Cystic fibrosis: a hereditary inflammatory process

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Cystic fibrosis: een erfelijk inflammatoir proces

(met een samenvatting in het Nederlands)

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Voor Jacco, Luc en Coen
Voor mijn ouders

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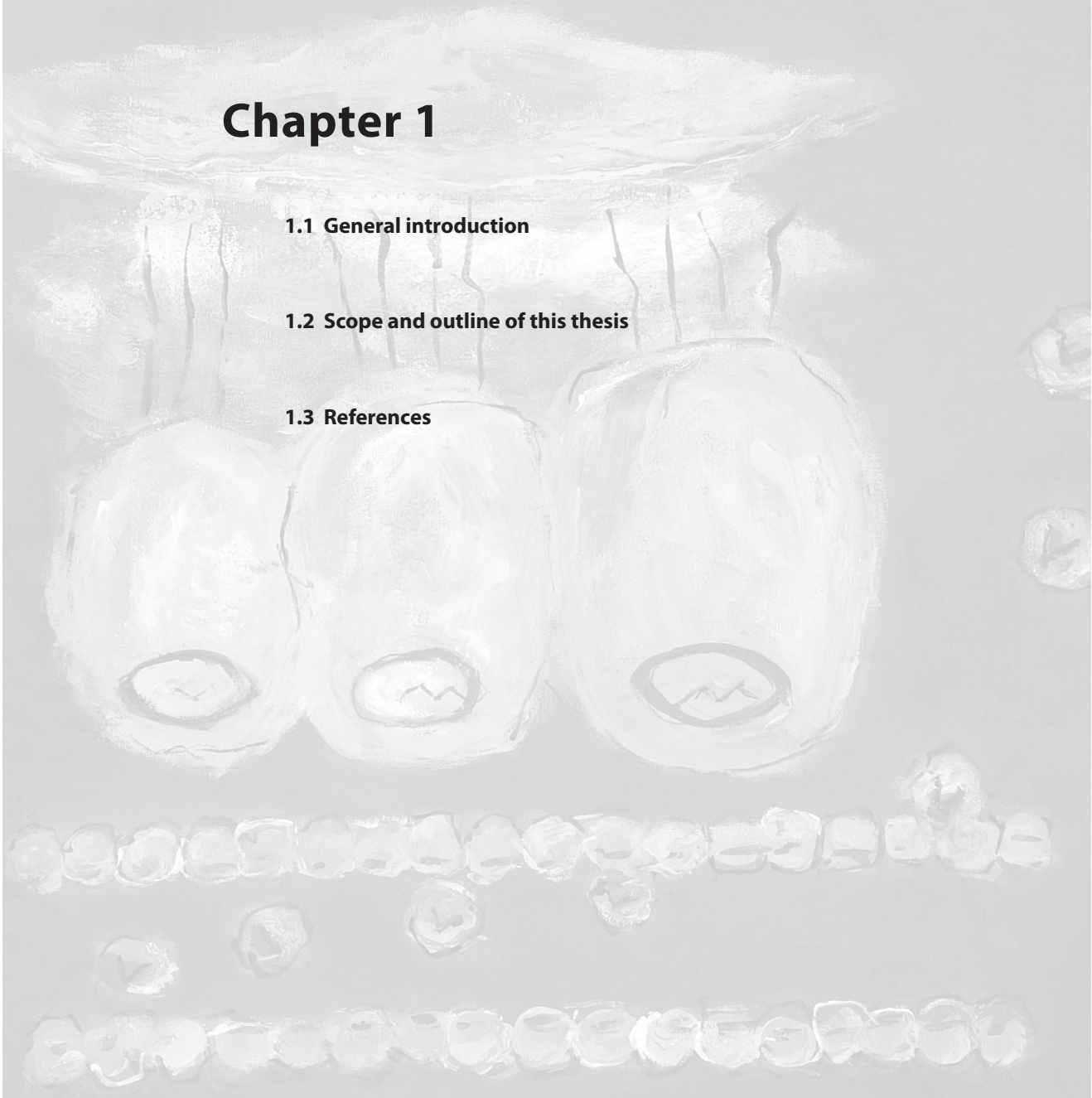
Chapter 1



1.1 GENERAL INTRODUCTION

CYSTIC FIBROSIS AND THE CYSTIC FIBROSIS TRANSMEMBRANE REGULATOR (CFTR)

Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder in the Caucasian population, with an overall birth prevalence in the Netherlands of 1 in 4750 live births from 1974 to 1994 (1). CF is caused by mutations of the CFTR gene which spans 250 kilo bases on chromosome 7 (2-4). Over 1300 CFTR mutations have been found (http://www.genet.sickkids.on.ca/cftr/) but the most frequent mutation is the class 2 mutation, caused by a deletion of phenylalanine at position 508 (ΔF-508) of the CFTR protein, accounting for 75% of the CFTR mutations in Dutch CF patients (5). Active transcription of the CFTR gene and CFTR mRNA transcripts are detectable in a variety of epithelial cells, but also in cells of non-epithelial origin like peripheral blood neutrophils, lymphocytes and alveolar macrophages (6).

The CFTR protein functions as a cAMP-modulated chloride channel on the apical surface of epithelial cells lining most exocrine glands and has a role in controlling the activity of an outwardly rectifying chloride channel (ORCC) (7-11). CFTR also regulates other membrane proteins and the most thoroughly documented regulatory role of CFTR is the negative regulation of the amiloride-sensitive epithelial Na^+ channel (ENaC) (12-16). When CFTR function is lost, the Na^+ conductance is markedly increased in human airways (17).

Once activated, CFTR has a role in mediating ATP release and regulating $[Ca^{2+}]_i$ and thus controlling the balance between secretion and absorption in airway epithelia (18). Besides the cAMP-dependent Cl^- conductance, human airway epithelial cells also express Ca^{2+}-activated Cl^- channels (CaCC) and the CaCC-mediated Cl^- secretion is preserved in CF airways. CaCC might thus provide a compensatory mechanisms to bypass the Cl^- secretory defect in CF (19). Airway epithelial cells of CF knock-out mice express a large endogenous Ca^{2+}-activated Cl^- conductance and are devoid of the severe lung disease seen in humans (20,21). Airway epithelial cells of CF patients also exhibit larger Ca^{2+}_i-dependent responses e.g Ca^{2+}_i-enhanced mucociliary clearance (22), triggered by luminal purinoceptor agonists (23,24). In CF airway epithelial cells there is an activation of the pro-inflammatory transcription factor nuclear factor-kappa B (NF-κB) and this activation is regulated by Ca^{2+}_i (25-28). It was suggested that the endoplasmic reticulum (ER) retention of the ΔF508 CFTR protein leads to a constitutive activation of NF-κB through an ER stress response (ER overload) (26). Recently it has been shown that the activation of NF-κB, and subsequently secretion of inflammatory mediators such as interleukin (IL)-8 is induced by chronic airway infection and inflammation (29).

Finally, CFTR also seems to play a role in epithelial HCO_3^- transport (30-32) and HCO_3^- is suggested to play a crucial role in the pH regulation of the airway surface liquid and thus in the defence mechanism of the lung.

CFTR AND INFLAMMATION

CF lung disease is characterized by an exaggerated, sustained and extended inflammatory response, with massive influx of neutrophils and high concentrations of IL-8 in bronchoalveolar lavage fluid (BALF) and by airway infection (particularly with *Pseudomonas aeruginosa* and *Staphylococcus aureus*) (33-40). In the airway epithelium, the mutant CFTR fails to initiate cAMP-dependent Cl^- secretion and causes a lack of inhibition of epithelium sodium channels (ENaC) with an increased Na^+ absorption. This causes an increased water absorption, leading to a decreased volume of the airway surface liquid (ASL), covering the airway epithelium (41,42). The decrease in ASL volume decreases efficacy of mucociliary clearance by coughing, leading to mucus stasis and chronic airway infection and neutrophil dominated inflammation (37,39,43).

Not only ASL plays an important role in the innate immune defence against all kinds of inhaled pathogens (such as bacteria) but also non specific airway defences such as defensins and nitric oxide (NO) are able to attack and neutralize small doses of bacteria under normal conditions and subsequently prevent infection. However, the major isoform of nitric oxide synthase (NOS) in airway epithelia, NOS2, is reduced in CF airway epithelium and appears to be directly related to defective CFTR function (44,45). This decreased NOS2 expression leads to a reduction in local NO production and this might contribute to an impairment of primary host defence in CF lungs.

CFTR also regulates the expression of several genes, including the chemokine regulated upon activation normal T cell expressed and presumably secreted (RANTES) (46) and the chemokine IL-8 (47). RANTES is expressed and secreted by a variety of cells such as airway epithelial cells and seems to play a role in the pathogenesis of airway inflammatory diseases. In CF patients BALF specimens contained significantly reduced levels of RANTES compared to BALF specimens from asthmatics (48) and CF airway epithelial cells express little or no RANTES protein or mRNA compared with non-CF airway epithelial cells (49). There is an increase in IL-8 secretion by CF airway epithelial cells through activation of the NF-κB pathway (25-28,50,51) and a reduced ability of CF airway epithelial cells and $CD4^+$T- lymphocytes to produce IL-10 in response to inflammatory stimuli (52,53). Both lung and blood neutrophils of CF patients also produce increased amounts of IL-8 and reduced amounts of the anti-inflammatory cytokine IL-1 receptor antagonist (54).

Besides the pro-inflammatory and anti-inflammatory disbalance in CF lungs and the locally produced bacterial toxins and inflammatory mediators, regulation of neutrophil activation, function and accumulation in the CF airways seems altered (55-57). CF neutrophils show different responses with regard to cytokine production, intracellular pH, myeloperoxidase-dependant oxidant generation, elastase release, adherence to airway epithelial cells, and migration compared to neutrophils from healthy controls or patients with bronchiectasis (54,58-62).

Several hypotheses have been postulated to clarify the pathophysiological mechanism linking mutant CFTR to pulmonary infection and inflammation. One of these hypotheses is the inflammation-first hypothesis. This hypothesis is supported by the finding that pulmonary inflammation is present in infants diagnosed by neonatal screening before infection. (33-37,63). Also in severe combined immunodeficient mice grafted with CF fetal trachea, CF airways were shown to be in a pro-inflammatory state before any infection is present (64).

Studies by other investigators dispute the inflammation-first hypothesis and emphasize that inflammation follows infection (39,65). Whether inflammation or infection comes first is still a matter of debate, but it is clear that the excessive inflammatory response not only fails to clear infection, but contributes to lung damage and progression of CF lung disease (see figure 1).

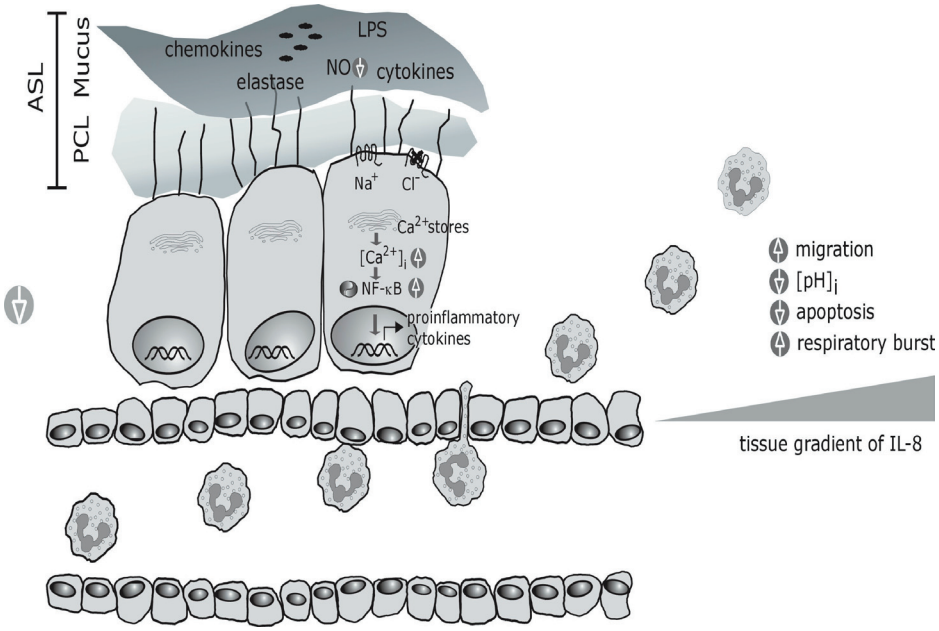


Figure 1. Role of airway epithelial cells and blood neutrophils in the chronic inflammatory response in the CF lung (see text for further explanation).

MONITORING CF RELATED LUNG DISEASE

In both clinical management and in clinical research trials, monitoring of pulmonary function tests (PFTs), especially FEV_1 measured by spirometry, is used to assess CF lung disease progression (66-68). Spirometry can be performed reliably from the ages of 5-6 years and several different pulmonary function measurement techniques have been developed that

can be performed in preschool children. One of these methods is the interrupter resistance technique (Rint), a method that measures resistance of the airways, lung tissue and chest wall. Rint can be performed in young children without sedation, but there are only a few studies evaluating Rint in young children with cystic fibrosis (69-71).

Structural lung damage can be assessed by an annual chest radiograph that can be scored according to several cystic fibrosis chest radiograph scoring systems, like the Shwachman-Kulczycki and the Chrispin-Norman scoring systems (72,73). Another tool to assess lung structure is high-resolution computed tomography (HRCT) scanning (74,75). HRCT scanning is more sensitive than chest radiographs, especially in the early detection of CF lung disease, however implementation in daily CF care is hampered by the higher radiation exposure to the patient compared to a chest radiograph, higher costs, and the need for sedation and control of breathing in young children.

MONITORING AIRWAY INFLAMMATION

Bronchoalveolar lavage fluid (BALF)

Airway inflammation plays a crucial role in the progressive lung damage seen in CF. In order to assess airway inflammation, several reliable techniques have been developed. In BALF, increased levels of pro-inflammatory cytokines like tumor necrosis factor (TNF)-α and IL-6 and chemokines like IL-8 have been described (33-37,53,63,76-77). Analysis of neutrophil count and levels of inflammatory mediators in BALF seem to provide an accurate picture of airway inflammation. Routine bronchoscopy with BAL is however invasive and unpleasant for the patient, thus limiting its use in daily clinical practice especially in longitudinal studies. Therefore less invasive, surrogate markers of inflammation, measured in peripheral blood and exhaled breath have been evaluated.

Exhaled breath

Nitric oxide (NO) is a free radical gas and messenger that is important in host defense, inflammation, vasodilatation and bronchomotor control (78). NO is formed from L-arginine by NO synthase (NOS) and the inducible isoform of NOS, called NOS2, is upregulated following an inflammatory stimulus. NO concentrations are increased in inflammatory lung diseases like asthma and brochiectasis (79,80) but are decreased in CF (81,82). There have been several explanations for this decrease in NO in CF lung disease. These include mechanical retention of NO in CF mucus (83), a lack of NOS2 expression in CF airway epithelium related to CFTR dysfunction (44,45), or an increase in arginase activity as has been shown in a recent study (84).

Exhaled carbon monoxide (CO) concentration, usually quantitated in end tidal breath (ETCO), has been described in several studies as a candidate marker for airway inflammation in lung diseases like CF (85-91). The predominant source of endogenously produced CO

(about 85%) is produced by the enzymatic degradation of heme by heme oxygenase (HO-1 and HO-2) (92-94). The enzyme HO-1 has been identified as a stress response protein and is induced by several conditions and agents like cytokines, oxidant stress and heat shock (95).

Inflammation in the lung in CF causes an increase in oxidative stress, which can cause an induction of HO-1 and subsequently an increase in exhaled CO.

Peripheral blood

In plasma and serum of CF patients, increased levels of TNF-α, IL-8, IL-6 and soluble adhesion molecules such as soluble intercellular adhesion molecule (sICAM)-1 and E-selectin are found, suggesting that the increased inflammatory activity in the CF lung is reflected in the systemic circulation (61,76,96-98). These studies were performed in CF patients with moderate to severe lung disease. Data on cytokine levels in blood of CF patients with mild lung disease are lacking.

Since neutrophils play a key role in CF inflammation, measurement of neutrophil preactivation (also called priming) and activation in peripheral blood can be of use in the assessment of inflammation. Priming and activation of neutrophils can be assessed for example by measuring myeloperoxidase-dependent oxidant production, by measuring phagocyte opsonin receptor response capacity or by measuring adhesion surface molecule expression e.g. $\alpha_m\beta_2$ integrin chain (CD11b/CD18) (59, 99-101).

ANTI-INFLAMMATORY THERAPY IN CF

Since inflammation plays a key role in CF lung disease, controlling the inflammatory process with anti-inflammatory drugs may be beneficial in managing CF lung disease. Despite potential benefits of anti-inflammatory therapy, both the decision to treat and the selection of the most appropriate therapeutic agent are controversial.

High dose ibuprofen significantly slowed the progression of lung disease without serious side effects (102) but this anti-inflammatory drug is not widely used in CF care. This is caused by the need for frequent serum ibuprofen measurements to avoid undesired effects associated with either too low or too high levels of ibuprofen, including perceived risk of gastric bleeding. Corticosteroids are very powerful, non-specific anti-inflammatory agents. The three main mechanisms through which corticosteroids display their anti-inflammatory and immunosuppressive effects are through direct effects on gene expression by the binding of glucocorticoid receptors to glucocorticoid-responsive elements (inducing annexin 1 and mitogen-activated protein kinase (MAPK) phosphatase 1), indirect effects on gene expression through interactions of glucocorticoid receptors with other transcription factors (blocking transcription of inflammatory proteins by NF-κB and activator protein 1), and glucocorticoid receptor-mediated effects on second-messenger cascades (activating phosphatidylinositol-

3-hydroxykinase (PI3K) finally resulting in production of NO) (103). In CF patients, alternate-day prednisone treatment reduced the frequency of pulmonary exacerbations and improved pulmonary function (104). The significant side effect profile (e.g. reduced linear growth and abnormal glucose tolerance) of oral glucocorticoids however have precluded their use on a routine basis in CF care (105-107). Inhaled corticosteroids (ICS) have localised anti-inflammatory actions in the airways and a relatively high safety profile. Therefore, several studies evaluating the effects of ICS in CF patients have been performed and reviewed (108-114). Most of these studies however have been performed in adolescent or adult CF patients with irreversible lung damage. The studies that have been performed in children are mostly of (too) short duration, using different dosages (possibly too low dosages) and different inhaled corticosteroids to be conclusive as to whether inhaled corticosteroids are beneficial in CF.

More recent studies describe the anti-inflammatory effects of oral macrolide antibiotics (e.g. azithromycin) on pulmonary function and pulmonary exacerbations (115,116). Currently there are several experimental and promising novel targets for anti-inflammatory therapy including modulation of calcium clearance and calcium influx mechanisms of neutrophils but clinical trials in CF patients are lacking (117).

1.2 SCOPE AND OUTLINE OF THIS THESIS

Inflammation is a hallmark of CF lung disease and neutrophils and airway epithelial cells are key players in the inflammatory cascade in CF lung disease. There are several invasive methods to analyze inflammation and less invasive alternatives for measurement of inflammation in CF are urgently needed. Controlling inflammation seems to be of great importance in improving morbidity and long term survival in CF patients. The studies described in this thesis are focused on young children with CF and address 3 main questions:

1. Is systemic inflammation already present in clinically stable young children with CF and if present is this pro-inflammatory phenotype intrinsic and CFTR-related or caused by environmental factors?
2. Can we measure inflammation in a non-invasive way in young children?
3. Can the inflammatory process be influenced by anti-inflammatory therapy, using inhaled corticosteroids, started at an early age?

In **Chapter 2** an overview of the role of airway epithelium and neutrophils in CF lung inflammation is presented. **Chapter 3** is focused on fMLP-induced intracellular calcium (Ca^{2+}_i) responses and migration of peripheral blood neutrophils of clinically stable CF patients and neutrophils differentiated from cord blood $CD34^+$ progenitor cells of a CF newborn and a healthy newborn to clarify whether neutrophils in CF display intrinsic CFTR-related altera-

tions. In **Chapter 4** we analyze possible measures of inflammation that can be assessed in peripheral blood. In **Chapter 4.1** neutrophil priming phenotypes using expression of the $\alpha_m\beta_2$ integrin chain and newly developed priming associated cellular markers (MoPhabs A17 and A27) in peripheral blood are assessed. In **Chapter 4.2** mulitplex cytokine profiles in plasma are measured and compared with healthy control children. **Chapter 5** describes ETCO measurements in clinically stable CF patients. **Chapter 6** is focused on the role of CF chest radiograph scoring systems in monitoring structural lung damage and disease progression. In **Chapter 6.1** the relationship between CF chest radiograph scoring systems, PFTs, and other clinical parameters of disease is described. **Chapter 6.2** describes longitudinal changes in chest radiograph scores and PFTs ($Rint_{exp}$ and spirometry) in young children with CF. In **Chapter 7** the effects of the inhaled corticosteroid, hydrofluoroalkane beclomethasone dipropionate (HFA-BDP, Qvar®) on lung function, inflammation in peripheral blood, chest radiograph scores, bacterial colonization, ETCO, and quality of life is evaluated in 57 young children with CF. Finally, the main findings and relevance for clinical practice are discussed in **Chapter 8,** followed by a summary in English and Dutch in **Chapter 9**.

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Chapter 2

The role of airway epithelium and blood neutrophils in the inflammatory response in cystic fibrosis

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ABSTRACT

Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, which accounts for the cAMP-modulated chloride conductance of airway epithelial cells. CFTR also regulates other membrane proteins like the negative regulation of the amiloride-sensitive epithelial sodium channel (ENaC). Mutations in the CFTR gene lead to hyperabsorption of sodium chloride and a reduction in the periciliary salt and water content which leads to impaired mucociliary clearance. It seems that a lack of functional CFTR leads to abnormal function of the NF-κB pathway in submucosal gland cells, causing an increased production of pro-inflammatory cytokines and the chemokine IL-8, and a pro-inflammatory environment. CFTR is also expressed in neutrophils and several neutrophil functions like cytokine production, migration, phagocytosis and apoptosis seem altered in CF. In this review we describe the role of airway epithelium and blood neutrophils in the viscious circle of inflammation and infection seen in CF.

1. INTRODUCTION

The hallmarks of the lung pathology in CF are bacterial colonisation and infection of the airways (particularly with *Pseudomonas aeruginosa* and *Staphylococcus aureus*) and an apparently exaggerated, sustained and extended inflammatory response, characterised by influx of neutrophils and high concentrations of interleukin-8 in bronchoalveolar lavage fluid (BALF) (1-8). Several pathophysiological mechanisms have been postulated to explain the pulmonary infections and inflammation. In this review we will focus on the role of mutant CFTR on various aspects of the airway epithelium including mucociliary clearance, submucosal gland cell products and bacterial binding. We will furthermore focus on the role of neutrophils in airway inflammation in CF, since these cells play a key role in the ongoing inflammatory response.

2. DEFENCE MECHANISMS BY AIRWAY EPITHELIUM

2.1. Mucociliary clearance

Airway epithelium is covered on its apical surface by a thin liquid layer called the airway surface liquid (ASL). The ASL is the first line of defence against inhaled pathogens and is mandatory for effective mucociliary clearance (9). ASL is composed of a mucus gel and a periciliary sol layer (PCL) (10) that are propelled upwards by coordinated ciliary beating. The PCL of the ASL is an aqueous solution with a height, equalling the height of extended cilia (~7µm), and a relatively low viscosity enabling effective ciliary beating and cell surface lubrication. The mucus layer is a gel-like aqueous layer in which soluble compounds are mixed with mucus polymers and aggregates of mucins and other high molecular-weight glycoproteins, proteoglycans, defence molecules (like lactoferrin, lysozyme and defensins), DNA, and actin. The two major mucins present in human respiratory mucosa are MUC5AC and MUC5B (11,12). The diversity of the carbohydrate side chains within the mucin gel allows entrapment of a wide variety of particles, like bacteria, for ultimate clearance from the airway (13).

Effective mucus transport requires a well defined PCL liquid layer and the capacity of the airway epithelium and mucus layer to maintain the PCL layer at the appropriate height (14). The mucus layer has the propensity to swell and shrink by accepting liquid from or donating liquid to the PCL layer as needed (15). The airway epithelium seems to have the capacity to both absorb liquid from the PCL and to secrete liquid into the PCL as necessary, a process that is believed to be mediated by isotonic (100-150 mM NaCl) volume transport (16-18). Still relatively little is known about this tightly regulated process such as: what are the sensors of ASL volume and how is information transmitted to the various effectors (ion channels) in the apical cell membrane (15). Under physiological conditions, the airway epithelium defence system, by virtue of endogenously produced antimicrobial peptides like lactoferrin and ly-

sozyme can suppress bacterial growth for 3 to 6 hours (19), after which bacteria are cleared from the airways by mucociliary clearance within 6 hours (13).

In CF, loss of CFTR function results in altered salt transport of the airway epithelium. This has lead to the proposal of basically two hypotheses, addressing the mechanism by which altered salt transport, associated with the loss of CFTR, leads to a breakdown of lung defence mechanisms in CF and to persistent endobronchial infections (20,21). The first hypothesis is the *isotonic* "low volume" hypothesis with resultant abnormalities in mucociliary clearance. This hypothesis proposes that a lack of CFTR inhibition of epithelium sodium channels (ENaC) causes increased Na^+ absorption. On top of this mutant CFTR also fails to initiate cAMP-dependent Cl^- secretion. This causes an increased water absorption, leading to a decreased volume of the PCL components of the ASL (see Fig.1) (22,23). The decrease in ASL volume also decreases mucociliary clearance and causes mucus stasis. The concentrated mucus adheres to the cell surface leading to a reduced efficacy of mucociliary clearance by coughing. The ciliated cells require an increased metabolic activity to sustain the excessive salt and water absorption and this, together with the accumulated mucus leads to hypoxia in adherent mucus plaques near the epithelial cell surface (24,25). The combination of mucus plaques and mucus hypoxia probably promotes the accumulation of bacteria. The innate antimicrobial peptides present in the mucucs are no longer able to control bacterial growth, and the formation of bacterial biofilms can result in the acquisition of chronic bacterial infection (26). An important argument in the low volume hypothesis is ENaC dysregulation. Several studies have shown that CFTR functions as a regulator of ENaC (27-30) but the exact molecular interaction between CFTR and ENaC remains to be elucidated.

The second and opposite hypothesis is the "compositional" hypothesis with hypertonic ASL salt concentrations in CF, inactivating salt-sensitive antimicrobial peptides (31). This hypothesis proposes that a lack of CFTR leads to defective cellular chloride absorption, leading to an increased Cl^- concentration of ASL in patients with CF. This increased Cl^- concentration of ASL inactivates the salt-sensitive antimicrobial peptides like defensins (31-33). More recently studies in mouse-models on the ASL ionic composition as well as studies in CF-patients however failed to detect differences in ASL ionic composition between normal and CF, i.e. both normal and CF ASL are isotonic (34-36). These results therefore would favour the isotonic "low volume" hypothesis but the "compositional" hypothesis can not be entirely refuted yet. It should be kept in mind that there are significant technical limitations of collecting and assaying ASL from the upper and lower airways and it is not feasible yet to perform the necessary experiments in vivo, in actual human airways.

2.2. Submucosal gland cell products

Serous cells secrete a variety of nonmucin products like lysozyme, lactoferrin, secretory IgA, peroxidase and protease inhibitors (38). They also secrete several defensins, salt-sensitive antimicrobial substances important in airway defence (31,39-40). CFTR is highly expressed in

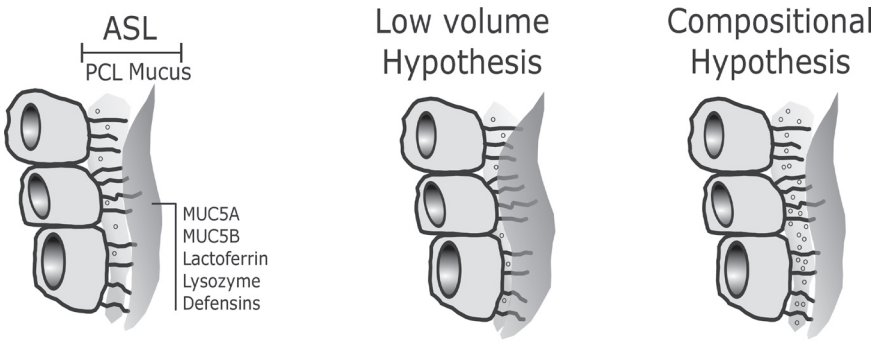

Figure 1. Hypothetical models on ASL dysregulation in CF (see text for further explanation).

the serous epithelial cells of submucosal glands (41) and these serous cells are considered to be the primary defence cells of the airway mucosa (38). The presence of CFTR in the secretory granules of serous cells (38) suggests that CFTR contributes mechanistically to secretion of glycoproteins, either by providing a regulated Cl^- conductance in secretory granule membranes or by contributing to cAMP-mediated secretory vesicle exocytosis. Loss of CFTR function may lead to an alteration in the macromolecular composition of the serous cell secretions and thereby also change mucus viscosity and may adversely affect mucociliary clearance (13,37,42-43).

In CF the bronchial and tracheal gland cells show an altered cytokine production profile with a significant decrease of IL-10 content and a reduced ability of CF epithelial cells (and $CD4^+$T- lymphocytes) to produce IL-10 in response to inflammatory stimuli (44-46). Furthermore there is a constitutive upregulation of the pro-inflammatory chemokine IL-8 by CF airway epithelial cells and an increased IL-8 mRNA expression in bronchial epithelial cells in young CF patients (44,47-52). The synthesis of all these proinflammatory cytokines and chemokines elevated in CF is regulated by the transcription factor nuclear factor-κB (NF-κB) (53). In epithelial cell lines derived from CF-patients a higher and constitutive activation of NF-κB has been described (49-50,54). It is suggested that a lack of functional CFTR leads to abnormal function of the NF-κB pathway (49,55). It is however also suggested that a decrease or absence in IL-10 locally, which is described in patients with CF (44,56), causes increased expression of IκB α/β kinases. IκB α/β kinases are required for NF-κB activation (49). The combination of reduced IL-10 and increased IκB α/β kinases may lead to uncontrolled NF-κB activation (see also Fig.2) (57).

2.3. Bacterial binding and colonization

CFTR appears to play an indirect, but also a direct role in the ability of CF pathogens like *S. aureus*, *H. influenzae* and *P. aeruginosa* to bind to epithelial membrane in cell culture systems

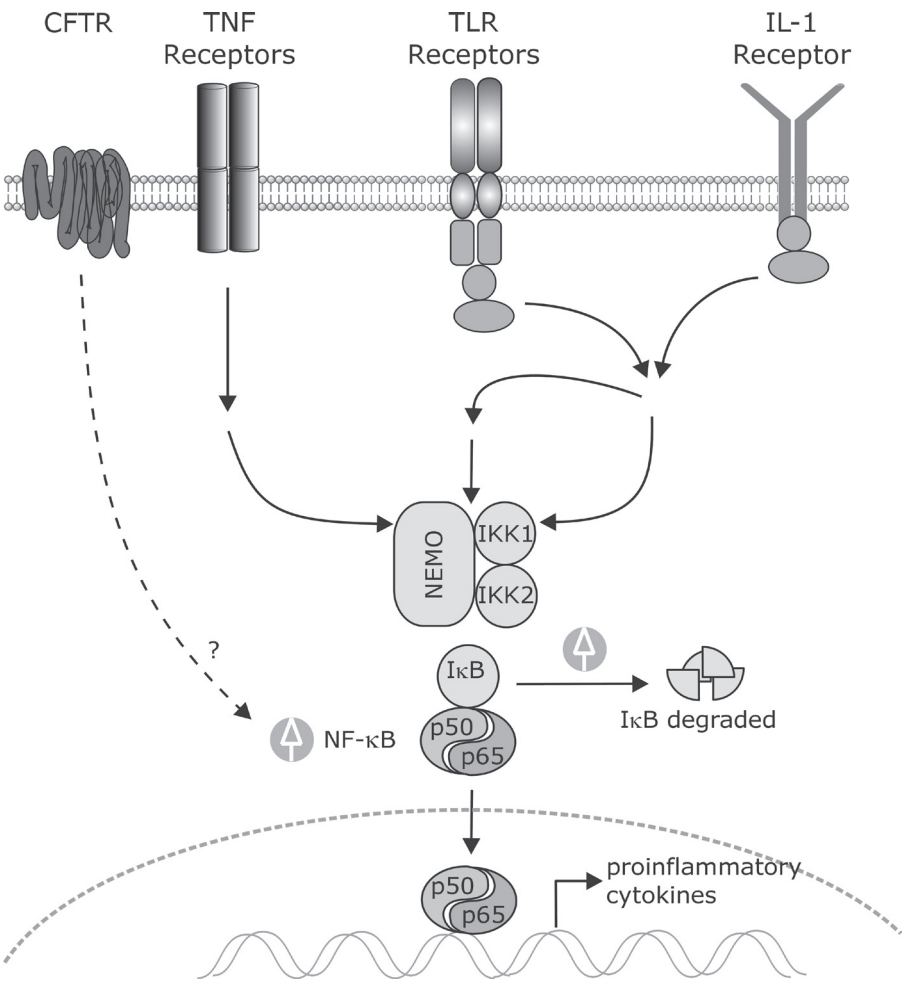

Figure 2. Central role of NF-κB in signalling routes of inflammation receptors. Pathways/activities found to be upregulated in CF indicated by circled arrowheads.

(58-60). The finding that significant numbers of bacteria do not bind to differentiated epithelia of native airway secretions (61-62) but are bound to injured epithelia (63-66) suggest that direct epithelial-bacterial cell interactions require antecedent epithelial injury. Bacterial-epithelial adhesion may be important once infection and inflammation have caused epithelial injury but appears less likely to play a critical role in the initiation of infection.

A direct role for CFTR is suggested by the finding that CFTR serves as a pattern recognition molecule for *P. aeruginosa* lipopolysaccharide-core oligosaccharide. Expression of mutant CFTR leads to a defective internalisation of *P. aeruginosa* and defective epithelial cell phago-

cytosis of these bacteria (67-69) The relative importance of epithelial phagocytosis in innate immune defense against *P. aeruginosa* is uncertain.

2.4. Neutrophils

Neutrophils are the major cellular players of the innate immune system, both by phagocytosis of infectious agents but also by their capacity to limit the growth of some microbes. Both mechanisms serve to keep an infection under control until adaptive (specific) immunological responses can develop.

2.5. Priming

Neutrophils exist in a dormant (resting) state unless they become activated. Over the last decade it has become clear that this "all or nothing" scenario is too simplistic and that various intermediate stages exist, such as the primed state. Priming is a mechanism whereby dormant neutrophils acquire a state of preactivation that generates a more powerful response to microbial activity (70). In patients with CF, neutrophils show an increased primed phenotype measured by expression of phagocyte opsonin receptors, like complement receptor (CR)1 (CD35) and CR3 (CD11b), response capacity, even in uninfected children with CF (71). These data, which are compatible with data from our own group (72) may point towards CF as a disease with autoinflammatory characteristics.

2.6. Cytokine and chemokine production

During an inflammatory response, chemotactic factors of different origin and pro-inflammatory cytokines signal the recruitment of neutrophils to sites of infection and/or injury. Not only CF epithelial cells, but also CF neutrophils display an abnormal release pattern of inflammatory mediators. Blood neutrophils from patients with CF constitutively secrete higher amounts of IL-8 and lower amounts of the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1RA) (73). Spontaneous release of IL-8 and IL-1RA by CF airway neutrophils is even higher, indicating a modification of the response by the local environment (73). The finding that spontaneous release of IL-8 was significantly lower in airway neutrophils from children with dyskinetic cilia syndrome however provides support for a genetic component.

2.7. Migration

Neutrophil migration to the site of infection/inflammation is a multistep process that consists of tethering (capture), rolling, slow rolling, firm adhesion and transmigration (see also Fig. 3) (74-75). The initial event is caused by the appearance of new adhesion molecules (E-selectin and P-selectin (pre-stored in the Weibel-Palade bodies) on the endothelium adjacent to the inflamed site. Expression of these adhesion molecules is induced by inflammation mediators released by damaged tissues, like TNF-α and bacterial lipopolysaccharide (LPS). In postcapillary venules, the slow flow rate allows for a short transient interaction (or tethering) between

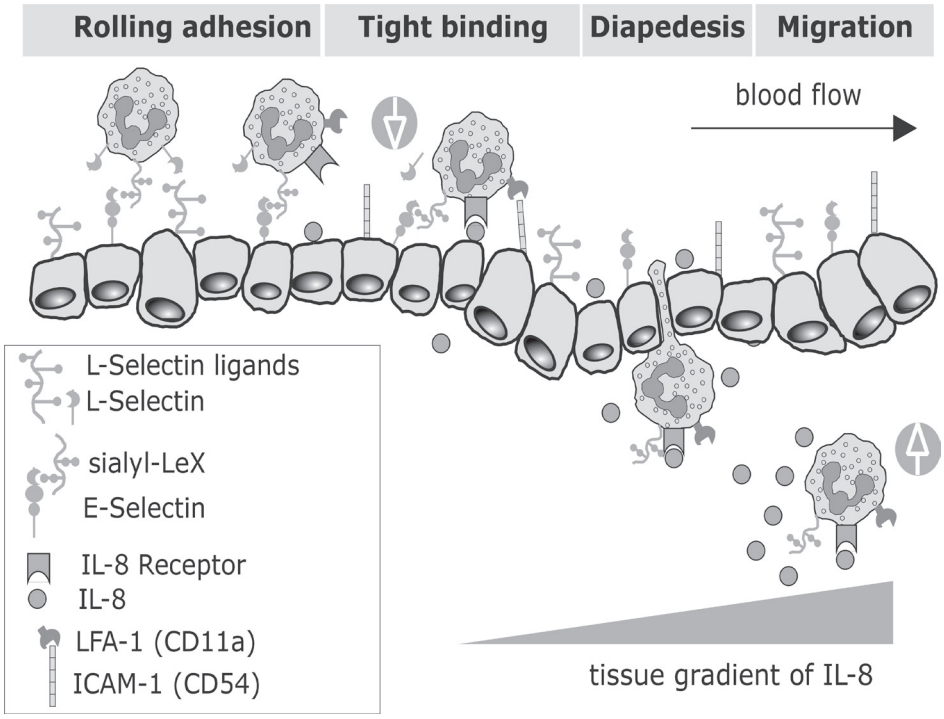

Figure 3. Cellular and molecular interactions during neutrophil migration. Pathways/activities found to be upregulated or downregulated in CF indicated by circled arrowheads. See text for further details.

the neutrophil and the endothelium. Neutrophils roll along microvascular walls via low affinity interaction of selectins (like L-selectin (CD62L), present on neutrophils and E-selectin on endothelial cells) with specific endothelial carbohydrate ligands. This leads to the activation of neutrophil-integrins (for example $\alpha_m\beta_2$ (CD11b/CD18, also known as CR3)) and subsequent firm adhesion to intercellular adhesion molecules on the surface of activated endothelial cells in postcapillary venules. Under the influence of a locally generated chemotactic gradient and by diffusion of chemoattractants from the infection site, neutrophils penetrate the endothelial layer and migrate through connective tissue to sites of inflammation/infection (transmigration), where they finally congregate and adhere to extracellular matrix components such as laminin and fibronectin. In the pulmonary capillaries however selectin-mediated rolling of neutrophils does not occur, presumably due to spatial constraints (76) and selectins are often even not required during the acute response of neutrophils (77-78).

Two different pathways have been described through which neutrophils can emigrate in the alveoli and distal bronchioles that are fed by the pulmonary circulation. Neutrophils can emigrate through a pathway that requires CD11/CD18 and one that does not require

CD11/CD18 (79). Neutrophil emigration in response to acute *P. aeruginosa* exposure occurs through adhesion pathways that require CD11/CD18. Via this pathway shedding of L-selectin only occurs after the neutrophil has emigrated from the circulation into the alveolar interstitium (80). In contrast, chronic *P. aeruginosa* exposure shifts the migration pathway to the CD11/CD18-independent route, and is accompanied by a decrease in the number of neutrophils migrating to the lung (81-82). In this pathway L-selectin shedding occurs in the vessels (80). Neutrophils from both CF and non-CF subjects showed similar up-regulation of CD11b, but CF neutrophils showed significantly less L-selectin shedding than control subjects upon stimulation with IL-8 or fMLP (83). It has therefore been suggested that the reduced L-selectin shedding observed in CF patients may reflect the maintenance of a heightened 'acute-type' (CD18-dependent) response to *P. aeruginosa*.

Migratory response towards IL-8 in neutrophils from clinically stable CF patients is increased (84). Neutrophils from acutely infected patients with CF however show decreased chemotaxis, possibly due to bacterial exoproducts, and show lower numbers of IL-8 receptors (85-86). Reduced responsiveness of CF neutrophils to IL-8 seems to be associated with receptor desensitisation as a result of exposure to high systemic levels of IL-8 and this might contribute to the persistence of chronic endobronchial infections (85-86).

2.8. Phagocytosis

Once neutrophils have transmigrated to the site of infection phagocytosis can begin. This is a complex process consisting of several morphological and biochemical steps. After recognition and particle binding to the phagocyte surface, ingestion (engulfment), phagosome origination, phagolysosome formation (fusion of phagosome with lysosomes), killing and degradation of ingested cells or other material proceed. Antimicrobial efficiency of neutrophils is dependent on the generation of reactive oxygen species (ROS) by assembly and activation of NADPH-oxidase. Activation of the oxidative metabolism, known as the respiratory burst first involves NADPH-oxidase, an enzymatic complex that generates superoxide anion (O_2^-) which can dismutate in H_2O_2. Generation of O_2^- leads to production of various reactive oxidants, including halogenated oxidants generated through the myeloperoxidase (MPO) pathway. MPO, an enzyme contained in azurophilic granules of neutrophils, catalyzes the H_2O_2-dependent oxidation of chloride (Cl^-) to hypochlorous acid (HOCl).

In neutrophils many functional responses, including triggering of secretion in azurophilic granules (containing neutrophil elastase (NE) and MPO), oxidant production and microbe killing, are pH dependent. Intracellular pH in CF neutrophils after phorbol ester activation is more acidic, indicating an intrinsic defect in CF neutrophil pH regulation possibly linked to mutant CFTR (87). This "hyperacidification" has also been described in trans-Golgi network in CF lung epithelial cells (88). Neutrophils of both CF homozygotes and heterozygotes display normal NADPH activity but increased myeloperoxidase-dependent oxidant activity (89).

CF neutrophils also release increased amounts of neutrophil elastase in response to TNF-α and IL-8 (90). NE plays a major role in the pathophysiology of chronic inflammation in CF (91-92). It directly contributes to tissue damage by degrading structural proteins, such as elastin, collagen, and proteoglycans, and has many other detrimental biological activities in the CF airways. NE enhances macromolecular secretion from serous gland cells and promotes hypertrophy and hyperplasia of the mucus-secreting apparatus and inhibits ciliary beating *in vitro*. NE also facilitates the persistence of infection by cleaving immunoglobulins, complement components and opsonin receptors, such as CR1, on the surface of phagocytes, and thus has an important impact on opsonophagocytosis (93). These effects may further impair mucociliary and bacterial clearance and exacerbate airway obstruction in CF patients.

2.9. Apoptosis

After succesfull elimination of an invading pathogen, the inflammatory response should come to an end in order to avoid further tissue damage. Therefore, neutrophils ultimately are removed by apoptosis. CF pathogens like *P. aeruginosa* and *S. aureus* stimulate epithelial expression of G-CSF and GM-CSF, thereby counteracting induction of apoptosis and thus prolonging the inflammatory response (94). The hyperacidification of CF cells as mentioned above also has an anti-apoptotic effect (95).

In summary there is a complex pathophysiological cascade leading to lung damage in CF, as summarized in figure 4. The resultant excessive inflammatory response not only fails to clear infection, but contributes to its persistence and is mostly responsible for lung damage and the progression of CF lung disease. Next to keeping control over the infections, it is therefore probably equally important to keep control over the (pulmonary) inflammation in CF.

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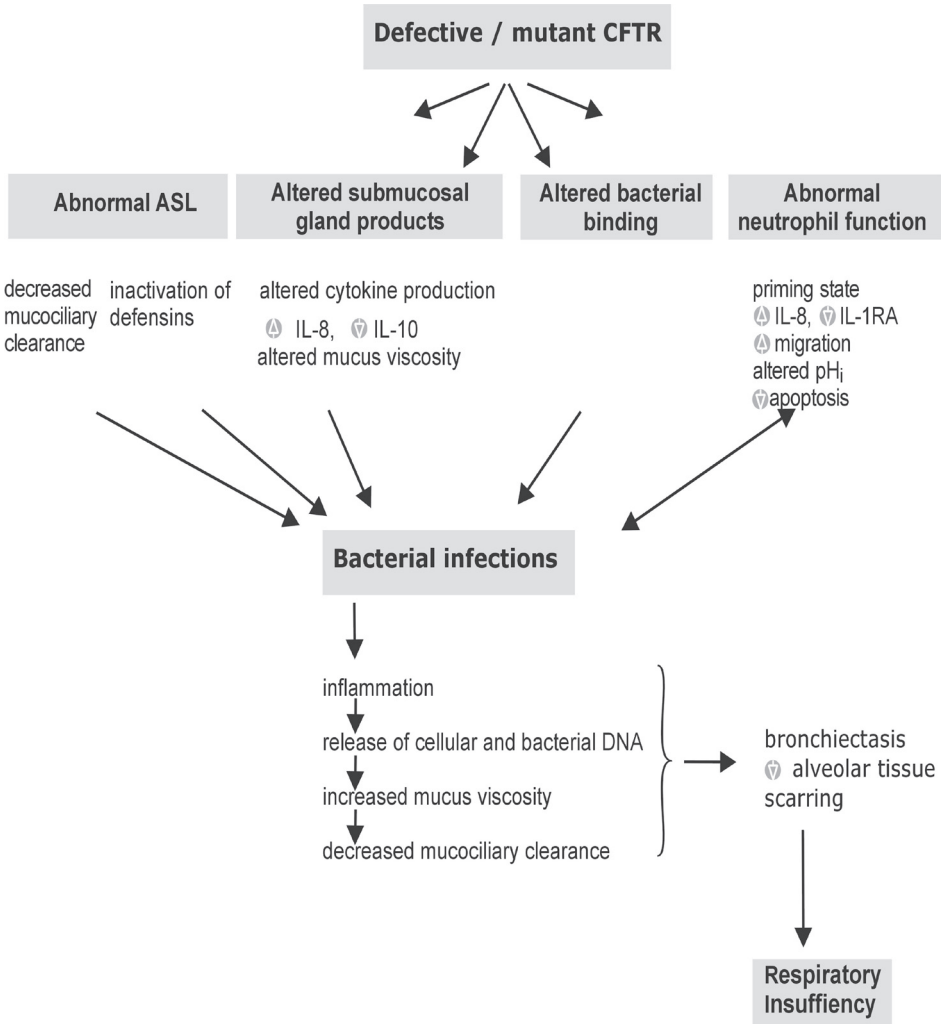

Figure 4. Summary of proposed pathophysiological links between defective/mutant CFTR, airway defence mechanisms and neutrophils in the development of CF lung disease. Defects in CFTR lead to abnormal airway surface liquid, altered submucosal gland cell products and to abnormal neutrophil functions. Via mechanisms outlined, bacterial infection results and supports the vicious circle of ongoing inflammation and infection in CF lung disease. This further impairs mucociliary clearance and promotes chronic infection.

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Chapter 3

Intracellular calcium mobilization response is increased in cystic fibrosis blood neutrophils

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Submitted

ABSTRACT

Rationale: Cystic fibrosis (CF) lung disease is characterized by a neutrophil-dominated inflammation. Intracellular calcium (Ca^{2+}_i) is an intermediate in cellular signaling pathways regulating important neutrophil functions.

Objectives: To test the hypothesis that CF neutrophils exhibit enhanced N-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced Ca^{2+}_i mobilization responses and migration compared to healthy control neutrophils.

Methods: Ca^{2+}_i mobilization induced by the innate immune activator fMLP was measured in blood neutrophils of 9 CF patients and 9 healthy controls using the fluorescent Ca^{2+} indicators fluo-3 and fura red. Ca^{2+}_i mobilization was also studied in *ex-vivo* differentiated neutrophils from cord blood $CD34^+$ progenitor cells of a CF newborn and a healthy newborn. Migration of blood neutrophils from 4 CF patients and 4 healthy controls were measured before and after activation with fMLP using time-lapse video microscopy.

Measurements and main results: FMLP induced more pronounced increases in Ca^{2+}_i responses in CF neutrophils compared to healthy control neutrophils with respect to Ca^{2+}_i mobilization (peak ratio 6.2 ± 0.9 versus 4.3 ± 0.7, p=0.001). Priming of healthy control neutrophils with the systemic immune activator TNF-α did not affect these Ca^{2+}_i responses. *Ex-vivo* differentiated neutrophils from a CF newborn already showed this phenotype illustrated by greater Ca^{2+}_i responses after stimulation with fMLP than cells from a healthy control newborn. The overall distance of migration was increased in CF neutrophils. Depletion of Ca^{2+}_i resulted in an abrogated migration.

Conclusion: CF blood neutrophils show enhanced fMLP-induced Ca^{2+}_i mobilization responses and migration and this pro-inflammatory phenotype is already present at birth.

INTRODUCTION

Cystic fibrosis (CF) is caused by mutations of the cystic fibrosis conductance regulator (CFTR) gene, resulting in a defective cAMP-dependent chloride ion (Cl^-) conductance and enhanced Na^+ absorption across CF airway epithelia. The abnormal ion transport in CF leads to a reduction in the volume of the airway surface liquid (ASL) (1) causing decreased mucociliairy clearance and mucus stasis. This in turn leads to chronic airway infection and neutrophil-dominated inflammation (2-4). Human airway epithelial cells express two types of Cl^- channels: the cAMP-dependent Cl^- conductance channel affected in CF, and Ca^{2+}-activated Cl^- channels (CaCC), which are preserved in CF airways. CaCC thus provide a compensatory mechanism to bypass the Cl^- secretory defect in CF and may (partly) restore the defective mucociliairy clearance (5). Airway epithelial cells of CF patients exhibit larger intracellular calcium (Ca^{2+}_i)-dependent responses, e.g. Ca^{2+}_i-enhanced mucociliairy clearance (6), but also Ca^{2+}_i-dependent interleukin (IL)-8 secretion (7), which enhances inflammation by recruitment of neutrophils.

Neutrophils are key players in airway inflammation in CF. Many of the pro-inflammatory activities of neutrophils such as activation of the respiratory burst induced by N-formyl-methionyl-leucyl-phenylalanine (fMLP) are initiated by changes in Ca^{2+}_i (8,9). Ca^{2+} has also been implicated in the regulation of migration of neutrophils on specific substrates such as fibronectin or vitronectin (10-12). Transient increases in cytosolic Ca^{2+}_i are needed for the release of integrins from their attachments and recycling to the front of the cells, leading to de-adhesion at the cell rear (10-12). However, migration of neutrophils can also be induced by depolymerization of microtubules, through the Rho/Rho-kinase pathway that is independent of increases in cytosolic Ca^{2+}_i (13). Therefore, identification of the exact role of Ca^{2+} transients in neutrophil migration still requires further studies (14). Recent insights into antagonism of changes in Ca^{2+}_i in activated neutrophils have led to the identification of potential novel targets for anti-inflammatory therapy in neutrophil-dominated inflammatory disorders such as CF and chronic obstructive pulmonary disease (COPD) (15).

Data from previous studies evaluating Ca^{2+}_i homeostasis in CF neutrophils have been inconclusive (16,17), yet, altered neutrophil functions with regard to modulation of intracellular pH (18), myeloperoxidase-dependent oxidant generation (19), adherence to airway epithelial cells (20), and directed migration to interleukin (IL)-8 in CF patients have been described (21). These findings combined with the demonstration of CFTR mRNA transcripts in various non-epithelial cells, including neutrophils (22), might lead to the hypothesis that CF neutrophils might display an intrinsic CFTR-related defect. Since Ca^{2+}_i –dependent responses are increased in CF airway epithelial cells and Ca^{2+}_i is an intermediate in cellular signaling pathways regulating important neutrophil functions, we hypothesized that neutrophils from CF patients have an altered Ca^{2+}_i mobilization response compared to healthy controls. Such a difference might have functional consequences such as an altered Ca^{2+}-mediated neutrophil activation in CF patients.

We studied Ca^{2+}_i mobilization and migration of blood neutrophils of CF patients and healthy controls. We next addressed the question whether the observed differences in Ca^{2+}_i responses can be explained by an intrinsic CFTR-related defect in neutrophils or by environmental factors such as a specific cytokine environment. We had the unique opportunity to study *ex-vivo* differentiated neutrophils, derived from cord blood $CD34^+$ progenitor cells, from a CF newborn and a healthy control newborn. Effects of environmental factors (priming neutrophils with tumor necrosis factor-alpha (TNF-α)) were studied in healthy control neutrophils.

MATERIALS AND METHODS

Study subjects

Nine children with CF (5 male, mean (SD) age 14.7 (2.9) years) attending the outpatient Pediatric Clinic of the Cystic Fibrosis Centre of the University Medical Centre Utrecht (UMC Utrecht), were included. Only children without signs of an acute pulmonary exacerbation at the time of blood sampling were included. Eight of the 9 CF patients were colonized with *Pseudomonas aeruginosa* and one with *Staphylococcus aureus*. Nine healthy controls (5 male, mean (SD) age 26.6 (4.2) years) were selected from the clinical and laboratory staff of the UMC Utrecht and had no signs of an infection at the time of blood sampling. The study was approved by the Medical Ethics Committee of the UMC Utrecht and informed consent was given by parents and children when applicable.

Isolation of human neutrophils

Neutrophils were isolated as described previously (23). In short, mononuclear cells were depleted from neutrophils by density gradient centrifugation over isotonic Ficoll (Pharmacia,Uppsula, Sweden). Erythrocytes in the pellet fraction were lysed in a hypotonic NH_4Cl solution and neutrophils were washed and resuspended in incubation buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM glucose, 1 mM $CaCl_2$ and 0.5% human serum albumin). The total number of cells was counted, and the viability was determined by trypan blue exclusion.

Detection of changes in Ca^{2+}_i

Neutrophils (5-10x10^6/ml) were loaded with a combination of 10 μM fura red and 4 μM fluo-3 (fluorescent Ca^{2+} indicators; Molecular Probes, Eugene, OR). Cells were incubated at 37°C for 30 minutes, then washed and resuspended in RPMI-1640 medium at a concentration of 5 x 10^6/ml and were kept in the dark at room temperature until analysis. Before starting analysis cells were diluted in Ca^{2+}-containing assay buffer (composed of 145 mM NaCl, 5 nM KCl, 1 mM $Na_2PO_4.2H_2O$, 0.5 mM $MgSO_4.7\ H_2O$, 5 mM glucose, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4). In all experiments, isolated neutrophils were measured in resting state for 21 seconds. Then

neutrophils were stimulated with fMLP in a final concentration of 10^{-7} M. Data were recorded for a period of up to 286 seconds. Changes in Ca^{2+}_i in fluo-3 and fura red loaded cells were analyzed on a FACS Calibur Flowcytometer (Becton Dickinson, San Jose, CA). Fluo-3 / fura red fluorescence was measured by excitation at 488 nm and emission at 530 (fluo-3) and 625 nm (fura red). As quantitative parameters of the Ca^{2+}_i responses, the fold increase (highest value divided by baseline value) in fluo-3 / fura red ratio (peak ratio) was calculated, as well as the area under the curve.

Analysis of neutrophil migration

Neutrophil migration experiments were performed as described previously (24). In short, glass cover slips were coated with HEPES buffer containing 0,5% human serum albumin. Purified neutrophils (10^6 /ml in HEPES buffer) were first incubated at 37°C for 15 minutes. Neutrophils were allowed to attach to the cover slip for 15 minutes at 37°C. Medium was removed and the cells were washed twice with HEPES buffer. The cover slip was then inverted in a droplet of medium containing 10^{-7} M fMLP and sealed with a mixture of beeswax, paraffin, and petroleum jelly (1 : 1 : 1, w/w/w). Cell tracking at 37°C was monitored by time-lapse microscopy and analyzed by a custom-made macro (Arithmetic Language for Images; ALI) in image analysis software (Optimas 6.1; Media Cybernetics, Silver Spring, MD, USA). Cell migration was monitored for 10 minutes saving an image every 20 seconds. Migration patterns were measured ex vivo (in HEPES buffer) and after stimulation with fMLP.

Isolation of human cord blood $CD34^+$ progenitor cells

Cord blood was collected from a healthy newborn donor and from a CF newborn after informed consent was provided according to the Declaration of Helsinki. $CD34^+$ progenitor cells were isolated as previously described (25). In brief, mononuclear cells were isolated from umbilical cord blood by density centrifugation over a ficoll-paque solution (density 1.077 g/mL). Magnetic activated cell sorting (MACS; Miltenyi Biotech, Auburn, CA) using a hapten-conjugated antibody against CD34, which was coupled to beads, was used to isolate $CD34^+$ cells. $CD34^+$ cells were cultured in Iscoves modified Dulbecco medium (IMDM; Gibco, Paisley, United Kingdom) supplemented with 10% fetal calf serum (FCS), 50 µM -mercaptoethanol, 10 U/mL penicillin, 10 µg/mL streptomycin, and 2 mM glutamine at a density of 0.3 x 10^6 cells/mL. Cells were differentiated towards neutrophils using stem cell factor (SCF) (50 ng/mL), fms-like tyrosine kinase (FLT)-3 ligand (50 ng/mL), GM-CSF (0.1 nM), interleukin (IL)-3 (0.1 nM), and granulocyte-colony stimulating factor (G-CSF) (30 ng/mL). After 6 days of culture only additional G-CSF (30 ng/ml) was added to the cells according to protocol till day 17. At day 17 neutrophils were differentiated and measurements of fMLP-induced Ca^{2+}_i responses were performed as described above.

Statistical analysis

Results of the magnitude and kinetics of the fMLP-induced Ca^{2+}_i responses are expressed as mean ± standard deviation. Neutrophil migration results are expressed as mean ± standard error of the mean (SEM). Since Ca^{2+}_i and migration experiments were performed in parallel on the same day for CF-patients and controls, paired sample t-tests were used to compare CF neutrophils with healthy control neutrophils. Results of the combined experiments were compared using Student's unpaired t-tests. A two-tailed p-value of equal to or less than 0.05 was considered significant. All statistical tests were performed by using the statistical software package for the Social Science (SPSS version 12.0, Chicago, Ill USA).

RESULTS

Induced changes in Ca^{2+}_i of peripheral blood neutrophils

FMLP-induced changes in Ca^{2+}_i mobilization responses were measured in neutrophils isolated from 9 CF patients and 9 healthy controls. Histograms expressing the mean fluo-3 / fura red ratio versus time of the 9 CF neutrophil experiments and 9 healthy control neutrophil experiments are shown in Figure 1. Basal levels of fluo-3 / fura red ratiometry were comparable between CF and healthy control neutrophils. CF neutrophils showed higher fMLP-induced increases in Ca^{2+}_i responses (peak ratio) as compared to healthy control neutrophils (6.2 ± 0.9

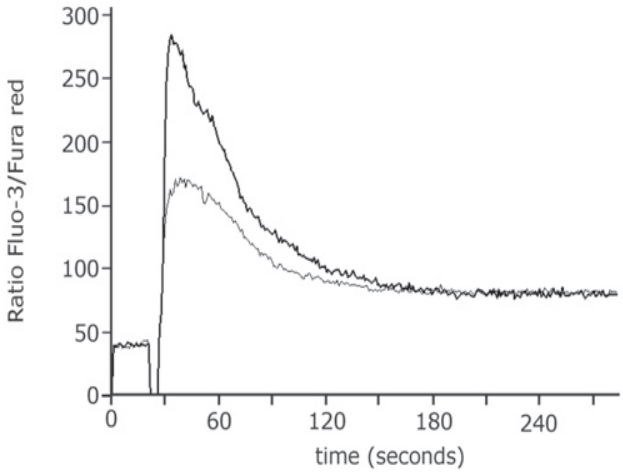

Figure 1. Mean fMLP-induced Ca^{2+}_i responses of neutrophils from 9 CF patients compared to 9 healthy control using fluo-3 / fura red ratiometry. Cells were stimulated with fMLP 10^{-7} M (heavy black line shows the mean Ca^{2+}_i response found in the CF patients and the thin black line the mean response of the healthy controls). The response found in CF patients was significantly different from the response found in control neutrophils ($p < 0.05$, n=9).

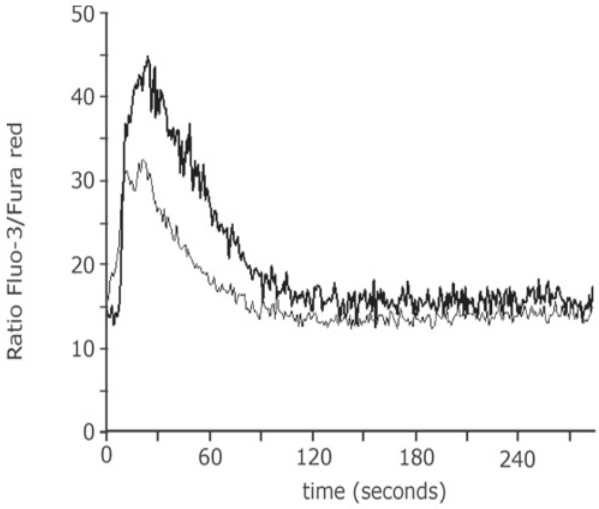

Figure 2. Mean fMLP-induced Ca^{2+}_i responses of neutrophils from 4 healthy controls with and without priming with TNF-α 100 IU/ml using fluo-3 / fura red ratiometry. Cells were stimulated with fMLP 10^{-7} M (thin black line shows the healthy control neutrophils without TNF-α priming and the heavy black line shows the healthy control neutrophils with TNF-α priming).

versus 4.3 ± 0.7, p=0.001). Area under the curve was 1.3 times greater in CF neutrophils versus healthy control neutrophils. The percentage of responding neutrophils was comparable between CF patients and healthy controls.

To assess whether the difference in the magnitude of the fMLP-induced Ca^{2+}_i responses could be secondary to the high cytokine levels originating from the CF-airways, serum and plasma, we measured the effects of TNF-α (100 IU/ml) on the fMLP-induced Ca^{2+}_i responses in neutrophils from 4 healthy controls. After loading of the cells with the Ca^{2+} fluorescent dyes part of the cells from each control were kept at 37ºC for 30 minutes without addition of TNF-α (unprimed cells) and part of the cells were primed at 37ºC for 30 minutes with TNF-α (100 IU/ml) (primed cells). Hereafter, fMLP-induced Ca^{2+}_i responses were measured in the unprimed and the primed cells as is shown in Figure 2. Neutrophils primed with TNF-α showed similar Ca^{2+}_i responses to unprimed cells, suggesting that increased cytokine levels such as TNF-α in CF plasma or serum have no major influence on Ca^{2+}_i responses in CF neutrophils.

Induced changes in Ca^{2+}_i in neutrophils differentiated from healthy and CF $CD34^+$ progenitor cells

We next studied Ca^{2+}_i mobilization in *ex-vivo* differentiated neutrophils from cord blood obtained from both a CF patient as well as a control child in order to circumvent potential effects of pro-inflammatory cytokines other than TNF present in the blood of CF patients on Ca^{2+}_i homeostasis. At day 17 of *ex-vivo* differentiation we measured Ca^{2+}_i mobilization (in quadrupli-

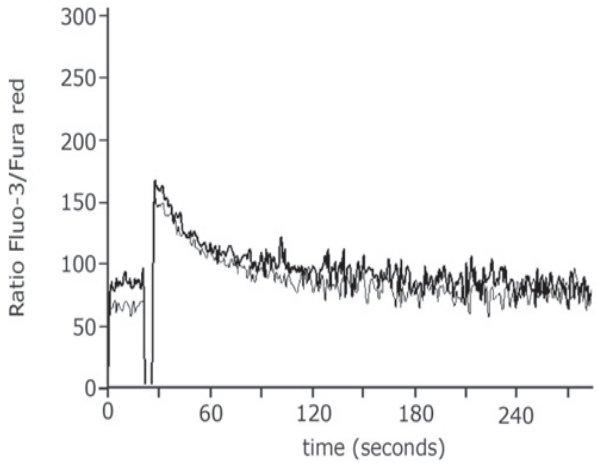

Figure 3. Mean fMLP-induced Ca^{2+}_i responses in neutrophils (differentiated from $CD34^+$ cord blood stem cells) from a CF newborn compared to a healthy control newborn using fluo-3 / fura red ratio (mean values of 4 experiments). Cells were stimulated with fMLP 10^{-7} M (heavy black line shows the CF patient and the thin black line the healthy control).

cate for each cell culture) with the differentiated neutrophils from both the healthy newborn and CF newborn source. In accordance with results found in peripheral blood neutrophils, fMLP-induced Ca^{2+}_i mobilization responses in *ex-vivo* differentiated CF neutrophils were also increased compared to healthy control cells (Figure 3). These data suggest an intrinsic defect in CF neutrophils which results in greater fMLP-induced changes in Ca^{2+}_i responses. It should be noted that the magnitude of the Ca^{2+}_i response, as measured by peak ratio, was much smaller in differentiated neutrophils either from CF or control cord blood, as compared to blood neutrophils.

Neutrophil migration

Having established a difference between CF and normal neutrophils in the Ca^{2+}_i response to fMLP stimulation, we subsequently analyzed neutrophil migration in response to fMLP. Neutrophil movement was measured on albumin-coated glass cover slips in the presence or absence of fMLP (100 nM) by time-lapse imaging during 10 minutes. Table 1 shows the average migration characteristics of 4 CF patients and 4 controls. Of every subject 30 individual neutrophils were analyzed.

Neutrophil migration was visualized by showing the tracks of individual cells (Figure 4A top panels) and the centered tracks (Figure 4A bottom panels) of a representative CF patient and a healthy control before and after stimulation with fMLP (100 nM). Average migration distance of neutrophils (track distance in μm) is shown for all CF patients and healthy controls ex vivo and after stimulation with fMLP (Figure 4B). In the absence of a stimulus, track

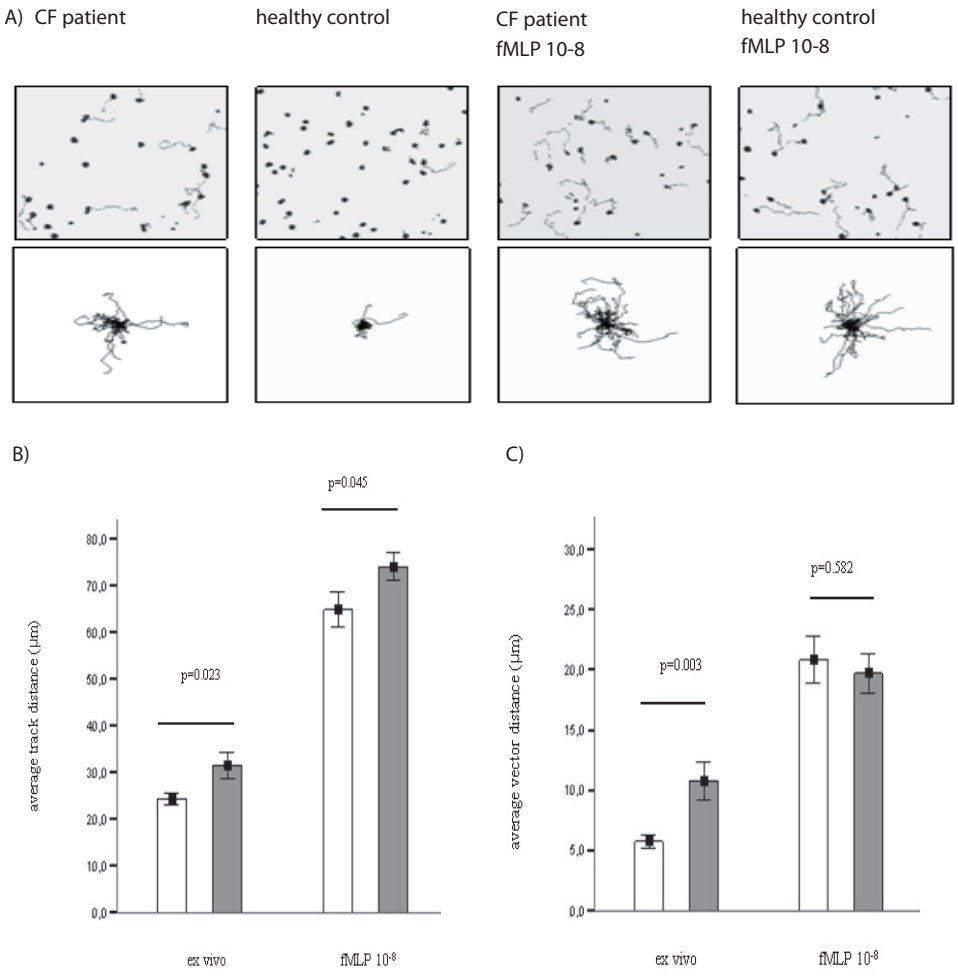

Figure 4. Migration of neutrophils was monitored by time lapse analysis. A) Migration tracks of individual cells are shown per cell (top row) and centered (bottom row) for a representative CF patient and healthy control ex vivo and after stimulation with fMLP 10^{-8}. B) Average track distance of 30 individual neutrophils of 4 healthy controls (white bar) and 4 CF patients (light grey bar) before and after stimulation with fMLP 10^{-8} are calculated and expressed as micrometers ± SE. C) Average vector distance of 30 individual neutrophils of 4 healthy controls (white bar) and 4 CF patients (light grey bar) ex vivo and after stimulation with fMLP 10^{-8} are calculated and expressed as micrometers ± SE.

distance was markedly increased in neutrophils from CF patients as compared to the healthy control neutrophils that showed little or no movement (31.5 ± 2.7 versus 24.2 ± 1.3, $p<0.05$). In the presence of fMLP, migration was increased in both CF neutrophils and healthy control neutrophils. Under these conditions a small but statistically significant increase in track distance was observed for CF neutrophils relative to healthy controls (73.9 ± 3.0 versus 64.8 ± 3.8, $p<0.05$) (see also Figure 4B). Vector distance in µm (which is a measure of neutrophil

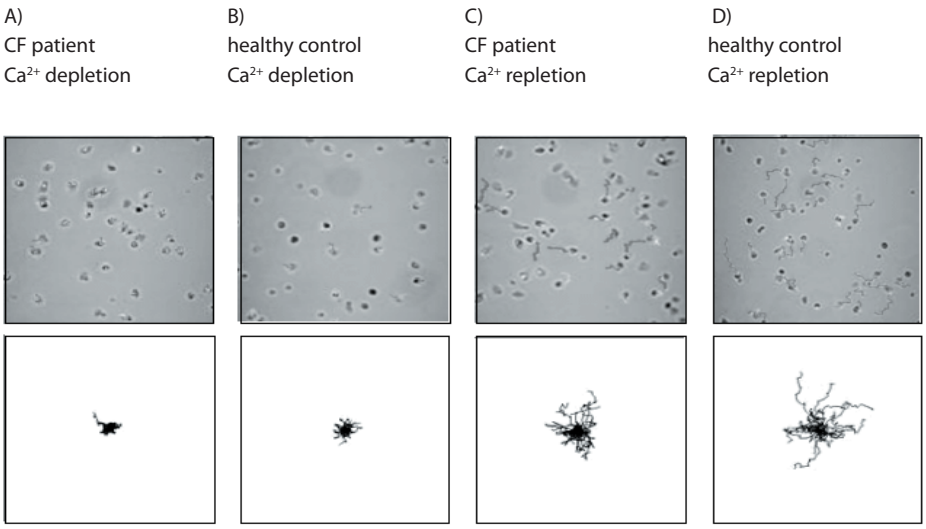

Figure 5. Migration tracks of individual cells are shown per cell (top row) and centered (bottom row) after stimulation with fMLP 10^{-8} for A) a CF patient after Ca^{2+} depletion B) a healthy control after Ca^{2+} depletion, C) a CF patient after Ca^{2+} repletion, and D) a healthy control after Ca^{2+} repletion.

displacement) was increased in CF neutrophils compared to healthy control neutrophils as is shown in Figure 4C (10.8 ± 1.6 versus 5.7 ± 0.6, $p<0.01$). fMLP-treated neutrophils from both CF patients and healthy controls showed a similar vector distance ($p=0.582$, figure 4C).

It has been demonstrated previously that migration of neutrophils on poly-D-lysine-coated glass requires transient increases in Ca^{2+}_i (10). We therefore performed neutrophil migration experiments with neutrophils from a CF patient and a healthy control in Ca^{2+} depleted HEPES buffer (as described in (26)). Ca^{2+} depletion completely abrogated neutrophil migration, even after stimulation with fMLP (Figure 5A and 5B) and repletion of Ca^{2+} to the HEPES buffer restored migration ability of neutrophils in both the CF neutrophils and healthy control neutrophils (Figure 5C and 5D). Magnification of the video microscopic images after Ca^{2+} depletion showed that both CF and healthy control neutrophils were still capable of morphological changes of cell shape but not of de-adhesion from the glass cover (results not shown).

DISCUSSION

In this study we found greater fMLP-induced increased Ca^{2+}_i mobilization responses in neutrophils from CF children compared to healthy controls. The difference in Ca^{2+}_i responses was most prominent in the first rapid phase resulting in a higher peak ratio after stimulation with fMLP, suggesting greater Ca^{2+} release from intracellular stores in CF neutrophils. In CF airway epithelial cells, an increased apical endoplasmic reticulum (ER) compartment with an

increased capacity to sequester Ca^{2+} in response to chronic luminal airway infection has been described and these findings lead to higher Ca^{2+}_i mobilization responses in CF airways (6).

However, gradual expansion of intracellular Ca^{2+} stores in CF neutrophils due to chronic infection and inflammation, does not seem reasonable, since neutrophils have a very short lifespan. In addition we evaluated the effect of environmental factors (e.g. presence of inflammatory cytokines) in CF on the fMLP-induced Ca^{2+}_i response by priming healthy control neutrophils with TNF-α. Concentrations of TNF-α are increased in CF plasma and serum, and TNF-α induces cachexia and weight loss, promotes neutrophil infiltration of the airways, and is inversely related to FEV_1 (27-29). Priming healthy control neutrophils with TNF-α did not lead to similar increases in fMLP-induced Ca^{2+}_i mobilization responses as seen in CF neutrophils. Increased TNF-α concentrations in CF plasma, serum, sputum, and bronchoalveolar lavage fluid (BALF) therefore are not likely the explanation for the increased fMLP-induced Ca^{2+}_i responses in CF neutrophils (27-30). All CF patients studied had positive sputum cultures with *Pseudomonas aeruginosa* or *Staphylococcus aureus*, without any signs of an acute pulmonary exacerbation. Both *Pseudomonas aeruginosa* and *Staphylococcus aureus* have been shown to bind to asialylated glycolipid receptor asialo GM1 (31), and this binding stimulates increases in Ca^{2+}_i in airway epithelial cells causing translocation of nuclear factor kappa B (NF-κB) (32). This in turn leads to activation of various proinflammatory cytokines (such as IL6 and TNF-α), chemokines (such as IL-8) and adhesion molecules (32). We cannot exclude that the *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteria found in sputum cultures in our patients caused an increase in (1) cytokines other than TNF-α, (2) chemokines and/or (3) adhesion molecules in the systemic circulation, which might lead to neutrophil priming in peripheral blood and a potentiation of the fMLP-induced Ca^{2+}_i response in CF blood neutrophils. However, studies showing that cytokines prime the fMLP-induced Ca^{2+}_i response as they prime the fMLP-induced activation of cytotoxic responses are lacking. Moreover, our experiments focused on modulation of this response with addition of TNF did not alter Ca^{2+}_i mobilization responses induced by fMLP (see Figure 2).

To circumvent the above mentioned potential effects of bacterial colonization on the fMLP-induced Ca^{2+}_i mobilization responses of neutrophils, we studied Ca^{2+}_i mobilization responses in neutrophils differentiated from CF and healthy control cord blood $CD34^+$ progenitor cells. In this system we also observed a higher response in CF differentiated neutrophils although the response was lower than in blood neutrophils. This might be explained by the difference in maturation (*ex-vivo* versus in vivo in the bone marrow) with possibly a lower number of fully functional fMLP receptors in the *ex-vivo* differentiated neutrophils compared to blood neutrophils. Taken together, our results suggest that there is an intrinsic CFTR-related defect in CF neutrophils and a relationship between CFTR dysfunction and Ca^{2+}_i homeostasis in CF neutrophils. Current understanding of the precise molecular mechanisms regulating Ca^{2+} influx in human neutrophils is incomplete. After an initial rise in Ca^{2+}_i that is caused by release from intracellular stores a further rise in Ca^{2+}_i is provided through store-operated calcium en-

try (SOCE) channels (33-35). Human neutrophils express several transient receptor potential (TRP) proteins that participate in the formation of at least two SOCE channels with different ionic permeabilities and regulatory characteristics (33,34). Recently the bioactive lipid lysophosphatidic acid (LPA) has been shown to trigger Ca^{2+} entry through another non-SOCE channel, providing evidence for alternative Ca^{2+} influx channels that can be activated without the signal of empty intracellular stores (35). While we are only just beginning to understand regulation of Ca^{2+} influx in human neutrophils, a potential role for CFTR in regulation of Ca^{2+} influx in human neutrophils is now emerging, but the underlying mechanisms remain to be established.

Ca^{2+}_i homeostasis has been implicated in the control of many granulocyte responses. However, the specific mechanisms by which changes in Ca^{2+}_i and extracellular Ca^{2+} are regulating neutrophil funtion are still poorly defined. Much emphasis has been given to the role of Ca^{2+}_i in migration of neutrophils but clear mechanisms have not yet been defined (14). Some aspects of the granulocyte migration process have been shown to be at least partially controlled by changes in Ca^{2+}_i (10,11,36). Here we describe an increase in track and vector distance of CF neutrophils *ex-vivo* compared to healthy controls. Stimulation with fMLP also resulted in a small but statistically significant difference in track distance in favor of CF neutrophils. Apparently, the altered Ca^{2+}_i homeostasis in CF neutrophils potentiates migration *ex-vivo* and might participate in the enhanced homing of neutrophils to CF lungs *in vivo*. Previous studies have shown that depletion of Ca^{2+}_i leads to inhibition of locomotion of neutrophils attached to fibronectin- and vitronectin-coated glass surfaces *ex-vivo* (10) and decreased the level of Ca^{2+}-dependent myosin II activation required for uropod retraction during neutrophil motility on adhesive surfaces (36). In our study we also found that calcium depletion abrogated neutrophil migration and repletion of calcium restored migration ability of both CF and healthy control neutrophils. These data point to a possible link between the greater fMLP-induced Ca^{2+}_i responses found in CF neutrophils and the increased migration, possibly through integrin recycling (10-12,14). Previously, increased migratory responses towards IL-8 have been shown for CF neutrophils (21). Pizurki and colleagues did not find increased neutrophil migration across CF airway epithelial monolayers but they tested migration of healthy control neutrophils and not of CF neutrophils (37).

In conclusion, we show that neutrophils from clinically stable CF patients and *ex-vivo* differentiated CF neutrophils from cord blood $CD34^+$ progenitor cells display increased fMLP-induced Ca^{2+}_i responses. Priming of healthy control neutrophils with TNF-α did not result in a similar Ca^{2+}_i response as seen in CF neutrophils, suggesting an intrinsic defect in CF neutrophils. Additional experiments addressing fMLP-induced Ca^{2+}_i responses in *ex-vivo* differentiated CF and healthy control neutrophils should provide further insight into whether or not CF neutrophils are intrinsically altered. These results might provide a rationale for anti-inflammatory therapy targeting on Ca^{2+}_i homeostasis in neutrophils of CF patients.

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Chapter 4.1

Gradual increase in systemic inflammation over time in children with cystic fibrosis: *Pseudomonas aeruginosa* acquisition increases the expression of the integrin $\alpha_m\beta_2$ on blood neutrophils

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Submitted

SUMMARY

Despite the neutrophil-dominated inflammation in CF lung disease, the airways in CF patients become colonized by different pathogens. Neutrophils migrate from the blood through a pathway involving the β_2 integrin $\alpha_m\beta_2$ (CD11b/CD18 or CR3) (CD18-dependent migration) or through an alternative uncharacterized pathway not involving $\alpha_m\beta_2$ (CD18-independent migration). We hypothesized that during the natural course of CF in children, the different stages of the disease are mediated by differences in phenotype of the systemic innate immune response. We therefore compared expression of $\alpha_m\beta_2$ and priming associated cellular markers MoPhabs A17 and A27 on blood neutrophils from 12 children with a first *Pseudomonas aeruginosa* acquisition (5 male, mean age 5.4 years) with those of 8 children with CF with a chronic *Pseudomonas aeruginosa* infection (5 male, mean age 7.3 years), 12 uninfected CF children (5 male, mean age 5.2 years), and 10 healthy control children (6 male, mean age 7.1 years). The expression of both α_m integrin chain (CD11b) and β_2 integrin chain (CD18) were significantly higher in CF children with a first *Pseudomonas aeruginosa* acquisition, and were significantly lower in CF children with a chronic *Pseudomonas aeruginosa* infection compared to all other groups. In contrast, expression of the MoPhabs A17 and A27 were significantly higher in all CF children, even in uninfected children, compared to healthy controls. We have demonstrated the existence of multiple priming phenotypes in the peripheral blood of children with CF, and these phenotypes are associated with different phases of the disease.

INTRODUCTION

Cystic fibrosis (CF) lung disease is characterized by chronic bacterial infection (especially with *Pseudomonas aeruginosa*) and chronic inflammation with massive influx of polymorphonuclear neutrophils. This is associated with high concentrations of interleukin-8 (IL-8) in bronchoalveolar lavage fluid (BALF) (1-8). Under these conditions neutrophils migrate out of the bloodstream to the site of infection in order to eliminate the infectious pathogens. Neutrophil migration from the peripheral blood to the site of inflammation is a multistep process that either involves the β_2 integrin $\alpha_m\beta_2$ (CD11b/CD18 also called Mac-1 or CR3) complex (CD18-dependent migration) or an alternative uncharacterized pathway that does not involve $\alpha_m\beta_2$ (CD18-independent migration) (9-12). In a rabbit model of *Pseudomonas aeruginosa* induced acute pneumonia, neutrophil influx occurred through the CD18-dependent pathway while in recurrent pneumonia migration occurred through the CD18-independent pathway (11). Also in *in vitro* human studies CD18-dependent migration was described in acute pulmonary infection but not in chronic infection (12).

Despite the neutrophil-dominated chronic inflammation in CF lung disease, the airways of CF patients become infected by different pathogens. Neutrophil motility, bacterial capture, and killing are impaired in the CF-like thickened mucus (13) and antimicrobial properties of the airway surface liquid (ASL) in CF are compromised and this is not mediated through a direct effect of the ASL on phagocyte function (14). Recently, reduced iC3b-mediated phagocytosis was demonstrated in CF BALF neutrophils derived from BALF (15). This reduced phagocytic capacity could not be attributed to a defect in $\alpha_m\beta_2$ (CR3) functioning or $\alpha_m\beta_2$ (CR3) cell surface density and a failure of neutrophil phagocytic priming during translocation to the lung was proposed as a possible explanation. Expression of α_m on blood neutrophils isolated from adult CF patients with moderate to severe lung disease and chronic *Pseudomonas aeruginosa* infection was comparable between stable CF patients, patients with a pulmonary infective exacerbation, and controls (16,17). Also in uninfected children with CF, α_m expression on blood neutrophils was comparable with healthy controls (18). However expression of $\alpha_m\beta_2$ on blood neutrophils have not been compared between children with CF with a first acquisition of *Pseudomonas aeruginosa* (acute infection) and children with chronic *Pseudomonas aeruginosa* infection.

We hypothesize that during the natural course of CF in children the different stages of the disease are mediated by differences in phenotype of the systemic innate immune response. We tested this hypothesis by measuring expression of $\alpha_m\beta_2$ on blood neutrophils from children with a first acquisition of *Pseudomonas aeruginosa* and compared these data with those obtained from children with CF with a chronic *Pseudomonas aeruginosa* infection, uninfected CF children, and healthy control children. We also assessed additional characteristics for the activation status of peripheral blood neutrophils in these groups by using recently developed and applied monoclonal phage antibodies, (MoPhabs) A17 and A27 (19-21). These antibodies

recognize cytokine-primed neutrophils in whole blood with a sufficient dynamic range to allow detailed priming studies in vivo and in vitro (19).

METHODS

Study subjects

Thirty-two children (18 girls, 14 boys) with CF attending the outpatient Paediatric Clinic of the Cystic Fibrosis Centre of the University Medical Centre (UMC) Utrecht for a routine check-up (mean age 5.8 ± 2.7 yr) were recruited. Genotyping had been performed and patients were categorized as either having a homozygous ΔF508 mutation (ΔF508/ΔF508), heterozygous ΔF508 mutation (ΔF508/other) or two other mutations (other/other). At the time of inclusion, all children were clinically stable. Blood samples were drawn for assessment of neutrophil activation status and serologic measurement of *Pseudomonas aeruginosa* antibodies. The demographic data are depicted in table 1.

Pseudomonas aeruginosa (PA) infection was assessed by sputum or oropharyngeal cultures (when children were unable to produce and expectorate sputum) and these were obtained on the same day as blood sampling. All bacterial cultures were performed according to standard procedures. We assessed in all patients whether they had *Pseudomonas aeruginosa* positive cultures in the past. Furthermore we performed serologic measurements of *Pseudomonas aeruginosa* antibodies in all children directed to three tested antigens: exotoxin A, elastase and alkaline protease on the same day as sputum culture. CF patients were divided into 3 groups based on the results of bacterial cultures and *Pseudomonas aeruginosa* antibodies.

Group 1, designated uninfected, comprised of 12 CF children with a mean (SD) age of 5.2 (2.2) years), no history of a PA positive culture and negative PA antibodies. These children had negative sputum cultures > 6 months before blood sampling.

Group 2, designated acute PA, comprised of 12 CF children (mean age of 5.4 (2.8) years with a first ever PA positive culture. Nine of these 12 children were positive for one or more of the tested PA antibodies.

Group 3, designated chronic PA, comprised 8 CF children (mean age of 7.3 (3.1) years) with a PA positive culture and PA positive cultures for >50% of months, when cultures had been performed in the past years (22). Five of the 8 children were positive for one or more of the tested PA antibodies.

Pulmonary function (spirometry; Masterlab Jaeger, Hochberg, Germany) was determined by the forced expiratory volume in 1 second (FEV_1) expressed as percent predicted using reference values by Zapletal (23).

Control subjects were recruited at the outpatient Paediatric Clinic of the UMC Utrecht. Ten children (mean age of 7.1 (3.3) years) without an inflammatory or infectious condition were

included. Neutrophil activation status was assessed using CD11b (clone D12, ref 347557), CD18 (clone L130, ref 347953), and MoPhabs A17 and A27.

This study was approved by the Medical Ethics Committee of the UMCU, Utrecht and written informed consent was obtained from all study participants.

Staining neutrophils for adhesion and activation associated receptors

After venapuncture whole blood anticoagulated with sodium heparin was immediately chilled on ice. Hereafter, whole blood was incubated for 30 min on ice and double stained with combinations of optimal concentrations of (1) fluorescein isothiocyanate (FITC) conjugated CD14 (clone MøP9, ref 345784) and phycoerythrin (PE) conjugated CD11b (CR3, clone D12, ref 347557) and (2) with PE conjugated CD14 (clone MøP9, ref 345785) and FITC conjugated CD18 (β2 integrin chain, clone L130, ref 347953). The antibodies were obtained from Becton Dickinson (San Jose, CA). After incubation cells were washed with phosphate-buffered saline (PBS) supplemented with 0.01% sodiumazide and 1% foetal bovine serum (PBS^{++}), followed by lysis of erythrocytes for 10 minutes using FACS Lysing Solution. After a final washing step with PBS^{++}, cells were resuspended in 200 µl 1 % paraformaldehyde in PBS and analyzed in a FACS Calibur Flowcytometer (Becton Dickinson). Neutrophils, monocytes and lymphocytes were differentiated based on side-scatter characteristics and CD14 expression. Median fluorescence intensity in arbitrary units (MFI in AU) of 20,000 events was assessed.

Staining of blood neutrophils with monoclonal phage antibodies A17 and A27

Blood samples were collected in sodium-heparin tubes. After venapuncture, blood was directly kept on melting ice and analysed within 1 hour. Each blood sample was divided in 4 aliquots of which two were kept on ice. The other two samples were incubated with TNF-α at a concentration of 100 IU/ml and these samples were kept on 37°C for 30 minutes. Hereafter, all samples were incubated in 100 µl of a 1:10 dilution with PBS containing either FITC-labelled MoPhab A17 or MoPhab A27 for 60 minutes on ice. After incubation cells were washed twice with PBS^{++} and erythrocytes were lysed for 10 minutes on ice using FACS Lysing Solution (Becton Dickinson, Mountain View, CA). Cells were resuspended in 200 µl 1% paraformaldehyde in PBS and analysed in a FACS Calibur Flowcytometer (Becton Dickinson). Neutrophils were identified according to their specific forward-scatter and side-scatter characteristics and expression levels of MoPhabs A17 and A27 were expressed as median fluorescence intensity in arbitrary units (MFI in au) of 20,000 events.

Quantitation of Pseudomonas aeruginosa antibodies

Serum anti-pseudomonas antibodies were determined using a commercially available ELISA-kit (Mediagnost, Reutlingen, Germany) (24). In short, the purified *Pseudomonas aeruginosa* antigens exotoxin A, elastase, and alkaline protease coated on albumin saturated microtiter plates were used to detect the respective antibodies in CF serum samples. Samples were clas-

sified as negative when extinction values were less than 20% of the mean positive control value and were classified positive when extinction values were >20% of the mean positive control value. Patients were considered serologically PA positive when at least one of the PA antigens tested positive.

Statistical analysis

Results of CD11b, CD18, and MoPhabs A17 and A27 were expressed as mean ± standard error (SE). Comparison of the 3 different groups of CF-patients and healthy control subjects was performed using a Kruskall-Wallis one-way ANOVA, which, if significant, was followed by non-parametric Mann-Whitney U-tests. A p-value of equal to or less than 0.05 was considered significant. All statistical tests were performed by using the statistical software package for the Social Science (SPSS version 12.0, Chicago, Ill USA).

RESULTS

Most CF children were homozygous for the ΔF508 mutation (see table 1). There was no statistically significant difference in age, gender, or lung function between uninfected, acute PA, and chronic PA CF children (table 1).

Striking differences were found in the expression of α_m (CD11b) and β_2 integrin chain (CD18) on blood neutrophils obtained from the different study groups. α_m Integrin chain (CD11b) expression was significantly increased in the acute PA CF children (children with a first PA acquisition) compared to (1) the chronic PA children (853.1 ± 67.6 versus 295.3 ± 43.8 MFI, p<0.001), (2) the uninfected children (853.1 ± 67.6 versus 531.3 ± 39.1 MFI, p=0.001) and (3) healthy controls (853.1 ± 67.6 versus 512.3 ± 60.4 MFI, p=0.001) as is shown in figure 1a. Interestingly, α_m integrin chain (CD11b) expression on neutrophils was significantly lower in

Table 1. Characteristics of the CF children (uninfected (children with no history of a PA positive culture and negative PA antibodies); acute PA (children with a first ever PA positive culture); and chronic PA (children with a PA positive culture and PA positive cultures for >50% of months)). Age and lung function test results are expressed as mean ± standard deviation (SD).

	uninfected (n=12)	acute PA (n=12)	chronic PA (n=8)
Age	5.2 ± 2.2	5.4 ± 2.8	7.3 ± 3.1
Sex (male: female)	5 : 7	5 : 7	5 : 3
FEV_1 % (n)	101 ± 26 (8)	84 ± 17 (8)	88 ± 16 (6)
Genotype			
ΔF508/ΔF508	9	7	7
ΔF508/ Other	3	5	1

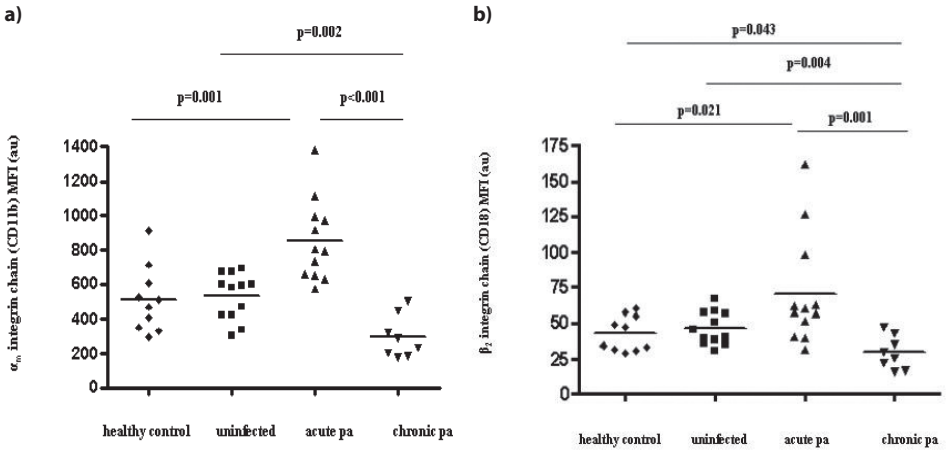

Figure 1. Expression of a) α_m integrin chain (CD11b) and b) β_2 integrin chain (CD18) on blood neutrophils from healthy controls (n=10), uninfected CF children (n=12), CF children with first PA acquisition (acute PA; n=12), and CF children with a chronic PA infection (n=8).

CF children with a chronic PA infection compared to healthy controls (p=0.006), uninfected CF children (p=0.002) and acute PA CF children (p< 0.001) (see figure 1a). Similar findings were obtained with CD18 antibodies. β_2 Integrin chain (CD18) expression was also significantly increased in the acute PA CF children compared to the chronic PA CF children (70.9 ± 11.2 versus 29.6 ± 4.2 MFI, p=0.001), healthy controls (70.9 ± 11.2 versus 42.9 ± 3.9 MFI, p=0.02) and the uninfected CF children (70.9 ± 11.2 versus 46.5 ± 3.3 MFI, p=0.05) (see figure 1b). CF children with a chronic PA infection had significantly lower expression of β_2 integrin chain (CD18) compared to the acute PA and uninfected CF children (p=0.001 and p=0.004 respectively) and the healthy controls (p=0.043) (see also figure 1b).

Monoclonal phage antibodies, A17 and A27, recognize epitopes on phagocytes that are induced by cytokines with a dynamic range allowing measurement of acute and chronic priming of phagocytes in peripheral blood of patients with chronic inflammatory diseases (17,19). The expression of these priming associated epitopes was measured on blood neutrophils ex vivo in the presence and absence of TNF-α (100 IU/ml). We found a significant increase in MoPhab A17 and MoPhab A27 expression ex vivo on neutrophils in all three groups of CF children compared to healthy controls (p-value <0.01, figure 2a and 2b). As has been described previously, priming with TNF-α causes up regulation of the A17 and A27 epitopes and induces maximum expression levels in vitro (19). Maximum A17 and A27 epitope expression was measured after priming with TNF-α to induce a fully primed phenotype. These experiments were performed to have an indication of how pronounced the partial priming phenotype is that is found in vivo in the absence of a stimulus. Both A17 and

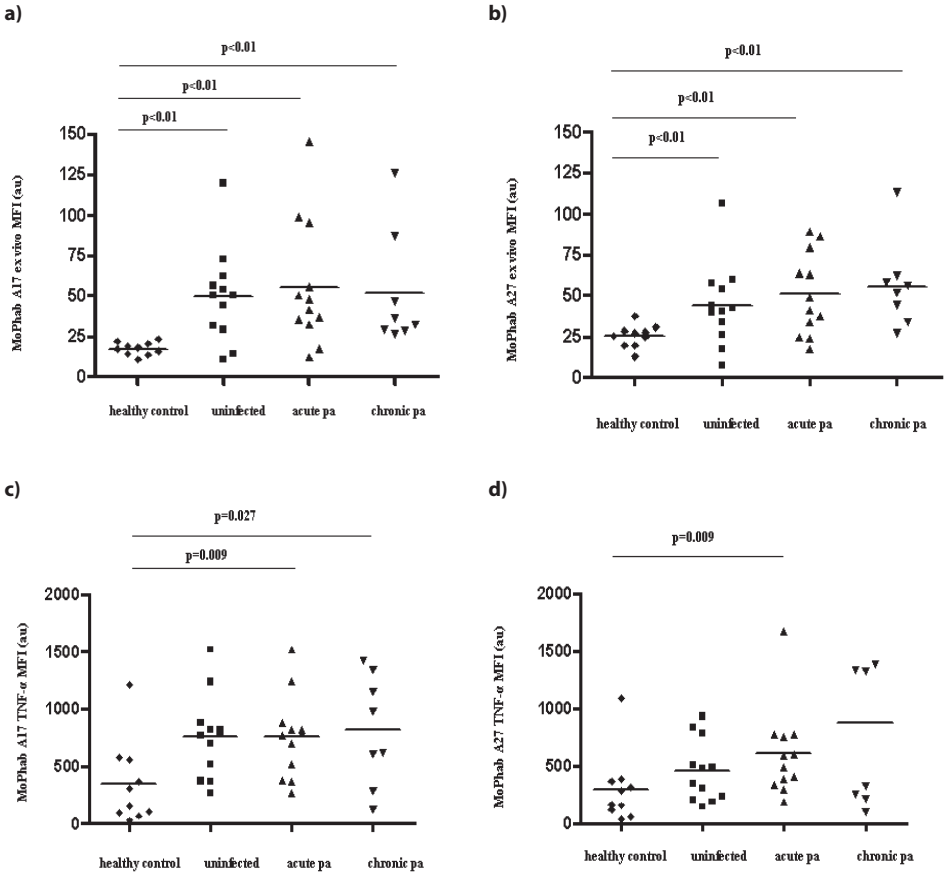

Figure 2. Expression of priming epitopes, recognized by FITC-labelled MoPhabs A17 and A27 (MFI in au) on neutrophils: a) A17 ex vivo; b) A27 ex vivo; c) A17 with TNF-α; d) A27 with TNF-α .

A27 epitope expression increased in healthy controls and CF children. Maximum expression levels of MoPhab A17 was significantly higher in acute PA CF children and chronic PA CF children compared to healthy controls (p=0.009 and p=0.027 respectively) but not compared to uninfected CF children (figure 2c). Maximum expression levels of MoPhab A27 were only significantly higher in acute PA CF children compared to healthy controls (p=0.009) but not in chronic PA CF and uninfected children (figure 2d).

Analysis of the labelling of neutrophils by A17 and A27 with this flow cytometric approach showed that the priming response was present in all neutrophils and was not restricted to a subpopulation of cells (figure 3a-d). Neutrophils in peripheral blood showed a partially primed phenotype ex vivo (3a and 3c) since expression of the priming epitopes could still be enhanced after addition of TNF-α in vitro (3b and 3d).

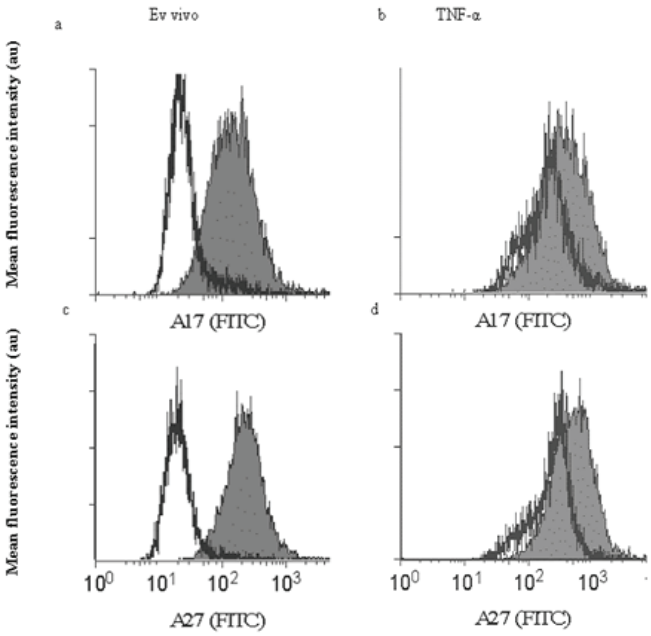

Figure 3. Figure 3a and 3b show the histograms of MoPhab A17 ex vivo and with TNF-α respectively of a CF-patient (filled histogram) and a healthy control (open histogram) and 3c and 3d show the histograms of MoPhab A27 ex vivo and with TNF-α respectively of a CF-patient (filled histogram) and a healthy control (open histogram).

DISCUSSION

In this study, we show an increased expression of the integrin $\alpha_m\beta_2$ (CD11b/CD18) on neutrophils obtained from the peripheral blood of CF children with a first *Pseudomonas aeruginosa* acquisition compared to CF children with a chronic *Pseudomonas aeruginosa* infection, uninfected CF children and healthy controls. These findings show that a first contact with *Pseudomonas aeruginosa* leads to priming of the systemic innate immune response. Mere expression of $\alpha_m\beta_2$ is not essential for increased functionality (25), because this integrin needs inside-out signals in order to gain maximal functionality. However, it is tempting to speculate that neutrophils with an enhanced α_m expression are optimally prepared for $\alpha_m\beta_2$ mediated homing after proper activation. This hypothesis is supported by the finding that in CF children with a chronic *Pseudomonas aeruginosa* infection low expression of $\alpha_m\beta_2$ was found compared to the other CF children and to healthy controls. Apparently, under these conditions neutrophils have migrated to the tissues leaving $CD18^{dim}$ and $CD11b^{dim}$ neutrophils in the blood. Under these conditions the expression of $\alpha_m\beta_2$ is similar between controls

and uninfected CF patients. Previous studies failed to differentiate between CF patients and controls using $\alpha_m\beta_2$ expression on blood neutrophils (15-18). CF patients described in these studies were, however, adolescent or adult patients with moderate to severe lung disease and chronic *Pseudomonas aeruginosa* infection (16,17), uninfected CF children (18), or CF patients with no infection or infection with mostly other pathogens than *Pseudomonas aeruginosa* (15). In these CF patients antibodies directed against $\alpha_m\beta_2$ can not differentiate in terms of pre-activation.

This is the first study comparing $\alpha_m\beta_2$ expression on blood neutrophils between CF children with a first *Pseudomonas aeruginosa* acquisition (acute PA infection) and a chronic *Pseudomonas aeruginosa* infection. We defined CF children with a first *Pseudomonas aeruginosa* acquisition and chronic *Pseudomonas aeruginosa* infection as accurately as possible by analyzing all previous sputum or oropharyngeal cultures and also by determining serum anti-pseudomonal antibodies. Nine of the 12 first *Pseudomonas aeruginosa* acquisition CF children and 5 of the 8 chronic *Pseudomonas aeruginosa* infection CF children had positive serology to one or more of the tested PA antibodies. Recently the diagnostic value of the ELISA-kit used in this study was evaluated in a large population from our centre (24). Especially in children below the age of 6 years (as were most children in our study), serology was of no additional value to diagnose *Pseudomonas aeruginosa* colonisation compared to bacterial culture. Transient positive serology was seen in the follow up study (24) and also in healthy controls positive serology has been described (26). We therefore conclude that classification of CF children with acute and chronic *Pseudomonas aeruginosa* infection in this study was as accurate as possible.

To better characterize neutrophils in terms of priming in all CF groups we also stained the samples with very sensitive priming markers A17 and A27 (19-21). In marked contrast to CD11b/CD18 antibodies, these monoclonal phage-antibodies clearly recognized priming of neutrophils in all CF groups even in uninfected CF children with completely normal lung function and normal levels of CD11b and CD18. When the results of the different priming markers used in this study are combined, different priming phenotypes of neutrophils can be found in the peripheral blood such as $CD11b^{bright}$ and $A17/A27^{bright}$ during first interaction with PA and $CD11b^{dim}$ and $A17/A27^{bright}$ during chronic disease. These priming phenotypes are partial as TNF-α activation in vitro clearly upregulates A17/A27 even more (see figure 2), and maximum expression of priming epitopes induced by TNF-α was significantly greater in CF children with acute and chronic *Pseudomonas aeruginosa* infection compared to healthy controls. Part of these priming phenotypes can be explained by increased levels of cytokines and chemokines such as TNF-α (27) and IL-8 (28) in plasma and serum. However, these increased levels were not found in children with very mild disease (27).

Interestingly also uninfected CF children demonstrated a systemic inflammatory response in the context of preactivated neutrophils in the absence of any detectable infection. Although we cannot exclude that these CF children had recently encountered a bacterial or

viral infection of the airways, evaluation of sputum and oropharyngeal cultures obtained in the last 6 months prior to blood sampling showed no sign of such a respiratory infection. Apparently very early in the pathogenesis of CF already a systemic activation of the innate immune response is present. Our data are consistent with studies showing priming of peripheral blood neutrophils from CF patients with moderately decreased pulmonary function in a functional context. Priming was demonstrated by measuring the ratio between unprimed and PAF-primed phagocyte responses to serum-opsonized zymosan and by L-selectin shedding *in vitro* (16,18). These findings suggest that the local inflammatory response can communicate with the systemic circulation and triggers priming and activation of peripheral blood neutrophils resulting in a systemic inflammatory response. Analysis of the labelling of neutrophils by CD11b/CD18 as well as by A17 and A27 showed that the priming response was present in all neutrophils and was not restricted to a subpopulation of cells (figure 3). However, nothing is known regarding the functional differences between neutrophils with these different priming phenotypes.

In conclusion, we have demonstrated the existence of multiple priming phenotypes in the peripheral blood of children with CF. These phenotypes are associated with different phases of the disease. They e.g. differentiate between CF children with a first *Pseudomonas aeruginosa* acquisition, CF children with a chronic *Pseudomonas aeruginosa* infection, and uninfected CF children. Aberrant (pre)activation of the innate immune response is likely to be deleterious for the tissues of CF patients and, therefore, the determination of the different priming epitopes will be helpful in better targeting new therapies focused on antagonism of the pronounced innate immune response.

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Chapter 4.2

Multiplex cytokine profile detection in young children with cystic fibrosis

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Submitted

ABSTRACT

Background: Cystic fibrosis (CF) patients with moderate to severe lung disease display signs of systemic inflammation measured by cytokine profiles in plasma. In young CF patients with mild lung disease it is however unclear whether signs of a systemic inflammatory response are present. We therefore measured plasma cytokine profiles in young, clinically stable CF patients with mild lung disease.

Methods:. Twenty-eight different cytokines, chemokines and soluble adhesion molecules were measured in 47 CF children, ages 1.7-12.2 years, using a multiplex immunoassay. Sputum cultures were obtained on the same day as blood sampling and children were categorized as uninfected (n=12), positive for *Staphylococcus aureus* and/or *Haemophilus influenzae* (n=26) or positive for *Pseudomonas aeruginosa* (n=9). Data were compared to those obtained from 20 healthy control children, ages 3.8-11.5 years.

Results: All CF children displayed a pro-inflammatory cytokine profile and especially interleukin (IL)-1α, IL-4, IL-12 and tumor necrosis factor-alpha (TNF-α) were significantly higher in CF children (p-values<0.001 or <0.05). Although interleukin-8 and oncostatin M (OSM) were also higher in CF children, CC-chemokines were significantly lower than in controls. In CF children bacterial sputum culture results did not influence plasma cytokine profiles, except for soluble vascular endothelial cell adhesion molecule-1 (sVCAM-1). sVCAM-1 concentrations were significantly lower in uninfected CF children compared to *Pseudomonas aeruginosa* positive CF children (2186.8 ±1207.7 versus 7170.21 ± 2129.7, p=0.025).

Conclusions: Young, clinically stable CF children display a derangement of inflammatory mediator profiles. Pro-inflammatory cytokines are increased in plasma while CC-chemokines are decreased.

INTRODUCTION

Airway inflammation in cystic fibrosis (CF) is characterized by massive influx of polymorphonuclear neutrophils and high concentrations of interleukin-8 (IL-8) in bronchoalveolar lavage fluid (BALF) which are present even in young, clinically stable patients (1-6). Neutrophil migration from the peripheral blood to the site of inflammation is a multistep process in which selectins (such as E- and L-selectin), endothelial cell adhesion molecules (such as intercellular adhesion molecule-1 (ICAM-1)) and integrins (such as CD11b/CD18) play a critical role (7,8). The first step in the process of migration to and activation in the tissues is neutrophil preactivation or priming (9,10). Neutrophil priming occurs in vivo in the peripheral blood by chemotaxins, cytokines or bacterial products (9,11).

Priming-inducing mediators such as IL-8 can be produced by bystander cells such as airway epithelial cells (12,13) but also by circulating and infiltrating inflammatory cells such as neutrophils and lymphocytes (14,15). Blood lymphocytes and mononuclear cells isolated from CF patients show selective cytokine dysregulation with reduced gamma interferon (IFN-γ) secretion, and increased IL-10 mRNA without increased production or secretion of IL-10 (15). Furthermore T lymphocytes from clinically stable CF patients produce high levels of IL-2 and low levels of IL-8 (16). Recently a Th2 type pulmonary immune response has been demonstrated in BALF of *Pseudomonas aeruginosa* infected CF patients with increased levels of IL-4, IL-13 and thymus and activation-regulated chemokine (tarc), and decreased levels of interferon-gamma (IFN-γ) that might be of influence on pulmonary outcome (17).

Although the most prominent inflammatory responses originate in the lung, increased levels of tumor necrosis factor (TNF)-α (18) and IL-8 (19,20) have been described in plasma and serum of CF patients with more advanced disease. Clinically stable CF patients also display elevated levels of soluble ICAM-1 (sICAM-1) and soluble E-selectin (sE-selectin) in serum and these levels increase even further during a pulmonary exacerbation (21). However, in CF patients with mild disease and normal lung function, local inflammatory responses have been observed in BALF (1-5) but until now studies did not reveal systemic inflammatory responses in these patients (20). Therefore, it is unclear whether in the early course of CF lung disease, inflammation is restricted to the lungs or whether there is a systemic inflammatory response from the beginning.

The most frequently used technique for detection of cytokines and chemokines in plasma is the enzyme-linked immunosorbent assay (ELISA). This technique however requires large sample volumes, and is time consuming. The Bio-Plex system employing the Luminex multi-analyte profiling technology (x-MAP™), allows individual and multiplex analysis of up to a hundred different mediators in a single well containing a sample volume of only 50 µl (22-24). We have set up a multiplex immunoassay (MIA) to detect up to 30 human soluble mediators, all related to chronic inflammation in plasma (24).

We hypothesized that even very young children with CF in clinically stable condition have a pro-inflammatory cytokine and chemokine plasma profile compared to healthy controls. We furthermore assessed the influence of bacterial sputum culture results on plasma cytokine, chemokine and soluble adhesion molecule profiles in CF patients.

MATERIALS AND METHODS

Study subjects

Forty-seven children with CF (28 male, mean (SD) age 6.1 (2.9), age range 1.7-12.2 years) attending the outpatient Paediatric Clinic of the Cystic Fibrosis Centre of the University Medical Centre Utrecht (UMC Utrecht), were included. Only children without signs of an acute pulmonary exacerbation at the time of blood sampling were included.

Thirty-four of the 47 children were able to perform pulmonary function tests (PFTs; forced expiratory maneuvers measured by a pneumotachograph (Masterlab Jaeger, Hochberg, Germany)). PFTs included forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) both expressed as percent predicted values using the reference data from Zapletal (25). Sputum culture or cough swabs (when children were unable to produce and expectorate sputum) were obtained on the same day as blood sampling. Furthermore we performed serologic measurements of *Pseudomonas aeruginosa* (PA) antibodies in all children to three tested antigens (exotoxin A, elastase and alkaline protease) on the same day as sputum culture using a commercially available ELISA-kit (Mediagnost, Reutlingen, Germany) (26). CF patients were divided into 3 groups based on the results of bacterial cultures and PA antibodies. Group 1, designated uninfected, comprised 12 CF children, who had a negative sputum culture and negative PA antibodies. Group 2, designated SA/HI, comprised 26 CF children with a *Staphylococcus aureus* and/or *Haemophilus influenzae* positive sputum culture, and negative PA antibodies. Group 3, designated PA, comprised 9 CF children with a PA positive culture. Eight of the nine children were also positive for one or more of the tested PA antibodies.

Genotyping had been performed and patients were categorized as either having a homozygous ΔF508 mutation (ΔF508/ΔF508), heterozygous ΔF508 mutation (ΔF508/other) or two other mutations (other/other).

Twenty healthy controls (11 male, mean (SD) age 7.7 (3.0) years, age range 3.8-11.5 years) were included either at the out patient clinic of the University Medical Center Utrecht (the Netherlands).

All heparinized blood samples were collected and blood was centrifuged to remove cells. Cell free plasma samples were stored frozen until analysis. The study was approved by the Medical Ethics Committee of the UMC Utrecht and informed consent was given by parents and children when applicable.

Multiplex Immunoassay

All plasma samples were measured using the Bio-Plex system as described previously (24). In short all assays were carried out directly in a 96 well 1.2 µm filter plate (Millipore, Billerica, MA, USA) at room temperature and protected from light. To exclude interference from heterophilic antibodies, immunoglobulins were removed using protein-L coated beads. A mixture containing 1000 microspheres per mediator (total volume 10 µl/well) was incubated together with a standard sample or blank for 1 hour at room temperature. Next, 10 µl of a cocktail of biotinylated antibodies (16.5 µg/ml each) was added to each well and incubated for an additional 60 minutes. Beads were then washed with phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA) and 0.5%-Tween 20 at pH of 7.4. After incubation of 10 minutes with 50 ng/well streptavidin R-phycoerythrin (BD Biosciences, San Diego CA, USA) and washing twice with PBS-1% BSA- 0.5%-Tween 20 pH 7.4, fluorescence intensity of the beads was measured in a final volume of 100 µl HPE-buffer. Calibration curves from recombinant protein standards were prepared using two-fold dilution steps in serum diluent (R&D Systems, Abingdon, United Kingdom). Samples were measured and blank values were subtracted from all readings. Measurements and data analysis were performed using the Bio-Plex system in combination with the Bio-Plex Manager software version 4.0 using five parametric curve fittings (Bio-Rad Laboratories, Hercules CA, USA).

Statistical analysis

Results are expressed as mean ± standard error (SE), unless otherwise specified. Comparisons of cytokine, chemokine and soluble adhesion molecule expression levels between CF patients and healthy controls were performed using non-parametric Mann-Whitney U-tests. Comparisons between the 3 groups of CF patients were performed using a Kruskall-Wallis one-way ANOVA, which, if significant, was followed by a non-parametric Mann-Whitney U-test. A p-value of equal to or less than 0.05 was considered significant. Geometric means were used to generate color profile figures.

All statistical analyses were performed using the statistical package for the social sciences (SPSS) software version 12.0 (SPSS, Chicago, IL, USA).

RESULTS

We first compared cytokine, chemokine and soluble adhesion molecule profiles between the entire group of CF children and healthy controls (shown in table 1). Many pro-inflammatory cytokines such as IL-1α, IL-2, IL-4, IL-5, IL-12 and TNF-α were significantly higher in CF children (p-values<0.001 or <0.05). IL-6 showed a tendency towards higher levels in CF children but this was not statistically significant (p=0.13, table 1). IL-18 levels were extremely low in CF children (table 1, p<0.001). Surprisingly only the chemokines CXCL8 (IL-8) and oncostatin

M (OSM) were significantly higher in CF children (table 1). All other chemokines such as macrophage inhibitory factor (MIF), CCL5 (regulated upon activation normal T cell expressed and secreted (RANTES)), CCL18 (pulmonary and activation regulated chemokine (parc)), and CCL17 (tarc), were significantly lower in CF children compared to healthy controls ($p<0.001$, table 1). Concentrations of sCD54/sICAM-1 were significantly higher in CF children.

Differences between healthy controls and CF patients were most prominent for IL-18, OSM and sCD54/sICAM-1. Median plasma concentration and individual results of IL-18, OSM, and sCD54/sICAM-1 for healthy controls and CF children are depicted in figure 1.

Cytokines such as IL-6, and soluble vascular endothelial cell adhesion molecule-1 (sCD106/sVCAM-1), are higher in plasma of CF patients but this did not reach statistical significance due to the large dynamic biological range of cytokine levels observed in all study subjects (CF children and healthy control children). To better visualize these subtle differences we digitized our data using geometric means creating a color profile of each mediator as described before (22,24) and as is shown in figure 2.

Table 1. Plasma cytokines, chemokines and soluble mediators in healthy controls and the CF children.

Mediator	Healthy controls (n=20)	CF children (n=47)	p-value
IL-1α	0.03 ± 0.0	1.5 ± 0.2	<0.001
IL-1β	1.2 ± 0.6	0.7 ± 0.4	0.042
IL-2	0.1 ± 0.06	0.3 ± 0.0	<0.001
IL-4	0.01 ± 0.0	2.8 ± 1.4	<0.001
IL-5	0.01 ± 0.0	0.7 ± 0.1	<0.001
IL-6	15.8 ± 7.6	31.5 ± 26.1	0.133
IL-10	0.3 ± 0.3	0.4 ± 0.3	0.620
IL-12	7.5 ± 2.4	11.1 ± 2.6	0.004
IL-13	1.9 ± 1.7	1.4 ± 0.8	0.431
IL-15	0.01 ± 0.0	1.0 ± 0.6	<0.001
IL-17	0.3 ± 0.2	0.7 ± 0.4	0.478
IL-18	132.9 ± 28.7	4.1 ± 1.9	<0.001
TNFα	1.1 ± 0.6	3.9 ± 2.9	<0.001
IFNγ	104.1 ± 55.3	79.2 ± 57.0	0.073
OSM	1.7 ± 0.4	15.4 ± 1.7	<0.001
MIF*	2.4 ± 0.4	0.6 ± 0.2	<0.001
CCL2	20.1 ± 10.0	8.5 ± 3.9	0.012
CCL3	56.1 ± 15.6	5.9 ± 3.8	<0.001
CCL5*	63.2 ± 0.4	36.5 ± 0.5	<0.001
CCL11	10.3 ± 3.0	6.2 ± 3.4	0.078
CCL17	43.8 ± 24.6	0.4 ± 0.2	<0.001
CCL18*	40.2 ± 5.3	21.8 ± 4.9	<0.001
CCL22	387.9 ± 52.4	20.0 ± 11.7	<0.001
CXCL8	0.2 ± 0.2	1.0 ± 0.5	<0.001
CXCL9	8.4 ± 3.0	1.6 ± 0.8	<0.001
CXCL10	98.4 ± 36.5	17.9 ± 10.7	<0.001
sCD54*	159.9 ± 5.9	392.4 ± 19.5	<0.001
sCD106*	566.2 ± 31.5	8942.0 ± 2638.8	0.381

All values (mean ± standard error of the mean) are expressed in pg/ml (* values in ng/ml).

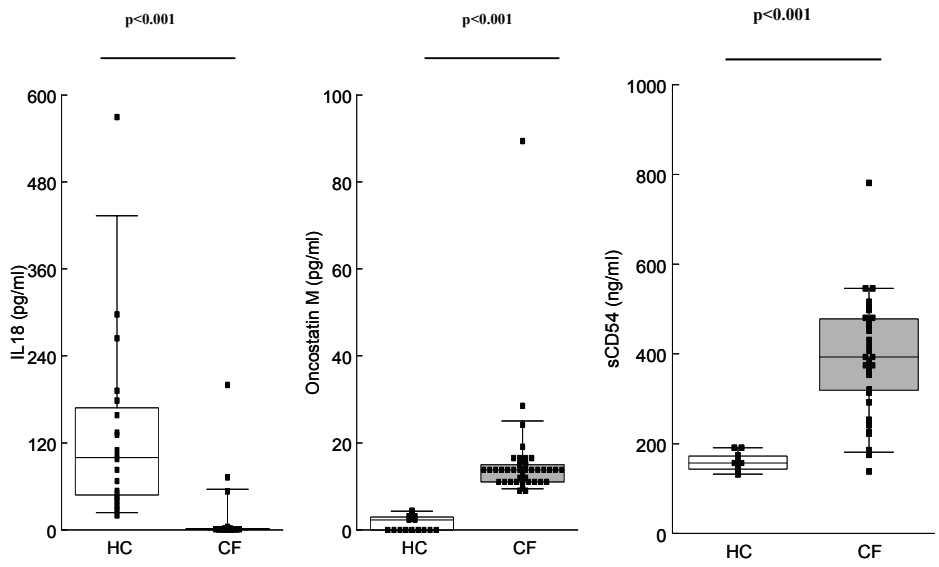

Figure 1. Plasma concentrations of IL-18, Oncostatin M (OSM) and soluble intercellular adhesion molecule 1 (sCD54/sICAM-1) of healthy controls (n=20) and CF children (n=47). Box and whisker plots show median plasma concentrations of healthy controls (white bars) and CF children (gray bars). Depicted are the results of each individual (squares).

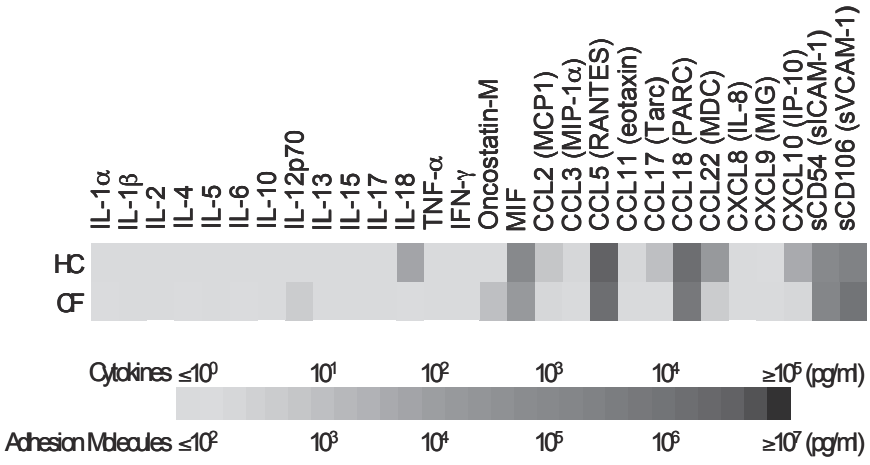

Figure 2. Cytokine, chemokine and soluble adhesion molecule profiles in plasma of healthy controls and CF patients.

Table 2. Patient characteristics of the 3 CF children subgroups (uninfected CF children; CF children with a *Staphylococcus aureus* and/or *Haemophilus influenzae* (SA/HI) positive sputum culture; CF children with a *Pseudomonas aeruginosa* positive sputum culture (PA)). Age is expressed as mean ± standard deviation (SD).

	CF uninfected (n=12)	CF SA/HI positive culture (n=26)	CF PA positive culture (n=9)
Age (years)	6.0 ± 3.0	5.8 ± 2.6	7.2 ± 3.4
Male/female	7 / 5	14 / 12	7 / 2
PFTs			
FEV_1 (%) (n)	99.9 ± 8.0 (9)	102.3 ± 3.5 (18)	89.7 ± 6.5 (7)
FVC (%) (n)	96.3 ± 6.0 (9)	97.3 ± 2.9 (18)	88.6 ± 6.0 (7)
Genotype			
Homozygous ΔF508/ΔF508	8	17	7
Heterozygous ΔF508/ other	4	7	1
Other/other	0	2	1

Furthermore we wondered whether cytokine, chemokine and soluble adhesion molecule profiles in the clinically stable CF children in this cross-sectional study would be influenced by bacterial culture test results. Therefore we subdivided the CF children into an uninfected group (n=12), a SA/HI positive group (n=26), and a PA positive group (n=9). Demographic characteristics for these 3 groups are shown in table 2. The majority of CF children were homozygous for the ΔF508 mutation. There were no significant differences with respect to age and PFTs between the 3 groups (table 2).

No significant differences in any of the cytokines, chemokines, or soluble adhesion molecules were found between the 3 groups, except for sCD106/sVCAM-1. Uninfected children showed significantly lower concentrations of sCD106/sVCAM-1 compared to PA positive CF children (2186.8 ±1207.7 versus 7170.21 ± 2129.7, p=0.025) but not compared to SA/HI positive children (p=0.09).

DISCUSSION

This is the first study describing such an extensive profile of cytokines, chemokines and soluble adhesion molecules in plasma of clinically stable young children with CF. We observed extensive differences between CF patients and healthy controls. In previous studies, individual cytokines, or combinations of a limited set of cytokines have been measured in plasma and serum of patients with CF using conventional ELISA techniques. The multiplex immunoassay allows for multiplex analysis of up to a hundred different mediators in a single sample (23). Therefore this technique can be a useful tool for measurement of cytokines in small sample volumes, such as samples from young paediatric patients.

Many studies have described the relationship between airway infection and local inflammatory response as reflected in BALF, sputum, and exhaled air (2,5,6,27). However most young children with CF are unable to expectorate sputum, bronchoalveolar lavage is invasive and exhaled breath measurements require breath manoeuvres difficult to perform by young children. Measurement of inflammatory markers in blood might provide a good alternative. In this study we describe elevated levels of different pro-inflammatory cytokines such as IL-1α, IL-2, IL-4, IL-5, IL-12 and TNF-α, even in young CF children with normal lung function. Both Th1 and Th2 cytokines were elevated in CF children and we found no difference in plasma cytokine profile with respect to these cytokines between CF children that were either infected with *Pseudomonas aeruginosa*, or *Staphylococcus aureus* and/or *Haemophilus influenzae*, or were uninfected. Levels of many cytokines were however low, and the clinical relevance of the increased values of some of these cytokines is not clear.

Concentrations of IL-18 however were significantly lower in CF children as has been described previously in BALF from CF patients (28,29) and serum (29). The exact cause for these decreased IL-18 protein levels remains to be elucidated but a role for *Pseudomonas aeruginosa* exotoxin A and IL-10 have been postulated (28-30). Decreased IL-18 might reduce IFN-γ production thus affecting Th1 immune response. We furthermore found significantly higher sCD54/sICAM-1 concentrations in CF children compared to healthy controls as was described previously in clinically stable CF patients (21). Although sCD106/sVCAM-1 was not increased in all CF children combined compared to healthy controls, we did find a significantly higher concentration of sCD106/sVCAM-1 in CF children that were infected with *Pseudomonas aeruginosa* compared to uninfected CF children.

In contrast to our findings in cytokine and soluble adhesion molecule profile, most chemokines (except CXCL8 (IL-8) and OSM) measured in this study were significantly lower in CF children. CF lung disease is a neutrophil-dominated inflammatory disorder and neutrophils are directed to the site of inflammation and infection by CXC chemokines such as CXCL8 (IL-8). However, most chemokines measured in this multiplex assay were part of the CC chemokine family and these chemokines attract different types of cells (such as T cells and monocytes) to the site of inflammation and infection. Question remains why these chemokines are lower in young CF patients compared to healthy controls and whether this is a finding only in young children. Previous studies have described several CC chemokines in CF patients. Cystic fibrosis transmembrane regulator (CFTR) has been shown to regulate the expression of several genes, including the chemokine CCL5 (RANTES) (31). In CF patients BALF specimens contained reduced levels of RANTES compared to asthmatics (32) and CF airway epithelial cells express little or no RANTES protein or mRNA compared with non-CF airway epithelial cells (33). This is in accordance with the lower plasma CCL5 levels found in our study. MIF in plasma was found to be increased in 6 chronically infected CF patients, but demographics of these patients were not described (34). It is possible that these CF patients were much older than the CF children included in

our study. Recently, CCL17 (tarc) in serum has been proposed as a marker for identification and monitoring of allergic bronchopulmonary aspergillosis (ABPA) in CF patients but plasma concentrations of CCL17 have not been described in young CF children without ABPA (35). It would therefore be very interesting to assess longitudinally whether the concentrations of these CC chemokines in plasma remain low or whether they increase with progression of lung disease, especially since the lungs of most CF patients become chronically infected with *Pseudomonas aeruginosa*.

In conclusion, even young, clinically stable CF children display a derangement of inflammatory mediator profiles. Pro-inflammatory cytokines are increased in plasma while CC chemokine concentrations are decreased. Our results underscore CF as a disease characterized by a complex general inflammatory process that involves activation of both innate and adaptive immunity. Cytokine signature measured in blood might provide the potential to assess risk factors related to pulmonary outcome in both individual and groups of CF patients. Long-term follow-up studies are needed to substantiate the clinical potential of measurement of such an extensive cytokine signature in CF patients.

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Chapter 5

End tidal carbon monoxide corrected for lung volume is elevated in cystic fibrosis patients

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ABSTRACT

Several factors influence levels of end tidal CO (ETCO). We studied determinants of ETCO corrected for inhaled CO (ETCOc) levels in healthy controls and compared ETCOc levels and determinants between healthy controls and cystic fibrosis (CF) patients. Thirty healthy controls, mean ± SD age 23 ± 6 years, and twenty clinically stable CF patients, aged 13.5 ± 3.5 years were included. ETCO was measured with the CO-STAT® End Tidal Breath Analyzer and determinants included lung volume (measured with the multiple-breath helium wash-in method), CO-diffusion capacity and different expiratory flow rates.

In healthy controls we found a significant correlation between ETCOc and lung volume ($r=0.64$, $p<0.05$) and with CO diffusion capacity uncorrected for alveolar volume ($r=0.48$, $p=0.02$). There was no expiratory flow rate dependency in either group. CF patients showed no difference in ETCOc levels compared to controls (mean 1.2 ± 0.4 ppm versus 1.3 ± 0.4 ppm, $p=0.32$) but CF patients had lower TLC-He than healthy controls. ETCOc corrected for lung volume was significantly higher in CF patients compared to controls ($p<0.001$).

We hypothesize that a possible increase in breath CO caused by airway inflammation might be masked by differences in lung volumes between controls and CF patients.

INTRODUCTION

Exhaled carbon monoxide (CO) concentration, usually quantitated in end tidal breath (ETCO), has been described in several studies as a candidate marker for airway inflammation in lung diseases like cystic fibrosis (CF)(1-7). Increased levels of ETCO described in these studies might reflect increased oxidative stress, which can cause an induction of the enzyme heme oxygenase (HO-1). This induced HO-1 has its origin in the respiratory epithelium of the bronchi and in airway macrophages and is considered to be an antioxidant enzyme (6-9). However, in a recent study, no increase in ETCO in patients with CF or asthma was found and there seem to be several pitfalls in measurements of exhaled CO that need to be considered (10).

ETCO can be quantified by a number of different techniques. Most techniques are based on electrochemical CO sensors that are inexpensive and give reproducible results but are susceptible to interference from a number of different breath components, for example, hydrogen (H_2) (8). Therefore, breath analyzers insensitive to H_2 or with separate H_2-sensors need to be used for obtaining reliable results (11,12). CO levels in exhaled air are influenced by levels of CO in inhaled ambient air and by active and passive tobacco smoking. Thus, it is important that ETCO measurements are corrected for inhaled air represented by room air CO (ETCOc) and that smoking is excluded. Under normal physiological conditions ETCO is largely produced by the oxidative degradation of heme and diffuses from the blood stream to alveolar air. This alveolar origin is supported by the lack of expiratory airflow dependency of ETCO concentration (10). It is unclear whether in patients with inflammatory diseases, like CF, an increase in ETCO concentration is caused by increase of the systemic CO production, or by production of CO in the lungs due to induction of epithelial HO-1 or possibly due to lipid peroxidative processes (13-15). Furthermore, a decrease of CO diffusion capacity or alveolar surface area might influence the amount of CO transferred to the alveolar air. In patients with CF a decline in lung volume and diffusion capacity occurs during the course of the disease because of fibrosis and this might influence ETCOc (15,17). Finally ETCOc can be influenced by a number of pathologic and non-pathologic conditions that increase the rate of hemoprotein degradation like anemia, hematomas and fasting (18).

The aim of this study was to investigate correlation of determinants such as lung volume, CO diffusion capacity and expiratory flow rate, with ETCOc measurements in healthy subjects using the relatively hydrogen insensitive CO-STAT® End Tidal Breath Analyzer (Natus Medical, Inc., San Carlos, CA). Furthermore we studied possible differences between healthy subjects and patients with CF with regard to these determinants.

METHODS

The study was approved by the ethics committee of the University Medical Center Utrecht where the studies were conducted.

Thirty (19 female) healthy, non-smoking volunteers, aged 22.8 ± 6.1 years (range 10-38 years), were included. Twenty patients with CF (9 female) in clinically stable condition i.e. without actual signs of pulmonary infections, defined as an increase in sputum production or cough, fever, anorexia or decline in lung function, (especially FEV1%) were recruited from the Pediatric and Adult Cystic Fibrosis Clinic at the University Medical Center Utrecht. CF patients were aged 13.5 ± 3.5 years (range 7-21 years), percent predicted forced expiratory volume in one second (FEV_1%) was 73 ± 25, percent predicted forced vital capacity (FVC%) was 84 ± 20 and percent residual volume as part of total lung capacity (RV/TLC%) was 37 ± 16. All patients were colonized with *Pseudomonas aeruginosa* and/or *Staphylococcus aureus* bacteria.

ETCOc measurements were assessed with the CO-STAT®. This is a computer-controlled instrument containing an infrared optical bench for the measurement of CO_2 and an electrochemical sensor for the measurement of CO and hydrogen. A single-use patient sampler, consisting of a flexible, 5-French nasal catheter with a filter cartridge is attached to the instrument and inserted 5 mm into the nostril. During 90 seconds of normal nasal breathing the subject's expired air is continuously sampled by the instrument for quantitation of the mean ETCO and CO_2. At the completion of the test the catheter is disconnected from the filter and the room air CO concentration is measured in order to correct ETCO for inspired CO (ETCOc). With this device, ETCOc can be measured easily and reproducibly, even in young infants, since the only requirement is spontaneous breathing (12).

Expiratory flow rate dependency was studied in a subgroup of 11 controls and 4 patients with CF, in order to distinguish between an alveolar and bronchial origin of ETCOc. Different expiratory flow rates were achieved by using an adjustable expiratory flow resistance, in series with a Lilly- type pneumotachometer. Measurements were performed without resistance and with increasing resistances, resulting in continuous expiratory flow rates of 0.05 $L \cdot s^{-1}$ and 0.2 $L \cdot s^{-1}$.

Lung volume (TLC-He) was assessed in all healthy subjects and patients with CF using the multiple-breath helium wash-in method (Masterscreen FRC, Erich Jaeger, Würzburg, Germany). In 23 out of 30 controls and in 5 out of 20 patients with CF CO diffusion capacity (DLco) was measured using a standardized single breath technique (Masterlab). All lung function measurements were performed according to the ATS/ERS standards. Other lung function parameters in patients with CF and controls were measured with spirometry and plethysmography (Masterscreen CS and Masterlab systems) and contained the following parameters: FEV_1%, FVC%, RV/TLC%, TLC-box and TLC-box %. For reference values data of Zapletal were used (19).

Statistical analysis

All values are represented as mean ± SD, except for box and whisker plots, where whiskers show the range and boxes show the 25th, 50th (median) and 75th percentiles.

Correlation between ETCOc and TLC-He and DLco was assessed with Pearson's correlation coefficient. Univariate regression analysis was used to assess the regression equation of ETCOc and TLC-He.

Mean values of ETCOc and of ETCOc corrected for TLC-He of controls and patients with CF were compared using the nonparametric Mann-Whitney U-test.

Statistical analysis was performed using the Statistical Package for the Social Science (SPSS version 10.1, Chicago, IL USA).

RESULTS

Group characteristics are described in table 1. Patients with CF were significantly younger than healthy controls and had significantly lower TLC-He values (also in percent predicted)

Table 1. Group characteristics

Characteristic	Controls (n=30) Mean ± SD	CF-patients (n=20) Mean ± SD
ETCOc (ppm)	1.3 ± 0.4	1.2 ± 0.4
Background CO (ppm)	0.3 ± 0.1	0.3 ± 0.1
Age (years)	22.8 ± 6.1	13.5 ± 3.5 †
Lung function parameters		
TLC-He (L)	5.81 ± 1.25	3.70 ± 1.37 †
TLC-He (%)	105 ± 10	86 ± 15 †
DLco (mmol/min/kPa)*	9.6 ± 1.9	5.9 ± 2.4 †
DLco/V_A (mmol/min/kPa/L)*	1.8 ± 0.22	1.96 ± 0.34
FEV1 (%)	111 ± 12	73 ± 25 †
FVC (%)	106 ± 10	84 ± 20 †
RV/TLC (%)	22.2 ± 5.3	37.3 ± 16.2 †
TLC-box (L)	5.48 ± 1.38	4.35 ± 1.11 †
TLC-box (%)	105 ± 12	103 ± 13
Serum IgG (g/L)		13.6 ± 4.5
CRP (mg/L)		8.6 ± 5.1

DLco (mmol/min/kPa)* was measured in 19 of 30 controls and in 5 of 20 CF-patients.
† $p<0.05$

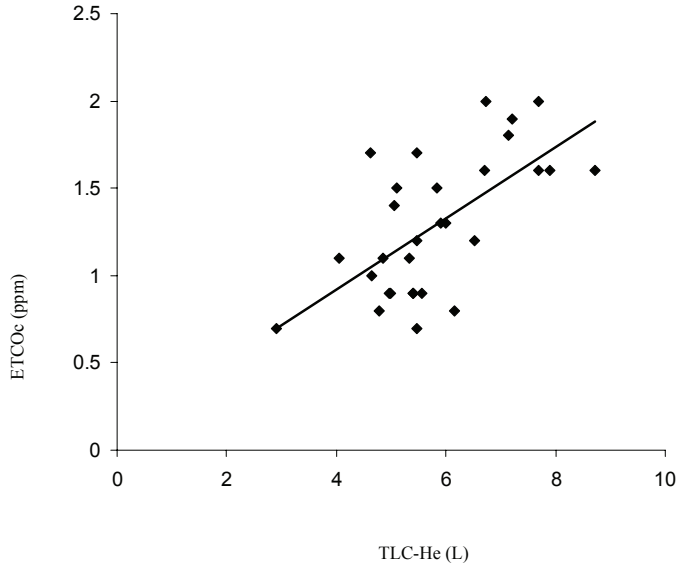

Figure 1. Relationship between ETCOc (ppm) and TLC-He (L) in 30 healthy subjects (ETCOc = 0.2043 times TLC-He +0.1035, r= 0.64)

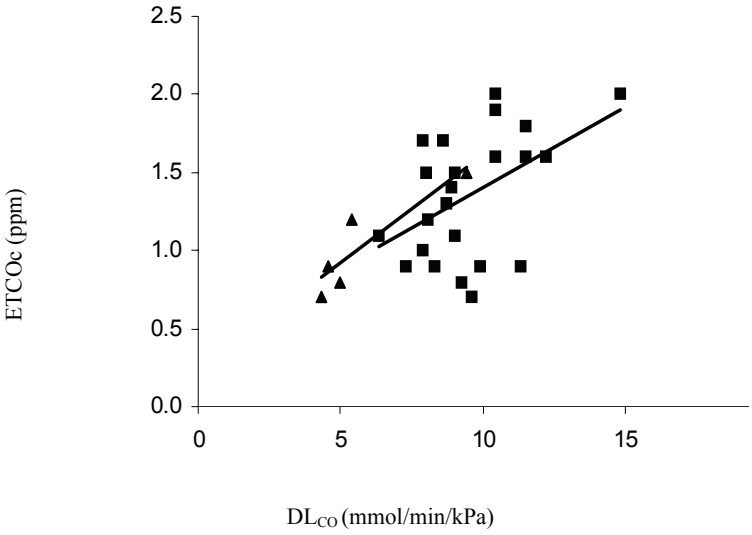

Figure 2. Relationship between ETCOc (ppm) and DLco (mmol/min/kPa) in 19 healthy controls (squares, r=0.48) and 5 CF patients (triangles, r=0.89).

ETCOc correlation with parameters in healthy controls

ETCOc in healthy controls showed a significant correlation with TLC-He (r=0.64, p<0.05), TLC-box (r=0.73, p<0.05) and with DLco uncorrected for V_A(r=0.48, p=0.021). Linear regression analysis was performed and figure 1 shows the regression line and equation for ETCOc and TLC-He. Figure 2 shows correlation between ETCOc and DLco for controls and CF patients. As indicated in figure 3, no significant differences in ETCOc at the two flow rates of 0.2 L·s $^{-1}$ and 0.05 L·s $^{-1}$ could be seen in the exhaled air of eleven healthy control subjects and four patients with CF.

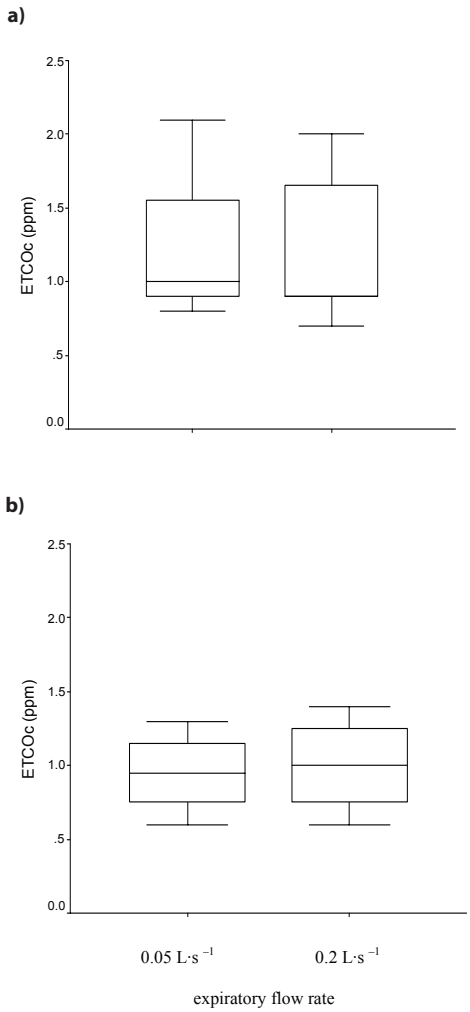

Figure 3. Comparison of expiratory flow rates of 0.05 L·s $^{-1}$ and 0.2 L·s $^{-1}$ and their influence on the ETCOc levels (ppm) in a) healthy controls (n=11) and b) patients with CF (n=4).

Comparison controls and patients with CF

Patients with CF showed no difference in ETCOc levels compared to controls (1.2 ± 0.4 versus 1.3 ± 0.4 ppm, p=0.32, figure 4). Since there was a strong relation between ETCOc and TLC-He in healthy controls and TLC-He was significantly lower in CF patients, we assessed ETCOc corrected for TLC-He (in %) in both groups by using the regression equation ETCOc=0.2043*TLC-He+0.1035 (figure 1). ETCOc corrected for TLC-He values were significantly higher in the patients with CF compared to controls (ETCOc (%) 143 ± 41% versus 100 ± 25%, p<0.001, figure 5).

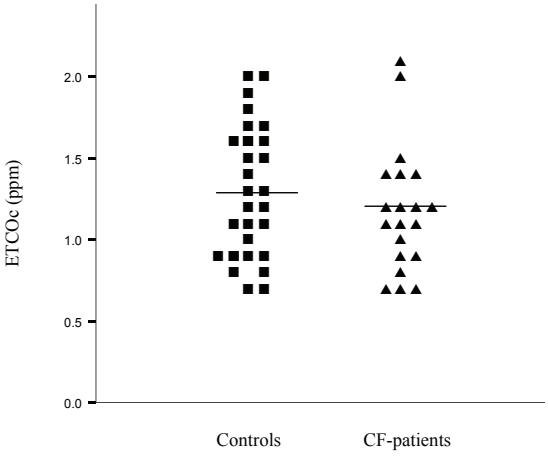

Figure 4. ETCOc (ppm) in healthy controls (n=30 squares) compared to patients with cystic fibrosis (n=20 triangles) p=0.32.

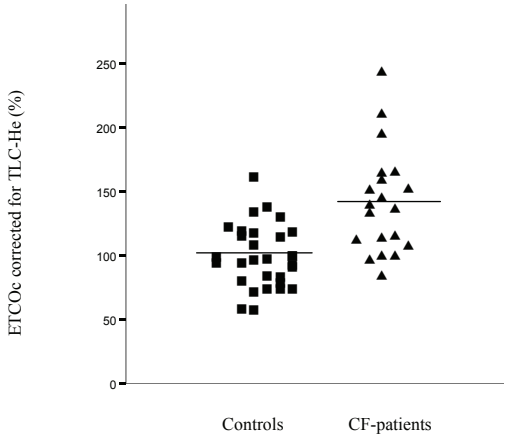

Figure 5. ETCOc corrected for TLC-He (%) in healthy controls (n=30 squares) compared to patients with cystic fibrosis (n=20 triangles) p<0.001.

DISCUSSION

In our study we found no significant difference in ETCOc levels between healthy controls and patients with CF. Using the CO-STAT analyzer we found that several physiological factors influenced ETCOc levels in healthy subjects. Most striking was the relation between ETCOc and total lung volume as measured with the multiple-breath helium wash-in method. Apparently, subjects with larger lung volumes, and probably larger lung surface areas produce higher concentrations of breath CO. Our study is the first to describe this relationship. Another physiological factor that might influence exhaled CO level, like CO diffusion capacity was significantly related to ETCOc in healthy volunteers and also in CF patients, although we must state that we only measured DLco in 5 CF patients. CO diffusion capacity corrected for alveolar volume was not significantly related to ETCOc. This is plausible since both ETCOc and CO diffusion are dependent on alveolar volume. In this study we found no expiratory airflow rate dependency in healthy controls which suggests an alveolar origin of CO as has been implied previously by Zetterquist and colleagues and by Kharitonov and colleagues (10,20).

Our findings in healthy controls suggest that the lack of difference in ETCOc levels between controls and patients with CF might be due to important differences in baseline characteristics. When ETCOc in both groups was corrected for lung volume we found a significantly higher ETCOc predicted in the patients with CF compared to controls (figure 5). Although values for ETCOc corrected for TLC-He were significantly higher in CF patients, the range of values for CF patients and healthy controls have significant overlap. In healthy subjects ETCOc also strongly correlated with TLC-box but since airtrapping (shown by significantly higher RV/TLC% in CF-patients) was increased in CF-patients, we chose to correct ETCOc for TLC-He which better reflects the actual ventilating part of the total lung volume. Progressive disease in CF is characterized by a decrease of functional alveolar volume and surface because of mucus plugging and air trapping. The progressive differences in TLC-He between patients and controls might mask differences in alveolar CO diffusion and or production. This is in line with data of Togores and colleagues who showed that levels of ETCO can be underestimated in smokers with severe airflow obstruction (21).

Previous studies have shown both increased ETCO levels (1-6) as well as equal levels of ETCO in patients with CF compared to healthy controls (10). Differences in ETCOc found in our study groups and the groups described in previous studies might be caused by the previous use of both different electrochemical sensors and/or measuring techniques, as well as the lack of correction for inhaled CO. ETCOc in our study was analyzed with the CO-STAT Analyzer that has a separate sensor for hydrogen and measures exhaled CO during spontaneous breathing (11,22). Levels of ETCO measured with other electrochemical sensors like the EC50 (Bedfont) were obtained after the following maneuver. Subjects inspired maximal from functional residual capacity, held their breath at total lung capacity for 15-20 seconds and then exhaled into the mouthpiece of the analyzer. Zetterquist and colleagues have shown

that exhaled CO levels increased by about 80% from baseline after a 10 seconds breath hold and that exhaled CO levels did not increase further after a longer breath-hold (10). It is possible that especially in patients with a ventilation-perfusion mismatch (as in CF) a 15 seconds breath-hold facilitates a prolonged diffusion of CO from the blood to the alveoli and thus results in higher levels of exhaled CO than when measured with the CO-STAT analyzer.

It has also been reported that other breath components like hydrogen, and hydrogen peroxide (H_2O_2) can interfere with ETCO measurements (11). Hydrogen peroxide in exhaled air of children with CF with an infectious exacerbation is elevated, but is not in patients with stable disease (23,24). Other substances like ethane and 8-isoprostane are increased in exhaled air in patients with CF and these correlate well with ETCO levels and may interfere with CO measurements (4,25). Another possible explanation might be differences in inhaled CO in the different studies although in all recent studies ETCO is corrected for background CO. Finally ETCOc is not measured orally, but nasally with the CO-STAT Analyzer. There is some discussion about whether or not CO is endogenously produced in the nose and paranasal sinuses. Andersson and colleagues described increased CO levels by breathing through the nose (26,27). Lundberg and colleagues however were unable to detect any CO signal in nasal air (28). The CO-STAT Analyzer has been validated against the Vitalograph Breath CO-monitor (Vitalograph Inc., Lenexa, KS) and when measuring ETCOc with different expiratory flow rates in this study, ETCOc was measured orally (22). We found no difference between nasally measured ETCOc and orally measured ETCOc with different flow rates. The use of another measuring method therefore cannot explain the differences in ETCOc found between our study and previous studies.

Elevated levels of ETCO in inflammatory lung diseases are believed to be caused by increased production in airway epithelium and is attributed to upregulation of HO-1. Although induction of HO-1 is predominantly caused by increased gene transcription rates, there has been no evidence yet that upregulation of HO-1 mRNA in vivo leads to a quantitative increase in enzyme activity or CO production (10,29-32). In healthy controls and in CF patients we found no expiratory flow dependency in ETCOc levels. Expiratory flow independence makes a contribution of CO from the bronchi less likely and confirms the alveolar origin of ETCO (10,20). The predominant source of endogenously produced CO (about 85%) is produced by enzymatic degradation of heme and we cannot exclude increased hemolysis in patients with CF, resulting in higher COHb and exhaled CO levels, since hemolysis or COHb was not investigated in this study. Finally, endogenously produced CO also originates from non-heme related release like lipid peroxidation and the release of CO by some bacteria but under normal physiological conditions this accounts for only 15% of the endogenously produced CO (33,34). The lungs of all patients with CF in this study were colonized with *Pseudomonas aeruginosa* and or *Staphylococcus aureus* bacteria. CO release however by these bacteria has not been described and it is not clear if bacterial CO release plays a role of importance in the endogenous production of CO in a disease like CF. Lipid peroxidation might play a role of

importance in additional production of ETCO, which is suggested by an increase of markers of lipid peroxidation in exhaled air and in plasma (4, 25-26,35-36).

In summary, in this study we have shown that levels of ETCOc are strongly related to lung volume in healthy controls and are expiratory flow independent in both controls and patients with CF. We found no difference in ETCOc between controls and patients with CF although ETCOc corrected for lung volume was significantly higher in patients with CF. An increase in CO caused by airway inflammation might be masked by differences in lung volumes between controls and patients with CF. In order to assess utility of ETCOc as a biomarker of airway inflammation in CF, future studies are needed. ETCOc and ETCOc corrected for TLC-He need to be measured longitudinally in the same group of CF-patients, in clinically stable condition and in times of pulmonary exacerbation.

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Chapter 6.1

Correlation of six different cystic fibrosis chest radiograph scoring systems with clinical parameters

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SUMMARY

In past decades several chest radiograph scoring systems for cystic fibrosis have been developed. This study was performed to compare interobserver variability of six different radiograph scores and to correlate them with clinical parameters.

Thirty chest radiographs of 30 patients with cystic fibrosis were scored with the Shwachman-Kulczycki scoring, the Chrispin-Norman scoring, the adjusted Chrispin-Norman scoring, the Brasfield scoring, the Wisconsin scoring and the Northern scoring system by two independent observers. Data on clinical parameters like lung function, nutritional status and infectious exacerbation rate, obtained simultaneously with the chest radiograph were reviewed.

Interobserver variability was low (Pearson's correlation coefficients 0.76 to 0.84, all p-values <0.01) and the scores had good limits of agreement (Bland and Altman). Correlation of radiograph score with clinical parameters was good for most pulmonary function test data (correlation coefficients from 0.72 to 0.78 for FEV_1% predicted and from 0.69 to 0.74 for FVC% predicted) and for infectious exacerbation rate (correlation coefficients from 0.68 to 0.73).

All six radiograph scoring systems, especially the Chrispin-Norman score, showed a low interobserver variability and correlated well with lung function tests, especially FEV1% predicted and infectious exacerbation rate and moderately with maximum work capacity and thoracic mobility.

INTRODUCTION

In the past decades several chest radiograph scoring systems have been developed to assess the severity of respiratory disease in cystic fibrosis (CF) (1-7). Among these are the Shwachman-Kulczycki scoring (1), the Chrispin-Norman scoring (2), the adjusted Chrispin-Norman scoring (4), the Brasfield (Birmingham) scoring (3), the Wisconsin scoring (6) and the Northern scoring system (7). Critical evaluation of most of these chest radiograph scoring systems has shown a good reproducibility among different observers.

Most of these studies have dealt with mutual comparison of only two or three different scoring systems. Data on correlation of radiograph scoring systems with lung function and maximum work capacity are scarce (3,6,8-12). The relationship of the scoring systems with other clinical parameters of prognostic importance in CF, like body mass index and rate of pulmonary exacerbations has not been described so far.

In this study we compared six different radiograph scoring systems scored by two independent observers and evaluated the clinical relevance of these systems in relation to lung function test data, nutritional status, thoracic mobility, maximum work capacity, infectious exacerbation rate and serum immunoglobulin G levels (IgG).

METHODS

Thirty chest radiographs of children with CF taken between January 2001 and May 2001 as a part of the annual routine check-up were studied. The children were randomly chosen from the database of the CF Centre of the Wilhelmina Children's Hospital and had to be at least 9 years old in order to be able to perform a maximal exercise test. At the time of their check-up all children were in a stable condition for at least one month without any clinical symptom of an infectious exacerbation (defined as a period of decline in lung function, increase in sputum production and cough, and need for oral or intravenous antibiotics).

Two observers (NT and NvP) scored all 30 chest radiographs according to six different chest radiograph scoring systems. They were unaware of the clinical condition of the patients. The six scoring systems used were the Shwachman-Kulczycki scoring, the Chrispin-Norman scoring, the adjusted Chrispin-Norman scoring, the Brasfield scoring, the Wisconsin scoring and the Northern scoring system. Radiographs were scored independently and in random order by each observer using a combined list with all items from the different scoring systems. Data on lung function, body mass index (BMI) as a parameter of nutritional status, thoracic mobility, maximum work capacity, infectious exacerbation rate over the previous six months and total IgG as a parameter of inflammation, simultaneously obtained with the chest radiograph, were reviewed. Lung function was measured with Jaeger Masterscreen CS and Masterlab systems (Wuerzburg, Germany) and contained the following parameters: percent

predicted forced expiratory volume in one second (FEV_1%), percent predicted forced vital capacity (FVC%), percent FEV_1 as part of vital capacity (FEV_1/VC%), percent predicted peak expiratory flow rate (PEFR%), percent predicted total lung capacity (TLC%) and percent residual volume as part of total lung capacity (RV/TLC%). For reference values data of Zapletal were used (13).

Nutritional status was calculated as body mass index (weight/height^2) and was expressed as the percentage predicted body mass index of the 50th percentile (p50) of age and sex matched healthy subjects (Dutch growth-research by TNO/LUMC 1997). Thoracic mobility was expressed as the difference (in centimeters) in thoracic circumference between maximal inspiration and expiration at the xyphoid process. Maximum work capacity was performed on a stationary bicycle ergometer (Lode, Groningen the Netherlands and Jaeger Oxycon Champion, Wuerzburg Germany). Results included Wmax (maximum power generated on the bicycle in Watt) and maximal oxygen uptake capacity per kilogram (kg) body weight ($V'O_2$max/kg in ml/min/kg). $V'O_2$max/kg was expressed as percent predicted (14-15). In this study an infectious exacerbation was defined as a period of decline in lung function, increase in sputum production and cough, and need for intravenous antibiotics in the six months prior to the chest radiograph. IgG was measured in serum (expressed in g/L).

Statistical analysis

The interobserver variability of the different radiograph scoring systems was assessed by Pearson's correlation coefficient and interobserver agreement was determined using Bland and Altman plots (16). With a Bland and Altman plot, the difference of the two scores of the two observers is plotted against the mean of the two scores. Since perfect correlation is obtained when the measurements lie along any straight line and perfect agreement is obtained when measurements lie along the line of equality Bland and Altman analysis was performed besides correlation.

The correlation of clinical data and the different scoring systems was assessed by Pearson's correlation coefficient. A p-value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics are described in table 1.

The interobserver correlation coefficient was 0.84 for the Shwachman-Kulczycki score, 0.84 for the Chrispin-Norman score, 0.84 for the adjusted Chrispin-Norman score, 0.82 for the Brasfield score, 0.80 for the Wisconsin score and 0.76 for the Northern score (all p-values <0.01).

Table 2 shows the means and limits of agreement between the two observers assessed by Bland and Altman plots. In relation to the scoring range of the six scoring systems, limits of agreement were best for the Chrispin-Norman score, the adjusted Chrispin-Norman score

Table 1. Patient characteristics (n=30)

Patient characteristic	Mean ± SD
Age (years)	13.8 ± 2.7
Sexe (m:f)	15:15
Lung function	
FEV_1% pred	72 ± 26
FVC% pred	83 ± 21
FEV_1/VC%	71 ± 12
PEFR% pred	83 ± 19
TLC% pred	100 ± 12
RV/TLC%	40 ± 15
BMI (%p50)	97 ± 13
Thoracic mobility (cm)	7.3 ± 1.7
Exercise tolerance	
Wmax (W)	134 ± 42
V'O_2max/kg (ml/min/kg)	38 ± 11
Exacerbation rate (number in last half year)	0.8 ± 1.2
Serum IgG (g/l)	13.1 ± 3.8

Values are presented as mean ± SD. BMI= body mass index, IgG= immunoglobulin G

Table 2. Mean and limits of agreement (Bland and Altman)

Scoring system	Mean	Limits of agreement
Shwachman-Kulczycki	-0.7	-4.8 – 3.2
Chrispin-Norman	-0.6	-6.0 – 4.8
Adjusted Chrispin-Norman	-1.0	-6.8 – 4.8
Brasfield	0.8	-2.0 – 3.6
Wisconsin	-6.7	-27.2 – 14
Northern	-0.2	-4.4 – 4.0

Limits of agreement are expressed as ± 2SD of the mean.

and the Brasfield score. There was a good agreement for all six scores between the two observers which means that the scores of observer 1 and 2 are comparable for the different scoring systems.

Table 3 shows the Pearson's correlation coefficients for the clinical parameters and the mean of the two observers of all six different radiograph scores. All six radiograph scores showed a good correlation with FEV_1%, FVC%, RV/TLC% and infectious exacerbation rate and a moderate correlation with Wmax and V'O_2max/kg and thoracic mobility. There were no marked differences in correlation with clinical parameters between the different scoring systems, although the adjusted Chrispin-Norman score seemed to have the best correlation with lung function.

Table 3. Correlation of radiograph scores with clinical parameters

	Shwachman Kulczycki	Chrispin-Norman	Adjusted Chrispin-Norman	Brasfield	Wisconsin	Northern
Lung function						
FEV_1% pred	0.73[b]	-0.77[b]	-0.78[b]	0.72[b]	-0.72[b]	-0.72[b]
FVC% pred	0.74[b]	-0.73[b]	-0.74[b]	0.69[b]	-0.69[b]	-0.72[b]
FEV_1/VC%	0.57[b]	-0.68[b]	-0.69[b]	0.62[b]	-0.63[b]	-0.55[b]
PEFR% pred	0.36	-0.45[a]	-0.46[a]	0.35	-0.37[a]	-0.35
TLC% pred	-0.22	0.34	0.33	-0.35	0.30	0.25
RV/TLC%	-0.71[b]	0.73[b]	0.74[b]	-0.70[b]	0.67[b]	0.72[b]
BMI (%p50)	0.36	-0.43[a]	-0.41[a]	0.31	-0.39[a]	-0.35
Thoracic mobility	0.45[a]	-0.42[a]	-0.44[a]	0.46[a]	-0.42[a]	-0.50[a]
Exercise tolerance						
Wmax	0.50[a]	-0.53[b]	-0.53[b]	0.41[a]	-0.47[a]	-0.45[a]
$V'O_2max$ (%pred)	0.39[a]	-0.43[a]	-0.45[a]	0.41[a]	-0.42[a]	-0.36
Exacerbations	-0.68[b]	0.71[b]	0.69[b]	-0.69[b]	0.73[b]	0.71[b]
IgG	-0.47[a]	0.30	0.34	-0.42[a]	0.30	0.43[a]

[a] correlation is significant at the 0.05 level (2-tailed)
[b] correlation is significant at the 0.01 level (2-tailed)
BMI= body mass index, IgG= immunoglobulin G

DISCUSSION

In this study we found a low interobserver variability of all 6 scoring systems for CF, especially for the Chrispin-Norman, the adjusted Chrispin-Norman and the Brasfield scoring system. We also found a good correlation of all six scoring systems with lung function (except for TLC%) and infectious exacerbation rate, especially for the adjusted Chrispin-Norman score. The correlation of the scoring systems with maximum work capacity and thoracic mobility was moderate. Poor correlations were found for nutritional status and for markers of inflammation (IgG).

Previous studies have already shown a low interobserver variability of the six radiograph scoring systems developed for CF (8,10-11,17-20). Neither one of the previous studies, however, compared more than 3 different scoring systems nor evaluated the correlation of the radiograph score with nutritional status, infectious exacerbation rate, thoracic mobility or serum IgG. Only a few of these previous studies assessed the correlation between radiograph scores and lung function tests (3,7-11,18). The Shwachman-Kulczycki, adjusted Chrispin-Norman and Wisconsin score have not been correlated with lung function. Only one study correlated the exercise tolerance with radiograph scores and found no significant correlation in the 15 patients studied whereas we found a moderate and significant correlation with maximum work capacity (12).

In our study FEV_1% had the best correlation with all six radiograph scores. FEV_1 is prognostically one of the most important lung function tests in the evaluation of morbidity and mortality of CF (21-23). From the age of approximately five years old most children can perform reliable lung function tests especially if these tests are performed with the assistance of qualified lung function personnel trained to work with children. Lung function tests are functional, non-invasive, easily reproducible and repeatable. If applicable they form an easy and reliable way to assess the disease status.

Chest radiography is recommended annually by the Cystic Fibrosis Foundation (24,25). Especially in young children, who are unable to perform lung function testing, the chest radiograph might be helpful in evaluating the disease status. However, conventional chest radiology seems insensitive to early lung abnormalities as seen in young children with CF. Several recent studies suggest that high-resolution computerized tomography scans are more sensitive in detecting minor pulmonary abnormalities in CF (26-30). Demirkazik and colleagues found a good correlation between the HRCT score according to Bhalla and pulmonary function namely FEV1% and FVC% and between HRCT score and the Shwachman-Kulczycki scoring system (28,31). Although previous study results seem promising, HRCT also has important disadvantages compared to conventional radiology. Very young children often need to be sedated which includes a risk, radiation dosage is much higher than with conventional radiology and the costs for HRCT are much higher. Current development techniques like a higher speed of the scanners and obtaining fewer scans at greater intervals can lower radiation dosage, costs and can make the use of sedation unnecessary in the future (26).

The plain chest radiograph in CF has an important role in the exclusion of acute complications like a pneumothorax and acute bronchopulmonary aspergillosis. Because a chest radiograph is relatively inexpensive and the radiation dosage is low it has been used as a valuable and additional tool alongside pulmonary function testing in longitudinal evaluation of disease progression. In case a chest radiograph is obtained, a simple scoring method with good interobserver variability like the Chrispin-Norman score is preferable.

CONCLUSION

Chest radiograph scores in CF, especially the Chrispin-Norman score, show a low interobserver variability and correlate well with lung function parameters (except TLC%) and infectious exacerbation rate and moderately with exercise tolerance and thoracic mobility. There is a poor correlation with nutritional status and serum IgG. A chest radiograph is useful in identifying complications in CF, like pneumothorax and acute bronchopulmonary aspergillosis and in the assessment of regional differences in disease status. The chest radiograph plays a complementary role in evaluating disease status.

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Chapter 6.2

Radiological and functional changes over 3 years in young children with cystic fibrosis

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Submitted

ABSTRACT

The aim of this study was to evaluate airway disease progression assessed by chest radiology, expiratory interrupter resistance ($Rint_{exp}$), and spirometry in young children with CF over a 3-year interval.

Two chest radiographs combined with two $Rint_{exp}$ measurements, in a 3 year interval, were performed in 21 preschool children (mean (SD) age 3.2 (0.9) years) and 30 school children with CF (mean (SD) age 7.2 (1.9) years). Chest radiographs were scored using five different CF scoring systems and $Rint_{exp}$ measurements were expressed as height adjusted Z-scores. Spirometry was assessed in school children and results were expressed as percent predicted.

Chest radiograph scores worsened significantly over the 3 year period and worsening was most prominent in preschool children. Most preschool and school children had $Rint_{exp}$ Z-scores within normal range at start and follow-up, and annual change in $Rint_{exp}$ Z-score was not significant. In school children only forced expiratory volume in 1 second as percentage of forced vital capacity (FEV_1%FVC) declined significantly during the study period. There was a moderate but significant correlation between chest radiograph scores and spirometry after 3 years.

In young children with CF, chest radiograph scores worsen significantly over time even while lung function remains stable.

INTRODUCTION

Chronic airway infection and inflammation in cystic fibrosis (CF) lung disease lead to structural lung damage, pulmonary dysfunction and eventually to respiratory insufficiency. CF lung disease starts at a very young age and ideally, treatment should also start at onset or even before onset of lung disease. Therefore sensitive measures of structural and functional lung damage are needed to objectively assess lung disease progression and to evaluate effect of treatment.

Structural lung damage in CF can be assessed by chest radiology and high resolution computed tomography (HRCT) scanning. An annual chest radiograph and the use of chest radiograph scoring systems are recommended by the European CF Consensus Committee (1) and several different CF chest radiograph scoring systems (2-6) have been developed. In older children with CF with moderate to severe disease, there is a good correlation between pulmonary function tests (PFTs, especially forced expiratory volume in 1 second (FEV_1)) and chest radiograph scores in cross-sectional studies (4,6,7). Longitudinal evaluation of bronchopulmonary disease in children with CF using chest radiology showed disease progression from about the ages of 5 years, even when spirometry still remained stable (8-10). However, in preschool children the sensitivity of chest radiographs as a measure of disease progression and the relationship with functional parameters is unclear.

Lung function can be assessed by PFTs (especially spirometry) and forced expiratory volume in 1 second (FEV_1) is still considered gold standard in daily CF practice. In most children spirometry is feasible from the age of 4-6 years, but standards for quality control are lacking for preschool children (11-13). Therefore alternative PFTs, such as the expiratory interrupter resistance ($Rint_{exp}$) measurement, have been developed for this age group (14-19). $Rint_{exp}$ measurements can be performed without sedation, are easily applicable in general practice, are effort-independent, and reference values for children between 3 and 13 years of age are available (17). $Rint_{exp}$ measurements might be of use in detecting early lung function abnormalities in children with CF. However, data on the value of Rint measurements in CF patients are not conclusive (11,16,17,19) and longitudinal measurements of Rint have never been related to structural damage assessed by chest radiology.

The purpose of our study was to evaluate the sensitivity of structural and functional parameters of lung disease progression in young children with CF. We prospectively studied disease progression in preschool (children aged < 5 years) and school children (children aged >5 years) using simple chest radiograph scores, $Rint_{exp}$ measurements, and spirometry.

METHODS

Study population

The CF Centre Utrecht of the University Medical Centre Utrecht (Utrecht, The Netherlands), a tertiary academic hospital, uses annual chest radiographs and PFT measurements (including spirometry and $Rint_{exp}$ measurements) to monitor lung disease in patients with CF.

We studied 21 preschool children and 30 school children who attended the CF Centre between April 2002 and June 2003 for their annual check-up, and had a routine chest radiograph and $Rint_{exp}$ measurement on the same day both that year and after 3 years. All school children performed spirometry at visit 1 and 2. Measurements were performed when children were clinically stable. Informed consent was obtained from the parents of all participating children.

Chest radiographs

Chest radiographs (anteroposterior and lateral view) were blinded and scored in random order according to the following five cystic fibrosis chest radiograph scoring systems: Chrispin-Norman scoring (2), adjusted Chrispin-Norman scoring (3), Wisconsin scoring (4), Northern scoring (5) and Brasfield (Birmingham) scoring system (6).

Previously it has been stated that a cut-off value of 5 for the Wisconsin score and 21 for the Brasfield score are associated with mild but potentially irreversible lung damage (10).

$Rint_{exp}$ and spirometry

In all children airway resistance was measured using the MicroRint® (Micro Medical Limited, Kent, UK), as described in previous studies (14,15,18). A successful $Rint_{exp}$ measurement consisted of a median $Rint_{exp}$ value of at least 5 out of 10 interruptions and $Rint_{exp}$ was calculated using the back extrapolation technique to t=0 ms after shutter closure during 100 ms (14,20). $Rint_{exp}$ values in children with CF were expressed as height adjusted Z-scores, using data from a Dutch healthy control population (18).

All school children performed spirometry at visit 1 and visit 2 (Masterlab, Hochberg, Germany). Spirometry results FEV_1, forced vital capacity (FVC), maximal expiratory flow at 50% of vital capacity (MEF_{50}), and the forced expiratory flow between 25% and 75% of expiratory vital capacity (FEF_{25-75}), were expressed as percentage of predicted values using the data from Zapletal (21). FEV_1 was also expressed as percentage of FVC (FEV1%FVC). Abnormal lung function was defined as a FEV_1 % pred < 85%.

Lung structure and lung function over time

Data obtained at the first visit are reported as chest $radiograph_1$, $Rint_{exp}$ $Z\text{-}score_1$ and PFT_1, and at the second visit as chest $radiograph_2$, $Rint_{exp}$ $Z\text{-}score_2$, and PFT_2. Δchest radiograph is the annual change for a chest radiograph scoring system (chest $radiograph_2$-chest $radiograph_1$/ time interval), $\Delta Rint_{exp}$ Z-score the annual change for $Rint_{exp}$ Z-score ($Rint_{exp}$ $Z\text{-}score_2$- $Rint_{exp}$

$Z\text{-score}_1$/time interval), and ΔPFT the annual change for PFTs (PFT_2- PFT_1/time interval). A positive value for Δchest radiograph indicates an increase of structural abnormalities for all chest radiograph scoring systems except for the Brasfield scoring system, where a negative value for Δchest radiograph indicates an increase of structural abnormalities. A positive value for $\Delta Rint_{exp}$ Z-score indicates an increase in resistance and thus a worsening of lung function. A negative value for ΔPFT indicates a decline in lung function.

Statistical analysis

To determine whether chest radiograph scores and/or $Rint_{exp}$ Z-score and/or PFTs changed significantly over time in preschool and in school children, Student's *t*-test for paired samples was performed. Mean differences between the parameters at visit 1 and 2 were expressed as mean annual decline by dividing the mean difference by time interval. The relationships between chest radiographs, $Rint_{exp}$ Z-scores, and PFTs and between Δchest radiograph, $\Delta Rint_{exp}$ Z-score, and ΔPFT were evaluated using the Spearman correlation coefficient. Data of preschool and school children were compared using Student's *t*-test for unpaired samples. Results were considered statistically significant when the p-value was <0.05. Data are presented as mean ± standard deviation (SD). Analysis was performed using the Statistical Package for the Social Science (SPSS version 12.0, Chicago, IL USA).

RESULTS

Study population

Characteristics of the preschool and school children are shown in table 1. Mild but potentially irreversible lung damage (Wisconsin score >5, Brasfield score < 21) was found in 10 of the 21 (48%) preschool children for both scores, and in 18 of the 30 (60%) school children for the Wisconsin score and 22 of the 30 (73%) school children for the Brasfield score at visit 1.

Most preschool children had $Rint_{exp}$ Z-scores that were within normal range (mean ± 2 Z-scores) both at visit 1 (9.5% had a $Rint_{exp}$ Z-score >2 SD) and at visit 2 (19% had a $Rint_{exp}$ Z-score >2 SD). In school children 13% had a $Rint_{exp}$ Z-score >2SD at visit 1 and 23% at visit 2 (figures 1A and 1B). Only 7 of the 30 (23%) school children had abnormal lung function (FEV_1 <85%) at visit 1.

Lung structure and lung function over time

All chest radiograph scores worsened significantly over time in preschool children (Wisconsin p<0.05 and all other scores p<0.001, see table 2). In school children all chest radiograph scores except the Wisconsin score worsened significantly over time (table 2). Preschool children showed a tendency towards a faster decline in chest radiograph scores than school children and this difference was statistically significant for the Northern and Brasfield scoring

Table 1. Patient characteristics (mean ± SD) of the preschool and school children with CF at visit 1.

	Preschool (n=21)	School (n=30)
Age (years)	3.2 ± 0.9	7.2 ± 1.9
Body height (m)	0.95 ± 0.09	1.24 ± 0.12
Body weight (kg)	14.0 ± 2.2	24.3 ± 5.9
PFTs		
$Rint_{exp}$ Z-score	0.14 ± 1.1	0.64 ± 1.4
FEV_1 % pred		95.1 ± 16.3
FVC % pred		92.4 ± 14.8
FEV_1%FVC		88.2 ± 7.7
MEF_{50} % pred		72.9 ± 27.1
FEF_{25-75} % pred		72.1 ± 29.3
Radiograph scores		
Chrispin-Norman	10.1 ± 3.9	11.2 ± 3.0
Adjusted Chrispin-Norman	11.2 ± 4.5	12.6 ± 3.1
Wisconsin	4.6 ± 1.9	5.2 ± 1.7
Northern	5.9 ± 2.0	6.9 ± 1.5
Brasfield	19.8 ± 1.9	18.8 ± 1.9

$Rint_{exp}$; expiratory interrupter resistance, FEV_1% pred; percent predicted forced expiratory volume in 1 second FVC % pred; percent predicted forced vital capacity, FEV_1%FVC; FEV_1 expressed as percentage of FVC, MEF_{50} % pred; percent predicted maximal expiratory flow at 50% of vital capacity, FEF_{25-75} % pred; forced expiratory flow between 25% and 75% of expiratory vital capacity

system (p=0.04 and p=0.03 respectively). This suggests that chest radiology is more sensitive to disease progression in preschool children compared to school children.

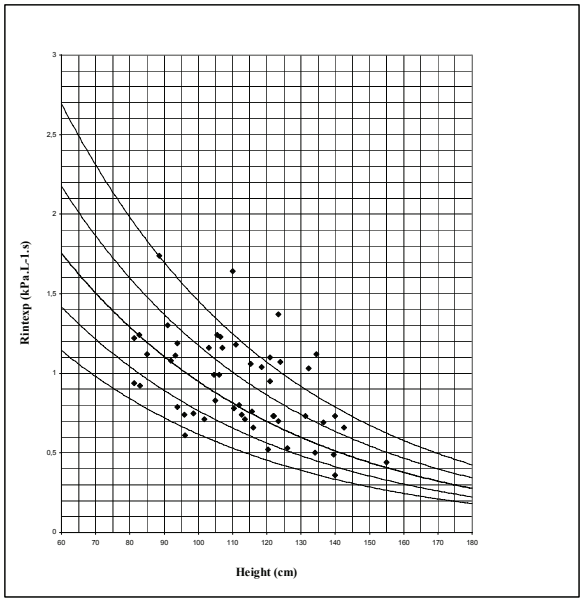
$Rint_{exp}$ Z-scores did not change significantly over time in preschool and school children. In school children only FEV_1%FVC worsened significantly over time (annual decline 2.14% (p<0.01)). When comparing preschool children with school children, $Rint_{exp}$ Z-scores appeared higher in school children but this difference was not statistically significant.

Correlation between lung structure and lung function

In preschool children there was no significant correlation between chest radiograph and $Rint_{exp}$ Z-score at visit 1 and visit 2 at cross-sectional data analysis. Longitudinal data analysis also showed no significant correlation between all Δchest radiograph scores and Δ$Rint_{exp}$ Z-score.

In school children we also found no significant correlation between chest radiograph and $Rint_{exp}$ Z-score at visit 1 and visit 2, or between chest radiograph and PFT at visit 1. There was however a significant but moderate correlation between the Chrispin-Norman and adjusted Chrispin-Norman score and FEV_1% pred, MEF_{50}% pred, FEF_{25-75} % pred and FEV_1%FVC at visit 2. The Wisconsin score correlated significantly but weakly with FEV_1% pred, FEV_1%FVC, and FEF_{25-75} % pred and the Brasfield score correlated significantly but weakly with FEV_1% pred,

A)



B)

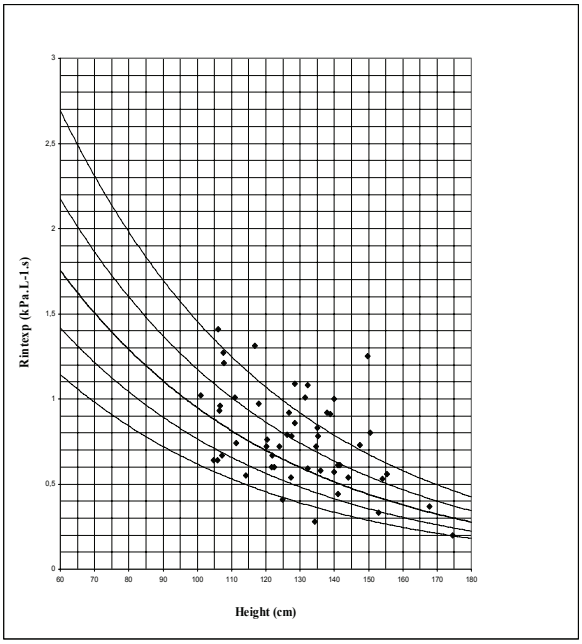


Figure 1. Absolute $Rint_{exp}$ values in preschool and school children with CF compared to the regression line (10 log $Rint_{exp}$ = 0.645-0.00668 x standing height (cm) kPa/L/s ± 1 Z-score and ± 2 Z-score lines) of the Dutch healthy control group a) at visit 1 and b) at visit 2.

Table 2. Mean annual change and p-value for different parameters in preschool and school children.

	Preschool children (n=21)		School children (n=30)	
	mean annual change	p-value	mean annual change	p-value
Age (years)	Interval 2.76		Interval 2.89	
Body height (m)	0.070	0.000	0.055	0.000
Body weight (kg)	2.84	0.000	2.71	0.000
PFTs				
$Rint_{exp}$ Z-score	0.05	0.755	0.13	0.188
FEV_1 % pred			-1.72	0.089
FVC % pred			0.17	0.852
FEV_1%FVC			-2.14	0.000
MEF_{50} % pred			-0.86	0.640
FEF_{25-75} % pred			-2.64	0.101
Radiograph score				
Chrispin Norman	1.19	0.000	0.51	0.030
Adjusted Chrispin Norman	1.50	0.000	0.71	0.005
Wisconsin	0.57	0.002	0.21	0.117
Northern	0.72	0.000	0.28	0.031
Brasfield	-1.02	0.000	-0.46	0.007

$Rint_{exp}$; expiratory interrupter resistance, FEV_1% pred; percent predicted forced expiratory volume in 1 second FVC % pred; percent predicted forced vital capacity, FEV_1%/FVC; FEV_1 expressed as percentage of FVC, MEF_{50} % pred; percent predicted maximal expiratory flow at 50% of vital capacity, FEF_{25-75} % pred; forced expiratory flow between 25% and 75% of expiratory vital capacity

Table 3. Correlation (r) between chest radiograph score and PFT data in preschool and school children with CF at visit 2.

	Chrispin-Norman	Adjusted Chrispin-Norman	Wisconsin	Northern	Brasfield
Preschool children					
$Rint_{exp}$ Z-score	0.39	0.39	0.02	0.21	-0.09
School children					
$Rint_{exp}$ Z-score	-0.00	0.02	-0.07	-0.18	0.10
FEV_1 % pred	-0.50**	-0.51**	-0.41*	-0.31	0.40*
FVC % pred	-0.26	-0.27	-0.14	-0.10	0.22
FEV_1%FVC pred	-0.39*	-0.41*	-0.39*	-0.27	0.31
MEF_{50} % pred	-0.38*	-0.40*	-0.31	-0.16	0.28
FEF_{25-75} % pred	-0.46*	-0.48**	-0.38*	-0.29	0.37*

$Rint_{exp}$; expiratory interrupter resistance, FEV_1% pred; percent predicted forced expiratory volume in 1 second FVC % pred; percent predicted forced vital capacity, FEV_1%/FVC; FEV_1 expressed as percentage of FVC, MEF_{50} % pred; percent predicted maximal expiratory flow at 50% of vital capacity, FEF_{25-75} % pred; forced expiratory flow between 25% and 75% of expiratory vital capacity

* $p<0.05$, ** $p<0.01$

Table 4. Correlation (r) between annual decline in chest radiograph score and spirometry in school children with CF.

	Δ Chrispin-Norman	Δ Adjusted Chrispin-Norman	ΔWisconsin	ΔNorthern	ΔBrasfield
ΔFEV_1 % pred	-0.61**	-0.55**	-0.60**	-0.25	0.39*
ΔFVC % pred	-0.52**	-0.48**	-0.43*	-0.14	0.29
ΔFEV_1%FVC pred	-0.24	-0.23	-0.29	-0.24	0.32
ΔMEF_{50} % pred	-0.58**	-0.52**	-0.54**	-0.37*	0.50**
ΔFEF_{25-75} % pred	-0.60**	-0.55**	-0.55**	-0.57**	0.63**

FEV_1% pred; percent predicted forced expiratory volume in 1 second, FVC % pred; percent predicted forced vital capacity, FEV_1%/FVC; FEV_1 expressed as percentage of FVC, MEF_{50} % pred; percent predicted maximal expiratory flow at 50% of vital capacity, FEF_{25-75} % pred; forced expiratory flow between 25% and 75% of expiratory vital capacity

* $p<0.05$, ** $p<0.01$

and FEF_{25-75} % pred at visit 2. Table 3 shows Spearman's correlation coefficient between chest radiograph scores and lung function for cross-sectional data analysis at visit 2.

Longitudinal data analysis in school children showed moderate but significant correlation between Δchest radiograph scores (except the Northern score) and ΔPFT (except Δ FEV_1%FVC). Best correlation was found between all Δchest radiograph scores (including the Northern score) and ΔFEV_1 and ΔFEF_{25-75} percent predicted values (table 4).

DISCUSSION

In this study we evaluated the capability of chest radiograph scores, $Rint_{exp}$ measurements, and spirometry to detect and monitor progression of lung damage in young children with CF. Chest radiograph scores worsened significantly over a 3 year period in both preschool and school children. Preschool children showed a tendency towards a faster decline in chest radiograph scores than did school children. The Crispin-Norman score and adjusted Chrispin-Norman score showed the greatest annual decline in both preschool and school children. On the other hand Wisconsin score did not change significantly in school children. At inclusion already 48% of preschool children and 60-73% of school children had a chest radiograph score that resembled mild but potentially irreversible lung damage (10).

In preschool children the parameters hyperinflation, peribronchial thickening and bronchiectasis worsened while in school children mostly nodular cystic lesions worsened (data not shown). In our study we compared radiograph scores in preschool and school children who were treated according to the same protocol and who were followed up for a similar period of time. In this study set up radiograph scores were more sensitive to disease progression than functional parameters, especially in young children. This suggests that in young children chest radiographs can be helpful in follow up of CF lung disease progression.

For several years now, use of HRCT of the chest is advocated since HRCT might be more sensitive than chest radiographs in detecting structural abnormalities in children with mild CF (22-24). Even in young children and infants with CF, HRCT can detect structural abnormalities (25,26). Several cross-sectional studies showed good correlation between HRCT scores and chest radiograph scores (22,23,27). Although HRCT is sensitive in detecting structural abnormalities (25), especially in young patients, implementation of sequential CT-scanning into daily CF-care is hampered by several factors. HRCT of the chest causes higher radiation dosage and a subsequent higher risk for cancer compared to chest radiographs (28), is more expensive, and young children need to be sedated (with control of breathing). Studies comparing changes in HRCT scores with changes in chest radiograph scores in children over a longer period of time are lacking. In our study we show that chest radiograph scores worsen in both preschool and school children while lung function was stable. Considering these results and current limitations of implementation of CT-scanning in daily CF care, there still seems to be a valuable role for chest radiograph scores in monitoring structural lung damage in daily clinical practice and possibly in long-term clinical trials.

Despite significant abnormalities of chest radiograph scores most preschool and school children had a $Rint_{exp}$ Z-score within normal range both at visit 1 and visit 2, and annual change in $Rint_{exp}$ Z-score was not significant. These findings suggest that $Rint_{exp}$ measurements do not sensitively differentiate between children with CF and healthy children and that longitudinal evaluation of $Rint_{exp}$ measurements are not sensitive enough to detect early progression of lung disease in CF. The between-occasion repeatability of Rint is poor and the variation in bronchial tone is high, limiting the usefulness of repeated $Rint_{exp}$ measurements in monitoring disease progression in CF (29). In accordance with our results, no consistent abnormal levels or increases in Rint were found in a 4- year prospective study of inspiratory respiratory resistance measured by interrupter technique in young children with CF (19).

We found no correlation between structural damage assessed by chest radiograph scores and lung function measured by $Rint_{exp}$. In school children we also found no correlation between chest radiograph scores and spirometry at visit 1 but we did find a moderate but significant correlation between most chest radiograph scores and spirometry at visit 2. A possible explanation for the moderate but significant correlation between chest radiograph scores and spirometry at visit 2 is that at visit 2 a larger part of the children had a FEV_1% pred <85%, suggesting more advanced lung disease, and consequently a better correlation with structural abnormalities, as has been shown previously (7). Longitudinal analysis of PFT decline in school children showed no statistically significant worsening except for FEV_1%FVC. A significant change in FEV_1/FVC ratio (expressed as Z-score) has been described previously by de Jong and colleagues in a larger group of older children with CF (30). In a different study by the same investigator no significant annual decline in percent predicted values of FEV_1/FVC ratio was described (25). Significant but slow deterioration of percentage predicted FEV_1/FVC ratio was however described in a longitudinal study assessing effect of mucoid *Pseudomonas*

aeruginosa infection on lung disease progression (9). Whether or not the FEV_1/FVC ratio is a sensitive measure in detecting early and mild lung disease in CF remains to be answered, since neither percentage predicted nor FEV_1/FVC ratio Z-score is usually included in analysis of pulmonary function in CF trials.

In conclusion, this study shows that there is a significant annual deterioration of routine chest radiograph scores in young children, and this decline is greatest for the Chrispin-Norman score and adjusted Chrispin-Norman score. A large proportion of preschool children already have abnormal chest radiographs while $Rint_{exp}$ Z-scores are within normal range in most children with CF**.** $Rint_{exp}$ is not a good measure to monitor pulmonary disease progression in groups of children with CF. Spirometry (except FEV_1%FVC) in school children remained stable in our studied group and correlated only moderately with chest radiographs. Radiograph scores therefore represent a more sensitive measure to evaluate lung disease progression in young children with mild CF lung disease.

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Chapter 7

Effect of inhaled beclomethasone dipropionate on lung function and inflammation in young children with cystic fibrosis: a randomized controlled trial

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Submitted

ABSTRACT

Rationale: Lung inflammation in cystic fibrosis (CF) eventually leads to structural and functional lung damage and respiratory insufficiency. Anti-inflammatory therapy, started early in the disease process, could be beneficial.

Objectives: To test the hypothesis that inhaled corticosteroids started at an early age diminish lung function decline and inflammation.

Methods: Randomized, double-blinded, placebo-controlled trial for 3 years using high dose beclomethasone dipropionate (HFA-BDP) in 57 young CF children. Eligibility criteria included ages 2-11 years, normal lung function, and clinically stable condition.

Measurements and main results: The mean difference in change in FEV_1%, was -2.5%/year (95 percent confidence interval -5.7 to 0.8, p=0.14) less decline in the HFA-BDP treated children compared to the placebo treated children. Mean difference in change in FVC % was also -2.5%/year (95 percent confidence interval -5.2 to 0.3, p=0.07) in the intention-to-treat analysis and -3.1%/year (95 percent confidence interval -6.3 to 0.9, p=0.03) in the per-protocol analysis in HFA-BDP treated children compared to placebo treated children. Other lung function outcome measures all showed a tendency towards a lesser decline in the HFA-BDP treated children but these differences were not statistically significant. There was also a tendency towards lower levels of neutrophil priming associated cellular markers and plasma cytokine concentrations in the HFA-BDP treated children, and there was a statistically significant lower concentration of sICAM-1 (ng/ml) in the HFA-BDP treated children versus the placebo treated children (362 ± 27 versus 450 ± 26, p=0.024). Bacterial colonization was comparable between HFA-BDP and placebo treated children.

Conclusion: In this study population there seems to be a positive effect of the use of HFA-BDP on lung function parameters and inflammation in peripheral blood.

INTRODUCTION

Cystic fibrosis (CF) lung disease is characterized by a neutrophil-dominated inflammation of the airways and endobronchial infection with bacteria like *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Inflammation is detectable in bronchoalveolar lavage fluid even in early infancy and in clinically stable patients (1-5). Chronic airway infection and inflammation leads to activation of nuclear factor kappa B (NF-κB), and subsequently to secretion of inflammatory mediators such as interleukin (IL)-8, interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) (6). If untreated, this inflammatory process irreversibly leads to damage of the airways, to bronchiectasis and ultimately to respiratory failure. Despite potential benefits of anti-inflammatory therapy, clinical effects are controversial.

Glucocorticoids are powerful, non-specific anti-inflammatory and immunosuppressive agents that have a direct effect on gene expression (such as induction of annexin 1), an indirect effect on gene expression (such as blocking transcription of NF-κB) and have a mediator function in second-messenger cascades (7). In CF patients alternate-day prednisone reduced the frequency of pulmonary exacerbations and improved pulmonary function (8). In a subsequent large multicenter trial, however, a significantly reduced linear growth (especially in boys), abnormal glucose tolerance and increased *Pseudomonas aeruginosa* colonization was found (9-11). This has led to the recommendation not to use oral corticosteroids for more than 24 months, implying that oral corticosteroids are no long term option in the anti-inflammatory treatment of CF patients.

Inhaled corticosteroids (ICS) have more localised anti-inflammatory actions in the airways and a relatively high safety profile. Several studies evaluating the effect of ICS in CF patients have been performed and reviewed (12-18). Most of these studies however have been performed in adolescent or adult CF patients with established irreversible lung damage, and the studies performed in children are mostly of (too) short duration using different dosages (possibly too low dosages), different ICS and different outcome measures. Recently a large multicenter study found it to be safe to withdraw ICS in CF patients, but the authors state in their discussion that they did not show whether starting ICS may be beneficial in reducing lung function decline and lung inflammation (19). Although these studies did not provide evidence to support the use of ICS in CF, ICS are still widely used in CF patients with a reported prescription percentage in paediatric patients in the UK of 52% (CF Trust Database) and in the USA of 41% (Epidemiologic Study of CF) (19). Early CF lung disease seems to be confined to the small airways and hydrofluoroalkane-beclomethasone dipropionate (HFA-BDP, Qvar®) has a smaller median particle size, with a greater proportion of the drug deposited in the small airways and less deposited in the throat (20,21). HFA-BDP might thus be beneficial in controlling lung damage in early stage CF lung disease.

We performed a three-year randomized, double-blinded placebo-controlled trial with relatively high dose of HFA-BDP in young children with CF. The primary aim of this study

was to assess the effect of HFA-BDP on pulmonary function. Another aim was to monitor the effect of HFA-BDP on inflammation in peripheral blood (assessed by neutrophil priming and plasma cytokine/chemokine profile), lung structure assessed by chest radiograph scores, and bacterial colonization.

METHODS

A randomized, double-blinded, placebo-controlled trial was conducted over a 3 years period. The study was approved by the Central Committee on Research involving Human Subjects (CCMO). Written informed consent of both parents was obtained in all cases. IVAX Pharmaceuticals (London, England) provided HFA-BDP and placebo aerosols and Trudell Medical International (London ON, Canada) provided the AeroChamber devices for aerosol inhalation but otherwise did not participate in the design and conduct of the study; in the collection, analysis, or interpretation of the data; or in the writing or review of the manuscript.

Patients

Children in clinically stable condition, with a confirmed diagnosis of CF by a sweat test and CF transmembrane regulator (CFTR) mutation analysis, were recruited from the Cystic Fibrosis Center of the University Medical Center Utrecht, the Netherlands. Patients were eligible for this study if they were 2 to 11 years old with, if feasible, a normal lung function defined as a forced expiratory volume in 1s (FEV_1) greater than 85% predicted, and if they were clinically stable. Exclusion criteria were *(1)* the use of anti-inflammatory therapy (systemic or ICS, ibuprofen), *(2)* other conditions besides CF influencing growth, *(3)* participation in another clinical trial. The study was conducted between March 2002 and December 2005.

Randomization and treatments

Patients were randomly assigned to inhalation of HFA-BDP twice daily 200 micrograms or placebo twice daily with an AeroChamber for a period of 3 years. All patients were recruited by the investigator (SWJ T-L). Both the coding of study medication and assignment of patients to treatment (HFA-BDP) / placebo groups was performed by the Pharmacological Department of the University Medical Center Utrecht. Patients were stratified by age (below or above 4.5 years old). Study protocol outlined that all adverse events were reported immediately to the principal investigator who would assess the clinical situation and recommend any deviation necessary from the treatment regimen. When during the study period, treatment with systemic or inhaled glucocorticoids was recommended by the child's physician, the study protocol was terminated for that child. Study protocol was also terminated when growth curves for height would decline by 2 standard deviation scores (SDS) during treatment.

Assessment of trial outcomes

Spirometry if feasible was performed after the administration of a bronchodilator at baseline, after one year, two years, and three years, respectively. The primary outcome measure was linear change in $FEV_{1,}$ expressed as percent predicted values (FEV_1%) using reference data from Zapletal *et al*, after 3 years of treatment (22). Secondary outcomes were other pulmonary function test results (PFTs); percent predicted forced vital capacity (FVC%), FEV_1 as percentage of FVC (FEV1%FVC), percent predicted maximal expiratory flow at 50% of vital capacity (MEF_{50}%), and percent predicted forced expiratory flow between 25% and 75% of expiratory vital capacity (FEF_{25-75}%).

Neutrophil activation status in peripheral blood was measured by neutrophil priming using adhesion and activation associated surface markers (CD11b/CD18) and monoclonal phage antibodies (MoPhabs) A17 and A27 as described previously (23) at baseline and after 1,2, and 3 years. Blood samples were collected in sodium-heparin tubes. After venapuncture, blood was directly kept on melting ice and analyzed within 1 hour. Each blood sample was divided into 5 aliquots. One aliquot of whole blood was incubated for 30 min on ice and double stained with combinations of optimal concentrations of (1) fluorescein isothiocyanate (FITC) conjugated CD14 (clone MøP9, ref 345784) and phycoerythrin (PE) conjugated CD11b (CR3, clone D12, ref 347557) and (2) with CD14 PE (clone MøP9, ref 345785) and CD18 FITC (β2 integrin chain,clone L130, ref 347953). The antibodies were obtained from Becton Dickinson (San Jose, CA). The other four aliquots were incubated in 100 ml of a 1:10 dilution with phosphate buffered saline (PBS) of either FITC labelled MoPhab A17 or MoPhab A27 for 60 minutes on ice. Two of the 4 aliquots were kept on ice and two samples were incubated with TNF-α at a concentration of 100 IU/ml and these samples were kept on 37°C for 30 minutes before cells were incubated with MoPhabs A17 or A27. After incubation cells were washed with PBS supplemented with 0.01% sodiumazide and 1% foetal bovine serum (PBS^{++}), followed by lysis of erythrocytes for 10 minutes on ice using FACS Lysing Solution (Becton Dickinson, Mountain View, CA). After a final washing step with PBS^{++}, cells were resuspended in 200 µl 1 % paraformaldehyde in PBS and all samples were analyzed in a FACS Calibur Flowcytometer (Becton Dickinson). Neutrophils were identified according to their specific forward-scatter and side-scatter characteristics and expression levels of CD11b, CD18, and MoPhabs A17 and A27 were expressed as median fluorescence intensity in arbitrary units (MFI in au) of 20,000 events.

Plasma cytokines (interleukin (IL-)1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, and TNF-α), chemokines (IL-8, thymus and activation-regulated chemokine (tarc), interferon (IFN)-γ, IFN- γ inducible protein of 10 kd (IP-10)) and soluble adhesion molecules (sCD106 (soluble vascular cell adhesion molecule-1 (sVCAM-1), and sCD54 (soluble intercellular adhesion molecule-1 (sICAM-1)) were assessed at baseline and after 3 years using a multiplex immunoassay (MIA) as described previously (24). Both baseline and 3 year samples were available in 22 HFA-BDP and 24 placebo treated children who completed the study protocol.

In all children sputum and/or cough swab cultures (when children were unable to expectorate sputum) were sent to the local laboratory for routine qualitative microbiologic analysis at all visits. We performed serologic measurements of *Pseudomonas aeruginosa* antibodies in all children to three tested antigens: exotoxin A, elastase and alkaline protease on the same day as sputum culture using a commercially available ELISA-kit (Mediagnost, Reutlingen, Germany) (25) at baseline in all children and after 3 years in the children who completed the study protocol.

Structural lung damage was recorded using routine chest radiographs (anteroposterior and lateral view) that were performed annually as part of CF check up. Baseline and 3-year follow-up chest radiographs were blinded and scored in random order according to the following three cystic fibrosis chest radiograph scoring systems: Chrispin-Norman scoring (26), adjusted Chrispin-Norman scoring (27), and the Wisconsin scoring system (28).

Airway inflammation measured by end tidal breath carbon monoxide (ETCOc) was assessed at baseline and after 3 years as described previously (29). ETCOc measurements in CF patients were expressed as height adjusted percentage predicted values since not all children were able to perform total lung capacity measurements (30).

Health-related quality of life (HRQOL) was assessed after 3 years of treatment in both treatment groups using the parent version and child version (6-11 years) of the Dutch translation of the Cystic Fibrosis Questionnaire (CFQ) (31).

Lung deposition

We performed a pilot study in 5 healthy controls and 5 CF patients to assess lung deposition of ICS, using a bioassay that evaluates the bioactivity of glucocorticoids in serum (32). Blood was sampled at baseline and then all subjects inhaled 500µcg of fluticasone propionate (FP) via metered dose inhaler (MDI) device. Blood was again sampled 15, 30, and 60 minutes after FP inhalation. To circumvent interference with endogenous cortisol, 1 mg dexamethasone was administered 10 hours prior to FP inhalation. Since FP absorbed from the gastrointestinal tract is almost completely inactivated by the very high hepatic first pass effect, the increase of bioavailable glucocorticoids in serum following FP inhalation is caused foremost by he lung-absorbed fraction.

Statistical analysis

Previous data from our center showed a mean (SD) FEV_1% predicted in CF children aged 5-13 years of 80 (10)%. Sample size was based on the ability to detect a treatment effect of 8% difference in linear change from baseline in FEV_1 in the HFA-BDP treated children compared to placebo treated children. Power analysis showed that 25 children per group would provide 80% power to detect this treatment effect with the use of a two-sided test (α=0.05). Descriptive statistics for the children with CF in the treatment group and placebo group were described for the time of inclusion (baseline measurements). Data are expressed as mean (SD) or mean (SEM).

Data were analyzed according to the intention-to-treat and per-protocol principle. The primary outcome measure, the linear rate of change in lung function, was assessed with the use of values obtained at baseline and at 1,2 and 3 years after randomization. The effect of the intervention was tested and estimated with repeated measures analysis using the SAS Procmixed module with random individual slopes and intercepts (SAS statistical package, SAS Institute Inc.Cary, NC, USA) (33). A negative regression coefficient for PFTs indicates worsening of disease in the placebo group compared to the HFA-BDP group. Other secondary outcomes, including other PFT results, neutrophil priming, and height SDS scores were also assessed using this model.

Plasma cytokines, chemokines and soluble adhesion molecule levels after 3 years of treatment were compared between groups using Mann Whitney's non-parametric testing. Chest radiograph scores, ETCOc % predicted, and Dutch CFQ for children and parents after 3 years of treatment were compared between groups using independent samples Student's *t*-tests.

Results of the glucocorticoid bioassay were evaluated using a general linear model for repeated measurements, testing the null-hypothesis that there was no difference between healthy controls and CF patients in glucocorticoid bioavailability.

A two-sided p-value of equal to or less than 0.05 was considered statistically significant. Analysis was performed using SPSS version 12.0 (SPSS Inc, Chicago, IL USA).

RESULTS

Demographic characteristics

Of the 107 patients eligible for participation in this study, 57 gave informed consent and underwent randomization between March 2002 and December 2002 (Figure1). Of the 57 patients included 28 were assigned to the HFA-BDP treatment regimen and 29 to the placebo regimen. Nine children did not complete the protocol, 5 in the HFA-BDP group and 4 in the placebo group. Of these nine children, 5 children were non compliant with therapy, one developed asthma and started treatment with ICS, two children developed an allergic bronchopulmonary aspergillosis (ABPA) and started with oral glucocorticoids, and one child showed progressive decline in height growth while using HFA-BDP (see also Figure 1). This child still showed a progression in decline of height growth after discontinuing study medication and showed an increase in height growth after starting nasogastric tube feeding.

At baseline, there were no differences between the treatment groups that could explain the treatment effects, as is shown in Table 1.

Pulmonary function test results

In table 2 effect of HFA-BDP on PFTs is shown for both the intention-to-treat and per-protocol analysis. The mean difference in our primary outcome measure, change in FEV_1 %, was -

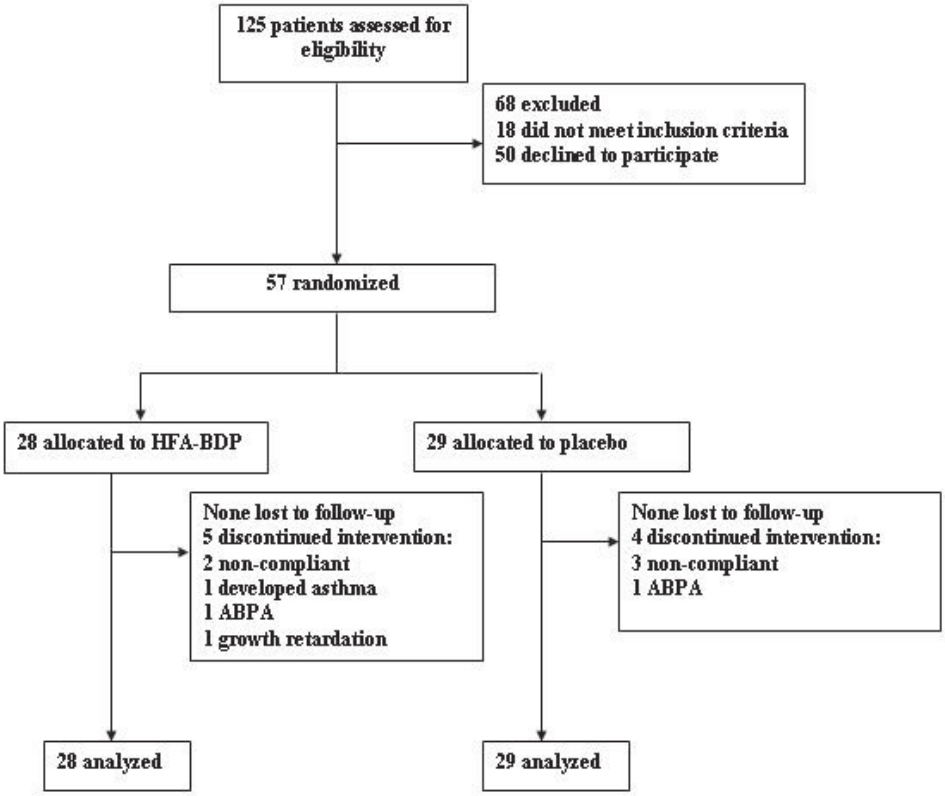

Figure 1. Trial profile.

2.5%/year (95 percent confidence interval -5.7 to 0.8, p=0.14) in the HFA-BDP treated children compared to the placebo treated children in the intention-to-treat analysis and -2.7%/year (95 percent confidence interval -6.3 to 0.9, p=0.15) in the per-protocol analysis. Although this difference was not statistically significant, HFA-BDP children clearly showed a tendency towards a lesser decline in annual FEV_1 % compared to placebo treated children (see also Figure 2a). HFA-BDP treated children showed an increase in FEV_1 % after 2 years of treatment and a decline in FEV_1 % after 3 years of treatment, while placebo treated children showed a decline in FEV_1 % after 1,2, and 3 years (figure 2a). Mean difference in change in FVC % was -2.5%/year (95 percent confidence interval -5.2 to 0.3, p=0.07) in the HFA-BDP treated children compared to the placebo treated children in the intention-to-treat analysis (see also Figure 2b). Mean difference in FVC% was -3.1%/year (95 percent confidence interval -6.3 to 0.9, p=0.03) in the per-protocol analysis and this difference was statistically significant. FVC% increases in HFA-

Table 1. Patient characteristics per treatment group at baseline. Values are means ± SD.

Characteristics	HFA-BDP (n =28)	Placebo (n=29)
Age (years)	5.6 ± 2.7	5.8 ± 2.9
Gender (m/f)	13/15	17/12
Height (cm)	111.1 ± 20.2	109.7 ± 29.1
Height SDS	-0.8 ± 1.0	-0.6 ± 0.9
Weight (kg)	20.3 ± 8.1	20.6 ± 6.8
Genotype		
ΔF508 homozygous (%)	19 (68)	18 (62)
ΔF508 compound heterozygous (%)	7 (25)	10 (35)
Other/other (%)	2 (7)	1 (3)
PFTs		
FEV_1 % (n)	97.3 ± 23.5 (18)	101.1 ± 18.2 (20)
FVC% (n)	90.8 ± 20.1 (18)	97.6 ± 15.2 (20)
FEV_1 %FVC (n)	91.2 ± 7.5 (18)	86.1 ± 12.1 (20)
MEF_{50}% (n)	92.1 ± 30.7 (18)	89.7 ± 33.8 (19)
FEF_{25-75}% (n)	86.3 ± 36.6 (18)	83.5 ± 41.3 (19)
Cultures showing P aeruginosa	6	7
Chest radiograph score		
Chrispin-Norman	9.5 ± 4.0	11.1 ± 3.0
Adjusted Chrispin-Norman	10.9 ± 4.3	12.8 ± 3.5
Wisconsin	4.0 ± 1.8	5.0 ± 1.9

Table 2. Mean annual differences in pulmonary function test (PFT) after use of a bronchodilator between placebo and HFA-BDP (intention-to-treat and per protocol analysis).

	mean annual difference (SE)	95% confidence interval	p-value
PFTs intention-to-treat			
FEV_1(%)	-2.45 (1.63)	-5.69 – 0.80	0.14
FVC(%)	-2.46 (1.37)	-5.17 – 0.26	0.08
FEV_1%/FVC	0.38 (1.01)	-1.62 – 2.39	0.71
MEF_{50} (%)	-2.18 (2.77)	-7.69 – 3.33	0.43
FEF_{25-75} (%)	-2.96 (3.66)	-10.23 – 4.31	0.42
PFTs per-protocol			
FEV_1(%)	-2.65 (1.80)	-6.25 – 0.94	0.15
FVC(%)	-3.09 (1.41)	-5.89 – -0.28	0.03
FEV_1%FVC	0.46 (1.15)	-1.83 – 2.75	0.69
MEF_{50} (%)	-0.59 (2.98)	-6.52 – 5.35	0.84
FEF_{25-75} (%)	-0.27 (3.96)	-8.17 – 7.63	0.95

BDP treated children after 1 and 2 years of treatment, but a decline in both groups is seen after 3 years of treatment (Figure 2b). Other PFT outcome measures all showed a tendency towards a lesser decline in the HFA-BDP treated children, except for FEV_1%/FVC since FVC% declined more rapidly than FEV_1%. These differences were not statistically significant (Table 2).

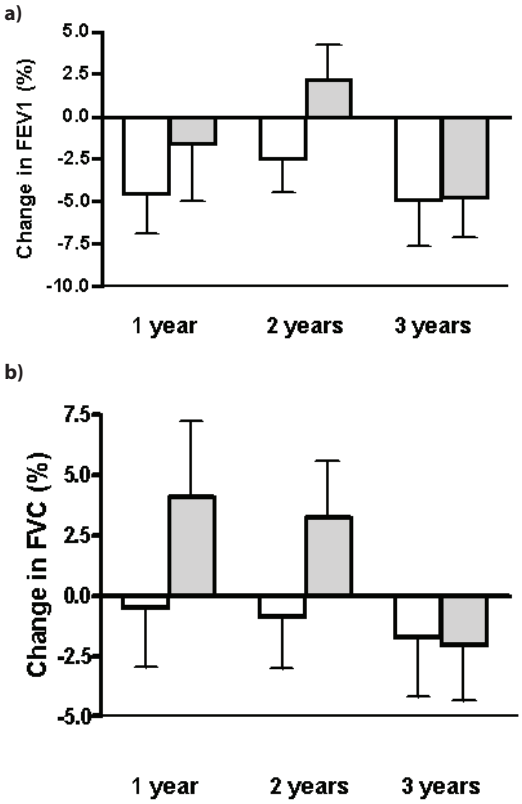

Figure 2. Mean (SE) percentage annual change in a) FEV_1% and b) FVC% for the placebo treated children (white bars) and the HFA-BDP treated children (grey bars) after 1, 2 and 3 years for the intention-to-treat analysis. T bars indicate standard errors.

Inflammation in peripheral blood

Neutrophil activation status in peripheral blood was assessed using CD11b, CD18 and MoPhabs A17 and A27. There was no statistically significant difference between HFA-BDP and placebo treated children although there was a tendency towards lower levels of the priming associated cellular markers (except CD11b) in the HFA-BDP treated children (Table 3).

Table 3. Mean annual differences in neutrophil activation status between placebo versus HFA-BDP (intention-to-treat analysis).

	mean annual difference (SE)	95% confidence interval	p-value
CD11b (mfi)	3.24 (27.50)	-51.39 – 57.88	0.91
CD18 (mfi)	-1.57 (5.29)	-12.08 – 8.94	0.77
A17 ex vivo (mfi)	-9.70 (6.08)	-21.78 – 2.37	0.11
A17 TNF-α (mfi)	-57.67 (38.87)	-134.86 – 19.56	0.14
A27 ex vivo (mfi)	-15.86 (11.75)	-39.21 – 7.48	0.18
A27 TNF-α (mfi)	-35.90 (47.20)	-129.64 – 57.83	0.45

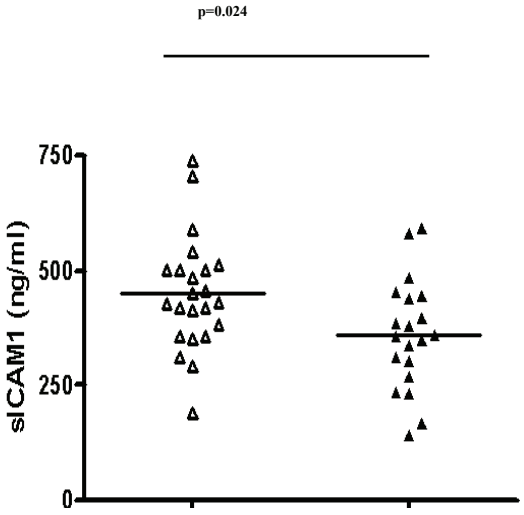

Figure 3. Plasma concentrations in pg/ml of sICAM-1 in 23 placebo treated children (open triangles) and 21 HFA-BDP treated children (closed triangles). Horizontal bars represent mean values.

In 22 HFA-BDP treated children and 24 placebo treated children that completed the study protocol IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-8, TNF-α, tarc, IFN-γ, IP-10, sCD106/sVCAM-1, and sCD54/sICAM-1 at baseline and after 3 years were analyzed. Most cytokines, chemokines and soluble adhesion molecules showed a tendency towards lower levels in the HFA-BDP treated children after 3 years. There was a statistically significant lower concentration of sICAM-1 (ng/ml) in the HFA-BDP treated children versus the placebo treated children (362 ± 27 versus 450 ± 26, p=0.024, see also figure 3) and a strong tendency towards statistically significant lower concentrations for IL-1α (pg/ml) (0.97 ± 0.05 versus 3.1 ± 1.1, p=0.066) and tarc (pg/ml) (0.41 ± 0.18 versus 1.36 ± 0.93, p=0.07). Both Th1 and Th2 cytokines and chemokines showed a tendency towards lower levels in HFA-BDP treated children.

Microbiology

In all children sputum cultures were performed at all visits and results were categorized as negative (no micro organisms), SA/HI (positive for *Staphylococcus aureus* and/or *Haemophilus influenzae*), or PA (positive for *Pseudomonas aeruginosa*). Table 4 shows result of cultures and *Pseudomonas aeruginosa* antibodies at baseline and after 3 years for HFA-BDP and placebo treated children. In the HFA-BDP treated children only one additional child became *Pseudomonas aeruginosa* positive in the 3 year follow-up compared to 2 children in the placebo treated group. Measurement of *Pseudomonas aeruginosa* antibodies did not lead to additional *Pseudomonas aeruginosa* positive children in either group compared to culturing.

Table 4. Sputum and/or oropharyngeal culture results (negative (no-micro organisms); SA/HI (*Staphylococcus aureus* and/or *Haemophilus influenzae*); PA (*Pseudomonas aeruginosa*) in HFA-BDP (n=28) and placebo (n=29) treated children at baseline and after 3 years of treatment, and results of *Pseudomonas aeruginosa* (pa) antibodies in HFA-BDP (n=28) and placebo(n=29) treated children at baseline and after 3 years of treatment (n=23 and n=25 respectively).

	HFA-BDP (n=28)		placebo (n=29)	
	baseline	3 years	baseline	3 years
Sputum culture				
negative	6	9	8	5
SA/HI	16	12	14	15
PA	6	7	7	9
PA antibodies				
exotoxin A	1	3	5	4
elastase	3	4	5	4
alkaline protease	1	2	3	1

Chest radiology

All chest radiograph scoring systems worsened significantly in the 3 year follow-up period in both HFA-BDP and placebo treated children. There was no statistically significant difference in chest radiograph score for the Chrispin-Norman, adjusted Chrispin-Norman or Wisconsin scoring system between the HFA-BDP treated children and the placebo treated children (p=0.36, p=0.35, and p=0.20 respectively).

Airway inflammation

ETCOc as a measure of airway inflammation was recorded at baseline and after 3 years of treatment using the CO-STAT® End Tidal Breath Analyzer (Natus Medical, Inc., San Carlos, CA). Mean (SE) ETCOc % predicted values at baseline were 124.0 (8.9)% in the HFA-BDP treated children and 109.6 (8.4)% in the placebo treated children (p=0.25). We found no difference between the 2 groups in change in ETCOc% after 3 years.

Quality of life

Dutch CFQ for parents after 3 years of treatment was assessed in 20 HFA-BDP and 20 placebo treated children. In the placebo treated group, 5 children were either too young or too old to complete this questionnaire, and in the HFA-BDP treated group 3 children were either too old or too young. Dutch CFQ for children with CF were assessed after 3 years in 15 HFA-BDP and 15 placebo treated children. CFQ scores of the children with CF and their parents were comparable between both groups for all domains as is shown in table 5. Parents rated the treatment constraints scale lower than their children as was described earlier (34).

Table 5. CFQ scores (mean ± SD) for children and parents in the HFA-BDP treated children (n=20) and placebo treated children (n=20).

	CFQ child		CFQ parents	
	HFA-BDP	Placebo	HFA-BDP	Placebo
Physical functioning	87.1 ± 13.3	85.6 ± 19.4	86.2 ± 18.1	85.9 ± 18.3
Emotional state	80.8 ± 9.1	84.6 ± 9.9	87.0 ± 15.8	83.4 ± 17.4
Social functioning	87.4 ± 9.8	87.6 ± 12.8		
Body image	93.8 ± 9.0	92.9 ± 12.7	90.1 ± 10.6	85.6 ± 18.5
Eating disturbances	61.2 ± 24.8	54.7 ± 21.4	68.4 ± 24.1	67.5 ± 30.3
Treatment constraints	90.3 ± 15.7	91.4 ± 9.8	78.5 ± 19.0	76.2 ± 19.3
Respiratory constraints	80.1 ± 11.8	76.3 ± 14.5	83.5 ± 16.3	86.9 ± 11.7
Digestion	75.3 ± 14.8	71.6 ± 17.8	74.1 ± 14.5	76.3 ± 14.1
Vitality			73.1 ± 13.9	69.3 ± 12.1
Weight			78.4 ± 24.9	75.1 ± 35.6
School			76.1 ± 23.9	87.3 ± 22.3
Health problems			75.1 ± 23.1	77.4 ± 23.2

Safety and adverse events

No treatment-related serious adverse events occurred. One child randomized to the HFA-BDP treatment regimen showed progressive decline in height growth. After discontinuing treatment protocol height SDS still declined and only after starting nasogastric tube feeding a catch-up in height growth was seen. In the HFA-BDP treated children it was clearly seen that height SDS declined in the first year with a mean (SE) decline in SDS of 0.28 (0.06) SDS. As is also shown in figure 4a catch-up in height growth reflected in an increase in height SDS is

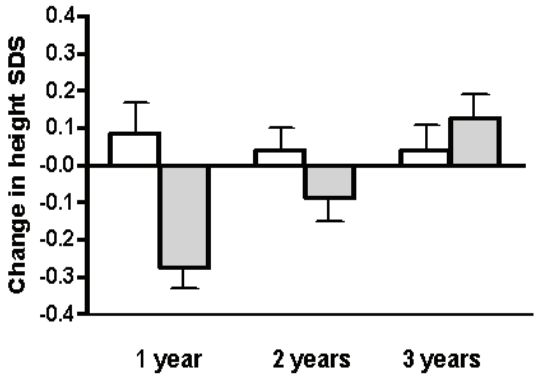

Figure 4. Mean (SE) percentage annual change in height SDS (intention-to-treat analysis) for the placebo treated children (white bars) and the HFA-BDP treated children (grey bars) after 1, 2 and 3 years. T bars indicate standard errors of the mean.

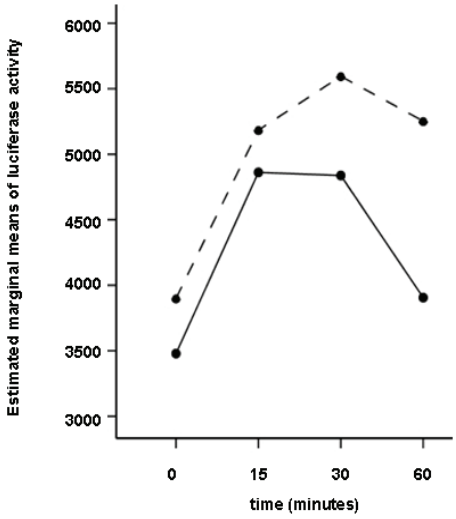

Figure 5. Estimated marginal means of the luciferase activity in 5 healthy controls (dotted line) and 5 CF patients (solid line) over time.

seen after respectively 2 and 3 years of treatment. Mixed model analysis of change in height SDS over 3 years in the HFA-BDP treated children versus placebo treated children showed a statistically significant mean (SE) annual increase in height SDS of 0.13 (0.04) (p=0.003) in favour of the HFA-BDP treated children. No difference was found in body mass index at baseline or after 3 years of treatment between both groups.

Lung deposition

There was no statistically significant difference in increase of bioavailable glucocorticoids in serum after inhalation of FP between healthy controls and CF patients (p=0.92) as is depicted in figure 5.

DISCUSSION

In this long-term trial we show that 3 years of treatment of young children with CF with HFA-BDP results in a 7.5% lesser mean decline in FEV_1 % predicted, and a 9.3% lesser mean decline in FVC % predicted in the per-protocol analysis. We started treatment in young children and children with FEV_1 % within the normal range (>85%), since it is plausible that the maximum benefit of anti-inflammatory therapy will be at an early age when lung function is still normal and lung damage due to chronic inflammation is still mild. Although HFA-BDP treated children showed an overall tendency towards a lesser decline in lung function pa-

rameters, especially in the first two years of treatment, these differences were not statistically significant (except for FVC% predicted). This study was powered for the primary outcome on the assumption that 50 children were required for an 8% improvement in FEV_1 % predicted, based on historical data from our center showing that the mean (SD) FEV_1 % predicted in CF children aged 5-13 years old is 80 (10) %. The mean (SD) FEV_1 % predicted in CF children in our study however turned out to be 95 (23) %. In other studies an improving rate of decline in lung function is also described in successive birth cohorts (35). The improving rate in lung function decline makes it even more necessary to carefully evaluate outcome measures and sample sizes. Despite the fact that our study had less power than originally planned, caused by the improving rate of lung function decline, we still found a strong tendency towards a lesser decline in all lung function parameters in HFA-BDP treated children.

Failure of effects of ICS on the preservation of lung function could be caused by a lack of penetration of the anti-inflammatory agent into the thick viscous mucus present in CF. We therefore treated CF children in this study with a dosage of HFA-BDP that was above the current licensed dose for children with asthma. To assess whether lung deposition of inhaled corticosteroids is indeed decreased in CF patients, we performed a pilot study using a bioassay that evaluates the bioactivity of glucocorticoids in serum (32). We found no differences in lung deposition between CF patients and healthy controls, suggesting that the dosage used in this study should be adequate in CF patients. Furthermore we found a decline in height growth in HFA-BDP treated children in the first year of treatment with an increase in height growth after 2 and 3 years, suggestive of HFA-BDP deposition in the lungs of CF children and systemic side-effects of the ICS.

Ideally inflammation should be measured in the target organ, the CF lung, but repeated measurements of inflammation in BALF or inhaled sputum is hampered by the invasive character and the difficulty to obtain approval from the Medical Ethics Committee. We therefore assessed inflammation in peripheral blood. HFA-BDP treated children also showed a tendency towards a decline in neutrophil activation status in blood, but these differences were not statistically significant. Both Th1 and Th2 cytokines, chemokines, and soluble adhesion molecules showed a decline in HFA-BDP treated children and these differences were significant for sCD54/sICAM-1, suggesting a tampering of the systemic inflammatory response seen in CF. sICAM-1 is increased in serum, even in stable CF patients, and pulmonary exacerbation even further increased the levels of sICAM-1 (36). ICAM-1 is expressed on multiple cell lineages and is upregulated at sites of inflammation and therefore might represent the chronic inflammatory process in CF. This study is the first to describe a decline in sICAM-1 in plasma after treatment with HFA-BDP.

As has been shown previously, chest radiographs show a good correlation with lung function parameters in children with moderate to severe lung disease (37) but in children with mild lung disease high resolution computed tomography (HRCT) scanning of the lungs seems more sensitive in detecting structural damage (38,39). We chose to assess chest ra-

diograph scores, since in our study many children were aged under 6 years. Some of these children would need repeated sedation for the annual HRCT, and would be exposed to a much higher radiation dosage with the consequent higher risk of cancer. HFA-BDP had no effect on progression of structural lung damage assessed by chest radiograph scores. After 3 years of treatment, most children in both groups still had normal lung function making it likely that structural damage assessed on a chest radiograph was too insensitive to measure a potential treatment effect. Finally, treatment with HFA-BDP had no effect on other secondary outcome measures such as ETCOc% and CFQ scores. This can be explained by the fact that CF children included in this study were young and still had relatively mild lung disease at the end of the treatment period.

In conclusion, this is the first study demonstrating a positive effect of the use of ICS in young children with CF on lung function parameters and inflammation in peripheral blood. Until anti-inflammatory therapy directed more specific at the neutrophil-dominated inflammation in CF lung disease has proven to be effective and safe, we believe that ICS can be useful as an anti-inflammatory agent in children with CF. Maximum benefit of ICS will be sorted when started at an early age, while lung disease is still mild. Further studies will need to elucidate the optimal dosage and the even longer term effects of ICS on rate of lung function decline and potential side effects such as height growth impairment.

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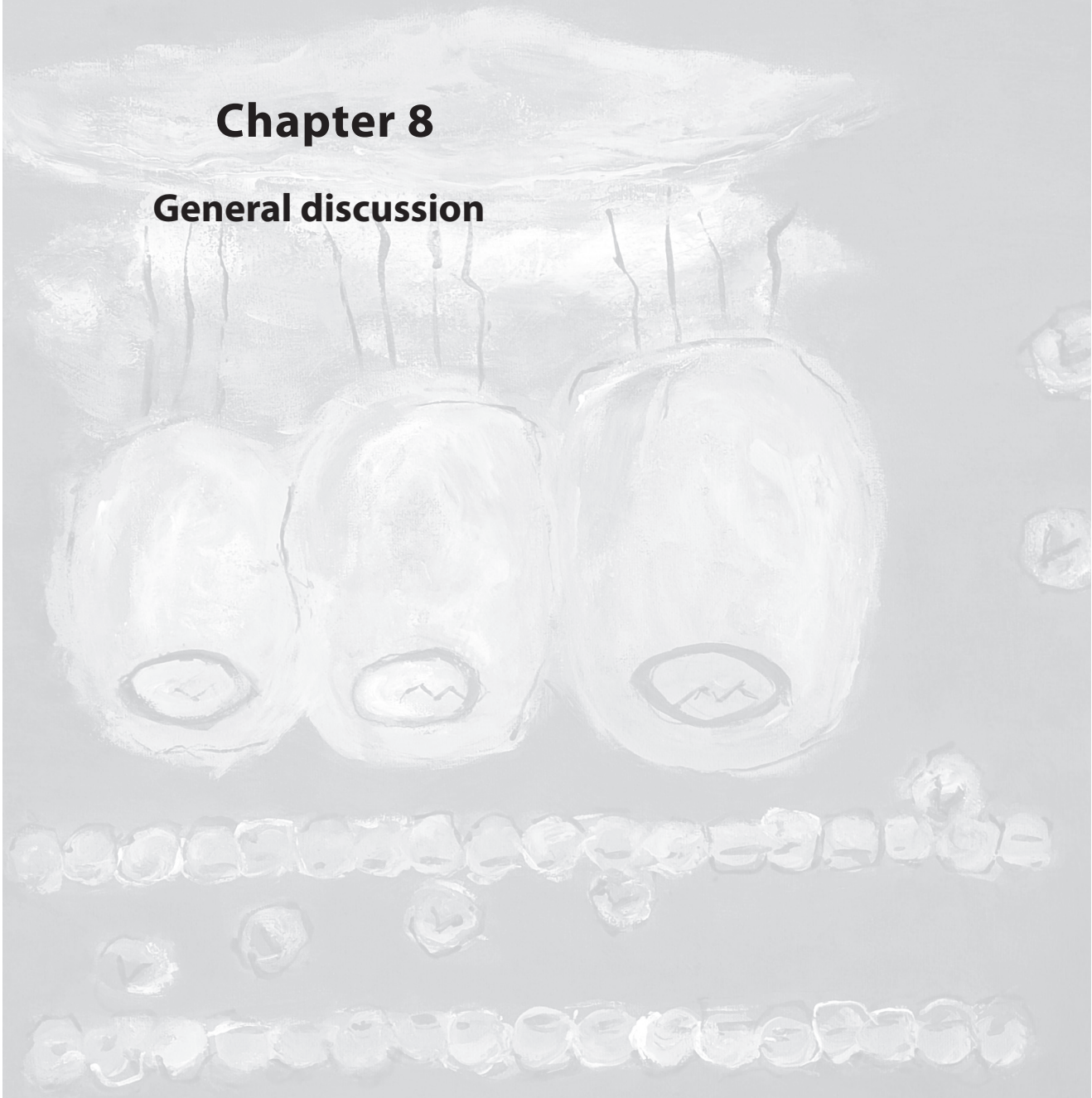
Chapter 8

General discussion



GENERAL DISCUSSION

CF lung disease is characterized by chronic inflammation with massive influx of polymorphonuclear neutrophils and chronic bacterial infection with specific pathogens such as *Staphylococcus aureus* and later on in the disease process *Pseudomonas aeruginosa* and other opportunistic pathogens. There has been much debate about whether infection precedes inflammation or *vice versa*. *In vitro*, animal, and human studies support the hypothesis that inflammation starts very early in the lungs of patients with CF, even without detectable infection. Since inflammation plays a crucial role in CF lung disease and leads to progressive lung damage, it seems beneficial to intervene in this inflammatory process.

The most important findings of this thesis are:

1. CF neutrophils exhibit a pro-inflammatory phenotype that is present even in clinically stable young children with normal lung function and negative sputum cultures. (**chapter 4**). CF inflammation starts early and a pro-inflammatory phenotype is already present in *ex-vivo* differentiated neutrophils from a CF newborn (**chapter 3**).
2. Inflammation can be measured systemically in peripheral blood and exhaled breath and is increased in CF children compared to healthy controls (**chapter 4 and 5**).
3. Structural lung disease can be assessed using chest radiograph scoring systems specific for CF. Even preschool children show progressive structural damage on an annual chest radiograph while lung function still remains normal (**chapter 6**).
4. Intervening in the inflammatory process using inhaled corticosteroids (HFA-beclomethasone dipropionate) diminishes lung function decline and reduces the pro-inflammatory cytokine profile in plasma (**chapter 7**)

Both airway epithelium and neutrophils play a critical role in the inflammatory process seen in CF lung disease. Defective CFTR protein in airway epithelial cells leads to defective Cl^- secretion and enhanced ENaC-mediated Na^+ absorption. This in turn leads to decreased airway surface liquid (ASL) volume, decreased mucociliairy clearance and mucus stasis. *In vitro* studies have shown that defective clearance of inhaled environmental particles and irritants can trigger release of chemokines from airway epithelia and/or macrophages (1). Furthermore in a Scnn1b-transgenic mouse model of CF lung disease it is suggested that defective mucus clearance, caused by dehydration of the ASL, can cause neutrophilic airway inflammation in the absence of bacterial infection (2). In the first part of this thesis we summarize the role of airway epithelium and neutrophils in inflammation in CF (**chapter 2**).

There are several studies addressing the possibility that an intrinsic CFTR-related neutrophil defect is partly responsible for the increased inflammatory response seen in CF lung disease (3-6). Altered neutrophil functions with regard to modulation of intracellular pH (3), myeloperoxidase-dependent oxidant generation (4), adherence to airway epithelial cells (5),

and directed migration in response to interleukin (IL)-8 in CF patients have been described (6). CFTR mRNA transcripts have not only been demonstrated in epithelial cells but also in cells of non-epithelial origin, such as peripheral blood neutrophils, lymphocytes and alveolar macrophages (7). This has led to the hypothesis that CF neutrophil functions might be intrinsically altered. Our study showed increased fMLP-induced Ca^{2+}_i mobilization responses in CF neutrophils, and these increased responses could not be mimicked when healthy control neutrophils were primed with the systemic immune activator TNF-α. Also in *ex-vivo* differentiated neutrophils from CF $CD34^+$ progenitor cells an enhanced fMLP-induced Ca^{2+}_i mobilization response was observed, supporting the hypothesis that CF neutrophils are intrinsically altered (**chapter 3**).

Although neutrophil-dominated inflammation is a hallmark of CF lung disease, monitoring inflammation is difficult, especially in young children. Ideally inflammation is measured in the lung as target organ. These measurements are hampered by the invasive character of obtaining bronchoalveolar samples or the inability for young children to perform the complex techniques required for lung function and sampling of exhaled breath condensate. New and non-invasive measures of inflammation are needed to monitor disease progression over time. Peripheral blood neutrophils from CF patients with moderately decreased pulmonary function exhibit a primed phenotype, measured by the ratio between circulating and PAF-primed phagocyte responses to complement-opsonized zymosan and by L-selectin shedding *in vitro* (8,9). These findings suggest that the local inflammatory response can communicate with the systemic circulation and triggers priming and activation of peripheral blood neutrophils resulting in a systemic inflammatory response. Using the newly developed monoclonal phage antibodies (MoPhabs) A17 and A27 we described an increased neutrophil priming state in peripheral blood in clinically stable, young CF children, with normal lung function and no signs of a pulmonary infection compared to healthy controls (**chapter 4.1**). Although the expression of the more conventional integrin chain $\alpha_m\beta_2$ (CD11b/CD18) on blood neutrophils was comparable between healthy controls and uninfected CF children, CF children with a first acquisition of *Pseudomonas aeruginosa* did show increased expression of the $\alpha_m\beta_2$ integrin (**chapter 4.1**). It seems that during the course of CF in children the different stages of the disease are mediated by differences in phenotype of the systemic innate immune response. Not only the neutrophil priming state is increased in CF children, also cytokines and chemokines are increased in CF children and adults with more advanced lung disease (6,10,11). We show that even young CF children with normal lung function and mild disease display a pro-inflammatory plasma cytokine profile. However, CC-chemokines are very low (**chapter 4.2**). Other non-invasive measures of lung inflammation include exhaled breath condensate measurements. These have been proposed as surrogate markers of lung inflammation. Although exhaled breath condensate measurements can easily be performed in older children, in the very young it remains difficult to obtain a large enough sample to analyze. Another drawback of using exhaled breath condensate as a surrogate measure of lung inflammation is the lack

of standardization and the lack of specific high-sensitivity immunochemical or colorimetric assays (12). Besides exhaled breath condensate, lung inflammation can also be measured in exhaled breath. These measurements can easily be performed even in very young children and neonates. We found that end tidal carbon monoxide (ETCOc) levels in CF children were increased when these measurements were corrected for total lung volume (TLC-He) (**chapter 5**). Previously we found a strong correlation between height and ETCOc but the relation between ETCOc and TLC-He was even stronger (13). These findings make implementation of routine ETCOc measurements in young CF children less useful since they are not yet capable of performing lung volume measurements. In older children and in research settings there still might be a valuable role for ETCOc measurements in monitoring lung inflammation.

CF lung inflammation leads to structural and functional lung damage over time. In young children it is difficult to assess lung damage since gold standard pulmonary function testing (spirometry) can only be performed reliably in children from the age of 5-6 years onwards. Structural lung damage is still mild at the beginning of CF lung disease and sensitive techniques are needed to detect this early stage lung damage. Therefore in the past decades, many studies have focused on application of high resolution computed tomography (HRCT) scanning of the lungs to monitor structural lung damage in CF (14-16). HRCT has been reported to be more sensitive than chest radiographs in detecting structural lung abnormalities (14-16). The routine use of HRCT in daily CF care is hampered by the higher radiation dosage compared to a chest radiograph and the subsequent higher risk for cancer, the higher costs, and the need for sedation of young children during the procedure. Therefore, we assessed the value of routine chest radiograph scoring systems specific for CF. We found a strong correlation between chest radiograph scores, lung function and infectious exacerbation rate (**chapter 6.1**). Furthermore we found a significant progression of structural lung damage, even in preschool children with mild lung disease, scored on a chest radiograph over a 3-year interval, while lung function measured by interrupter resistance still remained normal (**chapter 6.2**). These findings imply that chest radiographs still have a valuable role in monitoring structural lung disease progression over longer periods of time, even in young children with mild lung disease. Chest radiograph scores might be less valuable than HRCT sores in monitoring short-term treatment effects in research settings (17). Lung function measurements in young, preschool children include the interrupter resistance measurement (Rint). In CF children results and value of Rint measurements were inconclusive (18-21). We demonstrated that most preschool and school aged CF children have normal Rint values and these values remain normal during a 3 year follow-up period. Therefore, Rint is not a good measure to monitor pulmonary disease progression in groups of children with CF.

It is clear from the literature and from studies presented in this thesis that inflammation in CF starts at a very early age, can be measured systemically and that structural damage of the lung is even present in preschool children. Starting anti-inflammatory therapy at an early age might therefore be beneficial. Previous studies investigating the efficacy of inhaled corti-

costeroids (ICS) in treatment of CF lung disease were inconclusive (22-28). We demonstrated a positive effect of treatment with the ICS beclomethasone dipropionate (HFA-BDP, Qvar®) on lung function decline and the pro-inflammatory cytokine profile in plasma in young CF children over a 3 year period (**chapter 7**). Longer follow-up is needed to evaluate whether this positive effect on lung function and inflammation is persistent and what the optimal treatment dosage is to minimize side effects and maximize therapeutic effect.

SUGGESTIONS FOR FUTURE STUDIES

Is there an intrinsic inflammatory process in CF?

Neutrophil-dominated lung inflammation plays a central role in CF lung disease and causes structural and functional lung damage, evolving in disease-related morbidity and eventually mortality. There is much debate on whether inflammation in CF is intrinsic or not and whether CF neutrophils are intrinsically altered. Since neutrophils have a short life-span in the circulation (about 8 hours) experiments addressing this question remain difficult to execute. Even CF newborns detected by screening have already been in contact with various pathogens since their birth and their neutrophil compartment cannot be considered "naive". Neutrophils, that are differentiated *ex-vivo* from CF and healthy control $CD34^+$ progenitor cord blood stem cells seem to provide us with the opportunity to investigate "naive" CF and healthy control neutrophils. Future studies using these neutrophils might clarify the question whether or not CF neutrophils are intrinsically altered.

Can specific surrogate markers of pulmonary inflammation be identified?

In order to optimize treatment and to monitor (new) treatment effects, it is necessary to assess the inflammatory process from the beginning of the disease process, in a non-invasive manner. Although inflammation is preferably measured in the target organ, several studies show that the lung compartment communicates with the systemic circulation and that inflammation can be measured systemically (6-11).

In this study we demonstrate that indeed inflammation is already present systemically in very young children with mild lung disease. Long-term follow-up studies are needed to evaluate whether MoPhabs A17 and A27 can be used in daily CF care. It would be interesting to investigate the neutrophil priming state on regular intervals, using MoPhabs A17 and A27 and to see whether these priming markers can be used to monitor the disease process and are related to other disease parameters such as lung function, nutritional status and structural lung damage.

Plasma cytokine profiles, using the multiplex immunoassay, also seem promising in monitoring systemic inflammation in CF. Young children already have a pro-inflammatory cytokine profile but in contrast show low concentrations of chemokines of the CC-family that are in-

volved in attracting T-lymphocytes and monocytes. It would be useful to measure the plasma cytokine profiles longitudinally. It is interesting to speculate whether CF children that display high levels of the CC-chemokines are children that become infected later on or not at all, suggesting a possible link between plasma chemokine profile and adaptive immunity.

Although ETCOc measurements are easy to perform in young children, we only found increased levels when ETCOc levels were corrected for TLC-He. Long-term studies are needed to investigate whether ETCOc can be used as a measure of lung inflammation in older children and in research settings.

Value of chest radiograph in assessing structural lung damage?

In this study we demonstrate that structural lung damage can be assessed on a chest radiograph and that progression of these structural abnormalities, using CF chest radiograph scoring systems, are detectable in preschool children. Chest radiographs still have an important role in assessing structural lung damage in CF, especially since HRCT of the lungs has not yet been implemented in routine CF care.

Efficacy of ICS?

ICS slow down the progression of lung function decline and decrease the pro-inflammatory cytokine concentrations in plasma in young CF children. Treatment should be started at an early age when lung disease is still mild. Longer follow-up is needed to evaluate whether the results are persistent and whether the risk-benefit ratio remains in favor of the benefit. Studies are needed to assess the optimal dosage of ICS at which side-effects are minimal and efficacy is maximal. Furthermore, future studies should focus on more specific neutrophil-directed anti-inflammatory therapy, possibly directed at changes in Ca^{2+}_{i} in activated neutrophils (29).

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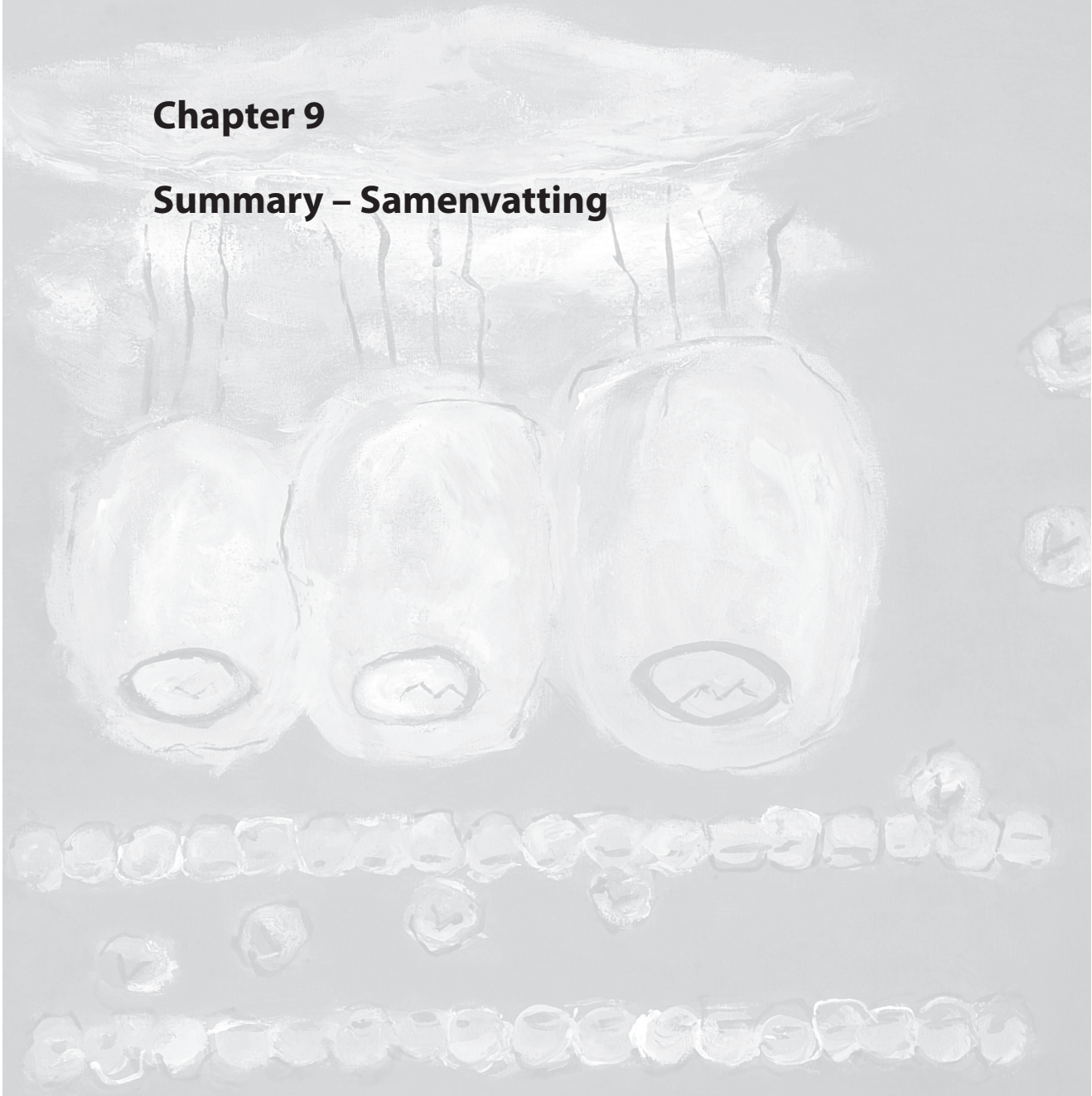
Chapter 9

Summary – Samenvatting



SUMMARY

Cystic fibrosis (CF) is the most common lethal hereditary disorder in the Caucasian population and is caused by mutations in the Cystic Fibrosis Transmembrane Regulator (CFTR) gene, localized on chromosome 7. CF is a multi-organ disease, but morbidity and mortality are mostly determined by CF lung disease. CF lung disease is characterized by an exaggerated, sustained and extended inflammation, with a massive influx of neutrophils and high concentrations of interleukin (IL)-8 in bronchoalveolar lavage fluid (BALF). Furthermore, patients with CF have chronic bacterial airway infection, particularly with *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Chronic inflammation and infection lead to structural lung damage and impairment of lung function, finally resulting in respiratory insufficiency and death. Both airway epithelium and neutrophils play a key role in the inflammatory process in CF lung disease. An overview of the role of airway epithelium and neutrophils in CF lung inflammation is presented in **Chapter 2**.

In this thesis we address three important questions:

1. Is systemic inflammation already present in clinically stable young children with CF and if present, is this pro-inflammatory phenotype intrinsic and CFTR-related or caused by environmental factors?
2. Can we measure inflammation in a non-invasive way in young children?
3. Can the inflammatory process be influenced by anti-inflammatory therapy (inhaled corticosteroids) started at an early age?

Many studies have shown that inflammation starts early in the disease process, even without detectable infection. This in combination with the finding of CFTR mRNA transcripts in blood neutrophils has led to the hypothesis that CF neutrophils are intrinsically altered and that inflammation in CF might be intrinsic. In **Chapter 3** fMLP-induced intracellular calcium (Ca^{2+}_i) mobilization responses and migration of peripheral blood neutrophils of clinically stable CF patients are assessed and compared with healthy controls. FMLP-induced Ca^{2+}_i mobilization responses were increased in CF neutrophils and these responses could not be mimicked when healthy control neutrophils were primed with the systemic immune activator TNF-α. We also evaluated fMLP-induced Ca^{2+}_i mobilization responses in neutrophils differentiated *ex-vivo* from cord blood $CD34^+$ progenitor cells of a CF newborn and a healthy newborn. These cells also displayed an enhanced fMLP-induced Ca^{2+}_i response, suggesting an intrinsic neutrophil defect in CF. Ca^{2+} plays a critical role in several neutrophil functions and regulation of neutrophil migration to the site of infection. We also found enhanced migration of CF neutrophils compared to healthy control neutrophils and this migration was abrogated when Ca^{2+} was depleted from the cells. These results suggest a possible link between the altered Ca^{2+}_i mobilization responses and the massive influx of CF neutrophils into the lungs.

Inflammation does not seem to be confined to the lungs in CF. Studies evaluating inflammation in peripheral blood have shown that inflammation can be monitored systemically in CF patients with moderate disease. Furthermore inflammation can also be measured in exhaled breath condensate and exhaled breath. These methods are non-invasive and can be performed in young children. **Chapter 4** is focused on the possible measures of inflammation that can be assessed in blood. We describe neutrophil priming phenotypes using expression of the $\alpha_m\beta_2$ integrin chain and newly developed priming associated cellular markers (MoPhabs A17 and A27) that recognise cytokine-primed neutrophils in peripheral blood. Expression of the MoPhabs A17 and A27 were significantly increased in all CF children, even in young uninfected patients with normal lung function. Although the expression of the more conventional integrin chain $\alpha_m\beta_2$ (CD11b/CD18) on blood neutrophils was comparable between healthy controls and uninfected CF children, CF children with a first acquisition of *Pseudomonas aeruginosa* did show increased expression of the $\alpha_m\beta_2$ integrin chain. We furthermore show that not only neutrophil priming state is increased in CF children, but that also young CF children with normal lung function and mild disease display a pro-inflammatory plasma cytokine profile, while in contrast CC-chemokines are very low. **Chapter 5** describes end tidal carbon monoxide (ETCO) measurements in 18 clinically stable CF patients compared to 20 healthy controls. ETCO levels in CF children are increased when corrected for total lung volume as is described in this chapter.

Chronic inflammation and infection not only cause functional lung changes but also structural lung damage. Structural lung damage can be assessed by chest radiographs. **Chapter 6** is focused on the role of CF chest radiograph scoring systems in monitoring structural lung damage and disease progression. We describe a good correlation between CF chest radiograph scoring systems, PFTs, and other clinical parameters of disease in adolescents with mild to moderate disease severity. Furthermore we show longitudinal changes in chest radiograph scores and PFTs ($Rint_{exp}$ and spirometry) in 51 young children with CF. Even preschool children show a statistically significant progression of structural lung damage, scored on a chest radiograph, over a 3-year interval. Lung function determined by interrupter resistance (Rint) however was normal for most children at the start and remained normal after 3-years of follow-up. Rint measurements do not seem useful in monitoring disease progression in groups of CF patients.

In **Chapter 7** the effects of a randomized, double-blinded, placebo-controlled trial with hydrofluoroalkane beclomethasone dipropionate (HFA-BDP, Qvar®) on lung function, inflammation in peripheral blood, chest radiograph scores, bacterial colonization, ETCO, and quality of life is evaluated in 57 young children with CF. Children treated for 3 years with HFA-BDP show a lesser decline in lung function (mean difference between HFA-BDP and placebo treated children 7.5% less decline over 3 years). HFA-BDP treated children also display lower plasma concentrations of pro-inflammatory cytokines, chemokines and soluble adhesion molecules than placebo treated children. Structural lung damage scored on an annual chest radiograph,

neutrophil priming, ETCOc, bacterial colonization and quality of life scores were comparable between the two groups.

The studies in this thesis focused on several issues regarding inflammation in CF lung disease and treatment of inflammation with ICS. In **Chapter 8** conclusions from these studies and the relevance for clinical practice of this thesis are discussed.

The main findings of this thesis are that:

1. CF neutrophils exhibit a pro-inflammatory phenotype that is present even in clinically stable young children with normal lung function and negative sputum cultures. CF inflammation starts early and a pro-inflammatory phenotype is already present in *ex-vivo* differentiated neutrophils from a CF newborn.
2. Inflammation can be measured systemically in peripheral blood and exhaled breath and is increased in CF children compared to healthy controls.
3. Intervening in the inflammatory process using inhaled corticosteroids (HFA-beclomethasone dipropionate) diminishes lung function decline and reduces the pro-inflammatory cytokine profile in plasma.

SAMENVATTING

Cystic fibrosis (CF) is de meest voorkomende letale erfelijke aandoening in de Kaukasische populatie. De ziekte wordt veroorzaakt door afwijkingen in het Cystic Fibrosis Transmembrane Regulator (CFTR) gen, gelegen op chromosoom 7. CF is een aandoening van meerdere organen maar de morbiditeit en mortaliteit worden voornamelijk bepaald door de ernst van de longziekte. CF longziekte wordt gekenmerkt door een zeer uitgebreide, hevige en persisterende inflammatie (ontstekingsreactie), met een massale instroom van neutrofielen (witte bloedcellen) in de long en verhoogde concentraties van pro-inflammatoire ontstekingseiwitten zoals interleukine (IL)-8 in bronchusspoelsel. Patiënten met CF hebben ook chronische bacteriële infecties van de long met specifieke bacteriën zoals *Pseudomonas aeruginosa* en *Staphylococcus aureus*. Deze chronische inflammatie en infectie leiden tot structurele en functionele longschade, met op termijn benauwdheid, respiratoire insufficiëntie en uiteindelijk zelfs overlijden. Zowel luchtwegepitheel als neutrofielen zijn belangrijke spelers in het inflammatie proces in de long en in **hoofdstuk 2** wordt hiervan een overzicht gegeven.

In dit proefschrift beantwoorden we 3 belangrijke vragen:

1. Is systemische inflammatie al aanwezig bij jonge kinderen met CF die klinisch stabiel zijn en zo ja, is dit pro-inflammatoire fenotype intrinsiek of wordt het verklaard door omgevingsfactoren?
2. Kunnen we inflammatie op een niet invasieve manier systemisch meten bij kinderen met CF?
3. Kunnen we het inflammatoire proces beïnvloeden door behandeling met inhalatiecorticosteroïden, indien deze op jonge leeftijd worden gestart?

In verschillende studies is aangetoond dat inflammatie al heel vroeg in het ziekteproces aanwezig is, zelfs als er geen infectie is aangetoond. Daarnaast komt CFTR mRNA niet alléén in epitheelcellen tot expressie, maar ook in neutrofielen uit perifeer bloed. Dit heeft tot de hypothese geleid dat neutrofielen van CF patiënten intrinsiek anders zijn dan neutrofielen van gezonde personen en dat de hevige inflammatie bij CF aangeboren zou kunnen zijn. In **hoofdstuk 3** wordt de intracellulaire calcium (Ca^{2+}_i) mobilisatie respons van CF neutrofielen en neutrofielen van gezonde controles met elkaar vergeleken. CF neutrofielen vertonen een verhoogde Ca^{2+}_i mobilisatie respons en deze respons wordt niet gezien als de gezonde controle neutrofielen eerst worden gestimuleerd met TNF-α. Daarnaast vertonen neutrofielen die *ex-vivo* gekweekt zijn uit $CD34^+$ voorloper cellen, geïsoleerd uit navelstrengbloed van een pasgeborene met CF ook een verhoogde Ca^{2+}_i mobilisatie respons vergeleken met neutrofielen van een gezonde pasgeborene. Deze bevindingen ondersteunen de hypothese dat CF neutrofielen intrinsiek anders zijn. Aangezien Ca^{2+} een belangrijke rol speelt in regulatie van verschillende functies van de neutrofielen waaronder ook migratie, hebben we ook migratie

van CF neutrofielen vergeleken met gezonde neutrofielen. CF neutrofielen vertonen een versterkte migratie zonder stimulus ten opzichte van gezonde neutrofielen. Indien Ca^{2+} werd verwijderd uit de buffer werd de migratie van de neutrofielen volledig stilgelegd. Dit suggereert een verband tussen de verhoogde Ca^{2+}_i mobilisatie respons en de massale instroom van neutrofielen in de long bij CF.

Inflammatie lijkt zich niet tot de long alléén te beperken bij CF. Verschillende studies hebben inflammatie in perifeer bloed gemeten en hebben aangetoond dat kinderen en volwassenen met CF met matige ziekte-ernst verhoogde inflammatiemarkers hebben in perifeer bloed. Daarnaast kan inflammatie ook op een niet invasieve manier worden gemeten in uitademingslucht en deze metingen kunnen al succesvol worden uitgevoerd bij zeer jonge kinderen en zelfs pasgeborenen. In **hoofdstuk 4** ligt de nadruk op het meten van inflammatie in perifeer bloed. We beschrijven pre-activatie (priming) van perifere bloed neutrofielen, gebruik makend van de conventionele priming marker $\alpha_m\beta_2$ integrin (CD11b/CD18) en van de recent ontwikkelde priming markers (MoPhabs A17 en A27). De expressie van MoPhabs A17 en A27 was significant hoger bij alle kinderen met CF vergeleken met gezonde kinderen en zelfs niet geïnfecteerde kinderen met CF, met een normale longfunctie, vertoonden een verhoogde expressie. De expressie van CD11b/CD18 echter was alleen verhoogd bij kinderen met een eerste *Pseudomonas aeruginosa* infectie. Niet alleen priming van neutrofielen is verhoogd bij jonge kinderen met CF, zij hebben ook een pro-inflammatoir cytokine profiel gemeten in plasma. Chemokines die behoren tot de CC-familie en die zorgen voor de aantrekking van T-lymfocyten en monocyten naar de plaats van infectie waren echter verlaagd. In **hoofdstuk 5** is onderzocht of koolmonoxide (CO), gemeten in uitademingslucht (ETCOc) een maat van inflammatie in de long zou kunnen zijn. De waarden van ETCOc zijn verhoogd bij kinderen met CF als deze gecorrigeerd worden voor het totale longvolume.

Chronische inflammatie en infecties veroorzaken longfunctieachteruitgang maar ook structurele longschade. De ernst van structurele longschade kan onder andere worden bepaald met een thoraxfoto. **Hoofdstuk 6** is gewijd aan de rol van de speciaal voor CF ontwikkelde thoraxfoto scoringssystemen in het vastleggen van structurele longschade. We beschrijven een goede correlatie van de verschillende CF thoraxfoto scoringssystemen met longfunctie en andere klinische parameters bij adolescenten met milde tot matige longziekte. Vervolgens hebben we gedurende een follow-up periode van 3 jaar gekeken naar de mate van progressie van structurele longschade gescoord met een thoraxfoto. Jonge kinderen met CF vertonen een statistisch significante verslechtering van de thoraxfoto gedurende de follow-up van 3 jaar, terwijl longfunctie (luchtwegweerstand gemeten met de MicroRint®) bij de meeste kinderen normaal was en ook bleef gedurende de 3 jaar. Deze maat van longfunctie lijkt dan ook niet geschikt om het beloop van longziekte van groepen kinderen met CF te vervolgen.

In **hoofdstuk 7** worden de resultaten van een 3 jaar durende, dubbelblinde, placebogecontroleerde, studie naar de effecten van het inhalatiecorticosteroid beclometason dipropionaat

(HFA-BDP, Qvar®) op longfunctie, inflammatie in perifeer bloed, thoraxfoto scores, bacteriële kolonisatie, ETCOc en kwaliteit van leven beschreven. HFA-BDP vertraagt de longfunctieachteruitgang gemiddeld met 7,5% over een periode van 3 jaar. Daarnaast vertonen de kinderen die zijn behandeld met HFA-BDP lagere concentraties van verschillende cytokines dan de placebo groep. HFA-BDP heeft geen effect op de andere uitkomstmaten.

In **hoofdstuk 8** worden de resultaten van de verschillende studies uit dit proefschrift en de klinische toepasbaarheid hiervan besproken.

De belangrijkste conclusies van dit proefschrift zijn:

1. CF neutrofielen hebben een pro-inflammatoir fenotype en dit komt al tot expressie bij klinisch stabiele jonge kinderen met een normale longfunctie, die geen tekenen van een bacteriële infectie van de longen hebben. Inflammatie begint vroeg in het ziekteproces en zelfs *ex-vivo* gedifferentieerde neutrofielen van een pasgeborene met CF vertonen een pro-inflammatoir fenotype.
2. Inflammatie kan systemisch worden gemeten in zowel perifeer bloed als uitademingslucht en kinderen met CF hebben verhoogde inflammatie markers vergeleken met gezonde kinderen.
3. Behandeling met inhalatiecorticosteroïden (beclometason dipropionaat) remt de longfunctieachteruitgang en verlaagt het pro-inflammatoire cytokine profiel gemeten in plasma van kinderen met CF.

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liever liefjes dan om het even wat…

Het is niet met vuurwerk en violen, niet met goud en diamant…
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CURRICULUM VITAE

Suzanne Terheggen-Lagro werd op 30 december 1973 geboren te Tiel. Zij genoot haar middelbare school opleiding op de Rijks Scholengemeenschap te Tiel, waar zij in 1992 haar Gymnasium-β diploma behaalde. Datzelfde jaar begon zij aan de studie Geneeskunde in Utrecht, waar zij in 1998 het artsexamen behaalde. Tijdens haar studie genoot zij een meekijkstage in Le Bonheur Children's Medical Center in Memphis Tennessee. Gedurende haar co-schappen verrichtte zij onderzoek naar het effect van laag moleculair gewicht heparine ter preventie van centrale lijn trombose op de afdeling Hematologie in het Universitair Medisch Centrum Utrecht onder begeleiding van Dr. A.W. Dekker. Als keuze co-schap verrichtte zij onderzoek bij de afdeling Kindergeneeskunde (Drs. E.A. Wauters) naar een nieuwe gehydrolyseerde zuigelingenvoeding. Na een jaar AGNIO-schap kindergeneeskunde in het Wilhemina Kinderziekenhuis direct aansluitend aan de studie geneeskunde, werd zij AGIO kindergeneeskunde in januari 2000 (opleider Prof. Dr. J.L.L. Kimpen). Na 1,5 jaar klinische opleiding in het Sint Antonius ziekenhuis te Nieuwegein (2000-2001, opleider Dr. J.A.A.M. van Diemen-Steenvoorde) werd zij AGIKO kindergeneeskunde. Vanaf juli 2001 heeft zij de klinische opleiding afgewisseld met promotieonderzoek dat geresulteerd heeft in dit proefschrift. Ze is sinds 2003 bestuurslid van de junior afdeling en de congrescommissie van de Nederlandse Vereniging voor Kindergeneeskunde.

Suzanne is getrouwd met Jacco Terheggen. Samen hebben zij 2 kinderen: Luc (2003) en Coen (2006).

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LIST OF ABBREVIATIONS

ASL	airway surface liquid
au	arbitrary unit
BALF	bronchoalveolar lavage fluid
bmi	body mass index
BSA	bovine serum albumin
Ca^{2+}	calcium
CaCC	calcium-activated chloride channel
CCL2	MCP-1 (monocyte chemoattractant protein-1)
CCL3	MIP-1α (macrophage inflammatory protein)
CCL5	RANTES (regulated upon activation normal T cell expressed and secreted)
CCL11	eotaxin
CCL17	tarc (thymus and activation-regulated chemokine)
CCL18	parc (pulmonary and activation-regulated chemokine)
CCL22	MDC (macrophage-derived chemokine)
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
CFQ	cystic fibrosis questionnaire
CO	carbon monoxide
CXCL8	IL-8 (interleukin-8)
CXCL9	MIG (monokine induced by interferon-gamma)
CXCL10	IP-10 (interferon-inducible protein 10)
DL_{CO}	diffusion capacity
ELISA	enzyme-linked immunosorbent assay
ENaC	epithelium sodium channel
ER	endoplasmic reticulum
ETCOc	end tidal CO corrected for inhaled CO
FEF_{25-75}	forced expiratory flow between 25% and 75% of expiratory vital capacity
FEV_1	forced expiratory volume in 1 second
FEV1%FVC	FEV_1 as percentage of FVC
FITC	fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
FVC	forced vital capacity
HFA-BDP	hydrofluoroalkane beclomethasone dipropionate
HO	heme-oxygenase
HRCT	high resolution computed tomography
HRQOL	health-related quality of life
ICS	inhaled corticosteroids
IFN-γ	interferon-gamma
IL	interleukin
LPS	lipopolysaccharide

MEF_{50}	maximal expiratory flow at 50% of vital capacity
MFI	median fluorescence intensity
MIA	multiplex immunoassay
MIF	macrophage inhibitory factor
MoPhab	monoclonal phage antibody
MPO	myeloperoxidase
NE	neutrophil elastase
NF-κB	nuclear factor-kappa B
NO	nitric oxide
NOS	nitric oxide synthase
OSM	oncostatin M
PBS	phosphate buffered saline
PCL	periciliary sol layer
PE	phycoerythrin
PEFR	peak expiratory flow rate
PFT	pulmonary function test
Rint	interrupter resistance
RV/TLC	residual volume as part of total lung capacity
sCD54	sICAM-1 (soluble intercellular adhesion molecule-1)
sCD106	sVCAM-1 (soluble vascular endothelial cell adhesion molecule-1)
SOCE	store-operated calcium entry
TLC	total lung capacity
TNFα	tumor necrosis factor-alpha