log-transformed (base e) if they were not normally distributed. For comparison of means between groups at the same time point, independent t-test, Mann-Whitney U Test or ANOVA were used. To compare proportions, a Chi square test was applied. For comparisons within groups between two different time points, paired t-test was used. For correlation analyses, the Pearson or Spearman Test was used (**Paper I – IV**).

In the cross-sectional studies, univariate linear regression analyses were used to explore the relation between vitamin D status/intake variables on one hand, and serum total IgG/lung function/glucose homeostasis parameters on the other hand. Subsequently, MLR and multiple logistic regression analyses were performed for serum total IgG/lung function/glucose homeostasis parameters as dependent variables and vitamin D parameters together with potential confounders as independent variables (**Paper I** and **II**).

Data is presented as the mean/beta/OR ± 95 % confidence interval (CI), or as mean ± standard deviation (SD). Where not normally distributed, descriptive data is shown as median (quartiles) (**Paper I – IV**).

All tests were two-sided and $p < 0.05$ was considered as significant (**Paper I – IV**).

In the RCT, clinically-relevant changes together with $p < 0.20$ were considered as tendency towards significance and may be used to calculate power for designing a larger study (**Paper III**).

5 RESULTS AND DISCUSSION

5.1 VITAMIN D SUPPLEMENTATION AND S25OHD LEVELS IN CF

Vitamin D in CF received very little attention in the past. CF patients used to be given multi-vitamin supplements only, s25OHD level was not monitored and vitamin D insufficiency was common among CF patients worldwide (119-124). Longitudinal data on vitamin D concentrations suggest a trend towards higher s25OHD levels over recent years, which may well reflect the somewhat improved attention to vitamin D by CF centers (129). In line with that, we found that the frequency of s25OHD measurements in patients followed at the Stockholm CF Centre increased steadily during years 2007-2009, indicating that the attention to vitamin D in the field of CF has been continuously improving (**Abstract I**).

Despite that vitamin D has been receiving more attention and supplementation guidelines have recently been revised (137), the majority of CF patients in various countries have continued to have suboptimal s25OHD levels (117, 128, 356, 357). We have analyzed s25OHD concentrations in 787 Scandinavian CF patients in the Scandinavian CF Nutritional Study, and confirmed that 84% had s25OHD level < 75 nmol/L (**Paper I, Table 1**), which is currently referred to as suboptimal.

The estimated median total daily vitamin D intake in the 389 Scandinavian CF patients who filled in food records was 17 mg, i.e. 680 IU (**Paper I, Table 1**). This is a very low intake, compared with the current European and US vitamin D supplementation recommendations in CF (137, 146). According to the latest European guidelines, ergocalciferol or cholecalciferol should be administered in the starting daily dose of 1000-2000 IU and 1000-5000 IU in patients < and > 1 year of age, respectively (146). The US CF Foundation has made a consensus recommendation that all individuals with CF < 1 year of age should be treated with an initial daily dose of 400–500 IU cholecalciferol, which may be increased to 800-1000 IU or 2000 IU under certain conditions. Patients > 1 year and < 10 years of age, should get an initial daily dose of 800-1000 IU cholecalciferol, that may be increased to 1600 – 3000 IU or 4000 IU in certain cases. CF patients that are older than 10 years should receive 800 – 2000 IU cholecalciferol per day as initial dose, which can be increased to 1600 – 6000, maximum 10000 IU per day (137).

5.1.1 Very high vitamin D doses are needed to increase s25OHD in CF

The results in **Paper I** clearly demonstrated that most of the Scandinavian CF patients, including the patients followed at the Stockholm CF Center, had suboptimal s25OHD levels and had too low total vitamin D daily intake. Notably, the above-mentioned guidelines, including the vitamin D dosing they recommend, are consensus based only, due to lack of evidence in the field of vitamin D supplementation in CF (328). Indeed, almost all vitamin D supplementation strategies tested in various CF populations have been unsuccessful at increasing s25OHD levels, indicating that establishing an efficient vitamin D supplementation regimen in CF might not be easy (121, 356, 358).

Positive news were brought by a retrospective cohort observational study from Canada where long-term cholecalciferol supplementation increased s25OHD levels in adult CF patients (128), followed by a RCT where 50000 IU cholecalciferol or ergocalciferol weekly for 3 months significantly raised s25OHD in CF (357). However, at Stockholm CF Center we prefer once-daily dosing and have other vitamin D preparations available on the Swedish market. Moreover, our CF population might differ from the CF patients enrolled in Atlanta by Khazai *et al.* in several aspects. Therefore we have designed the pilot RCT (**Paper III**), where one of the goals was to establish an effective vitamin D supplementation strategy, which would primarily be adopted for Stockholm CF patient population. Hopefully, the strategy could then secondarily be extrapolated to other, similar populations. In this study, 16 CF patients were randomized to receive a starting dose of 5000 IU or 7143 IU/day (< or ≥ 16 years of age) ergocalciferol or cholecalciferol or to serve as controls. Three months of supplementation were followed by two of washout. The vitamin D dosing was further individually adjusted throughout the supplementation period in order to reach s25OHD of 100 – 125 nmol/L.

In this trial, all enrolled patients had tot-s25OHD < 75 nmol/L measured at the most recent visit before the baseline study visit. At baseline, the three study groups did not differ in either form of s25OHD (tot- or free-). The control group did not change their tot-s25OHD throughout the study, whereas patients allocated to receive cholecalciferol had higher tot-s25OHD than the controls at the end of supplementation (p=0.019), and patients receiving ergocalciferol had a tendency for increase in tot-s25OHD as compared with control patients (p=0.106) (**Paper III, Figure 2A-C**). In order to achieve this, the patients allocated to the ergocalciferol and cholecalciferol arms had ingested a total mean (SD) dose of 771173 (296870) and 598066 (132126) IU, respectively. In other words, to increase tot-s25OHD, the mean daily dose of ergocalciferol and cholecalciferol had to be increased up to 15650 and 8184 IU, respectively, and still, only two of nine patients reached the goal s25OHD concentration of 100 nmol/L.

Thus, we have shown that a very high vitamin D dose was needed to increase tot-s25OHD in Stockholm CF Center patients. Current European guidelines for vitamin D supplementation in CF recommend starting daily dose of 1000-5000 IU in patients > 1 year of age with further individual adjustment (146). Based on the pilot RCT in this thesis (**Paper III**), we suggest that the initial daily vitamin D dose for CF patients ≥ 6 years living in Scandinavia should be higher than the currently endorsed 5000 IU.

As expected, tot-s25OHD correlated closely with total vitamin D dose at the end of the supplementation. This observation indicates that oral vitamin D supplementation given on daily basis has the potential to modulate s25OHD levels in CF and that the response is dose-dependent (**Paper III, Supplementary Figure 2E**).

5.1.2 Cholecalciferol is more effective than ergocalciferol at increasing s25OHD in CF, but induces transient increase in DBP and calcium

Ergocalciferol absorption has been demonstrated to be significantly lower in CF patients than in healthy control subjects (132) and ergocalciferol has been shown to have a very low efficiency at increasing the circulating s25OHD levels in CF (356). Cholecalciferol supplementation, on the other hand, has been suggested as successful in this respect (128). Later on, ergocalciferol and cholecalciferol supplementation were directly compared in a well-designed RCT, which confirmed the lower efficacy of ergocalciferol (357). In this study, 100% of adult CF patients who ingested 50000 IU of vitamin D3 weekly for 3 months reached s25OHD levels > 75 nmol/L, and only 60% of those receiving ergocalciferol in the same dose reached s25OHD > 75 nmol/L (357). The pilot RCT included in this thesis (**Paper III**) confirms these findings in Stockholm CF patients. Ergocalciferol was partially effective, as all the patients in both vitamin D arms had tot-s25OHD > 75 nmol/L at the end of the supplementation period, but none of the patients allocated to the ergocalciferol study arm reached the goal of 100 nmol/L, whereas 2/5 (40%) of the cholecalciferol patients reached this goal (**Paper III, Figure 2A-C**).

It has been reported that the effectiveness of ergocalciferol supplementation in CF is transient (359). In the pilot RCT in this thesis, patients receiving any form of vitamin D decreased free-s25OHD during the washout period (at 16- and 20-weeks visits), when they had significantly lower free-s25OHD levels than at baseline (**Paper III, Figure 2D-F**). This suggests that consistent adherence with vitamin D supplements is important in order to ensure optimal s25OHD concentrations in a stable, long-term manner.

Patients receiving cholecalciferol initially increased vitamin D-binding protein (DBP) (**Paper III, Figure 2G**), which later negatively affected free-s25OHD concentration. On the other hand, DBP levels in CF have been reported to be low (131), and debated as one of the reasons for vitamin D insufficiency in CF. This would suggest that the observed DBP increase upon cholecalciferol supplementation might be beneficial. However, conclusions about this cannot be drawn from **Paper III** in this thesis, and further research is needed to answer this question.

Hypercalcaemia and its consequences have become a safety concern of vitamin D supplementation (360, 361) and long-term effects of high-dose vitamin D supplementation in CF have not been investigated (328). In the pilot RCT included in this thesis, albumin-corrected calcium increased initially in the cholecalciferol group at 1-week visit and the increase reappeared at the last study visit, compared with baseline (**Paper III, Figure 2H**). At the last study visit, the change in albumin-corrected calcium from baseline was positively correlated with total vitamin D dose ingested (**Paper III, Figure 2I**). Secondary analyses showed that only patients reaching the highest concentration of free-s25OHD increased calcium (**Paper III, data not shown**). However, none of the patients reported any symptoms of vitamin D toxicity throughout the study and all the measured calcium concentrations were within normal reference range. Taken together, the 3-month supplementation with individualized high vitamin D dosing based on s25OHD monitoring was safe *per se* but led to calcium increase in

dose-dependent manner; thus, long-term consequences of increased circulating calcium concentrations induced by long-term high-dose vitamin D supplementation in CF remain to be investigated.

5.1.3 Sun exposure is an important determinant of s25OHD levels in Stockholm CF patients

Tot-s25OHD values in CF were shown to exhibit a clear season- and sunlight-dependent pattern (126, 135, 136). Tot-s25OHD concentrations were significantly higher during 'Months with high UVB exposure' (May-October) than during 'Months with low UVB exposure' (November-April) (136). This confirmed the results of our retrospective study of Stockholm CF patients where the mean tot-s25OHD during May-October (n=150; 60.2 nmol/L) was higher than the mean tot-s25OHD during November-April (n=157; 44.3 nmol/L, $p<0.001$) (**Abstract I**). In line with this, the current CF Foundation vitamin D supplementation recommendations endorse the yearly screening for vitamin D status to be done preferably at the end of winter (137). On the other hand, tot-s25OHD levels in the large cohort of Scandinavian CF patients were not determined by latitude zone (zone 1: 54°–59°; zone 2: 60°–65°; zone 3: 66°–71° north), dark (23rd September to 19th March) or light (20th March to 22nd September) part of the year, or by belonging to a specific CF center (**Paper I and II**). We defined the summer season as May–October and the winter season as November–April as it was previously described in relation to fluctuating s25OHD levels in healthy Scandinavian population (338). The summer season did not show to be a significant determinant of tot-s25OHD levels in the MLR model in this patient dataset (**Paper I**). On the other hand, in the Stockholm CF patients enrolled into the pilot vitamin D supplementation RCT, tot-s25OHD concentrations correlated with sun exposure throughout the study (**Paper III, Supplementary Figure 2D**). Collectively, these data indicate that season determines s25OHD levels specifically in Stockholm CF patients, rather than in the whole Scandinavian CF population. One could speculate that Stockholm CF patients may have relatively low vitamin D intake compared with Norway (where cod liver oil- and fish-eating traditions are strong) and more pronounced sun- and water-seeking behaviour than Danish CF patients. This, together with more even sunshine distribution throughout the year in Denmark could maybe explain the discrepancy.

5.2 CIRCULATING ACTIVE VITAMIN D LEVELS DO NOT INCREASE UPON VITAMIN D SUPPLEMENTATION IN CF

In the vitamin D supplementation RCT, neither patients in the intervention groups, nor controls changed their circulating $1,25(OH)_2D$ at any of the time points (**Paper III**). Free-s25OHD, tot-s25OHD and the active vitamin D form were positively and tightly inter-correlated at 1-week visit. However, these correlations weakened gradually with time and at the end of supplementation, circulating active vitamin D was negatively correlated with total vitamin D dose per kg BW (**Paper III, Supplementary Figure 2F**). This is not surprising and could simply be interpreted as a confirmation of the well-known negative feedback regulation of systemic active vitamin D levels in CF.

However, results of the *in vitro* cell culture experiments included in this thesis (**Paper IV**) would suggest something else as a possible explanation. In these experiments, we aimed to assess the ability of CFBE cells to convert inactive $25OHD_3$ to the active $1,25(OH)_2D$ by growing them for 22 or 48 hours, and then adding $25OHD_3$ to the media. The tot-s25OHD concentration in the media was the same as the target tot-s25OHD concentration in the pilot RCT, i.e.100 nmol/L. After one day of incubation, media was harvested and $1,25(OH)_2D$ concentration was measured. The same was done with the HBE cells as control non-CF bronchial epithelial cells. Adding $25OHD_3$ increased the concentration of $1,25(OH)_2D$ in the media from HBE cells. Specifically, there was significant increase in $1,25(OH)_2D$ concentration where 350000 or 500000 HBE cells were seeded (**Paper IV, Figure 1B-C**). Similarly, the $1,25(OH)_2D$ concentration in the media with 500000 CFBE cells seeded increased upon $25OHD_3$ addition (**Paper IV, Figure 3A**). However, the amplitude of the increase in $1,25(OH)_2D$ (mean ± SD) was only 12.0 ± 8.0 pmol/L, whereas the increase in HBE cells was by 33.2 ± 12.4 pmol/L. Translated into the language of statistics, increase in $1,25(OH)_2D$ in wells with HBE cells was higher than increase in wells with CFBE cells ($p < 0.01$; **Paper IV, Figure 3B**). Ethanol, which $25OHD_3$ was solved in, did not influence the $1,25(OH)_2D$ concentration (**Paper IV, Figure 1C, Figure 3A**). These results can only be extrapolated to bronchial epithelial cells, and indicate that these cells may have impaired ability to activate vitamin D. However, if one could go further and speculate that this defect is universal for all cells carrying CF phenotype, it could mean that the ability of kidney cells to activate vitamin D would be decreased in CF as well, which in turn would explain why the patients in the pilot RCT did not increase circulating active vitamin D concentration upon high-dose vitamin D supplementation.

5.3 VITAMIN D SUPPLEMENTATION DOES NOT AFFECT PTH IN CF

PTH correlates with s25OHD (362) and some reports indicate that it responds to vitamin D administration in CF (357, 363). Tot-s25OHD ≥ 87.5 nmol/L in CF has recently been suggested to decrease the risk of having a PTH level associated with secondary hyperparathyroidism (362). In the pilot RCT in this thesis, PTH tended to decrease at 8 weeks of the intervention in patients reaching Peak 2 tot-s25OHD concentration as compared with those reaching the lowest tot-s25OHD peak ($p=0.17$; **Online data supplement for Paper III, data not shown**). There were neither any changes in PTH in the patients reaching the highest peak tot-s25OHD levels, nor any differences between the randomized groups in PTH throughout the study.

These findings should be highlighted in the context of the current debates on vitamin D supplementation and the recommended s25OHD levels in CF. The group designing the European guidelines for vitamin D supplementation in CF have expressed the fear that too low PTH levels induced by aggressive vitamin D supplementation could block bone formation (146), referring to a study suggesting that PTH in the high normal range may promote bone formation in children (364). However, the results of the vitamin D supplementation RCT in this thesis (**Paper III**) do not support the cautious attitude of the working group who created the European guidelines, as there was only tendency for decreased PTH values in patients who moderately increased s25OHD, and no change in PTH in patients reaching the highest s25OHD concentrations; on the other hand, this RCT was very small-scale and not powered for detecting changes in PTH. Thus, no firm conclusions can be made.

5.4 VITAMIN D INDUCES A COMPLEX SPECTRUM OF IMMUNO-MODULATORY EFFECTS IN CF

5.4.1 Vitamin D has effects on the innate immunity in CF

Triggering receptor expressed on myeloid cells (TREM) family seem to fine-tune the innate immune response (72). TREM-1 is a receptor induced by LPS on monocytes and neutrophils and its activation results in secretion of proinflammatory cytokines (71). Soluble TREM-1 (sTREM-1) in plasma was suggested to originate from shedding of neutrophil TREM-1 (365). In CF, both the expression of TREM-1 on monocytes and the plasma sTREM-1 concentration are low. Moreover, when stimulated *ex vivo* with LPS, CF monocytes upregulated neither the membrane-anchored TREM-1 nor the soluble form sTREM-1, which was described as they are locked in an endotoxin tolerance state (74). Indeed, plasma LPS levels are high enough to induce an endotoxin tolerance state in non-CF monocytes *in vitro* (73). The active $1,25(OH)_2D3$ induced TREM-1 on airway epithelial cells and monocytes/macrophages (271, 272), which may be one of the mechanisms how vitamin D boosts the innate immune response. In the vitamin D supplementation RCT in this thesis, patients randomized to receive ergocalciferol decreased plasma LPS at 8-weeks of supplementation (**Paper III, data not shown**) and patients reaching higher active vitamin D peak tended to have lowered levels of plasma LPS at the end of supplementation (**Paper III, Supplementary Figure 2A**). Accordingly, the change from baseline in tot-s25OHD was negatively correlated with change in LPS at the end of supplementation (**Paper III, Figure 4E**). In line with that, changes in free-s25OHD were positively correlated with changes in sTREM-1 at the end of supplementation (**Paper III, Figure 3G**). In that way, vitamin D enhanced the impaired innate immune response, which in turn seemed to have favourable clinical impact, because changes in sTREM-1 were positively correlated with changes in the lung function parameters PEF and FEV1 (**Paper III, Figure 3H**). Finally, change in LPS was negatively correlated with change in sTREM-1 at the end of supplementation (**Paper III, Figure 3I**), which indicates that the changes in LPS and sTREM-1 are interconnected.

Another mechanism how vitamin D induces the innate immunity *in vitro* is via direct upregulation of the cathelicidin-related antimicrobial peptide LL-37. LL-37 is important for lung or gut mucosal innate immunity against bacteria (366, 367). Vitamin D induces LL-37 expression in airway epithelial cells (269) and s25OHD has been correlated with LL-37 (368). In the pilot RCT in this thesis, the LL-37 plasma concentration did neither change upon vitamin D supplementation, nor was associated with changes in s25OHD levels.

CD14 is a TLR co-receptor, involved in innate immunity (369). It is indirectly induced by vitamin D (370). Soluble CD14 (sCD14) levels increase concomitantly with the expression of membrane-bound CD14 (369). In the pilot RCT, plasma sCD14 increased in patients reaching peak 2 tot-s25OHD at the end of supplementation (**Paper III, Supplementary Figure 4A**). At the end of supplementation, sCD14 decreased from baseline in patients reaching peak 3 tot-s25OHD compared with patients reaching peak 2 tot-s25OHD (**Paper III, Supplementary Figure 4B**). This could be interpreted

as bell-shaped association between tot-s25OHD concentration and sCD14, and would suggest that too high tot-s25OHD concentrations are not optimal.

In contrast with sTREM-1 and sCD14, which were enhanced in the pilot RCT, vitamin D negatively influenced acute phase reactants and proinflammatory cytokines. Already at baseline, tot-s25OHD was negatively correlated with CRP, sedimentation rate (SR), antitrypsin, orosomucoid, haptoglobin, IL-1β and IL-6 (**Paper III, Supplementary Figure 3 A-D**). Upon cholecalciferol supplementation and in patients reaching the highest tot-s25OHD peak, CRP tended to decrease during the supplementation period and returned to baseline during the washout period (**Online data supplement for Paper III, data not shown)**. At the end of supplementation, change in CRP from baseline was inversely correlated with change in free-s25OHD from baseline (**Paper III, Supplementary Figure 3E**). In line with that, vitamin D supplementation in patients with active tuberculosis augmented decrease in CRP induced by intensive antituberculous therapy (287). However, in CF, a single descriptive study performed found CRP to be inversely associated with vitamin A, but not with vitamin D (371). Hence, this is the first report in the CF field that vitamin D supplementation might lower CRP in CF, but our study was clearly underpowered to detect significant changes in CRP. In addition, we did not measure high sensitivity-CRP and many measured CRP values were below the detection limit < 0.1 mg/L, which may also help to explain why the observed changes did not reach statistical significance.

SR and acute phase reactants (antitrypsin, orosomucoid, haptoglobin) shared similar pattern of changes over time. At the end of supplementation and at 1 month of washout, change in SR was inversely correlated with change in tot-s25OHD from baseline (**Paper III, Supplementary Figure 3F**). In patients reaching the highest tot-s25OHD peak, the haptoglobin concentration was significantly decreased at the 8-week visit (**Paper III, Supplementary Figure 3G**). In patients receiving either form of vitamin D, the concentration of free-s25OHD dropped below the baseline level at 1 month of washout, and simultaneously their orosomucoid concentration increased significantly (**Paper III, Supplementary Figure 3H**). Importantly, these changes may have favourably affected lung function; for example, change in antitrypsin was negatively correlated with change in FEV1 at 1 month of washout (**Paper III, Supplementary Figure 3I**). In other words, dampening the acute phase reactants may be associated with improvement in lung function and *vice versa*.

CF patients who received a bolus dose of 250000 IU cholecalciferol had reductions in TNF-α and IL-6, whereas there were no significant changes in IL-1β and IL-8 (330). In tuberculosis, adjunctive vitamin D supplementation augmented the decrease in IL-6 and TNF- α induced by intensive-phase anti-tuberculous therapy (287). In the pilot RCT in this thesis, we observed that patients allocated to serve as controls, patients reaching tot-s25OHD < 90 nmol/L and patients reaching the lowest tot-s25OHD peak increased TNF-α at 1-week visit and IL-8 at 4-week visit. Accordingly, patients reaching the lowest free-s25OHD peak increased TNF-α at the 8-week visit, whereas patients reaching peak 2 free-s25OHD decreased TNF-α at the 8-week visit. Patients randomized to the ergocalciferol arm decreased IL-1β at 4-week visit, compared with controls, and patients receiving any form of vitamin D increased IL-1β at 1 month of washout (**Paper III, data not shown**). Finally, change in IL-1β from the baseline was negatively correlated with change in free-s25OHD at the end of the study (**Paper III, Supplementary Figure 3J**). This is in contrast with recent *in vitro* findings where active vitamin D induced IL-1β expression in macrophages (275). One explanation

could be that the *in vivo* situation is more complex that that in the test tube. CF patients suffer from high degree of chronic inflammation and their macrophages are chronically exposed to high plasma LPS concentration in CF. This may have changed their phenotype and behaviour (73). Both the proinflammatory environment and the CF-specific macrophage dysfunction could maybe explain the discrepancy in IL-1β dynamics.

By influencing pulmonary macrophages, the invariant natural killer T cells (iNKT cells) can improve the course of lung inflammation caused by *Pseudomonas aeruginosa* or *Mycobacterium tuberculosis* (83, 84). Recently it has been demonstrated that VDR is required for NKT cell development (372). However, NKT cell numbers could not be corrected by later intervention with vitamin D in mice (373). In the pilot RCT in this thesis, patients receiving vitamin D did not increase iNKT cell frequency, nor were changes in s25OHD associated with changes in iNKT cell frequency (**Unpublished data**). Thus, it seems that vitamin D supplementation does not increase frequency of iNKT cells in CF patients, which would confirm the findings from mice experiments. Intriguingly, however, iNKT cell frequency positively correlated with almost all lung function parameters at baseline: FVC (r=0.703; p=0.007; **Figure 5A**), FEV1 (r=0.621; p=0.024), PEF (r=0.681; p=0.021; **Figure 5B**), FEF25% (r=0.667; p=0.035), FEF50% (r=0.587; p=0.045) (**Unpublished data**). It remains to be determined, what are the chicken and the egg. NKT cells have been shown to make both protective and pathogenic contributions to inflammatory diseases (374). It appears that they are generally protective during Th1-mediated pathologies and harmful during Th2-mediated diseases (86). Abundances of NKT cells in the airways of asthma patients (375) and in the lungs of patients with COPD were reported (376). The question is whether NKT cells get recruited into the infected CF lung, decreasing their numbers in the circulation with increasing disease severity, or whether greater circulating NKT cell pool is protective and therefore associated with better lung function.

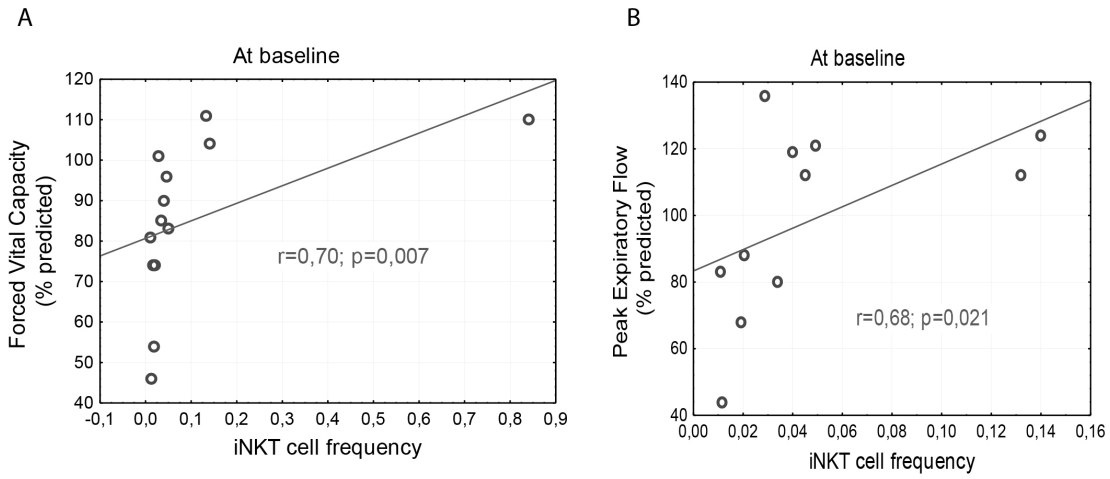

Figure 5. At baseline of the pilot RCT in this thesis (**Paper III**), iNKT cell frequency was positively correlated with lung function parameters, for example FVC (A) or PEF (B).

It would also be worth examining whether iNKT cells could become a future marker of long-term prognosis in CF. Indeed, it would be very useful to find a novel long-term biomarker in CF. Today, there are a spectrum of various blood biomarkers available in

CF for monitoring the current disease activity, that increase during exacerbations and decrease in response to treatment, such as CRP, neutrophil elastase anti-proteinase complex, IL-6, myeloperoxidase, lactoferrin, and calprotectin as reviewed in (377). However, there are only a few markers of long-term prognosis in CF, and they have considerable limitations (378). The lung function parameter FEV1 is the best available widely used surrogate outcome measure in CF, as lung disease is the main determinant of prognosis in CF (353, 354). Hence there have been attempts to define markers that would explain and predict variation in lung function. For example, serum total IgG levels represent a marker of the chronic inflammatory process in CF and correlate inversely with the patients' lung function and long-term prognosis (355, 379). However, there are no successful attempts published where IgG would discriminate between survival and the composite endpoint of death/lung transplantation. Recently a new serological marker has been described as promising predictor of long-term prognosis in CF, and showed superiority over the currently widely used Leeds classification in discriminating between survival and death/lung transplantation (380). The marker is called BPI-ANCA, which stands for anti-neutrophil cytoplasmic antibodies specific for bactericidal/permeability-increasing protein. However, BPI-ANCA does not discriminate perfectly, and neither the serology nor Leeds criteria add additional prognostic information regarding development of end-stage lung disease when the degree of lung function impairment is known. Thus, the search for biomarkers of long-term prognosis continues and iNKT cell frequency might be one of them. The role of iNKT cells for immunity of the lung has been recently highlighted by an outstanding review (86), which may become a starting point for more research in the area.

5.4.2 Vitamin D decreases IL-8 and may influence leucocyte migration to infection site in CF

One of the proinflammatory cytokines elevated in CF lungs is IL-8 (58). It is a major cytokine playing role in the pathophysiology of CF, as it is inversely correlated with oxygen saturation (381) and IL-8 gene variants were shown to modify CF lung disease severity (382). On top of that, IL-8 has been receiving major attention as the major chemokine recruiting neutrophils into the CF lungs (383), and IL-8 sputum concentration correlates with current bacterial lung colonization and degree of lung damage (384). In the pilot RCT, patients randomized to receive any form of vitamin D decreased IL-8 at the end of supplementation, and IL-8 remained decreased at both 1 month and 2 months of washout, as compared with baseline (**Paper III, Figure 3A**). In line with that, patients reaching the highest tot-s25OHD peak decreased IL-8 at the end of supplementation. Moreover, patients who received cholecalciferol, those who reached peak tot-s25OHD > 90 nmol/L and those who reached the highest free-s25OHD peak had decreased IL-8 plasma concentration at 1 month of washout, compared with baseline (**Unpublished data**). These results are in contrast with a study published by Grossmann et al., where U.S. CF patients did not change IL-8 concentration after receiving a bolus dose of 250000 IU cholecalciferol (330). One possible explanation of the discrepancy could be that once-daily supplementation regimen may well have different effects than one bolus dose, and/or that the patients allocated to receive vitamin D in the pilot RCT in this thesis ingested a total vitamin D dose that was several times higher than the 250000 IU.

While IL-8 is a major chemoattractant of neutrophils, MCP-1 is a potent monocyte attractant, implicated in the control of microbial growth and invasion (385). Another powerful chemokine driving Th1 inflammation and recruiting cytotoxic immune CXCR3-positive cells, such as T cells, NK cells, B cells, macrophages, and DCs, is IP-10 (CXCL10). *In vitro* studies show that VDR agonists are able to inhibit its release (386). In our pilot RCT, the patients who reached tot-s25OHD < 90 nmol/L did not change their MCP-1, whereas patients reaching tot-s25OHD > 90 nmol/L and patients reaching the highest free-s25OHD peak increased plasma MCP-1 already at 4 weeks of supplementation, and kept it increased at the end of supplementation and at 1-month of washout (**Paper III, Supplementary Figure 4C**). Similarly, patients reaching peak 2 free-s25OHD increased IP-10 at 4 weeks and 8 weeks of intervention, compared with patients reaching the lowest free-s25OHD peak (**Paper III, Supplementary Figure 4D-E**).

As the major attractant substances recruiting neutrophils and monocytes into the CF lung were affected by vitamin D supplementation and/or changing s25OHD levels, one might hypothesize that neutrophil and/or monocyte counts would be affected by vitamin D as well. As expected, tot-s25OHD was negatively correlated with leucocyte, neutrophil and monocyte counts in patients enrolled into the pilot RCT already at baseline (**Paper III, Supplementary Figure 1A-C**). At the end of supplementation, change from the baseline in free-s25OHD was inversely correlated with changes in neutrophil and monocyte counts (**Paper III, Figure 3B-C**) and at 1 month of washout, change in tot- or free-s25OHD continued to be inversely correlated with change in neutrophil counts (**Paper III, Figure 3D-E**). In summary, these results have created the hypothesis that vitamin D contributes to the regulation of the recruitment of innate immune cells from the circulation to the local infection sites in CF patients. This might theoretically have positive clinical effect, as it would focus the immune response to the infection site where it is most needed.

5.4.3 Vitamin D ameliorates the adaptive immune response in CF

Dendritic cells (DCs) are orchestrators of the immune response and can divert it towards Th1, Th2 or Treg cell response. They are one of the primary targets of the immunomodulatory actions of the active form of vitamin D, as reviewed elsewhere (303). *In vitro* experiments have taught us that the active vitamin D downregulates the expression of MHC-II and costimulatory molecules on DCs (302). This may translate into decreased T cell activation; for example, following stimulation with 25OHD, DCs from Crohn's disease patients displayed a diminished capability to activate T cells when exposed to LPS *in vitro* (387). The pilot RCT included in this thesis confirmed that a similar situation occurs in CF patients upon vitamin D supplementation *in vivo*. Specifically, patients receiving any form of vitamin D reduced their HLA-DR expression on CD8+ T cells at the end of supplementation (**Paper III, Figure 4A**). Moreover, patients receiving cholecalciferol decreased PD-1 expression on CD8+ T cells (**Paper III, Figure 4B**) and the expression of the costimulatory molecule CD40 on DCs at the end of supplementation, which remained decreased until the end of the study (**Paper III, Figure 4C**). Patients reaching total s25OHD > 90 nmol/L at 8- or 12-week visit decreased CD40 expression on DCs (**Paper III, Figure 4D**), PD-1 on CD4+ T cells (**Paper III, Figure 4E**), CD86 expression on DCs (**Paper III, Figure 4F**), and HLA-DR (**Paper III, Figure 4G**) and PD-1 on CD8+ T cells (**Paper III, Figure 4H**). These results show that the original *in vitro* findings published a few years ago can be

extrapolated into the *in vivo* environment of chronic inflammation in CF. We can even go further than that and say that the response is dose-dependent. There are number of findings that support this notion. For example, the total vitamin D dose per kg bodyweight was inversely correlated with change in CD8 T cell expressing HLA-DR frequency (**Paper III, Figure 4I**). At the end of the study, the change in tot-25OHD and total vitamin D ingested dose per kg BW tended to inversely correlate with change in frequency of CD4 T cells expressing HLA-DR (**Figure 6A-B**). Accordingly, the change in free-s25OHD tended to be inversely associated with change in the frequency of CD4 T cells expressing HLA-DR at the end of supplementation (**Figure 6C**).

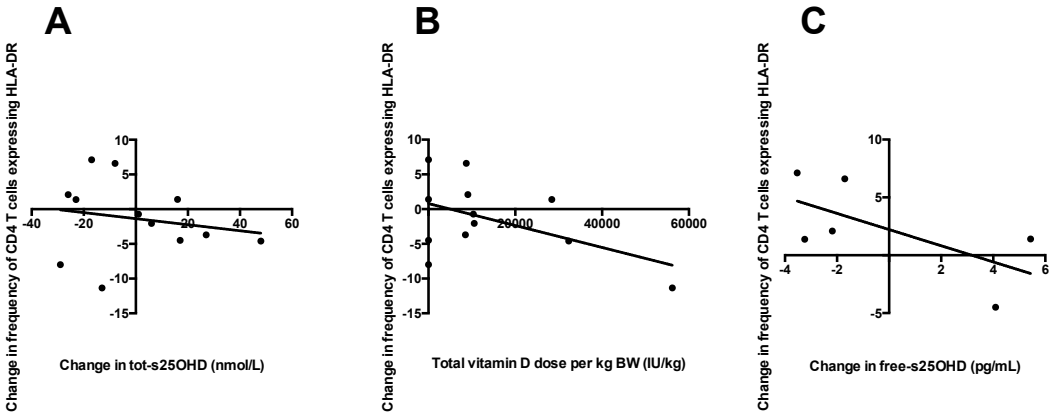

Figure 6. In the pilot RCT in this thesis (**Paper III**), change in tot-s25OHD (A), total ingested vitamin D dose per kg BW (B) and change in free-s25OHD (C) tend to negatively correlate with change in CD4 T cell expressing HLA-DR frequency. This supports the notion that the immunomodulatory effect of vitamin D is dose-dependent.

Calcipotriol proved to have selective effect on T-cell subsets with major reductions of CD45RO+ and CD8+ T cells (388). In the RCT included in this thesis, the CD4+/CD8+ ratio and CD4+ and CD8+ count remained unaffected by vitamin D supplementation or increasing s25OHD levels. This means that vitamin D supplementation affected T cells solely qualitatively, without shifting the CD4+/CD8+ balance or changing the quantity of these subsets (**Unpublished data**).

To test the hypothesis whether vitamin D could dampen the exaggerated antibody response in CF, we evaluated the relationship between vitamin D and serum total IgG in the database of the collected nutritional data on 898 Scandinavian CF patients. To explore the dataset, univariate linear regression analyses were done first. These showed significant negative correlations between IgG and s25OHD, food vitamin D intake per kg BW, supplemented vitamin D intake, supplemented vitamin D intake per kg BW, total vitamin D intake and total vitamin D intake per kg BW (**Paper I, Supplementary Table 1, Supplementary Figure 1**). To correct for potential confounders, we have built an MLR model where we controlled for age, gender, genotype, CFRD, season, infection/colonization status, corticosteroid treatment, macrolide treatment, pancreatic function and z-BMI. In this model, the negative associations remained statistically significant with s25OHD (**Paper I, Table 2, Figure 1**), supplemented vitamin D intake per kg BW (**Paper I, Table 3, Supplementary Figure 3**) and total vitamin D intake per kg BW (**Paper I, Table 3, Figure 2**) as independent variables. These negative relationships between vitamin D variables and serum total IgG described in the large cohort of Scandinavian CF patients are solely associations, even if they are independent. Whether the relationship is causative or not could only be determined by

doing a RCT. Therefore we started planning for a 4-year vitamin D supplementation study and have done a pilot RCT to establish an effective vitamin D supplementation strategy. This pilot RCT was not designed to detect changes in serum total IgG (which was only one of the secondary outcomes), so we were surprised to see that ergocalciferol supplementation decreased serum total IgG concentration at 8 and 12 weeks of supplementation (**Paper III, Figure 5B**), whereas the patients reaching the highest tot-s25OHD increased IgG at 8 weeks of supplementation compared with patients reaching peak 2 tot-s25OHD (**Paper III, data not shown**). Similarly, whereas the control group increased IgM at 8-week visit, patients receiving ergocalciferol decreased IgM both at the 8- and 16-week visit, compared with controls (**Paper III, Figure 5C-D**). Accordingly, tot-25OHD was negatively correlated with serum total IgA at baseline (**Paper III, Supplementary Figure 1D**) and change in IgA was negatively correlated with change in tot- and free-s25OHD from baseline, both at the end of supplementation and at 1 month of washout (**Paper III, Figure 5A**). The changes in IgG in the pilot RCT (**Paper III**) would speak for a bell-shaped relationship between tot-s25OHD and serum total IgG. This is in discrepancy with the results of the cross-sectional study in Scandinavian CF patients (**Paper I**), where we saw a linear negative association even after correcting for confounders. However, most of the population enrolled into that study were vitamin D insufficient and the absolute majority of the patients had tot-s25OHD in the range of 0 – 100 nmol/L (**Paper I, Figure 3**). Therefore, the linear relationship is true for this tot-s25OHD concentration range. Due to the almost absent group of patients with tot-s25OHD > 100 nmol/L in the Scandinavian database, no conclusions can be drawn about that concentration range from that study. Notably, the association between tot-s25OHD and IgE has been shown to be bell-shaped, with the lowest IgE concentrations at s25OHD within 100 – 125 nmol/L in a large cross-sectional study (389). If there is analogical relationship between IgG and s25OHD remains to be determined; the pilot RCT in this thesis generated results that are in support of that.

A number of *in vitro*, animal and human studies suggest inverse relationship between s25OHD and IgE. For example, $1,25(OH)_2D3$ inhibited anti-CD40- plus IL-4-mediated IgE production *in vitro* (390) and inflammatory crosstalk between NK cells and eosinophils via IL-15/IL-8 axis could be modulated by vitamin D (391). In mice with allergic asthma pre-treated with $1,25(OH)_2D3$, the immunotherapy could significantly inhibit the infiltration of eosinophils into lung tissues and bronchoalveolar lavage fluid and decreased levels of serum IgE when compared with untreated animals (392). Moreover, topical $1,25(OH)_2D3$ increased the regulatory capacity of CD4+CD25+ cells from the skin draining lymph nodes to suppress Th2-mediated allergic airway disease (393). In 616 children with asthma, vitamin D levels were inversely associated with total IgE and eosinophil count in MLR models (394). In CF patients with ABPA, active vitamin D3 attenuated Th2 responses to *Aspergillus fumigatus* mounted by CD4+ T cells *in vitro* (333). The inverse relation between IgE and vitamin D was evident even in the pilot RCT in this thesis (**Paper III**). IgE was mainly dependent on free-s25OHD status and circulating active vitamin D concentration, as changes in plasma total IgE were negatively correlated with changes in free-s25OHD and with changes in active vitamin D at 4 weeks of supplementation (**Paper III, Supplementary Figure 5E**). Moreover, vitamin D intake and sun exposure were clear determinants of IgE levels, because there was an inverse correlation between plasma total IgE, and both total vitamin D dose ingested and sun exposure at the end of supplementation (**Paper III, Supplementary Figure 5F-G**). At the end of the study, plasma total IgE was negatively associated with total vitamin D dose ingested per kg BW (**Paper III,**

Supplementary Figure 5H). Taken together, these results indicate that vitamin D might help to inhibit IgE-mediated hypersensitivity reactions against allergens including *aspergillus species*. A relevant question that should be addressed in future studies is whether improving vitamin D status would decrease the risk for and incidence of IgE-mediated allergic reactions to antibiotics in CF patients.

Collectively, these results indicate that vitamin D may dampen the excessively activated adaptive immune response in pre-transplant CF patients in a dose-dependent manner. In similar fashion, vitamin D might have a clinically meaningful suppressive immunomodulatory effect in post-transplant CF patients as well. We have examined a cohort of 41 lung transplanted CF patients, and found that the cumulative dose of hydrocortisone needed during the first week after transplantation was inversely related to their cumulative vitamin D supplement dose ($r = -0.52$, $p < 0.05$, **Abstract II**). Interestingly, there seemed to be an interaction with cyclosporine metabolism, because the active vitamin D concentration in the circulation was positively correlated with cumulative dose of cyclosporine A one year after lung transplantation ($r = 0.93$, $p < 0.01$, **Abstract II**). This is in line with findings in renal transplant recipients (395), and suggests that awareness of the complex immunomodulatory effects of vitamin D is to be recommended in order to optimise the management of CF patients both before and after lung transplantation.

5.4.4 Vitamin D decreases TGF-beta without affecting T-regulatory cell frequency and IL-10 concentration in CF

T-regulatory cells (Tregs) are essential for regulating immune response against pathogens, acting primarily via TGF-β (396) and IL-10 secretion (397). Vitamin D and Tregs have been described to be positively associated in various disease groups. For example, vitamin D status correlates positively with the frequency of CD4+FoxP3+ T cells in the respiratory tract of severe asthma patients (398). In patients with chronic pancreatitis, changes in serum $1,25(OH)_2D$ and 25OHD correlated with increases in levels of CD4 Tregs (399). In HIV patients, increases in $1,25(OH)_2D$ were associated with an expansion of activated CD4+ cells and Tregs (400). Additionally, vitamin D3-induced CD141+ DC-like cells had the capacity to induce immune tolerance via induction of potent Tregs (401). Thus, we hypothesized that vitamin D supplementation in CF patients enrolled into the pilot RCT (**Paper III**) would lead to upregulation of Tregs in these patients. Surprisingly, the frequency of $FoxP3^{pos}CD127^{low}$ Tregs did not change upon vitamin D supplementation (**Paper III, Supplementary Figure 5B**), nor was it associated with s25OHD levels (**Paper III, Supplementary Figure 5C-D**). Interestingly, the changes in Treg frequency were positively correlated with changes in CD86 expression on DCs at the end of supplementation (**Figure 7A**), and with changes in CD4 T cell expressing PD-1 frequency at the end of the study (**Figure 7B**). This suggests that, in the setting of chronic inflammation in CF, Tregs follow changes in the activation of DCs and T cells, and vitamin D primarily affects DCs and T cells rather than Tregs. It may be seen as positive that Treg frequency remained unchanged throughout the study despite the evident decrease in DC- and T cell- activation. This would suggest that vitamin D decreases the exaggerated activation of the adaptive immune system but simultaneously keeps the Treg frequency intact.

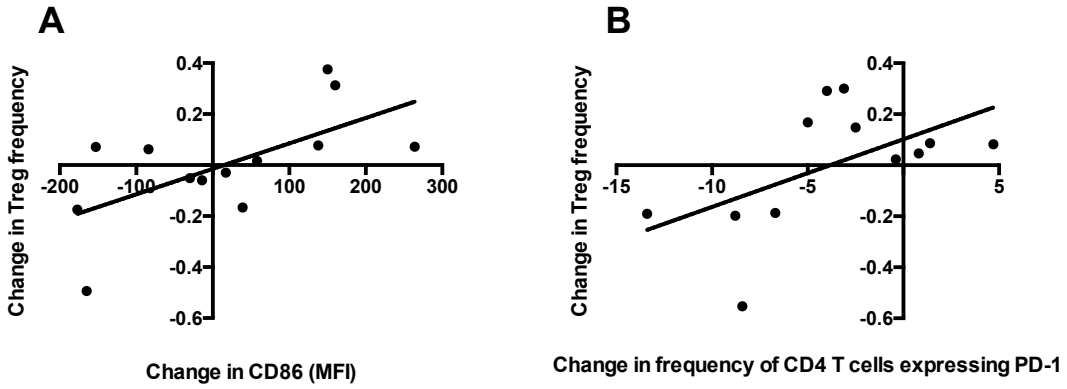

Figure 7. In the pilot RCT in this thesis (**Paper III**), changes in Treg frequency were positively correlated with changes in CD86 expression on DCs at the end of supplementation (A), and with changes in frequency of CD4+ T cell expressing PD-1+ at the end of the study (B). Tregs seem to follow the proinflammatory changes in CF, maybe in order to prevent excessive immune system activation.

CF is characterized by IL-10 deficiency (58) and attempts are being done to induce IL-10 in CF, as a promising way of positively modifying CF lung disease (402). Active vitamin D induces CD4+FoxP3+ and IL-10+ Tregs *in vitro* (298, 398). Therefore, we hypothesized that vitamin D supplementation would induce IL-10 and could be a safe and simple way of at least partially correcting the IL-10 deficiency in CF. It was disappointing to observe no changes in IL-10 upon vitamin D supplementation or upon s25OHD increase in the pilot RCT in this thesis (**Online data supplement for Paper III, data not shown**). This was in agreement with the cross-sectional findings generated in the Scandinavian CF Nutritional Study, where we measured IL-10 serum levels in 124 randomly chosen CF patients followed at Stockholm CF Center. Pearson's correlation analysis showed that s25OHD and log-transformed IL-10 values do not correlate (**Paper I**; $r = 0.053$; $p = 0.561$). Taken together, vitamin D does not seem to be associated with IL-10 in CF. It remains to be clarified what are the underlying reasons for those observations. It is generally recognized that IL-10 deficiency is a part of the dysregulated immunological profile in CF. That was the case in our dataset too, since large proportion of the patients enrolled into the trial had undetectable plasma IL-10 levels at baseline. The question is why there is the intrinsic IL-10 defect in CF, and whether this might have to do with the inability of vitamin D supplementation to increase IL-10 levels in CF. One speculation could be that the intrinsic IL-10 defect is caused by impaired ability of CF cells to activate vitamin D. The *in vitro* studies included in this thesis show that CFBE cells display a significantly lower ability to activate vitamin D than the non-CF HBE cells (**Paper IV, Figure 3B**). Moreover, this seemed to translate further downstream and result in absent increase in LL-37 upon stimulation of the CFBE cells with 25OHD (**Paper IV; Figure 4A**). Theoretically, another downstream consequence of the decreased ability to activate vitamin D could be the low IL-10 levels seen in CF. In that case, if the IL-10 levels are from the beginning extremely low or absent, and 25OHD turns into the active form inefficiently, even a very high-dose vitamin D supplementation may not be sufficient to influence the low IL-10 concentration. On the other hand, some other read-outs like T cell or DC activation or IgG levels may be more sensitive to vitamin D actions even if

the activation of vitamin D occurs at a lower pace (as the levels of these read-outs are very high from the beginning).

In CF children's plasma, TGF-β1 is positively associated with pseudomonas infection and lung disease, and is reduced in response to therapy (403). In the pilot RCT in this thesis (**Paper III**), patients reaching peak 2 and peak 3 tot-s25OHD reduced their plasma TGF-β concentration at the end of supplementation, compared with patients reaching the lowest tot-s25OHD peak (**Paper III, Supplementary Figure 5A**). Similarly, patients reaching peak 3 free-s25OHD decreased TGF-β plasma concentration at the end of supplementation, compared with peak 1 free-s25OHD patients (**Online data supplement for Paper III, data not shown**). This is in contrast with *in vitro* data showing that addition of active vitamin D3 increased DC expression of TGF-β and reduced Th2 responses by CD4 T cells isolated from CF patients with ABPA (333). Moreover, cholecalciferol supplementation in healthy adults induced expansion of peripheral CD4 Tregs (404). An explanation of these discrepancies could be that under exaggerated proinflammatory conditions *in vivo* vitamin D may decrease TGF-β secondarily via primarily dampening the excessive inflammation. Suppressing TGF-β may be in certain contexts beneficial, as TGF-β increased rhinovirus replication and decreased interferon secretion *in vitro* (405), and genetic variation in TGF-β1 modifies CF lung disease (406). In CF, TGF-β was recently shown to inhibit CFTR biogenesis and prevent functional rescue of deltaF508 CFTR in primary differentiated human bronchial epithelial cells (407). Therefore, the observed TGF-β decrease might be one of the mechanisms explaining the improvements in lung function and quality of life upon vitamin D supplementation (please see chapter 5.6 below).

5.5 VITAMIN D MAY IMPROVE GLUCOSE HOMEOSTASIS IN CF

A role for vitamin D in the pathogenesis of *diabetes mellitus* is supported by numerous *in vitro* and epidemiological studies, and there are excellent reviews covering this topic (308, 309). Vitamin D supplementation has been associated with a decreased incidence of T1D (310) and s25OHD is associated with T2D (408). The exact molecular pathways behind the associations have not been fully elucidated yet. In T1D, vitamin D has been suggested to act via its immunomodulatory properties (311, 409, 410). In T2D, vitamin D may increase both insulin secretion and peripheral insulin sensitivity (311, 411). As CFRD is primarily caused by beta cell dysfunction and secondarily by increased insulin resistance in the periphery, we hypothesized that low vitamin D status would be associated with worse glucose homeostasis. We tested this hypothesis using data collected in the Scandinavian CF Nutritional Study and chose HbA1c as a proxy for glucose homeostasis (**Paper II**). In this study, HbA1c was positively associated with s25OHD, s25OHD < 30 nmol/L, s25OHD < 50 nmol/L, and degree of vitamin D insufficiency, and negatively associated with supplemented vitamin D intake per kg BW and with total vitamin D intake per kg BW. When we analysed children and adults separately, vitamin D status was associated with HbA1c only in children (**Paper II, ESM Table 2**). To control for potential confounders, we built an MLR model with country, age, sex, genotype, lung function, long-term corticosteroid treatment, pancreatic insufficiency, and liver dysfunction as independent variables, and HbA1c as

dependent variable. In this model, the independent variables s25OHD < 30 nmol/L (**Paper II, ESM Table 3**), s25OHD < 50 nmol/L, and vitamin D insufficiency degree (**Paper II, Table 2**) remained significant determinants of HbA1c values. Strikingly, again, when analyzing children and adults separately, none of the studied vitamin D variables was associated with HbA1c in adults, whereas s25OHD < 30 nmol/L remained significantly associated with HbA1c values in children (**Paper II, ESM Table 4**). Association between vitamin D status and HbA1c was reported later on in several other populations (412-414). However, vitamin D supplementation in healthy individuals did not improve glucose homeostasis as assessed by HbA1c and the Homeostatic Model Assessment-Insulin Resistance index in several placebo-controlled trials (314, 414-416). Thus, despite being extensively studied, there is not sufficient evidence for vitamin D insufficiency as a causal factor in the pathogenesis of *diabetes mellitus*, which implies that the associations between HbA1c and s25OHD we described in the paediatric Scandinavian CF population should be interpreted with caution. It may be the case that vitamin D insufficiency is only a "smoke over the fire", i.e. a biomarker/bystander of some other causative agent in the etiology of CFRD that was not included in the MLR model.

In the pilot RCT, patients who specifically agreed with that, did a 3-hour OGTT test at baseline, at the end of supplementation and at the end of the study. **Preliminary data** show that change in free-s25OHD at the end of supplementation correlated negatively with blood glucose area under curve (AUC), and positively with change in the beta cell function index HOMA-%B (**Figure 8A-B**). The change in HOMA-%B was negatively associated with change in LPS level in the circulation at the end of supplementation (**Figure 8C**). Simultaneously, vitamin D dose given during the last month of the supplementation (IU/week) was negatively correlated with peak insulin concentration during the OGTT at the end of supplementation (**Figure 8D**). Of note, the change in insulin sensitivity index (ISI) measured by Stumvoll was negatively associated with change in free-s25OHD at the end of supplementation (**Figure 8E**). This index is dependent on BMI, and decreases with increased BMI (see the Methods section). Change in free-s25OHD at the end of supplementation was positively correlated with change in BMI (**Figure 8F**), which could explain the association of free-s25OHD with ISI measured by Stumvoll.

Taken together, the results indicate that vitamin D in CF may increase beta cell function measured by HOMA-%B index, which may be a consequence of decreased inflammation as it is associated with decrease in LPS levels. Vitamin D may also lower glucose AUC in 3-hour OGTT, which might indicate improved glucose tolerance. This happens together with simultaneously decreased peak insulin concentration during the OGTT, which would indicate that insulin sensitivity could be improved or that insulin production might become more evenly distributed. Notably, these results suggest that the associations described in **Paper II** may well be causative.

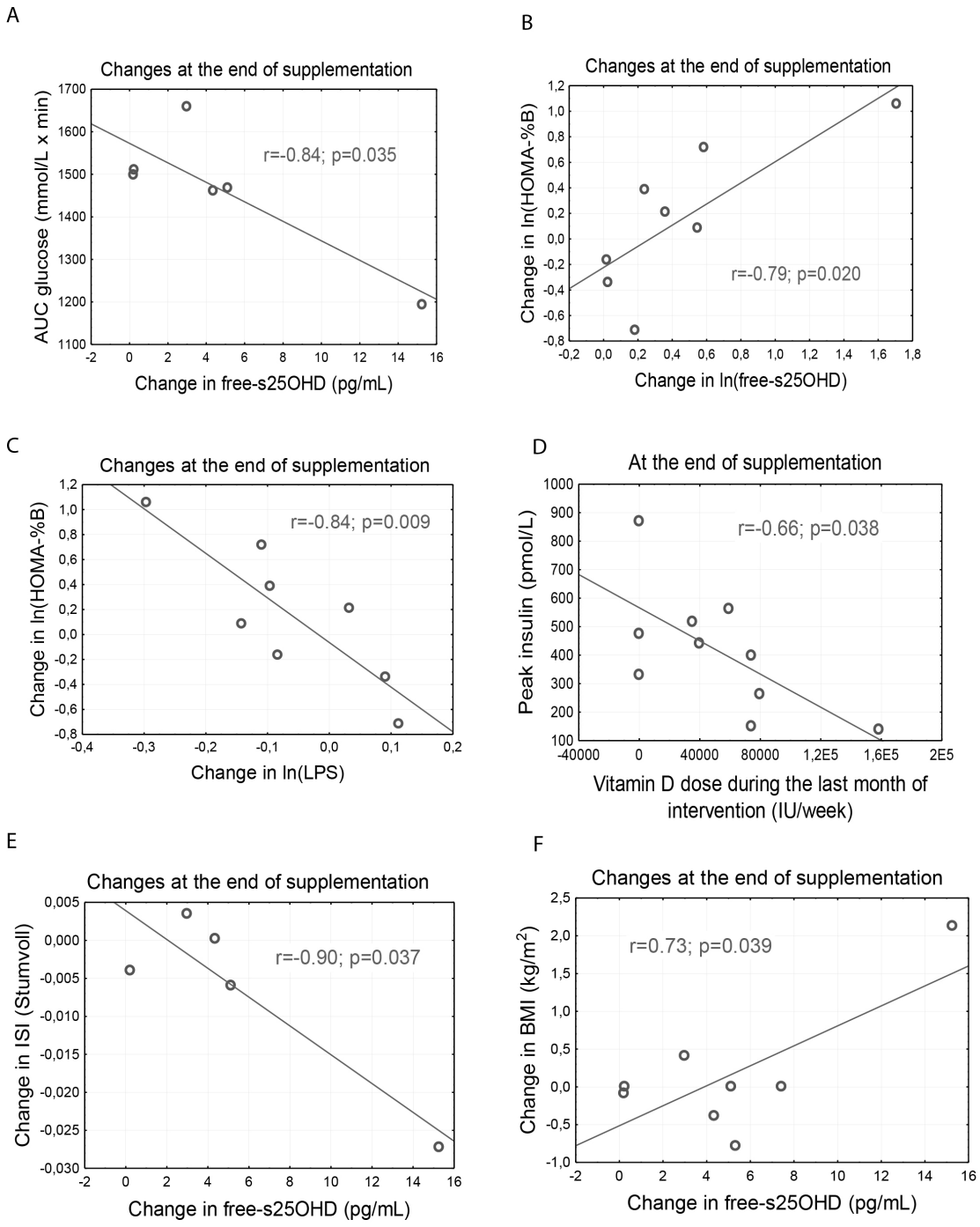

Figure 8. In the pilot RCT in this thesis (**Paper III**), vitamin D supplementation and increase in free-s25OHD were associated with changes in several glucose homeostasis parameters.

5.6 VITAMIN D SUPPLEMENTATION IN CF MAY HAVE POSITIVE CLINICAL IMPACT

As discussed above, s25OHD and/or vitamin D intake are negatively associated with serum total IgG and HbA1c, and positively associated with FEV1 after controlling for confounders in robust MLR models in the Scandinavian CF patients (n=898; **Paper I** and **II**). Moreover, the pilot RCT (n=16) showed that vitamin D supplementation in CF improves vitamin D status measured by the circulating tot-s25OHD level, may decrease PTH, increase beta cell function, and clearly induces complex immunological changes (**Paper III**). The *in vitro* cell experiments indicated that vitamin D most probably does get activated in the peripheral tissues of CF patients to some extent, even if less effectively than in non-CF tissues, which means that there is some potential for the described downstream actions of the active vitamin D (**Paper IV**). The crucial question that necessarily follows and inevitably will have to be addressed at some point is: *Do the associations and changes in surrogate markers translate into clinically relevant changes?* In the end, this is what really matters to the clinicians and the CF patients, because only clinically meaningful changes in hard outcomes can make a difference for the well being of the patients and decrease their need of other treatment(s).

For this reason we evaluated whether vitamin D insufficiency could be an independent risk factor for pathological OGTT result and/or CFRD development, which were chosen as clinically meaningful endpoints available in the Scandinavian CF Nutritional Study database (**Paper II**). As expected, patients having the diagnosis of CFRD at inclusion into the study had lower s25OHD, total vitamin D intake, and supplemented and total vitamin D intake per kg BW than patients without CFRD. Next step was to see if patients who got CFRD diagnosis before inclusion into the study differed from those getting CFRD diagnosis at the time point of inclusion into the study. Interestingly, patients with known CFRD, as opposed to new CFRD, had low vitamin D intake. However, the two subgroups did not differ in s25OHD, which was low in both subgroups. Accordingly, patients with pathological OGTT results had lower s25OHD than patients with physiological OGTT results. Patients with HbA1c-defined diabetes had lower supplemented and total vitamin D intake per kg BW than patients not meeting the definition of diabetes according to HbA1c (**Paper II, ESM Table 5**). In line with all of these results, the Chi-square test confirmed that inferior serum vitamin D status is associated with a greater proportion of patients with CFRD, both known and new, pathological OGTT results, or HbA1c-defined diabetes (**Paper II, ESM Table 6**). The next step was to control for all known risk factors for CFRD development, which included country, age, sex, genotype, lung function, long-term corticosteroid treatment, pancreatic insufficiency, and liver dysfunction. In this robust MLR model, s25OHD < 30 nmol/l and degree of vitamin D insufficiency remained significant independent risk factors for CFRD diagnosis (**Paper II, Table 3, ESM Table 7**). Similarly, s25OHD < 50 nmol/l was associated with CFRD, but statistical significance was not reached (p=0.058; **Paper II, Table 3**). In contrast, neither pathological OGTT nor HbA1c-defined diabetes remained independently associated with vitamin D after correcting for confounders (**Paper II, ESM Table 8**). Taken together, this would support a role for vitamin D in the etiology of CFRD *per se*, and

not in development of isolated impaired glucose tolerance. Vitamin D may co-act in synergy with some other agent(s)/factor(s) that lead to eventual manifest CFRD.

Several previous studies have found a positive association between s25OHD and FEV1 (117, 417, 418). In asthma, PEF rate values were found to be positively associated with s25OHD levels (419) and in children with asthma treated with inhaled corticosteroids vitamin D deficiency was associated with poorer lung function (420). In CF, a trend towards increased recovery of lung function was observed in patients receiving one oral bolus of 250000 IU cholecalciferol (331). A very recent retrospective observational study reported s25OHD < 75 nmol/L to be associated with lower FEV1 in CF adolescents and with greater frequency of pulmonary exacerbations in CF children (332). In line with that, in the Scandinavian CF Nutritional Study database we found significant positive correlations between FEV1 and s25OHD, supplemented vitamin D intake per kg BW and total vitamin D intake per kg BW (**Paper I, Supplementary Table 2, Supplementary Figure 2**). To rule out confounders, we designed an MLR model with FEV1 as dependent variable and with all the potential confounders (i.e. age, gender, genotype, CFRD, season, infection/colonization status, corticosteroid treatment, macrolide treatment, pancreatic function and z-BMI) as independent variables. In this model, s25OHD remained a significant determinant of FEV1 values (**Paper I, Supplementary Table 3, Figure 3**). Here again, this was only an independent association that supported the hypothesis that vitamin D may have positive effect on lung function in CF, but no conclusions could be made about causality. Moreover, the absolute amplitude of this association was small. It was not surprising in the context of the well known other strong lung function determinants in CF patients, but it still questioned the clinical relevance of this finding. Therefore, we were curious to see whether vitamin D supplementation given in the pilot RCT would improve lung function of the patients (**Paper III**). As expected, already at baseline s25OHD was positively correlated with lung function parameters FEV1, FEV%, FEF50% and FEF75% (**Paper III, Supplementary Figure 1F-H**). Patients allocated to serve as controls, patients reaching the lowest tot- or free-s25OHD peak, and patients reaching the tot-s25OHD peak < 90 nmol/L decreased their FEF25% at 4- and 8-week visits (**Paper III, Figure 6D**), and decreased their FEV% at 8-week visit (**Paper III, data not shown**). Patients allocated to the cholecalciferol group improved their FVC at the last study visit compared with baseline (p=0.043; **Paper III, Figure 6E**), whereas those receiving ergocalciferol did not change their lung function throughout the study. Moreover, patients receiving cholecalciferol improved their FEV% at the last study visit, compared with the control group (**Paper III, data not shown**). Notably, the pilot RCT was not powered to detect changes in lung function, but it still indicated a lung function improvement in patients taking cholecalciferol. Since patients allocated to ergocalciferol supplementation received much higher total vitamin D dose than cholecalciferol patients, and did not improve in lung function, we hypothesized that the improvement in lung function is strictly dependent on increase in the circulating tot- or free-s25OHD concentration. Indeed, patients reaching the highest peak of free-s25OHD improved their FEF25% at 8-weeks visit compared with those reaching peak 2 or peak 1 free-s25OHD (**Paper III, Figure 6F**), and so did patients reaching the highest tot-s25OHD peak (p = 0.003) and patients reaching > 90 nmol/L tot-s25OHD (p = 0.040) compared with peak 1 tot-s25OHD and patients reaching < 90 nmol/L tot-s25OHD,

respectively (**Paper III, Supplementary Figure 2B-C**). In patients reaching the highest tot-s25OHD peak, FEF25% was still increased at 1-month washout, compared with patients reaching peak 1 tot-s25OHD (p=0.047; **Paper III, Supplementary Figure 2C**). At that time point, the change in tot-s25OHD from baseline was positively correlated with changes in FEV1 and FVC (**Paper III, Figure 6G-H**), whereas there were no correlations between the total ingested vitamin D dose and changes in lung function throughout the study. The cross-sectional results derived from the Scandinavian CF Nutritional Study delivered the same message: s25OHD remained a significant determinant of FEV1 values after correcting for confounders (**Paper I, Supplementary Table 3, Figure 3**), whereas none of the various vitamin D sources of intake showed significant relation to FEV1 in the MLR model (**Paper I, Supplementary Table 4**). Taken together, all the results suggest that it is rather the actual circulating s25OHD concentration that is available to the peripheral tissues that is determinant of lung function in CF, rather than the amount of vitamin D intake *per se*. We also observed a considerable inter-individual variation in the vitamin D doses required to increase s25OHD levels. Altogether, these data highlight the importance of s25OHD concentration monitoring.

Overall, only a few studies have evaluated the effect of vitamin D treatment on health-related QoL. Despite correction of the hypocalcaemia, QoL was impaired in the vitamin D treated group of patients with autosomal dominant hypocalcaemia, compared with the vitamin D-untreated patients (421). On the other hand, lower s25OHD concentrations were independently associated with lower score on the Mental Component Summary of the Short Form-12, but not with the Physical Component Summary of the Short Form-12 in a cohort of incident dialysis patients (422). In Crohn's disease patients, vitamin D deficiency was associated with lower QoL assessed by Short Inflammatory Bowel Disease Questionnaire (423). Both high PTH levels and incident fragility fractures, which are commonly accompanied by vitamin D deficiency, were reported to be associated with low QoL (424, 425). In CF, vitamin D insufficiency was associated with depressive symptoms in CF (336) and there was a tendency for reduced vitamin D in depressed CF patients (335). In the Scandinavian CF Nutritional Study, we found vitamin D to be negatively associated with serum total IgG, and positively with FEV1 (**Paper I**). In addition, vitamin D supplementation in the pilot RCT induced a whole spectrum of various immunological changes that might theoretically be beneficial, and several of them were closely correlated with improvement in lung function parameters (**Paper III**). Therefore, we expected that vitamin D supplementation or elevated s25OHD concentration would improve QoL in the pilot RCT.

In a large national, multi-center study, the CFQ-R questionnaire consistently discriminated between patients seen for sick-versus-well visits, and among stages of disease severity based on lung function (347). However, CFQ-R has not been used to evaluate changes in QoL upon vitamin D supplementation in CF yet. Thus, we were first to use this tool, and already at baseline s25OHD was positively correlated with several QoL domains including the Respiratory score (**Paper III, Supplementary Figure 1E**). At the end of supplementation, children reaching peak 2 free-s25OHD decreased their QLP-Treatment Burden score compared with children reaching peak 1

free-s25OHD (**Paper III, Supplementary Figure 5I-J**), which suggests that vitamin D supplementation may increase treatment burden in CF. Importantly, patients reaching the highest tot-s25OHD and children reaching Peak 2 tot-s25OHD improved their QoL- and QLP-Respiratory scores, respectively (n = 2 in each group), at the end of supplementation by more than the minimum clinically relevant difference of five (**Paper III, Figure 6A-B**). Moreover, the effect of vitamin D on the Respiratory score seems to be dose-dependent, as change from baseline in free-s25OHD at the end of supplementation was positively correlated with change in QoL-Respiratory score (**Paper III, Figure 6C).** In summary, these results consistently indicate that vitamin D does not only influence surrogate markers but also impacts clinically meaningful read-outs, such as CFRD diagnosis, lung function and QoL. However, specific vitamin D supplementation may also increase treatment burden, particularly experienced by parents of CF children.

6 CLINICAL IMPLICATIONS AND FUTURE RESEARCH

There is currently no agreement between Europe and US on the recommended optimal s25OHD levels in CF. European guidelines are more conservative and claim that s25OHD > 50 nmol/L is optimal (146), whereas US CF Foundation guidelines see 75 nmol/L as the cut-off for optimal s25OHD concentration in CF (137). **Paper I** described a linear negative association between s25OHD and serum total IgG within the s25OHD concentration range 0 – 100 nmol/L. Within the same range, s25OHD was positively associated with the lung function parameter FEV1. This may indicate that increasing s25OHD up to 100 nmol/L could be beneficial with respect to lung function and the chronic inflammation process in CF. In **Paper II** we described an inverse linear association between s25OHD and HbA1c within the same s25OHD concentration range, but the clinical relevance of decreasing HbA1c in non-diabetic population is unclear. S25OHD < 30 nmol/L was an independent risk factor for CFRD diagnosis and there was similar tendency for s25OHD < 50 nmol/L, which would speak for s25OHD of 50 nmol/L as the optimal level for s25OHD in order to minimize the risk for CFRD development. However, the population of patients who had s25OHD > 75 nmol/L in the database was relatively small, so the study may have been insufficiently powered to detect the effect of this concentration cut-off. **Paper III** has shown that some surrogate parameters might be related with s25OHD in a bell-shaped fashion, with best values when 92 nmol/L < s25OHD < 97 nmol/L. This would indicate that too high s25OHD concentrations are not optimal, which is in line with literature (389). **Paper IV** confirmed that s25OHD at concentration 100 nmol/L does convert into active vitamin D by CF bronchial epithelial cells, but less effectively than by the corresponding non-CF cells. We did not test lower s25OHD concentrations; however, if the ability of CF cells to activate vitamin D *in vivo* is as low as we observed it *in vitro*, it may well be the case that relatively high circulating s25OHD concentrations are needed to ensure sufficient local active vitamin D concentrations. This might speak in favor of the concentration s25OHD = 100 nmol/L as optimal in CF. In summary, the papers in this thesis did not bring any conclusive evidence about the optimal s25OHD concentrations to be recommended for CF patients, but the results point collectively to s25OHD levels at 92 – 100 nmol/L as the concentrations that might have the greatest health benefits from various aspects. In order to achieve these concentrations, the initial dosing in Scandinavian CF patients ≥ 6 years of age may need to be higher than that currently recommended for CF patients in Europe (146) or the US (137). In the pilot RCT (**Paper III**), we aimed to achieve the goal s25OHD concentration of 100 nmol/L. The daily cholecalciferol dose had to be increased up to the average of 8184 IU and the daily ergocalciferol dose had to be increased up to the average of 15650 IU; and still, only two of nine patients receiving vitamin D reached the goal concentrations. We also observed considerable variation in the doses needed to increase s25OHD among the CF patients, which confirms the current recommendations that say that vitamin D dose should be individually adjusted.

The current recommendations for vitamin D supplementation favor cholecalciferol over ergocalciferol due to its greater efficiency at increasing s25OHD levels (137,

146). As expected, despite given in almost double doses compared with cholecalciferol, ergocalciferol did not significantly increase s25OHD in the pilot RCT (p=0.106; **Paper III**). Patients receiving ergocalciferol and not cholecalciferol decreased plasma IgG and IgM. We hypothesize that ergocalciferol might predominantly act locally via immune-modulation of the gut-associated lymphoid tissue. We propose that ergocalciferol may be a non-inferior substitute for the currently recommended cholecalciferol, but needs to be given in higher doses. Unlike cholecalciferol, ergocalciferol did not increase albumin-corrected calcium, indicating that it might be a useful immunomodulatory substance with very low risk of causing hypercalcaemia. On the other hand, several endpoints in the RCT were strictly and exclusively related to s25OHD levels, including IgA and lung function (**Paper III**). A possible explanation for that could be that circulating s25OHD needs to reach the lungs to affect them, which implies that significant elevation of s25OHD is necessary to optimize the health benefits of vitamin D. Using ergocalciferol only may make this very difficult; thus, the best option could maybe be combining ergocalciferol with cholecalciferol supplementation.

At present, tot-s25OHD is internationally agreed and used as marker of vitamin D status, and has been intensively studied as a correlate of the immunomodulatory and calcium/glucose-regulatory actions of vitamin D. In the pilot RCT (**Paper III**), changes in free-s25OHD were more tightly correlated with changes in the surrogate immunological parameters than tot-s25OHD. Moreover, changes in QoL-Respiratory score were closely associated with free-s25OHD rather than with tot-s25OHD. This indicates that following free-s25OHD instead of tot-s25OHD could be advisable in some cases, as it may well be a better correlate of the immunological and clinical changes induced by vitamin D. In practice, it would mean only two extra measurements (DBP and albumin concentration) and deliver more information than tot-s25OHD.

Some immunological parameters behaved upon vitamin D treatment in CF patients in opposite manner to that consistently described in *in vitro* studies. Specifically, we described clear discrepancy of *in vitro* and *in vivo* findings for Tregs, TGF-β and IL-1β. This could maybe be attributed by the surrounding inflammatory environment in CF *in vivo*. CF is characterized by proinflammatory shift. Therefore, TGF-β levels are relatively high and fluctuate, reaching highest values during lung exacerbations (403). Via dampening the cellular and antibody-mediated adaptive immune response, vitamin D maybe decreased the need for the counter-regulatory mechanisms including TGF-β (**Paper III**). This highlights the importance of clinical studies and confirms that the effect of vitamin D is very complex and may depend on the cytokine milieu and state of immune activation, or maybe even on other environmental and genetic factors. There is very little known about interactions of vitamin D with these factors, and future research will hopefully address these questions.

Studies in this thesis gathered comprehensive data, which consistently suggest that vitamin D supplementation may be beneficial in CF patients, with regard to beta cell function, chronic inflammation and the dysfunctional innate immunity, which is part of the CF phenotype, as well as with regard to clinically meaningful outcomes such as lung function, QoL and risk of developing CFRD (**Paper I, II and III**). However, these studies are only descriptive epidemiological studies (**Paper I and II**) and one small-

scale pilot RCT (**Paper III**). Therefore, their results should rather be seen as hypotheses-generating, and not evidence-generating. The changes observed in **Paper III** were inter-correlated, fit well into the puzzle, and some of them were reversible (returned to baseline after withdrawal of vitamin D), which would speak for causality. On the other hand, in some instances we solely observed correlations with changes in the circulating s25OHD, with no significant differences between the treatment arms, which might mean that the findings are confounded; for example, when patients get more stable and feel healthier, they go out in the sun more often, and thus their s25OHD increases. In Scandinavia, however, this would only be possible to happen during the summer season; and the patients were recruited into the trial evenly throughout the whole year. In summary, the beneficial effects suggested by the studies in this thesis should be interpreted with caution and confirmed in carefully designed, large, long-term placebo-controlled intervention studies.

Another exclamation mark is safety and risk-benefit ratio. Safety issues were not in focus of this thesis. Only one study of the four included in this thesis addressed the question of safety (**Paper III**). In this trial, safety was assessed only roughly by monitoring patient-reported symptoms, s25OHD and albumin-corrected plasma calcium levels at each of the seven study visits. We could conclude that none of the patients reached toxic calcium or s25OHD concentrations, neither developed symptoms consistent with vitamin D toxicity syndrome. Thus, when s25OHD is monitored and supplementation individually adjusted, the risk of acute toxicity seems to be small. However, long-term safety of high-dose vitamin D supplementation in CF is currently a frequently debated concern and none of the studies in this thesis addressed that question. Thus, it remains an unexplored area. Hopefully more studies will help to clarify this issue in the future. We are planning a larger-scale 4-year vitamin D supplementation trial where we will evaluate safety as one of the most important secondary outcomes.

Another issue is the treatment burden-benefit ratio of vitamin D supplementation in CF. Even in relatively stable CF patients, the treatment burden is very high, and increases with disease severity and exacerbation frequency. The results of **Paper IV** indicate that CF cells might have impaired ability to activate vitamin D. Follow up studies are needed to confirm these observations by using other approaches, such as comparing additional CF and non-CF respiratory epithelial cell lines, or CFBE cells and wt-CFTR-corrected CFBE cells. In order to provide more conclusive results, experiments with primary respiratory epithelial cells from CF patients or deltaF508 CF mice will be crucial. Nevertheless, if the preliminary results provided in **Paper IV** prove to be true, they might mean that the relative benefit of vitamin D supplementation in CF patients is lower compared to non-CF persons, and that very high s25OHD circulating levels may be needed for clinically meaningful effects. On top of that, **Paper III** confirmed that very high vitamin D dosing is needed to increase s25OHD in CF. One patient left the trial due to bad taste of the drug, and parent-reported treatment burden assessed by CFQ-R questionnaire increased at the end of supplementation. Apparently, the CF patients and/or their parents did not find taking the extra vitamin D very easy, and the results indicate that the increase in treatment burden may be relevant and should be studied in more detail by trials that would be specifically designed for that. Finally, the

treatment burden-benefit ratio should be evaluated in order to decide whether high-dose extra vitamin D supplementation is worth the effort or not.

In conclusion, this thesis increased the understanding of vitamin D in the context of CF. The results indicate that vitamin D most likely has multifunctional effects in the CF population, as regards immunity, inflammation, glucose homeostasis, lung function and quality of life (**Figure 9**). These effects seem to be beneficial. In addition, this thesis opens the door for further research on vitamin D in the field of CF and in other contexts as well. For example, the immunomodulatory effects described here could be relevant for many infectious and inflammatory diseases. The glucose-lowering effects suggested for vitamin D in CF patients by this thesis may support similar hypotheses that had been created for T1D and T2D. The positive influence on lung function could maybe be extrapolated and tested in other respiratory diseases, such as asthma, COPD or primary ciliary dyskinesia. On top of that, it may well be the case that non-CF diseases would benefit from vitamin D supplementation even more than CF. Indeed; the preliminary results produced by the cell experiments in this thesis indicate that cells harbouring mutated CFTR may have decreased ability to activate vitamin D. This needs to be confirmed by further experiments. However, if it is true, then the beneficial effects of vitamin D should have bigger amplitude in non-CF individuals.

I hope that this thesis will provide a solid basis for further research by elucidating the known, the unknown and the intriguing in the field of vitamin D and CF.

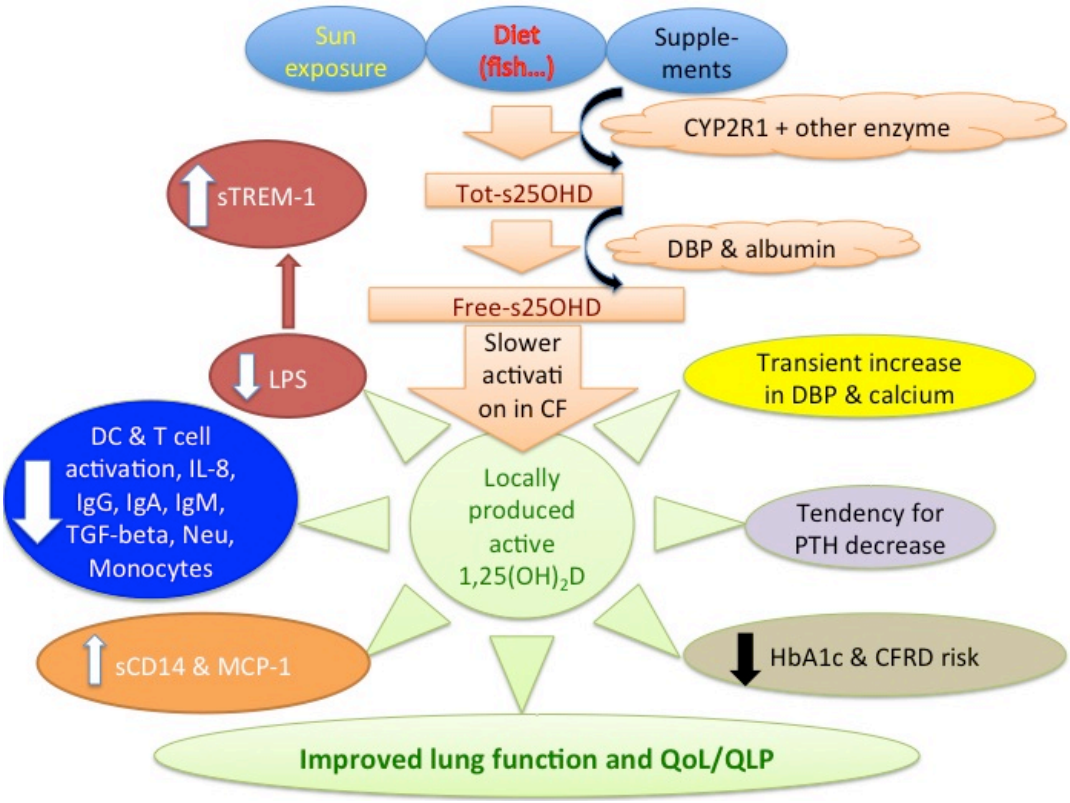

Figure 9. Multifunctional effects of vitamin D in CF suggested by results of this thesis.

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Figure 10. A Bird. Painted by the little artist Adela Pincikova, 2013.

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9 ABSTRACTS

9.1 ABSTRACT I

Vitamin D Status in Stockholm Cystic Fibrosis Patients in 2007-2009

Terezia Pincikova, Lena Hjelte

Stockholm Cystic Fibrosis Center, Karolinska University Hospital Huddinge, Karolinska Institutet, Stockholm, Sweden

Background: No evidence of benefit or harm of vitamin D supplementation exists in the CF population. Intervention trials are necessary, which require baseline data on vitamin D status at the respective latitude.
Objective: To assess the vitamin D status in all CF patients living in Stockholm region (n=133; 59° 20' North latitude), its development over 2007-2009 and seasonal variation.
Methods: Descriptive statistics, independent t-test and univariate linear regression were used to determine and compare serum 25-hydroxyvitamin D (s25OHD) levels, seasonal variation (summer: May-Oct; winter: Nov-Apr) and vitamin D insufficiency (s25OHD < 75 nmol/L) prevalence.
Results: Over years 2007-2009, number of measurements of s25OHD increased, whereas mean measured s25OHD remained stable (52.5; 52.7 and 51.3 nmol/L). Each year, 78-80% measured values were below the lower sufficiency level. In 2009, more measurements were done in winter (61.5 vs. 44.6 and 44.1 %; p=0.017 and p=0.009). Mean s25OHD from measurements done in summer (n=150; 60.2 nmol/L) was higher than those from winter (n=157; 44.3 nmol/L), p<0.001. Vitamin D insufficiency prevalence was lower during summer season (68.0% vs. 89.8%; p<0.001). However, summer season explained 9% and 7% of variation in s25OHD and vitamin D insufficiency.
Conclusion: Without coordinated intervention, vitamin D status of CF patients does not change. Summer season is associated with better vitamin D status, but it explains only a minor part of its variation. Majority of Stockholm CF patients have insufficient s25OHD levels both in winter and in summer.
Significance: To reach the recommended optimal s25OHD levels, vitamin D supplementation intervention is necessary both during summer and winter. During winter higher doses might be needed, though.

Reference:
Pincikova, T. and Hjelte, L., 2010. Vitamin D Status in Stockholm Cystic Fibrosis Patients in 2007-2009. In: Journal of Cystic Fibrosis 2010; Volume 9: Suppl 1. Abstract nr 353, Page S91.

9.2 ABSTRACT II

Interaction of Fat-soluble Vitamins with Immunosuppressant Drugs in Lung Transplanted CF Patients

T. Pincikova[1], L. Mared[2], L. Hjelte[1]

1 Stockholm CF Center, B 59, Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm, Sweden
2 Heart and Lung Center, Skåne University Hospital, Lund, Sweden

Context: Recent literature suggests that metabolism of fat-soluble vitamins undergoes changes in organ transplantated patients. Cyclosporine A was found to produce elevations in serum $1,25(OH)_2D$ levels in animal models and kidney transplanted patients. Literature also indicates that vit D supplementation decreases the need for immunosuppression. The role of vit D in the prevention of post-transplant osteoporosis remains unclear.
Aims: Patient records of 41 lung transplanted CF patients at CF centres in Lund and Stockholm were used to investigate the metabolism of fat-soluble vitamins, importance of vitamin D for post-transplant bone health and its interactions with immunosuppressant drugs.
Results: S-retinol and α-tocoferol levels increase after lung transplantation ($p<0.001$ and $P<0.005$ respectively), whereas S-25OHD and $1,25(OH)_2D$ levels do not change. Despite vitamin D supplementation, median S-25OHD is insufficient both before and after transplantation. Whole body, spinal and femoral BMD correlate with P-calcium ($p<0.05$ in all). Vitamin D supplementation is not associated with higher P-calcium or BMD after transplantation. However, vitamin D supplement dose correlates negatively with PTH ($r=-0.88$; $p<0.05$). S-$1,25(OH)_2D$ one year after transplantation is positively correlated with cumulative dose of cyclosporine A ($r=0.93$; $p<0.01$). Cumulative dose of hydrocortisone needed during the first week after transplantation is inversely related to the cumulative vitamin D supplement dose ($r=-0.52$; $p<0.05$).
Conclusions: The observed changes in vitamin A and E metabolism, and the interference of vitamin D with immunosuppressant drugs may have an impact on immune modulation and bone health in lung transplanted CF patients.

Reference:
Pincikova, T., Mared, L., Hjelte, L., 2011. Interaction of Fat-soluble Vitamins with Immunosuppressant Drugs in Lung Transplanted CF Patients. In: Journal of Cystic Fibrosis 2011; Volume 10: Suppl 1. Abstract nr 306, Page S77.