Prognosis in Cystic Fibrosis: Trends and Predictors

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Prognosis in Cystic Fibrosis: Trends and Predictors

Prognose van Taaislijmziekte: Trends en Voorspellers

(met een samenvatting in het Nederlands)

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Voor mijn ouders
Voor Florien

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Chapter 1

General introduction

Cystic fibrosis (CF) is a multisystem disease affecting the digestive system, sweat glands, and the reproductive tract, but progressive lung disease continues to be the major cause of morbidity and mortality. Patients with CF have abnormal transport of chloride and sodium across the respiratory epithelial cell membrane, resulting in thickened, viscous airway secretions [1,2]. Patients develop chronic infection of the respiratory tract with a characteristic array of bacterial flora, leading to progressive respiratory insufficiency and eventual respiratory failure [3].

TRENDS

CF is the most common lethal autosomal recessive disorder in Caucasian populations. More than 40 years ago the estimated birth prevalence in the Netherlands was 1 in 3,600 live births [4]. Because of improved methods of genetic counseling and an increased number of newborns of nonwestern descent, the CF birth prevalence in the Netherlands may have decreased over the past decades.
During the past decades, improved nutritional management and dietary recommendations, new airway clearance techniques, new antipseudomonal antibiotics, improved surgical techniques for meconium ileus, and the establishment of specialized care centers have resulted in dramatic improvements in CF survival in many countries [5-8]. Recently, the improvement in survival was illustrated by different national cohort survival studies from the US and UK [5,9-11], but recent data from the Netherlands are lacking [4].
Data on prognosis in general can be important in decision making regarding treatment. Especially in the end-of-life setting these data are of utmost importance. The prognosis for patients with end stage CF and respiratory failure requiring invasive mechanical ventilation (IMV) for acute respiratory failure (ARF) has been unfavorable [12]. Therefore, mechanical ventilation for ARF in patients with CF has long been discouraged. In recent decades, however, management of CF [5,13-15] and intensive care treatment principles [16-18] have changed dramatically. These developments necessitate re-evaluation of outcome of ARF in CF patients. However, so far no study on the outcome of ARF has been performed in Europe.

PREDICTORS

There is a wide range in the severity of CF lung disease and survival, with some patients facing death or lung transplantation during childhood while others have very mild disease well into adulthood. Furthermore, some CF patients suffer from severe complications such as CF-related liver disease (CFRLD), CF-related diabetes (CFRD) or nasal polyps early in life, whereas others will never develop these complications.
A better understanding of risk factors for an adverse pulmonary course and for complications of CF is the basis for early diagnosis, and an important step in targeting

populations for early intervention and prophylactic treatments. Early intervention by dietary therapy was shown to result in a better prognosis [19,20]. Starting antibiotic prophylaxis in good time can postpone permanent pulmonary damage [21], and the use of ursodeoxycholic acid for CFRLD is a successful treatment at an early stage [22].
Knowledge of genetic risk factors may optimize indications for close monitoring of pulmonary disease and lung transplantation. Nowadays, a number of patients may die while on the waiting list for pulmonary transplantation because their prognosis was too optimistically estimated, while other patients may be transplanted too early which results in a deterioration of prognosis [23].
This dramatic variability in severity of lung disease has long been recognized, and when CFTR was identified as the causative gene for CF, it was assumed that different mutations in CFTR would form the main basis for the variability. It was quickly demonstrated, however, that although certain aspects of the CF phenotype (such as pancreatic insufficiency) are determined by CFTR genotype, many other aspects of the disease are not [24]. Although more than 1500 different disease-causing mutations in the CF transmembrane regulator (CFTR) genotype have been identified, more than 50% of all individuals with CF are actually homozygous for mutation ΔF508 [25]. Individuals homozygous for the ΔF508 mutation demonstrate a full spectrum of lung-disease severity, from very mild to very severe, despite having the same CFTR genotype [26].

CLINICAL PREDICTORS

A great number of environmental factors such as *P. aeruginosa* (PA) [27], *B. cepacia* [28], nutritional support [29], early (antibiotic) treatment [30,31], tobacco use [32] and socioeconomic status [33] have been shown to be associated with the phenotype of CF.
Siblings with CF share the same CFTR mutations, are generally exposed to the same environment, and have similar quality of care. However, in countries where up till now no newborn screening takes place (as in The Netherlands), younger sibs are mostly earlier diagnosed with CF than their older sibs. Early asymptomatic diagnosis is associated with better lung function, nutritional status, and survival [34-38]. This would suggest an advantage for the younger sib. On the other hand, since cross-infection is known to occur between patients with CF [39,40], it can be hypothesized that younger siblings might acquire certain human adapted respiratory pathogens, like PA, at an earlier age due to early exposure. PA infection is associated with deteriorating lung function and increased morbidity [41,42], which may result in a more severe CF phenotype in the younger sib. The question is whether the beneficial effect on pulmonary disease of earlier diagnosis in younger CF siblings outweighs the potential negative effects of exposure to CF pathogens from older siblings.
Other common complications of CF include CFRLD and nasal polyps. CFRLD is becoming more prevalent and is now considered the third leading cause of death

in CF patients [43]. Hepatomegaly may cause mechanical effects on lung function. Furthermore, essential fatty acid deficiency has been suggested to influence lung function [44] besides being associated with CFRLD [45]. This fatty acid deficiency could therefore be a common factor for both deterioration of lung function and liver involvement [46]. However, it is not clear whether patients with CFRLD also suffer from more severe lung disease. Various studies have investigated potential risk factors for CFRLD but results from these studies are far from conclusive [46-54].
So far, no study has investigated the effect of nasal polyps on lung function in CF patients. Furthermore, it might be relevant to know whether symptoms and clinical characteristics can predict the presence of sinonasal polyposis in patients with ear, nose and throat (ENT) symptoms; however, this has also not yet been investigated.
Apart from lung function, aerobic capacity (peak oxygen uptake; VO_2peak) is a major determinant of well-being and of survival in CF patients [55,56]. Several studies have shown that VO_2peak in patients with CF is associated with lung function [57-61] and nutritional status [57,59,60]. However, the association of genetic markers, presence of inflammation and infections with VO_2peak is not well established.
In summary, the association of several clinical determinants with lung function and prognosis of CF is unknown and requires investigation in a large clinical cohort of CF patients prospectively followed in recent decades, since improved CF care may have altered past associations and conclusions.

GENETIC PREDICTORS

There has been growing interest in trying to identify non-CFTR genes that act as modifiers of CF lung disease phenotype. This interest has been stimulated by a few important observations. One is that monozygous twins have a significantly higher concordance in severity of lung disease than dizygous twins, suggesting that even when CFTR genotype is identical, differences in other genes can influence phenotype [62]. Second, studies in CFTR-knockout mice have identified a clear genetic modifier for intestinal manifestations of CF [63]. The total contribution of all of these so-called modifier genes to variation in CF lung disease severity, independent of CFTR genotype, was estimated to range from 54 to 100% [64].
The lung disease in CF is characterized by failure of lung defense, which leads to airway infection with persistent inflammation and eventual airway destruction [65]. Genes that modify the host response to infection are therefore obvious candidates to modify CF lung disease. Inflammation is, in part, a response to bacterial infections but it is known that infants with CF without detectable bacteria from bronchoalveolar lavage fluid (BALF) already have evidence of inflammation [66]. The wide variability in inflammatory response and subsequent tissue damage points to inflammatory cytokines as potential modifier genes in CF.
Mannose-binding lectin (MBL), a key factor in innate immunity, is a circulating host defense protein acting as a recognition molecule against a wide variety of infectious agents [67]. MBL is encoded by a gene (MBL2) characterized by a high degree of

functional polymorphisms, with some genotypes resulting in low concentrations of MBL that have been associated with non-infectious diseases such as systemic lupus erythematosus and rheumatoid arthritis [68], but also with susceptibility to infections [69]. Since acute and chronic pulmonary infections contribute significantly to CF morbidity and mortality, it is conceivable that MBL deficiency also contributes to the course of CF lung disease. In 1999, an association was described between MBL2 low-producer genotypes and decreased lung function in CF [70]. Subsequently, various studies reported conflicting data on the effects of MBL on lung function. However, most of these studies were limited by the small numbers of subjects and their cross-sectional design.

Another class of potential modifiers of disease progression in CF includes genes encoding toll-like receptors (TLRs). TLRs recognize different pathogen associated molecular patterns (PAMPs) and have prominent roles in the activation of innate and adaptive immune responses to infection [71]. In non-CF populations, associations have been described between other TLR single nucleotide polymorphisms (SNPs) and susceptibility to bacterial infections, as well as inflammatory entities like asthma and atherosclerosis [72-76]. Evidence for the role of TLR polymorphisms in CF patients is lacking so far.

Finally, SNPs have been demonstrated to regulate the transcription rate of a number of IL genes that could act as CF modifier genes. In CF patients, many cytokines are involved in the inflammation and dysregulated responses [77]. Concentrations of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and IL-8, were found to be elevated in the sputum and BALF of patients with CF compared to healthy controls [77], whereas anti-inflammatory cytokines, including IL-10, were observed to be relatively downregulated in CF airway cells [78]. However, it is unclear how SNPs in IL genes ultimately affect CF lung disease.

THE UTRECHT CF CENTER

The CF Center Utrecht of the University Medical Center Utrecht is the largest CF Center in the Netherlands and currently treats about 250 children and 120 adults with CF living in the central region of the Netherlands. All patients undergo a routine yearly multidisciplinary examination, including a standard history, physical examination, sputum culture, and pulmonary function, maximal exercise and blood testing. Furthermore, all pediatric patients undergo an endoscopic examination of the nose, and in all patients with elevated liver enzymes and/or clinical hepatomegaly a liver ultrasound is performed. Since 2000, all data from this multidisciplinary evaluation are recorded in an electronic database. In 95% of all patients CFTR genotyping was performed.

This large well-defined single-center population provides an optimal setting to study predictors for CF phenotype. Furthermore, because of the prospective data collection, data could be analyzed both cross-sectionally and longitudinally.

AIMS AND OUTLINE OF THIS THESIS

The prognosis of patients with CF is constantly improving and varies widely between patients. Therefore, the studies presented in this thesis aim to answer three main questions:

1. What are the actual birth prevalence and survival (trends) of CF patients?
2. What are clinical predictors of CF lung disease and CF-related complications?
3. What are genetic predictors of CF lung disease?

Ad question 1

The study in **chapter 2** determines the actual birth prevalence and survival in CF patients in the Netherlands over the past decades. In **chapter 3** we investigate the outcome of assisted ventilation in CF patients with acute respiratory failure, and search for risk factors associated with poor outcome.

Ad question 2

The study in **chapter 4** analyzes the effect of birth order on lung function and CF-related complications in siblings with CF. In **chapter 5** and **chapter 6** we investigate the association between CF lung disease and CFRLD and nasal polyposis, respectively. The aim of **chapter 7** was to identify independent predictors of aerobic capacity in children and adolescents with CF.

Ad question 3

In **chapter 8** we present an overview of the role of modifier genes in CF. We analyze the effect of polymorphisms in the MBL2 gene, TLR genes and IL genes on CF lung disease in **chapter 9, 10 and 11**, respectively.

In **chapter 12** a multivariate analysis is performed of all predictors identified in this thesis. A general discussion on investigating prognosis in CF is presented in **chapter 13**, and **chapter 14** provides a summary of this thesis in English and in Dutch.

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Chapter 2

Birth prevalence and survival in cystic fibrosis; A national cohort study in the Netherlands

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ABSTRACT

Background: Birth prevalence and survival in patients with cystic fibrosis (CF) in the Netherlands were last investigated > 30 years ago. However, since then the birth prevalence may have decreased because of genetic counseling and an increased number of newborns of non-European descent. Although survival of CF patients has increased worldwide, a significantly lower median age at death was recently reported in the Netherlands compared with data from the United States.
Objectives: To analyze birth prevalence and survival in CF patients in the Netherlands, and to compare this survival data with US CF data.
Design: Survey of all CF patients living in the Netherlands, and analysis of Dutch CF mortality statistics using data from the Dutch central statistics office, *Statistics Netherlands* (Voorburg, the Netherlands), and a comparison with Cystic Fibrosis Foundation (Bethesda, MD) patient registry data.
Setting: All CF centers in the Netherlands and the United States.
Participants: All CF patients treated in the Netherlands on January 1, 2001, and all persons who died of CF between 1974 and 2000, and an equivalent US population.
Measurements: Birth prevalence and birth cohort-specific survival.
Results: The overall birth prevalence of CF for 1974 to 1994 was 1 in 4,750 live births, which is a considerable decrease compared with 1961 to 1965 (1 in 3,600 live births). Estimated survival to 30 years increased from 6% in the 1950-to-1954 cohort, to 36% in the 1970-to-1973 cohort. Exact survival could be calculated from 1974 onwards. Survival to 15 years increased from 72% from the 1974-to-1979 cohort, to 91% in the 1985-to-1989 cohort. Survival in the United States in the 1980-to-1984 cohort was better compared to the Netherlands, but this difference has disappeared over subsequent cohorts.
Conclusions: The actual birth prevalence of CF in the Netherlands is clearly lower than it was 30 years ago. Survival in CF has dramatically improved. The difference in survival between the Netherlands and the United States, as observed in the cohorts born > 20 years ago, has disappeared.

INTRODUCTION

In the Netherlands, the birth prevalence of cystic fibrosis (CF) is estimated to be 1 in 3,600 [1]. This birth prevalence was estimated in a 1961-to-1965 birth cohort by means of an inquiry among medical specialists, and by analysis of hospital admission data, death certificates, and data of the national CF Foundation (*Statistics Netherlands*, Voorburg, the Netherlands). Because of improved methods of genetic counseling and an increased number of newborns of nonwestern (*i.e.*, Moroccan, Turkish, Indonesian, Surinamese, and Dutch Antillean) descent, the CF birth prevalence in the Netherlands may have decreased over the past decades. Survival in CF in the Netherlands was also determined > 30 years ago [1], but survival has clearly improved since that time.

From 1974 onwards, reliable CF mortality data has been obtained from the Dutch national statistics office, *Statistics Netherlands*. Furthermore, nearly all CF patients are currently treated at one of the seven specialized CF centers in the Netherlands. Apart from improvement of care and life expectancy [2,3], centralized care has resulted in a better registration of patients with CF. The improved registration of both living CF patients and CF mortality enabled us to analyze CF birth prevalence and survival since 1965.

Fogarty *et al.* [4] compared median age at death from CF in 10 countries in North America, Europe, and Australasia. They described significant differences in survival between these countries, showing favorable results in the United States compared to Europe, including the Netherlands. However, a cross-sectional measure is unsuited for capturing subtle longitudinal survival trends [5].

In the last decade, national cohort survival data from US and UK CF centers, countries with relatively similar populations, have been published [5-8]. The question arises whether the Dutch cohort survival differs from survival from US and UK cohorts. This study estimates the birth prevalence of CF in the Netherlands and analyzes the survival in CF in birth cohorts from 1950 to 1989. Furthermore, the Dutch cohort survival data are compared with those from US and UK CF centers.

MATERIALS AND METHODS

From 1995, nearly all Dutch CF patients are treated at one of seven CF centers in the Netherlands. Data on all CF patients living in the Netherlands on January 1, 2001, were obtained from these CF centers; duplicates were avoided by checking birth dates and postal codes. Patient numbers were similar to the number of patients known to the Dutch CF Foundation, which is estimated to represent > 98% of all Dutch CF patients. Since the 1990s, CF patients are treated according to consensus guidelines published by the Dutch CF Foundation (Baarn, the Netherlands), which are similar to those propagated and suggested by the Cystic Fibrosis Foundation (Bethesda, MD) [9]. In all patients included in the present study, diagnosis was made by clinical evidence (pulmonary and/or GI manifestations typical for CF), confirmed

by either an abnormal sweat chloride test result or CF genotype. In the Netherlands, there is neither mandatory prenatal screening nor newborn screening for CF.
Mortality data were obtained from the Department of Mortality Statistics of the Dutch central statistics office *Statistics Netherlands* [10]. With the introduction of the International Classification of Diseases in 1974, CF was classified as a separate disease; therefore, mortality data on CF are available from 1974. There is no registration of elective terminations of pregnancies with the diagnosis of CF. In order to test the completeness of mortality statistics, data on 50 CF patients who died during the last 15 years (37 at the University Medical Center Utrecht [UMCU], 4 at home, and 9 at other hospitals) were retrieved from the hospital records of the UMCU. All 50 deaths could be found in the CF mortality statistics of *Statistics Netherlands*.
Using the survey data of living CF patients and the CF mortality statistics, the actual patient numbers could be calculated for each annual cohort after 1974. Annual survival data in these cohorts could be calculated from the mortality statistics. The survival of the pre-1974 cohorts can only be calculated for those who have survived after 1974. The size of the original cohorts was estimated by assuming a CF birth prevalence of 1 per 3,600 live births, as observed in the period from 1961 to 1965 [1].
Five-year cohorts are used to give a reasonable sample size. Because for patients born after 1973 the exact survival could be calculated, the cohorts from 1970 to 1973 and from 1974 to 1979 were 4-year and 6-year cohorts, respectively. Because the numbers of patients are small, particularly in the early cohorts, survival rates were provided for both sexes together.

Statistical analysis
The life table was calculated using statistical software (SPSS version; 11.5; SPSS; Chicago, IL). Survival was analyzed using Cox proportional hazards model with age as the time scale.

RESULTS

Table 1 gives total Dutch live births and estimated numbers of CF live births (based on a birth prevalence of 1 in 3,600) [1] for different birth cohorts from 1950 to 1994. Actual numbers of CF live births for the birth cohorts from 1974 to 1994 are also shown. **Figure 1** presents actual annual births of patients with CF between 1974 and 2000. The number of CF live births in the 1995-to-1999 cohort was 162; however, because of a diagnostic delay, this is probably an underestimation of the actual number of CF patients [6].
The average overall birth prevalence of CF in 1974 to 1994 was 1 in 4,750 live births (95% confidence interval [CI], 1 in 5,100 to 1 in 4,440 births). **Figure 2** presents the estimated (cohorts born before 1974) and actual (cohorts born after 1974) survival curves for the different cohorts. The estimated survival (SE) to 30 years increased from 6% (1%) in the 1950-to-1954 cohort, to 36% (3%) in the 1970-to-1973 cohort.

Table 1. Total Dutch live births (in thousands) and estimated numbers of CF live births (calculated as total births/3,600) for different birth cohorts from 1950 to 1994.*

Dutch cohorts	Total Dutch live births, x 1,000	Estimated CF live births, No.	Actual CF live births, No.
1950–1954	1,146	318	
1955–1959	1,174	326	
1960–1964	1,233	343	
1965–1969	1,208	336	
1970–1973	875	243	
1974–1979	1,065	296	245
1980–1984	877	244	167
1985–1989	925	257	199
1990–1994	985	274	200

*The actual number of CF live births for the birth cohorts from 1974 to 1994 is based on a survey of CF patients alive on January 1, 2001, and mortality statistics from 1974 to 2000.

For the 1974-to-1979, 1980-to-1984, and 1985-to-1989 cohorts, the estimated survival (SE) to 15 years was 72% (3%), 77% (3%), and 91% (2%), respectively. The corresponding Cox proportional hazard ratios of the 1974-to-1979 and 1980-to-1984 cohorts, relative to the 1985-to-1989 cohort, were 3.5 (95% CI, 2.1 to 6.0) and 2.7 (95% CI, 1.5 to 4.7), respectively.

Figure 3 shows the improvement of survival rates from age 1 year in the subsequent birth cohorts, both in the Netherlands and in the United States (US data based on Cystic Fibrosis Foundation patient registry) [7]. In the 1980-to-1984 cohort, a clear

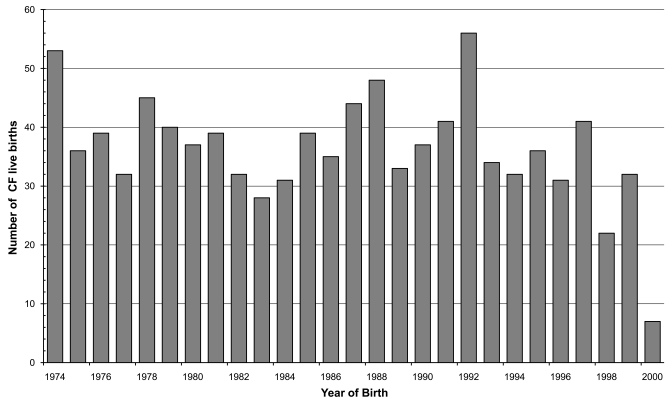

Figure 1. Annual births of CF patients between 1974 and 2000 based on a survey of CF patients alive on January 1, 2001, and mortality statistics from 1974 to 2000. Because of a diagnostic delay, calculated births after 1995 may be unreliable.

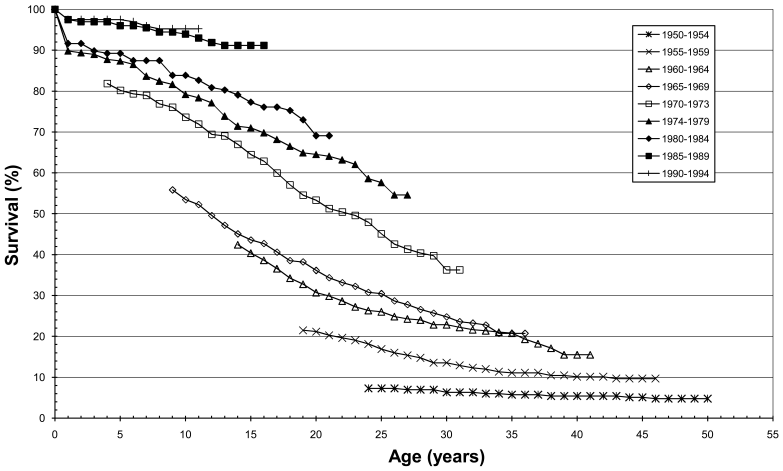

Figure 2. Survival of different cohorts of patients with CF born between 1950 and 1989. The size of the original cohorts born between 1950 and 1973 was estimated by assuming a CF birth prevalence of 1 in 3,600 live births [1].

difference in survival rate was observed in favor of the American patients; this difference, however, disappeared over the subsequent cohorts.
In the UK, for the 1980-to-1982 birth cohort, survival rates for boys and girls 10 years of age and 15 years of age were 88%/83% and 83%/77%, respectively [6]. In the 1980-to-1982 Dutch cohort, survival to these ages was 82% and 76%, respectively (both sexes combined). In a 1983-to-1985 cohort, survival to age 10 years in UK boys/girls was 90%/90%, vs 89% for Dutch CF children.

DISCUSSION

We have presented data on birth prevalence and survival of CF patients from 1974 and gave estimates for the period from 1950 to 1973. In 1977, ten Kate *et al.* [1] published the birth prevalence of CF among live births in the Netherlands for the years 1961 to 1965. He identified CF patients by means of an inquiry among medical specialists and by analysis of hospital admission data, death certificates, and data of the national CF Foundation. This elaborate study yielded a birth prevalence of approximately 1 in 3,600 live births. The birth prevalence shown by ten Kate *et al.* [1] is significantly higher than the birth prevalence found in our study (average birth prevalence from 1974 to 1994, 1 in 4,750 live births). Several factors may explain this difference.
Firstly, the completeness of data collection may play a role. Our data depend on patient data collected by the seven Dutch CF centers and on CF mortality data collected by *Statistics Netherlands*. Since 1995, nearly all CF patients are treated at one of the seven specialized CF centers in the Netherlands; all of these centers transferred pa-

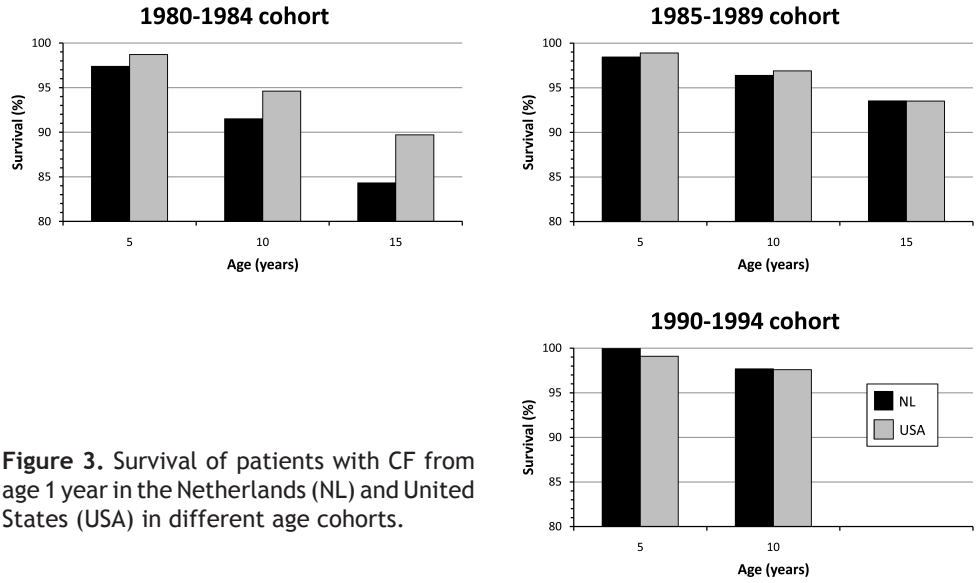

Figure 3. Survival of patients with CF from age 1 year in the Netherlands (NL) and United States (USA) in different age cohorts.

tient data to us. A comparison of our patient data with the number of patients known to the Dutch CF Foundation (estimated to represent > 98% of all Dutch CF patients) demonstrated that our data on living patients are at least complete for 98%.

A check on the completeness of the mortality statistics demonstrated that the mortality data of all 50 investigated CF patients could be found in the mortality statistics of *Statistics Netherlands*. Although the registration of mortality statistics seems to be complete, there might be underreporting due to prediagnosis mortality. However, in the study of ten Kate [1], there may be less reliable recordings of living subjects (he assumed patients with "probable" and "possible" CF to have CF) and dead subjects (no separate registration of CF by *Statistics Netherlands* because of lack of CF International Classification of Diseases coding) [1].

Secondly, in the last decades, there has been an increasing immigration to (and to a lesser degree of emigration from) the Netherlands. Since most immigrants were from nonwestern countries with a lower CF fibrosis transmembrane conductance regulator (CFTR) mutation carrier frequency (*e.g.*, Indonesia, Surinam, Dutch Antilles, Turkey and Morocco), this might have resulted in a lower CF birth prevalence over the last decades. In 1972 the number of inhabitants with a nonwestern background (first and second generation from Turkey or countries in Africa, South America and Asia, except Indonesia and Japan) in the Netherlands was 1% [10]. In 2000, 16% of all newborns had at least one nonwestern parent (first generation) [10]. On the extreme assumption that from 1961 to 1965 0% of all newborns had a nonwestern parent and that the CFTR mutation carrier frequency in nonwestern parents is 0%, the birth prevalence of 1 in 3,600 from 1961 to 1965 [1] would decrease to 1 in 4,300 in the year 2000. This number is still different from a birth prevalence of 1 in 4,750 live births. Furthermore, the CFTR mutation carrier frequency in nonwestern

parents is probably not completely zero [11]. However, only data on first-generation nonwestern parents are available. Consequently, the actual number of children with nonwestern parents is substantially higher, therefore, the birth prevalence of CF may be < 1 in 4,300.

Thirdly, the increased and improved prenatal diagnosis and genetic counseling might have resulted in a lower CF birth prevalence. Scotet *et al.* [12] found a 22.6% global reduction of CF prevalence at birth after the introduction of prenatal diagnosis in couples related to a child with CF. Although prenatal diagnosis of CF has only been performed in the last 15 years, the general awareness that CF is an inheritable disease might have influenced family planning from an earlier date.

In a more recent study, de Vries *et al.* [13] found a CF carrier frequency of 1 in 32 in Dutch blood donors, corresponding to a birth prevalence of approximately 1 in 4,000. This is lower (although not statistically significant) than the birth prevalence of 1 in 3,600 this group had published earlier [1], but higher than the birth prevalence of 1 in 4,750 in our study. Farrell *et al.* [14] and Gregg *et al.* [15] observed a lower CF birth prevalence in a CF neonatal screening project than expected from carrier frequency. This may support the view that family planning influences the birth prevalence of CF. However, the birth rate of 1 in 4,690 live births for the pre-1990 cohorts together (from 1974 to 1989) was not significantly higher than the birth prevalence of 1 in 4,920 for 1990 to 1994. Furthermore, also in a recent study [16] in Italy, no change was found in the birth prevalence of CF between 1988 and 2001.

In summary, it does not seem justified to assume that the actual CF birth prevalence in the Netherlands is still 1 in 3,600. Instead, a prevalence of 1 in 4,750 seems more realistic.

National cohort survival data of the United States [5] and United Kingdom [8] and data on median survival of western countries, including the Netherlands [4], have demonstrated a dramatic improvement in survival of CF patients during the past decades. Our study shows a similar dramatic improvement of survival for Dutch cohorts over the past decades. All successive cohorts demonstrate a higher survival rate than the previous one cohort, both for the estimated 1950-to-1973 cohorts and the 1974-to-1994 cohorts.

Because there is no single national registry of clinical or microbiological data of all Dutch patients, we were unable to directly analyze the cause of the observed improving survival of patients. Potential contributors to the better survival include improved nutritional management and dietary recommendations, new airway clearance techniques, new antipseudomonal antibiotics, and improved surgical techniques for meconium ileus [5,17]. During the last decade, new pulmonary therapies were introduced, including more aggressive antibiotic treatments, dornase-alfa and tobramycin for inhalation [5]. The establishment of specialized care centers may also have helped survival [2,3]. However, other factors may also play a role. The representation of patients with mild disease is likely to increase over time due to an increase in the diagnosis of CF in patients with mild disease [5]. However, because no Dutch CFTR mutation data have been published since 1994, a shift in severe/mild ratios of CFTR genotype could not be investigated [18].

Trends toward earlier diagnosis in CF might have led to more aggressive early care. Although the mean age of diagnosis in the Netherlands decreased from 27 months in the 1950s to 18 months in the 1960s, it appears to have stabilized between 14 and 18 months in later decades [13,19]. Because, unlike in some other western countries, no national prenatal or newborn screening for CF in the Netherlands has been implemented, also in the last decade the age of diagnosis remained at approximately 14 months (unpublished data from the UMCU CF patient registry). Therefore, the improvement in survival in the Netherlands in the last decades is not due to earlier diagnosis.

Fogarty *et al.* [4] compared the median age at death from CF in 10 different countries and found substantial differences in survival. Median age at death was significantly higher in the United States compared with the Netherlands. However, median age at death is an indirect marker of current mortality experience. In a disease such as CF (for which life expectancy is consistently increasing), median age at death will invariably underestimate median survival [5].

The North American Cystic Fibrosis Foundation publishes yearly survival data of CF patients from the age of 1 year [7]. Comparing the CF survival data between the United States and the Netherlands, a better survival could be demonstrated in the US patients compared to the Dutch patients in the 1980-to-1984 cohort (**Figure 3**); however, this difference has disappeared over the subsequent cohorts. Also, the difference in survival between the United Kingdom and the Netherlands found in the 1980-to-1982 cohort appeared to have disappeared for later cohorts. These findings stress the necessity of longitudinal survival studies in diseases for which life expectancy is consistently increasing, since cross-sectional studies fail to capture subtle changes in survival [5].

Apart from differences in the extent of underdiagnosis between nations [4], a number of other factors may explain the observed differences in survival in the older cohorts in the different countries. These factors include differences in socioeconomic status and variations in the distribution and virulence of pathogens [4,20,21]. However, these factors are not significantly different between the Netherlands, the United States, and the United Kingdom.

Another explanation may be the difference in distribution of CF genotypes [22]. In the Netherlands, the ΔF508 prevalence is 77% and the prevalence of severe mutations is 85% (percentage not determined, 12%) [18]. These percentages are higher than in the United States but similar to the United Kingdom [18].

Different studies [2,3] reported that management of CF in specialized CF centers results in a better clinical outcome and survival. The introduction of specialized CF clinics differs internationally and might therefore partially explain the international difference in survival. Since approximately 1995, almost all Dutch CF patients are treated at one of the seven CF centers. The long-term effects of this centralized care will become apparent over the coming years.

In Denmark (with a population similar to the Netherlands), approximately 75% of all patients attend the Copenhagen CF center. Major differences in CF care between Denmark and the Netherlands include early institution of centralized care (since

1968), aggressive use of antibiotics in patients with chronic *Pseudomonas aeruginosa* infection and cohort isolation, separating patients with *P. aeruginosa* from patients without such colonization [23]. In the Copenhagen CF center, a much better survival has been reported compared to our data and the US and UK data, with > 80% survival to 45 years from 1989 to 1993 [23]. Both the introduction of cohort isolation and the aggressive use of antibiotics have resulted in a lower incidence and prevalence of chronic *P. aeruginosa* infection in Denmark [24]. However, although it is well accepted that chronic *P. aeruginosa* infection is a major risk factor for mortality in CF [25], the plausibility of the Danish survival data has been debated because of small patient numbers [26].

In conclusion, the actual birth prevalence of CF in the Netherlands is lower than the birth prevalence of CF estimated 30 years ago. Similar to other countries, the survival of patients with CF has dramatically increased over the past decades. The difference in survival between the Netherlands and the United States, as observed in the cohorts born > 20 years ago, has disappeared.

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Chapter 3

Outcome of assisted ventilation for acute respiratory failure in cystic fibrosis

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ABSTRACT

Objectives: To assess outcome of assisted ventilation in cystic fibrosis (CF) patients with acute respiratory failure (ARF), to identify risk factors associated with poor outcome and to compare long-term outcome of CF children who were mechanically ventilated for ARF with unventilated CF controls.
Design: Retrospective cohort study.
Setting: Two large CF centers in the Netherlands.
Patients: CF patients who required assisted ventilation for ARF and unventilated CF controls.
Interventions: None.
Measurements and results: Thirty-one CF patients required assisted ventilation for ARF between January 1990 and March 2005. All five children (under 2 years of age) and seven adults (27%) survived. In the total population, age was a statistically significant risk factor for poor outcome ($P = 0.02$). In adult CF patients who required invasive mechanical ventilation, acute on chronic respiratory failure was associated with poor outcome. In children who required mechanical ventilation for ARF, lung function and CF-related complications 5 years later were not significantly different compared with controls matched for age, gender and genotype.
Conclusions: CF patients younger than 2 years old, who are ventilated because of ARF, have a good prognosis and their long-term outcome seems identical to unventilated CF controls. ARF in adult CF patients still is associated with high mortality, especially among patients with acute on chronic respiratory failure.

INTRODUCTION

Mechanical ventilation for acute respiratory failure (ARF) in patients with cystic fibrosis (CF) has long been discouraged because of a poor outcome [1]. In recent decades, however, life expectancy has increased significantly [2], and management of CF [3-6] and intensive care treatment principles [7-9] have changed dramatically.
These developments necessitate re-evaluation of outcome of mechanical ventilation in CF patients [10-12]. Mortality in adults varies between 45% and 80%. Although different studies tried to identify risk factors associated with poor outcome, results are not conclusive [10,11]. Results of mechanical ventilation in infants and children with CF are reported as favorable, but little is known about their long-term outcome [11,13].
This study aimed to assess outcome of assisted ventilation in CF patients with ARF, to identify risk factors associated with poor outcome and to compare long-term outcome in children who were mechanically ventilated for ARF with unventilated CF controls. Some of the results of this study have been published as an abstract [14].

MATERIALS AND METHODS

Setting

This study was performed at the University Medical Center Utrecht (Utrecht, the Netherlands) and at the Haga Teaching Hospital (The Hague, the Netherlands). These centers provide care for approximately half the Dutch CF population. Treatment strategies are similar in both centers [15].

Subjects

All CF patients who had been admitted to the intensive care unit (ICU) between January 1990 and March 2005 were identified from the electronic hospital databases. Medical records of these patients were reviewed. Only patients who were admitted to the ICU for ARF were included. ARF was defined as respiratory deterioration requiring assisted ventilation, due to an acute illness in a previously stable patient [11]. Assisted ventilation could be non-invasive (NIV) via nasal or full face mask, or invasive (IMV) via endotracheal tube. Patients with pneumothorax, hemoptysis or allergic reaction without respiratory exacerbation according to the criteria of Fuchs *et al.* [16] were not included, because of significantly better prognosis [12]. Patients with a history of lung transplantation (LTX) were excluded.

Ventilatory support

Children only received IMV. Adults were initially treated with NIV to avoid adverse effects of tracheal intubation [7,9]. When adequate gas exchange could not be reached, IMV was offered and initiated after informed consent.

Risk factors for poor outcome

To identify risk factors for mortality, the following data were recorded: age, sex, body mass index (BMI), best forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) in the year prior to ICU admission, presence of chronic respiratory failure, mode of ventilation, steroid use before admission, pancreatic insufficiency, history of CF-related diabetes and CF-related liver disease, history of hemoptysis or pneumothorax, sputum microbiology, and presence of allergic broncho-pulmonary aspergillosis.

Chronic respiratory failure was defined as consistent increase in arterial partial pressure of carbon dioxide above 45 mm Hg during spontaneous breathing of room air in the period prior to the acute exacerbation [17]. Sputum microbiology samples were collected at hospital admission. Arterial blood gasses on admission were not available for most subjects and could therefore not be included as potential risk factor for poor outcome.

Long-term outcome in children

To assess long-term outcome in infants who survived assisted ventilation for ARF, a retrospective follow-up study was performed. Cases were matched with controls (five per patient) with the same gender, genotype and age (± 1 year) without a history of ARF. Lung function, anthropometrics (standard deviation scores (SDS) of height and BMI) [18] and presence of CF-related complications 5 years after admission to the ICU were compared between cases and controls.

Statistical analysis

The association between potential risk factors and outcome was quantified using univariate logistic regression analysis. Differences in demographic and clinical variables between cases and controls 5 years after IMV were analyzed using Fisher's exact test and Mann–Whitney test.

RESULTS

In the inclusion period, 83 CF patients were admitted to the ICU. Details on indications for admission are displayed in **Supplementary Table 3A**. Thirty-one patients fulfilled the criteria of ARF. Their demographic and clinical data are summarized in **Table 1**. Two patients had a readmission for ARF > 1 year after the first. Only the first admission was included in the analysis. Two adults (one receiving IMV, one NIV) underwent LTX during their stay in the ICU. Because they probably would have died otherwise, they were analysed as non-survivors.

The 31 patients consisted of five children (aged between 2 months and 18 months), two adolescents (aged 15 years and 16 years) and 24 adults (aged ≥ 18 years). All five children were admitted because of pneumonia. Because of the age distribution, two groups of patients were defined: "children" (aged < 2 years) and "adults"

Table 1. Summary of demographic and clinical data of 31 cystic fibrosis patients admitted to the intensive care unit with acute respiratory failure requiring mechanical ventilation.

	Children	Adults
Number of patients	5	26
Mean age (range)	6 months (2–18)	26 years (15–41)
Male gender (%)	3 (60)	16 (62)
Endotracheal intubation (%)	5 (100)	17 (65)
FVC (% predicted) (SD)	-	39 (12)
FEV_1 (% predicted) (SD)	-	25 (8)
BMI (kg/m^2) (SD)	-	18 (3)

FVC indicates forced vital capacity; FEV_1 forced expiratory volume in 1 s; BMI, body mass index; and SD, standard deviation.

(aged ≥ 15 years). Detailed patient characteristics of the adults are displayed in **Supplementary Table 3B**.

Outcome

All five children survived (100%). Seven of 26 adults (27%) survived. When the two patients who underwent LTX were excluded from analysis, survival in adults was 29%. Nine adults received only NIV; 17 adults underwent endotracheal intubation. Mortality was 56% in patients receiving NIV and 82% in patients receiving IMV. After discharge from ICU, all patients survived more than 1 year.

Risk factors for poor outcome

In the total population, age was a significant risk factor for poor outcome ($p = 0.02$). Results of univariate logistic regression analyses in adults are shown in **Table 2**. None of the demographic or clinical data could predict mortality significantly. This did not change when the patients who underwent LTX were excluded from analyses.

Because mortality and presence of potential risk factors differed between patients receiving IMV and NIV, a subgroup analysis was performed. In patients receiving only NIV, no significant predictors of outcome were found. In patients receiving IMV, acute on chronic respiratory failure was a significant predictor of mortality (odds ratio [OR] = 26.0, 95% confidence interval [CI] 1.12–604). The association remained significant when the patient who underwent LTX was excluded.

Long-term outcome in children

No differences in long-term outcome were observed between cases and controls (**Table 3**). Since one infant was born in 2001, no 5-year follow-up data for this child were available.

Table 2. Comparison of demographic and clinical data between adult cystic fibrosis patients who survived and who died after assisted ventilation for acute respiratory failure. The capacity of the demographic and clinical data to predict outcome is also shown.

	Survivors	Non-survivors[a]	Odds ratio	P-value
N	7	19		
IMV versus NIV (%)	3 (43)	14 (74)	3.73	0.15
Acute on chronic (%)	5 (71)	17 (95)	3.40	0.28
Age (years) (SD)	26 (6)	26 (7)	1.01[b]	0.90
Male gender (%)	5 (71)	11 (58)	0.55	0.53
FVC (% predicted) (SD)	40 (19)	39 (9)	0.99[b]	0.83
FEV_1 (% predicted) (SD)	25 (11)	25 (7)	0.99[b]	0.83
BMI (kg/m^2) (SD)	17 (2)	19 (4)	1.15[b]	0.33
Steroid use (%)	2 (29)	7 (37)	1.46	0.70
Pancreatic insufficiency (%)	7 (100)	19 (100)	-[c]	-
CF-related diabetes (%)	3 (43)	10 (53)	1.48	0.66
CF-related liver disease (%)	3 (43)	6 (32)	0.62	0.59
History of haemoptysis (%)	2 (29)	9 (48)	2.25	0.40
History of pneumothorax (%)	2 (29)	7 (37)	1.46	0.70
P. aeruginosa (%)	6 (86)	19 (100)	-[c]	-
B. cepacia (%)	0 (0)	2 (11)	-[c]	-
ABPA (%)	0 (0)	2 (11)	-[c]	-

IMV indicates invasive mechanical ventilation; NIV, non-invasive ventilation; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; BMI, body mass index; CF, cystic fibrosis; ABPA, allergic broncho-pulmonary aspergillosis; and SD, standard deviation.

[a] Group includes two patients who underwent LTX.

[b] Odds ratio per unit of variable.

[c] Odds ratio could not be calculated.

DISCUSSION

This is the first European study in which outcome of assisted ventilation in CF patients with ARF was assessed. All children survived, and their long-term outcome was similar to that of non-ventilated CF-controls. In adults, ARF still was associated with a high mortality, despite improvements in ICU and CF management.

Two other studies [11,13] reviewed survival of assisted ventilation in children with CF. In both studies, as in our study, a favorable outcome was found. Assisted ventilation may result in long-term complications [19]. In infants this was assessed in only one other study, published in 1989 [13]. In that study, with a variable follow-up time, clinical status was identical between five ventilated children and their controls. In our study, lung function and CF-related complications 5 years after IMV did not differ between cases and controls, matched for age, gender and genotype. However,

Table 3. Lung function, anthropometrics and presence of cystic fibrosis-related complications in four children 5 years after invasive mechanical ventilation for acute respiratory failure and their non-ventilated controls matched for age, gender and genotype.

	Patients (N = 4)	Controls (N = 20)	P-value
Age (years)	5.6	5.6	0.88
FVC (% predicted)	96	95	0.88
FEV_1 (% predicted)	96	102	0.35
History of hemoptysis (%)	0	0	-
History of pneumothorax (%)	0	0	-
Height (SDS)	-0.1	-0.8	0.14
BMI (SDS)	0.1	0.0	0.91
Pancreatic insufficiency (%)	100	100	-
CF-related diabetes (%)	0	0	-
CF-related liver disease (%)	25	10	0.44
P. aeruginosa (%)	0	20	0.46
B. cepacia (%)	0	0	-
ABPA (%)	0	5	0.83

FVC indicates forced vital capacity; FEV_1, forced expiratory volume in 1 s; BMI, body mass index; CF, cystic fibrosis; ABPA, allergic broncho-pulmonary aspergillosis; and SDS, standard deviation score.

the number of children in our study is small, and most CF-related complications develop later in life. Conclusions regarding long-term outcome must therefore be made with caution.

In three recent studies, mortality in adults requiring ICU admission varied between 45% and 80% [10-12]. The reason for ICU admission and the possibility to perform LTX varied widely between studies. Despite these differences, all three studies found that most adults with ARF died, unless LTX was performed [12].

With the high mortality in adult CF patients, predictors for outcome are urgently needed. Sood *et al.* found no significant predictors [12]. Berlinski *et al.* found age as a significant risk factor [11]. Also in our total population, age was significantly associated with mortality. However, within the group of adults, age did not affect outcome. In a recent Australian study, $FEV_1 < 24\%$ of predicted and BMI < 18 kg/m^2 were associated with poorer outcome [10]. However, in that study, 25% of patients did not have ARF, but disorders with comparatively good outcome, and specifically these patients on average had higher FEV_1 and BMI.

In patients requiring IMV in our study, acute on chronic respiratory failure was a significant risk factor for mortality. Patients with chronic respiratory failure generally are in a poor clinical condition, and the capacity to recover from an acute exacerbation probably is substantially lower.

Like the recent American and Australian studies [10-12], our study was limited by its retrospective nature and by the relatively small number of patients included, es-

pecially where it concerns the pediatric patients. A prospective multi-center study, with well-defined treatment strategies and inclusion criteria, in centers having ample experience with NIV is needed to identify clear risk factors for poor outcome and to compare efficacy of NIV with IMV.
In conclusion, CF patients aged 2 years or younger who are ventilated because of ARF have a good prognosis. Assisted ventilation should not be withheld from these patients. ARF in adult CF patients still is associated with high mortality, especially in patients with acute on chronic respiratory failure. For these patients, mechanical ventilation should be undertaken with caution.

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Chapter 4

Differences in disease severity among siblings with cystic fibrosis

Submitted

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ABSTRACT

Background: Siblings with cystic fibrosis (CF) share many genetic and environmental factors, but often have different phenotypes due to differences in prognostic relevant aspects. Younger sibs are mostly earlier diagnosed with CF than their older sibs, but are often infected with *P. aeruginosa* (PA) at an earlier age than their older counterparts.

Aims: To compare lung function, PA colonization, nutritional status and survival during the first two decades of life between siblings with CF.

Methods: A retrospective cohort study of 52 sibling pairs was performed in two Dutch CF centers. Data were analyzed both cross-sectionally and longitudinally using random effects models, Kaplan-Meier curves and modified log-rank tests.

Results: Median age at diagnosis was significantly higher in the older sib compared with the younger sib (3.0 and 0.2 years, respectively, P<0.0001). At age 20 years, FEV_1 in older sibs was 19.4% (95% CI: 5.9-32.9%) lower than in younger sibs. Annual decline in FEV_1 (SE) between the age of 5 and 20 years was significantly higher in the older compared with the younger sibs. In the younger sibs group, FEV_1 at age 20 years was significantly better in those who had a diagnosis before the age of 6 months (difference 22.9%, 95% CI: 0.1-45.8%). In the first 10 years of life the younger sibs tended to be earlier colonized with PA than their older counterparts. No differences in nutritional status and survival were observed.

Conclusion: In this sibling cohort study, an early diagnosis of CF was associated with better lung function during the first two decades of life. Although younger siblings tended to be colonized with PA at an earlier age, they showed better lung function outcomes. This underscores the importance of early diagnosis with newborn screening in the prevention of long-term deleterious effects on lung function.

INTRODUCTION

There is considerable variation in the clinical course of disease in cystic fibrosis (CF), even in patients with the same CF genotype [1], suggesting the influence of other factors such as environment, quality of care and modifier genes [2-4].
Siblings with CF share the same CFTR mutations, are generally exposed to the same environment, and have similar quality of care. However, in countries where no newborn screening (NBS) takes place (as in the Netherlands), younger sibs are mostly earlier diagnosed with CF than their older sibs [5,6]. Early asymptomatic diagnosis is associated with better lung function [7-10], nutritional status [11-14], and survival [7,9,14-16]. This would suggest an advantage for the younger sib.
On the other hand, since cross-infection is known to occur between patients with CF [17,18], it can be hypothesized that younger siblings might acquire human adapted respiratory pathogens at an earlier age. *P. aeruginosa* (PA) infection is associated with deteriorating lung function and increased morbidity [19,20], which may result in a more severe CF phenotype in the younger sib.
To investigate the eventual effects of birth order, we studied differences in lung function, PA colonization, nutritional status, and survival between non-twin sibs with CF, aged up to 20 years.

METHODS

The study population consisted of all sibling pairs treated at the CF Centers of the University Medical Center Utrecht (Utrecht, the Netherlands) and the Haga Teaching Hospital (The Hague, the Netherlands). These centers provide care for approximately half the Dutch CF population (~600 patients). Treatment strategies are similar in both centers [21].
In families with more than two affected siblings, only the first two siblings were included in the study. Twins were excluded. Patients born before 1970 or without clinical data before the age of 20 years available were also excluded.
All data were retrospectively collected from patient's medical charts at each center. The diagnosis of CF was confirmed in all patients with sweat testing and/or DNA analysis for CF mutations.
The primary outcome measure was lung function (forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC)). Secondary outcome measures included age at diagnosis, nutritional status (height and weight), PA colonization and survival.
FEV_1 and FVC were measured by a pneumotachograph and converted to percentage of predicted values [22,23]. For cross-sectional analysis, the highest FEV_1 measure within the last year of available data for the younger sib was used. This value was matched with the highest FEV_1 of the older sibling during the year in which he/she reached the same age as the younger sib. Longitudinal measures were derived from

all years of available lung function data for each study subject, using the best FEV_1 measurement per year.
Z-scores for height and weight were calculated using standard growth diagrams for the Dutch population [24]. Matching of height and BMI measures between older and younger sibs was similar to those performed for lung function. Data on lung function and nutritional status were available from 1991 onwards.
PA colonization was considered to be present when more than 50% of the PA cultures in the preceding 3 years were positive [25]. Sputum cultures were performed at least once a year.
Based on their functional effects, CFTR mutations were divided in 5 classes [1]. Class I, II and III mutations result in absence of or non-functional CFTR, whereas classes IV and V mutations, allowing some function of CFTR, usually result in less severe disease manifestations [26]. Patients with two class I, II or III mutations were considered to have a severe CFTR genotype, whereas patients with one or two class IV or V mutations were considered to have a mild genotype [1].

Statistical analysis

Differences between siblings were calculated using paired Student's t-test for normally distributed continuous variables, Wilcoxon signed-rank test for not-normally distributed continuous variables, and McNemar test for discrete variables. In order to test if continuous variables were normally distributed, a Kolmogorov-Smirnov test was performed. The level of significance was 0.05.
To estimate the difference between the older and younger sib in rate of decline of FEV_1 we used a random effects model for repeated measurements (SAS version 9.1, PROC MIXED). A linear rate of decline was assumed. Birth order, age, an interaction term for these, and gender were entered as covariates in the model. The intercept and age were specified as random effects to allow for individual differences [27].
Kaplan-Meier curves and modified log rank tests were used to assess whether there were differences in survival and PA colonization by birth order. Specifically, a Cox proportional hazards model (the probability model implicit in the log rank test) was used, with a term denoting sibling pairs, to test for the significance of birth order in the model. This model takes into account the paired nature of the siblings when calculating the standard error for the birth order coefficient.

RESULTS

In total, 52 families with at least two sibling pairs with CF met the inclusion criteria. The mean age difference between the two siblings was 3.7 ± 2.2 years.
Patient characteristics are shown in **Table 1**. Median age at diagnosis was significantly higher in the older sib compared with the younger sib (3.0 and 0.2 years, respectively, $P<0.0001$).
In 37 pairs (71.2%) the older sib was diagnosed first, in 11 pairs (21.2%) the younger sib was diagnosed first, and in 4 pairs (7.7%) sibs were diagnosed at the same time.

Table 1. Baseline demographics of the study population.

Variable	Younger sib	Older sib	P-value*
N	52	52	
Males (%)	21 (40.4)	20 (38.5)	1.00
Pancreatic insufficiency (%)	48 (92.3)	48 (92.3)	1.00
Age at diagnosis (yrs), median (range)	0.2 (0.0-10.7)	3.0 (0.0-13.7)	<0.0001
Presenting symptoms of CF:			
Meconium ileus (%)	11 (21.2)	8 (15.4)	0.51
Failure to thrive (%)	22 (42.3)	26 (50.0)	0.50
Respiratory symptoms (%)	20 (38.5)	29 (55.8)	0.12
Genotype:			
Homozygous ΔF508 (%)	28 (53.8)	28 (53.8)	
Heterozygous ΔF508 (%)	15 (28.8)	15 (28.8)	
Other (%)	5 (9.6)	5 (9.6)	
Not determined (%)	4 (7.7)	4 (7.7)	
Genotype classes:			
Class I-III (%)	39 (75.0)	39 (75.0)	
Class IV-V (%)	9 (17.3)	9 (17.3)	
Not determined (%)	4 (7.7)	4 (7.7)	

*From McNemar test for PA colonization, paired t-test for all other analyses.

Table 2. Disease severity outcome measures at last visit for the younger sib and at the visit for the older sib at which he/she had the same age as the younger sib (N=47). Data are presented as mean (SD), unless otherwise specified.

	Younger sib	Older sib	P-value*
Age (years)	15.2 (6.5)	15.2 (6.5)	1.00
FEV_1 (% predicted)	81.3 (22.9)	74.2 (32.4)	0.11
FVC (% predicted)	88.5 (17.6)	82.0 (25.8)	0.11
FEV_1/FVC	77.2 (13.2)	73.7 (13.7)	0.08
P. aeruginosa colonization (%)	33 (63.5)	34 (65.4)	1.00
Height for age (z-score)	-0.76 (0.98)	-0.61 (1.13)	0.31
Weight for height (z-score)	-0.23 (0.92)	-0.12 (1.17)	0.55
BMI (kg/m^2)	18.6 (2.8)	18.8 (3.1)	0.66

BMI indicates body mass index; FEV_1,forced expiratory volume in 1 s; FVC, forced vital capacity.
*From McNemar test for PA colonization, paired t-test for all other analyses.

Eight of the younger sibs (15.4%) were diagnosed antenatally or shortly after birth before the development of symptoms because of CF in the older sib.
Older sibs tended to have respiratory symptoms at diagnosis more often (55.8% and 38.5%, respectively, P=0.12) compared with younger sibs (**Table 1**).
In four sibling pairs no CFTR genotype was determined. Of the remaining 48 patients, 39 (81.3%) had a severe CFTR genotype class.
Lung function at last clinical visit for the younger sib tended to be higher than the matched lung function for the older sib (**Table 2**). FEV_1 analyzed per 5-year intervals demonstrated a similar FEV_1 at the age of 5, 10 and 15 years. However, at the age of 20 years, FEV_1 in older sibs was 19.4% (95% CI: 5.9-32.9%) lower than in younger sibs (**Figure 1**).
We also analyzed lung function longitudinally. In a multivariate random effects model correcting for gender, annual lung function decline (SE) between the age of 5 and 20 years was significantly higher in the older compared with the younger sib (2.1% (0.28) and 1.6% (0.29) of predicted per year, respectively, P<0.0001).
In order to test the hypothesis that the better clinical outcome in the younger sibs is caused by earlier diagnosis of CF, we analyzed the effect of age at diagnosis on lung function. Since the association between age at diagnosis and lung function could be confounded by severity of CFTR genotype [28], this analysis was only performed in patients with a severe CFTR genotype. FEV_1 analyzed per 5-year intervals demonstrated a significantly higher FEV_1 at the age of 20 years in sibs diagnosed before

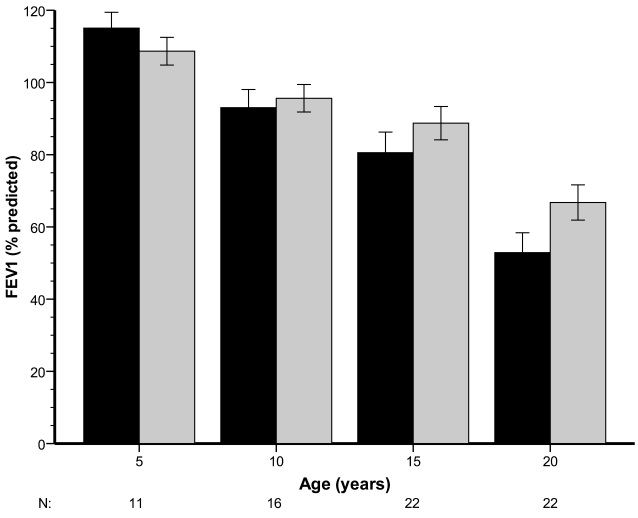

Figure 1. Lung function measured at five-year intervals. Numbers of sibling pairs per age category are displayed below the X-axis. FEV_1 at the age of 20 years was significantly lower in older siblings (black) compared with their younger counterparts (grey) (difference 19.4%, 95% confidence interval: 5.9-32.9).

6 months of age compared with those diagnosed after 6 months of age (difference 22.9%, 95% CI: 0.1-45.8%, P=0.05). However, no significant differences were observed at the age of 5, 10 and 15 years.
PA colonization at last visit was not different between the older and younger sibs (**Table 2**). Also, when differences in age at PA colonization between younger and older sibs were analyzed longitudinally, younger sibs were overall not earlier colonized with PA (Cox proportional hazard 1.60, 95% CI: -0.73-3.52). However, the younger sibs tended to be earlier colonized in the first 10 years of life than their older counterparts (P=0.08), but this difference disappeared in the second decade (**Figure 2**).
Height, weight and BMI did not differ between the older and the younger sibs, both when analyzed cross-sectionally (**Table 2**) and longitudinally.
Two of the older sibs and none of the younger sibs received a lung transplant before the age of 25 years. Three of the older sibs and one of the younger sibs died before the age of 25 years. Survival curves for older and younger sibs are displayed in **Figure 3**. Survival (no death and no lung transplant) was not significantly worse in the older sibs compared with the younger sibs (Cox proportional hazard: P=0.21).

DISCUSSION

This large study on long-term differences in lung function, PA colonization, nutritional status, and survival between older and younger sibs with CF demonstrated that older siblings had a stronger decline in lung function and a poorer lung function in the second decade of life than their younger siblings with CF. Early diagnosis was

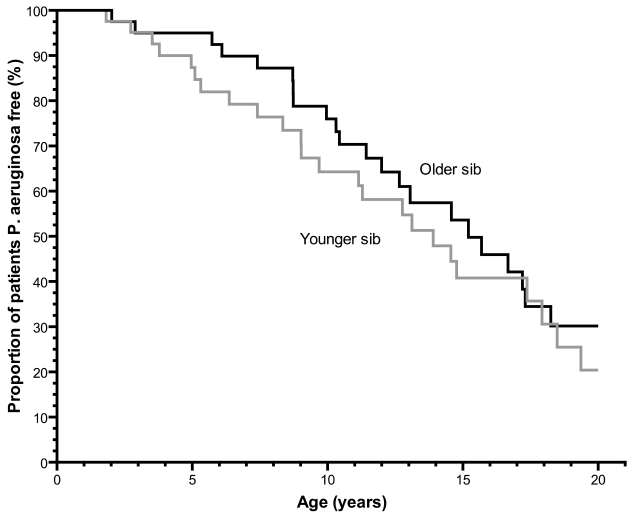

Figure 2. Kaplan-Meier curves of age at PA colonization by birth order, measured in 39 sibling pairs. Cox proportional hazard P=0.24.

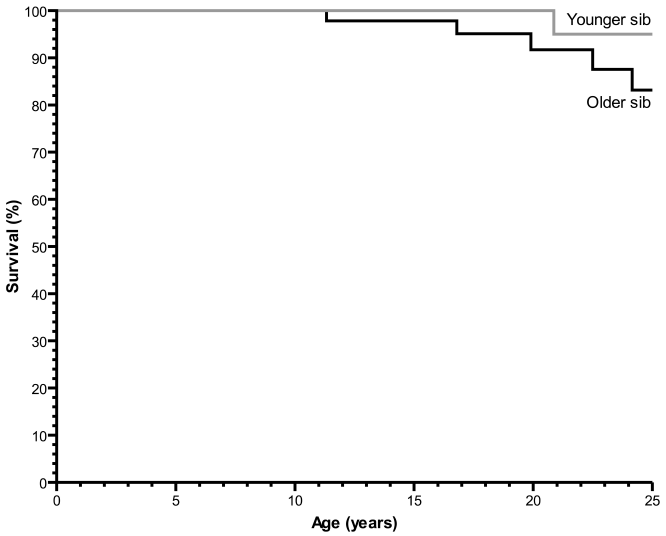

Figure 3. Kaplan-Meier survival plots for older and younger sibs with CF. Cox proportional hazard test P=0.21.

associated with better lung function outcome. PA colonization was not significantly different between younger and older sibs, but younger sibs tended to be earlier colonized in the first 10 years of life. Older and younger siblings did not appear to have a different height, weight and survival.

In this study, we hypothesized that younger siblings might be at risk of more accelerated lung disease because of exposure to cross-infection from older siblings. On the other hand, we postulated that younger sibs would be earlier diagnosed and would benefit from earlier therapy. It was shown that younger sibs tended to be earlier colonized with PA in the first 10 years of life, but that this difference disappeared in the second decade. Despite the higher PA colonization in the first decade, this did not result in a significantly worse lung function in the first decade, probably due to an earlier diagnosis. In the second decade no difference between PA colonization was found between the sibs, and younger sibs had better lung function. This suggests that the net effect of earlier diagnosis in younger CF siblings outweighs the negative effects, including the exposure to CF pathogens from older siblings.

The beneficial effect of younger age at diagnosis on lung function was confirmed by our subgroup analysis in the younger sibs: patients diagnosed before 6 months of age had a significantly better lung function in the second decade of life. This is in accordance with the better prognosis associated with early asymptomatic diagnosis of CF through NBS [7-10,29]. In these early diagnosed asymptomatic patients, early antibiotic therapy, nutritional supplementation, and intensive physiotherapy can delay the progression of lung disease in CF [29].

A better lung function in younger siblings with CF was also found in a relatively old and small study by Orenstein *et al*. [30]. On the other hand, two recent studies

could not find significant differences in FEV_1 and FVC between older and younger sibs with CF [5,6]. However, lung function was only measured at the age of 7 and 10 years [6], and at the age of 8 years [5], respectively. The age of 10 years may be too young to identify significant differences between the two siblings, which was demonstrated by our longitudinal analysis (difference only found in second decade of life).

Similar to other studies [5,6], we did find a strong familial tendency for PA colonization. This was probably caused by cross-infections between the siblings. The importance of cross-infections among CF patients within families has already been confirmed by DNA fingerprinting and serology [18,31]. In accordance with other studies [5,6], we demonstrated an earlier age at PA colonization in the younger siblings than in the older siblings. However, in all studies the interpretation is limited by the age-of-diagnosis bias. Patients are only tested for typical CF pathogens if they already have a CF diagnosis. Therefore, a positive culture at time of diagnosis prevents accurate determination of true age of infection. Since the older child in a family is always diagnosed at an older age than the younger sibling, regardless of who is the index case, older siblings are more likely to have positive cultures at time of diagnosis, simply due to more time to acquire the pathogens. The age-of-diagnosis bias could only be overcome by a study of CF siblings diagnosed through NBS, but NBS is not yet available in the Netherlands.

Similar to other sibling studies [5,6], we did not demonstrate any significant differences in height or BMI between older and younger sibs, although one of the earlier sibling studies demonstrated a marginally significantly higher height z-score in younger siblings than in older siblings [5]. Other studies [13,29,32] have shown dramatic differences in nutritional status at diagnosis between those diagnosed through screening and those diagnosed as a result of clinical signs and symptoms. However, these differences can be reduced once appropriate dietary intervention, including the provision of pancreatic enzyme replacement therapy, is commenced [13]. That we could not detect any advantage of early diagnosis on nutritional status in younger might be because of a significant catch-up growth achieved in the early years of life.

We found no significant differences in survival between older and younger sibs, but the number of patients investigated may have been too small to detect these differences. Other sibling studies did not investigate differences in survival between older and younger sibs [5,6,30]. However, most of the studies on the effect of NBS found that early diagnosis through NBS is associated with improved child survival [7,9,14-16].

In conclusion, in this sibling cohort study, an early diagnosis of CF was associated with better lung function during the first two decades of life. Although younger siblings tended to be colonized with PA at an earlier age, they showed better lung function outcomes. This underscores the importance of early diagnosis with newborn screening in the prevention of long-term deleterious effects on lung function.

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Chapter 5

Cystic fibrosis related liver disease:
Risk factors and effect on lung function

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ABSTRACT

Background: The incidence of cystic fibrosis (CF)-related liver disease (CFRLD) and its risk factors are still debated. Furthermore, the effects of CFRLD on lung function are unknown.
Aims: To determine the prevalence of CFRLD, to identify risk factors for the development of CFRLD, and to analyze the effect of CFRLD on lung function in a large single-center population of children with CF.
Methods: To estimate the incidence and risk factors for CFRLD, a retrospective cohort study was performed in 238 children (123 males) with CF. CFRLD was diagnosed using uniform diagnostic criteria, based on annual systematic clinical, laboratory, ultrasonography screening. The incidence of CFRLD and risk factors for CFRLD were studied using a multivariate Cox's regression analysis, including variables such as history of gender, meconium ileus, severe genotype, pancreatic insufficiency and CFTR gene mutations. A case control study was performed to analyze the decline in lung function after diagnosis of CFRLD.
Results: The cumulative incidence of CFRLD was 16.8%, 33.4%, and 37.5% at the age of 10, 15 and 18 years, respectively. Severe CFTR genotype was a significant risk factor for the development of CFRLD (P=0.004). The development of CFLRD tended to be associated with male gender and pancreatic insufficiency, but these associations were not significant (P=0.11 and P=0.07, respectively). Annual decline in lung function was not significantly different between patients with and without CFRLD. Patients with CFRLD even had a better lung function five years after diagnosis compared with age, gender and genotype-matched controls (P=0.01).
Conclusions: CFRLD occurs early in the course of the disease, mainly in the prepubertal period. Severe CFTR genotype is an important risk factor for the development of CFRLD. Lung function does not seem to be negatively influenced by the presence of CFRLD.

INTRODUCTION

With improved survival in patients with cystic fibrosis (CF) [1], CF-related liver disease (CFRLD) is becoming more prevalent and is now considered the third leading cause of death in these patients [2].
The development of CFRLD is probably the result of impaired secretory function of the biliary epithelium resulting in thickened, inspissated secretions in the bile ductules, which may cause obstruction and progress to the development of portal fibrosis, bridging, and eventually cirrhosis [3].
The reported prevalence of CFRLD varies widely, ranging from 2 to 68% in children and adolescents [4-11], mostly due to differences in the criteria used for the diagnosis of CFRLD. Other explanations for the discrepancies include the age of the study population and the study design (cross-sectional or longitudinal).
Identification of risk factors for the development of CFRLD is an important step in targeting populations for early intervention and prophylactic treatments [12]. Potential risk factors identified by different studies include male gender, history of meconium ileus, pancreatic insufficiency, and severe cystic fibrosis transmembrane regulator (CFTR) genotype, but results are far from conclusive [4-8,10,11,13,14].
Patients who develop CFRLD are at risk of developing several extrahepatic complications, including malnutrition [5], diabetes [15,16], and osteoporosis [17]. It is not clear whether patients with CFRLD also suffer from more severe lung disease. Hepatomegaly may cause mechanical effects on lung function. Furthermore, essential fatty acid deficiency has been suggested to influence lung function [18] and also seems to be associated with CFRLD [19]. This deficiency could be a common factor for both deterioration of lung function and liver involvement [8]. Recently, children with CFRLD were found to have a significantly lower lung function, but no worse chest radiography and clinical scores compared with children without CFRLD [5].
The aim of the present study was to determine the prevalence of CFRLD, to identify risk factors for the development of CFRLD diagnosed using uniform diagnostic criteria [13], and to analyze the effect of CFRLD on lung function in a large single-center population of children with CF.

METHODS

Patients

The Cystic Fibrosis Center of the University Medical Center Utrecht (Utrecht, the Netherlands) currently provides care for about 250 children with CF from the central region of the Netherlands. The diagnosis of CF in all children in the study was made by means of a sweat test and/or CFTR genotyping. All patients aged between 5 and 18 years were included.
All patients received treatment according to national guidelines [20].

Measurements

All patients undergo a routine yearly multidisciplinary examination. Since 1998, all results have prospectively been recorded in an electronic database. If CF-related complications (CFRLD, PA colonization) were already present, they were recorded retrospectively.

Patients were classified as pancreatic-sufficient or pancreatic-insufficient on the basis of their need for pancreatic enzyme replacement. Standard deviation scores for height and weight were calculated using standard growth charts for the Dutch population [21]. The pulmonary function (forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC)) were measured by a pneumotachograph and converted to percentage of predicted values [22]. PA colonization was considered to be present when more than 50% of the PA cultures in 3 consecutive years were positive [23]. Sputum cultures were performed at least once a year.

Based on their functional effects, CFTR mutations were divided in 5 classes [24]. Class I, II and III mutations result in a more severe CF phenotype compared with class IV and V [24].

CF patients were considered to have developed liver disease if at least 2 of the following conditions were present on at least 2 consecutive examinations spanning a 1-year period [13]: (1) clinical hepatomegaly (increase in liver span and consistency, with liver edge palpable more than 2 cm below the costal margin on the mid-clavicular line), confirmed by ultrasonography; (2) abnormal serum liver enzyme levels, consisting of elevation above the upper normal limits of 2 of the following: aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyltransferase (GGT); (3) ultrasound abnormalities other than hepatomegaly (*i.e.*, increased, heterogeneous echogenicity, nodularity, irregular margins, splenomegaly). Ultrasonographic pattern of steatosis did not represent a diagnostic criterion.

To estimate the cumulative incidence and risk factors for CFRLD a retrospective cohort study was performed. A case control study was performed to analyze the decline in lung function after diagnosis of CFRLD. CFRLD patients were pair-matched for date of birth (±0.5 year), sex and severity of CFTR genotype (severe/mild) with CF patients without CFRLD. 'Age at diagnosis' in the control group was the age at which the pair-matched case was diagnosed with CFRLD.

Statistical methods

Unpaired Mann-Whitney U and Kruskal-Wallis tests were used to compare not normally distributed continuous data, and unpaired T-tests and ANOVA to compare normally distributed continuous data. In order to test if continuous variables were normally distributed, a Kolmogorov-Smirnov test was performed. Chi-square and Fisher's exact tests were used to compare dichotomous variables. For the case-control study differences between patients with and without CFRLD were calculated using paired Student's t-test for normally distributed continuous variables, Wilcoxon signed-rank test for not-normally distributed continuous variables, and McNemar test for discrete variables.

The Kaplan-Meier method was used to estimate cumulative incidence of CFRLD (1-survival without CFRLD). Survival curves according to risk factors were compared using the Log-Rank test. The effects of risk factors associated at univariate analysis (P<0.25) with the development of CFRLD were tested multivariately using the Cox proportional hazard model.
To estimate the difference between cases and controls in rate of decline of FEV_1 after diagnosis of CFRLD we used a random effects model for repeated measurements (SAS version 9.1, PROC MIXED). A linear rate of decline was assumed. Time after diagnosis (years), the presence of CFRLD, an interaction term for these, and age at diagnosis were entered as covariates in the model. The intercept and time after diagnosis were specified as random effects to allow for individual differences [25].

RESULTS

Incidence of CFRLD

Table 1 shows the different parameters in the whole population (N=238), and in the 69 patients who developed CFRLD as compared with those who did not. In 225 patients CFTR genotype was determined. A severe genotype was found in 190 patients (84.4%).

Table 1. Characteristics of CF patients with and without CFRLD. Data are presented as mean (SD), unless otherwise specified.

	All patients (N=238)	CFRLD (N=69)	No CFRLD (N=169)	P-value
Male gender	123 (51.7)	41 (59.4)	82 (48.5)	0.13[b]
Age at diagnosis of CF (months)[a]	5.5 (0-155)	4.0 (0-135)	5.8 (0-155)	0.20[c]
Age at diagnosis < 3 mo	94 (39.8)	29 (42.6)	65 (38.7)	0.57[b]
History of meconium ileus	30 (12.6)	11 (15.9)	19 (11.2)	0.32[b]
CFTR genotype				
ΔF508/ΔF508	147 (62.3)	46 (67.6)	101 (60.1)	0.10[b,d]
ΔF508/other	61 (25.8)	15 (22.1)	46 (27.4)	
Other/other	15 (6.4)	1 (1.5)	14(8.3)	
Not determined	13 (5.5)	6 (8.8)	7 (4.2)	
CFTR class				
Severe	190 (79.8)	60 (87.0)	130 (76.9)	0.009[b,d]
Mild	20 (8.4)	0 (0.0)	20 (11.8)	
Unknown	15 (6.3)	3 (4.3)	12 (7.1)	
Not determined	13 (5.5)	6 (8.8)	7 (4.2)	
Pancreatic insufficiency	230 (96.6)	69 (100)	161 (95.3)	0.11[e]

[a] Median (range).
[b] Chi-square test.
[c] Mann-Whitney U test.
[d] Not determined genotypes excluded from analysis.
[e] Fisher's exact test.

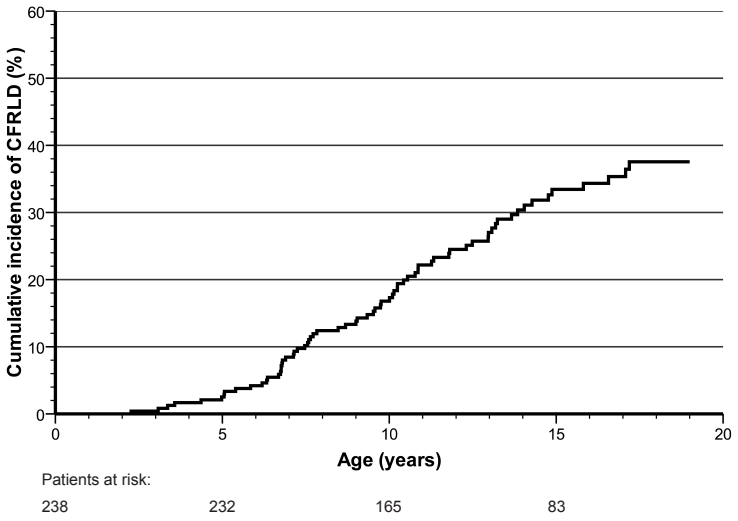

Figure 1. Kaplan-Meier plot of the cumulative incidence of CFRLD.

Cumulative incidence of CFRLD is shown in **Figure 1**. At the age of 10 and 15 years 16.8% and 33.4%, respectively, of the patients suffered from CFRLD. After the age of 15 years incidence rate declined, and at 18 years CFRLD was present in 37.5% of the CF patients.

Risk factors for CFRLD

There was a strong association between CFRLD and CFTR genotype severity: none of the patients with a mild genotype developed CFRLD (**Table 1**). Patients who developed CFRLD were more often male (59.4% vs. 48.5%, P=0.13), were younger at diagnosis (4.0 vs. 5.8 months, P=0.20) and were more often pancreatic insufficient (100% vs. 95.3%, P=0.11), but none of these associations was significant.
Severe genotype was also associated with cumulative incidence of CFRLD (Log Rank P=0.004, **Figure 2**) and again a trend was observed for gender (P=0.11) and pancreatic insufficiency (P=0.07). Multivariate assessment of risk factors for the de-

Table 2. Multivariate analysis of factors associated with CFRLD.

	HR	95% CI	P-value
Male gender	1.48	0.92-2.39	0.11
History of MI	1.29	0.68-2.46	0.44
Severe genotype[a]	4.50	1.41-14.4	0.01[b]
Age at diagnosis of CF	0.995	0.986-1.003	0.23

CFRLD indicates CF-related liver disease; MI, meconium ileus; HR, hazards ratio; and CI, confidence interval.
[a] Severe genotype vs. mild/unknown genotype. Not determined genotypes were excluded.
[b] Only significant factor in multivariate analysis.

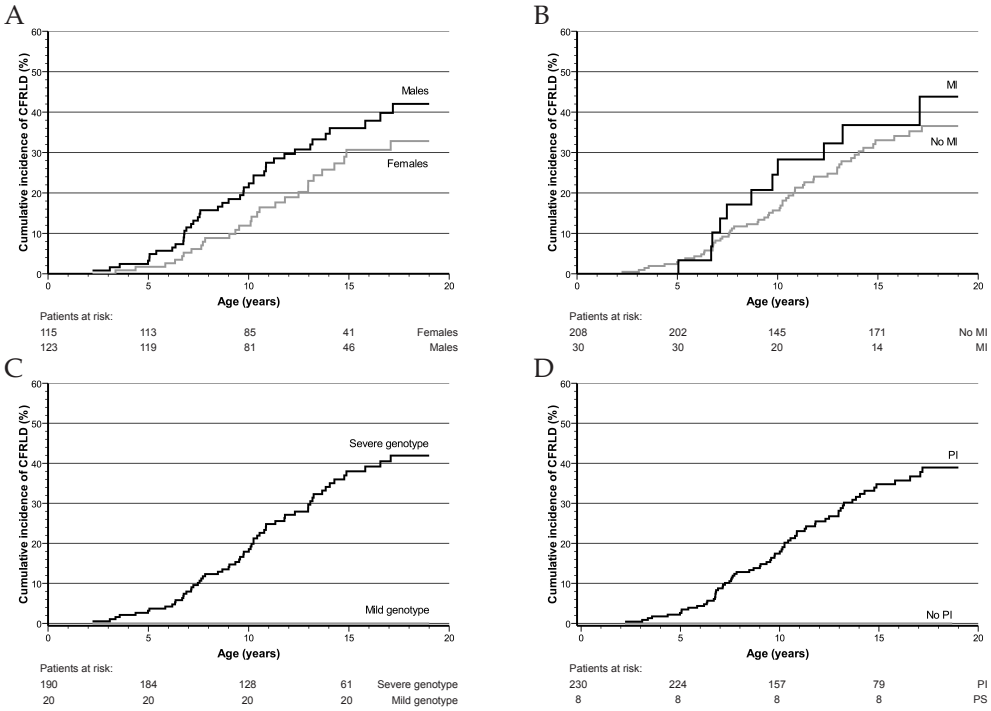

Figure 2. Kaplan-Meier plots for cumulative incidence of CFRLD in different groups of CF patients. (A) Gender, P (log rank)=0.11); (B) History of meconium ileus (MI), P=0.43; (C) Genotype, P=0.004; (D) Pancreatic sufficiency (PS)/ insufficiency (PI), P=0.07.

velopment of CFRLD by proportional hazard Cox regression showed that severe genotype was the only factor independently associated with CFRLD (hazard ratio: 4.50, 95% confidence interval: 1.41-14.4, **Table 2**).

Effects of CFRLD

To analyze the effect of CFRLD on lung function, a case-control study was performed. Since the development of CFRLD was associated with age, gender and CFTR genotype, cases were pair-matched for date of birth (±0.5 year), gender and CFTR genotype, with CF patients without CFRLD.

Forty-seven patients fulfilled the inclusion criteria and had one lung function measurement at diagnosis and at least two annual lung function measurements after diagnosis. All of them had a severe genotype and 28 of them were male (59.6%). Mean age (SD) in cases and controls was 9.9 (3.4) and 9.2 (3.9) years, respectively (P=0.4). Decline was analyzed using a random effects model, correcting for age at diagnosis. Annual decline in FEV_1 (SE) was 2.0% (0.6%) and 2.9% (0.6%) in patients with and without CFRLD, respectively (P=0.25). Annual decline in FVC (SE) was 0.6% (0.5%) and 1.6% (0.5%), respectively (P=0.19). We also performed a subgroup analysis in 24 patients with CFLD and splenomegaly. In this subgroup, annual decline in FEV_1

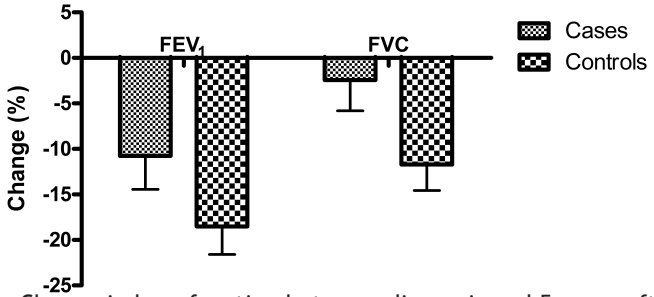

Figure 3. Change in lung function between diagnosis and 5 years after diagnosis in cases and controls.

(SE) was 1.5% (0.8%) and 3.5% (0.8%) (P=0.08) and annual decline in FVC (SE) was 0.1% (0.7%) and 2.0% (0.7%) (P=0.07), respectively.

In 25 patients and their controls lung function data at diagnosis (t0) and 5 years after diagnosis (t5) were available. FEV_1 (SD) in patients with and without CFRLD was 90.0% (21.2%) and 83.7% (26.9%) at diagnosis (P=0.20), and 79.3% (20.4%) and 65.2 (25.3%) 5 years after diagnosis (P=0.01), respectively. FVC (SD) in patients with and without CFRLD was 90.7% (15.8%) and 90.0% (19.9%) at t0 (P=0.88), and 88.2% (15.4%) and 78.3 (22.1%) at t5 (P=0.03), respectively.
Decline in FEV_1 between t0 and t5 in patients with and without CFRLD was 10.8% (18.3%) and 18.5% (15.5%), respectively (P=0.13). Decline in FVC between t0 and t5 in patients with and without CFRLD was 2.4% (11.7%) and 18.5% (14.2%), respectively (P=0.05) (**Figure 3**).

DISCUSSION

This study aimed to determine the prevalence and risk factors for CFRLD and to analyze the effect of CFRLD on pulmonary status in a large single-center population of CF patients.
Although studied retrospectively, data were prospectively collected as all patients were followed annually by the same team with a systematic clinical assessment, determination of serum liver enzymes levels, and of ultrasound examination of the liver. This permitted the precise determination of the onset of liver involvement, which was defined using uniform criteria [13].
As CFRLD develops progressively over time, estimation of cumulative proportions of patients developing CFRLD using the Kaplan-Meier approach is the best method to describe the natural history of CFRLD. However, until now most studies were cross-sectional and used different diagnostic criteria, making valid comparisons impossible.

Two studies used similar diagnostic criteria and also determined incidence of CFRLD using Kaplan-Meier models [7,13]. In accordance with these studies, the majority of our patients developed CFRLD in the first 10 to 15 years of life [7,13]. The cumulative incidence of 37.5% at age 18 years was higher than the cumulative incidence of 29% found by Colombo *et al*. [13], but lower than the cumulative incidence of 41% found by Lamireau *et al*. [7]. However, in the first study [13] the prevalence of severe CFTR genotypes (a strong risk factor for CFRLD) was much lower than in our population (59% and 84%, respectively). In the latter study the prevalence of CFRLD could be overestimated because only one of the three diagnostic criteria had to be present on two consecutive occasions (clinical hepatomegaly, elevated liver enzymes, and ultrasound abnormalities) [7], compared to two criteria in our study and the study by Colombo *et al*. [13].

Search for possible risk factors for liver involvement in CF has produced controversial results [4-8,10,11,13,14]. To a large extent, this was due to differences in study design, definition of CFRLD and size of the study population.

In the present study, CF patients with a severe CFTR genotype were 4.5 times as likely to develop CFRLD as those with a mild or unknown genotype. Severe CFTR mutations are associated with complete absence of CFTR activity at the apical membrane of cholangiocytes [26], while mild mutations are associated with residual CFTR activity. Thus, severe mutations may result in more reduced bile fluidity and alkalinity, and consequently increased plugging of intrahepatic bile ducts by inspissated biliary secretions. An association between severe genotype and CFRLD was also found in two other studies [11,13]. However, several studies could not find an association between CFRLD and specific mutations, including ΔF508 [4,7,8,11,27]. Therefore, the development of CFRLD does not seem to be influenced by a specific 'CFRLD mutation' in the CFTR gene, but rather by a group of mutations that result in no functional CFTR activity.

Other genes than CFTR, such as mannose-binding lectin, α1-antitrypsin, gluthation S- transferase and transforming growth factor β1 genes, may act as modifier genes to influence the severity of CFRLD [28-30]. However, the individual effect of these genes on the development of CFRLD is mostly small and none of the published associations have been replicated.

In our study, males tended to have an increased risk to develop CFRLD compared with girls. Most other studies also showed a preponderance of males among CFRLD patients, but in the majority of these studies associations were trends [4,6,7,11], rather than actually significant [10,13]. There is no good explanation for this (weak) association, but it was suggested that the association represents an over-representation of males, especially in cross-sectional studies, given their survival advantage [3]. Another explanation is the possible role for endocrine factors, including estrogens, in the development of biliary epithelium proliferation in response to liver damage [31].

In accordance with several studies [6,8,11,14], but in contrast with others [5,7,13], we could not demonstrate an association between a history of meconium ileus and the development of CFRLD. The reasons for these discrepancies are unclear. Potential

explanations include genetic differences between the populations and differences in surgical techniques for meconium ileus. In the Italian study, the majority of patients with meconium ileus had undergone significant bowel resection [13]. Furthermore, in an Irish case-control study all CFRLD cases with meconium ileus had undergone intestinal resection compared with only 11.8% of the controls with meconium ileus [5]. After surgery, patients with meconium ileus are at increased risk for poor nutrition early in life and prolonged total parenteral nutrition [5,32].

None of our patients with pancreatic sufficiency developed CFRLD, but because of the small number of these patients, this association did not reach significance. A substantial lower prevalence of CFRLD among pancreatic sufficient patients was confirmed by several other studies [4,7,11]. The association is not surprising, since there is a very strong association between pancreatic sufficiency and mild CFTR genotype [33].

There was no significant association between age at diagnosis and CFRLD. One study demonstrated an association between CFRLD and early age at diagnosis when analyzed cross-sectionally, but not when analyzed longitudinally [7]. One case-control study found a later age at diagnosis in patients with CFLRD [5], but this was not confirmed in other studies.

Patients with CFRLD had no significantly different lung function at diagnosis of CFRLD compared with age, gender, and genotype-matched controls. However, patients with CFRLD tended to have a slower decline in lung function in the years after diagnosis and had a better lung function 5 years after diagnosis compared with controls.

One study also found a tendency toward better lung function in patients with CFRLD [8], while two studies could not find an association between CFRLD and lung function [7,11]. However, one case-control study found a poorer lung function in patients with CFRLD, but this was not reflected in chest radiography and clinical scores [5]. These four studies did not compare lung function at diagnosis and the years thereafter with pair-matched controls [5,7,8,11]. Instead, they analyzed lung function at different years after diagnosis of CFRLD and lung function tests were often performed at different ages in patients with and without CFRLD. Furthermore, gender and severity of CFTR genotype are not only associated with CFRLD but also with lung function [33,34], hereby introducing potential confounding. We overcame this problem by matching for gender and severity of CFTR genotype.

Why patients with CFRLD have a slower decline in lung function, or at least do not have an increased decline in lung function, remains speculative. One explanation is that the presence of CFRLD in some of our patients could have led to more frequent visits to a physician, better compliance, and better overall pulmonary care in patients with CFRLD compared with those who did not have CFRLD. Another explanation could be less lung inflammation due to the immunomodulatory properties of ursodeoxycholic acid [35].

In conclusion, this study shows that CFRLD occurs early in the course of the disease, mainly in the prepubertal period, involving 37.5% of patients by 18 years of age. Severe CFTR genotype seems to be an important risk factor for the development of

CFRLD. A trend towards an increased incidence of CFRLD was found in male patients and in patients who are pancreatic insufficient. In our cohort meconium ileus and age at diagnosis were not important risk factors for CFRLD. Furthermore, lung function was not negatively influenced by the presence of CFRLD. Moreover, our patients with CFRLD tended to have better lung function compared with controls, but the reasons for this finding remain speculative.

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Chapter 6

Children with cystic fibrosis: who should visit the otorhinolaryngologist?

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ABSTRACT

Background: Sinonasal complications are common in children with cystic fibrosis (CF). Generally, those children with persistent symptoms of sinonasal polyposis are referred to an otorhinolaryngologic (ORL) physician for sinus surgery. Several studies have reported differences in clinical characteristics between CF patients with and without sinonasal polyps.
Objectives: To predict the presence of sinonasal polyposis in children with CF on the basis of symptoms and clinical characteristics and so to select those children who might benefit from referral to an ORL physician.
Methods: A survey of data from a database on the results of yearly multidisciplinary examinations of 140 children with CF was performed. The main outcome measure was the presence of sinonasal polyposis.
Results: In the total population of 140 children, no combination of ORL symptoms and clinical characteristics could accurately predict the presence of sinonasal polyposis. In a subgroup of 73 children with a history of nasal symptoms, independent predictors for the presence of sinonasal polyposis were male sex, age 10 years or older, presence of rhinorrhea, and a forced vital capacity 70% or more of the predicted value. The area under the receiver operating characteristic curve of a scoring rule including these independent predictors was 0.77. The positive and negative predictive values of this rule were 0.86 and 0.71, respectively.
Conclusions: A scoring rule including the independent predictors sex, age, symptoms of rhinorrhea, and forced vital capacity values could reasonably classify children with CF and nasal symptoms into a category with increased risk for sinonasal polyposis, thus facilitating the decision on ORL referral.

INTRODUCTION

Otorhinolaryngologic (ORL) complications are common in children with cystic fibrosis (CF), with reported prevalences of 92% to 100% for sinusitis [1,2] and 32% to 45% for nasal polyposis [3-6]. This raises the question whether the ORL physician should be routinely involved in the management of children with CF.
The above question could be answered in the affirmative if a treatment effective in preventing or limiting progression of nasal complications in children with CF were available. So far, there is no effective medical therapy, and the effectiveness of surgical therapy in CF is limited. Generally, sinus surgery is recommended to treat CF in children with persistent symptoms of sinonasal polyposis [2,7,8]. Uncontrolled studies of functional endoscopic sinus surgery in such children have reported up to 100% symptom improvement of sinonasal polyposis [8].
To select those children who may benefit from treatment by the ORL physician, it is important to know whether symptoms and clinical characteristics can predict the presence of sinonasal polyposis in patients with ORL symptoms. So far, it is not known to what extent ORL symptoms that are highly prevalent in children with CF correlate with the presence of sinonasal polyps [7]. Regarding clinical characteristics, several studies have reported differences between CF patients with and without nasal polyps [5,9-11]. Nasal polyps were more prevalent in male patients [9], and patients with CF and nasal polyposis had a relatively good pulmonary [10,11] and nutritional status [10].
At our tertiary care pediatric hospital, a large population of children with CF undergo a yearly multidisciplinary examination, including a standard ORL history and endoscopic examination of the nose. This is an optimal setting to study the prevalence of ORL complications and to find predictors for the presence of sinonasal polyposis.

METHODS

Evaluation scales and values

The Cystic Fibrosis Center Utrecht of the Wilhelmina Children's Hospital (Utrecht, the Netherlands) treats children with CF from the central Netherlands. All children 4 years or older undergo a routine yearly multidisciplinary examination regardless of their health status. Since October 1998, all results have been routinely recorded in an electronic database.
During the ORL evaluation, a standardized ORL history is taken. Patients are asked about nasal obstruction, rhinorrhea, postnasal drip, headache, facial tenderness, and loss of sense of smell. Apart from a routine ORL examination, nasal endoscopy is carried out using a 2.2-mm flexible endoscope; the presence of nasal polyps and bulging of the lateral nasal wall are noted. Since bulging is a sign of sinusal polyposis in infants and children [4], nasal polyposis and bulging of the lateral nasal wall

were analysed as a single entity (sinonasal polyposis) in the present study. For each patient, the first ORL evaluation registered in the database was used.
For each ORL symptom and several clinical variables, the predictive value for the presence of sinonasal polyposis was calculated. Clinical variables that were used in the present analysis include age, sex, nutritional status, the presence or absence of diabetes, pulmonary function, and inflammatory mediators (*i.e.*, C-reactive protein [CRP] and IgE). Information on the nutritional status was based on percentages of predicted values for height and weight by using standard growth diagrams for the Dutch population [12]. The pulmonary functions (forced expiratory volume in 1 second [FEV_1], forced vital capacity [FVC] and peak expiratory flow [PEF]) were measured by a pneumotachograph and converted to percentage of predicted value using the Zapletal reference values [13].

Statistical analysis

The association between the presence or absence of sinonasal polyposis and symptoms and clinical characteristics was quantified using univariate logistic regression analysis. Subsequently, predictors that were univariately associated with the outcome (odds ratios with $P<0.15$) were included in stepwise fashion according to the ease and frequency with which they are obtained in clinical practice in a multivariate logistic regression model to evaluate their independent value in the prediction of sinonasal polyposis [14]. Predictors from the model with P values greater than 0.10 were excluded such that a reduced model was derived that included independent predictors of sinonasal polyposis. The selected variables were dichotomized when necessary. Reliability (goodness of fit) of the models was estimated using the Hosmer-Lemeshow test. The prognostic capacity to discriminate between patients with and without sinonasal polyposis was estimated using the area under the receiver operating characteristic curve (ROC area). An ROC area of 1.0 corresponds to a model that perfectly predicts sinonasal polyposis; an ROC area of 0.5 corresponds to a model with random predictive accuracy.
To estimate the prognostic capacity of the final model in other groups of similar patients, the model was validated by random bootstrapping techniques [14]. The final model was transformed into a scoring rule by dividing the regression coefficients of the included predictors by the smallest one and rounding them subsequently to the nearest integer. A total risk score was computed for each patient by assigning points for each predictor present. Predictive values for each category of the scores were calculated.

RESULTS

The ORL evaluation was completed in 140 patients (71 boys, 69 girls), with a mean (SD) age of 11.2 (4.0) years. Nasal polyps and bulging of the lateral wall were found in 56 (40%) and 27 (19%) children, respectively. Nasal polyps and/or bulging of

the lateral nasal wall, indicative of sinonasal polyposis, were present in 70 patients (50%).

Table 1 summarizes the results of the univariate logistic regression analysis of ORL symptoms and clinical variables for the presence of sinonasal polyposis. Significant predictors (P<0.15) were male sex; symptoms of rhinorrhea and postnasal drip; high FEV_1, FVC, and PEF values; and low CRP levels.

Table 1. Univariate logistic regression of symptoms and clinical variables with the presence of sinonasal polyposis.

Clinical variable	Polyposis group (N=70)	Control group (N=70)	Total (N=140)	Odds ratio	P-value
Male sex, %	57	44	51	1.68	0.13‡
Age, mean, y	11.3	11.0	11.2	1.02†	0.72
Height, % predicted	97	97	97	1.01†	0.79
Weight, % predicted	96	96	96	1.00†	0.87
Symptoms, % (N=140)					
Nasal obstruction	27	24	26	1.16	0.70
Rhinorrhea	36	19	27	2.44	0.02‡
Postnasal drip	29	16	22	2.15	0.07‡
Headache	19	14	16	1.37	0.49
Facial tenderness	6	6	6	1.00	>0.99
Hyposmia	20	13	16	1.69	0.26
Any complaint	59	46	52	1.68	0.13
Diabetes, %	0	4	2	0.00	0.73
Pulmonary function, % predicted (N=116)					
FEV_1	84	79	81	1.01†	0.11‡
FVC	89	83	86	1.02†	0.09‡
PEF	93	84	88	1.02†	0.04‡
Inflammatory mediators, mean					
CRP, mg/L (N=100)	5	12	8	0.93†	0.04‡
IgE, kU/L (N=89)	120	252	193	1.00†	0.38

FEV_1 indicates forced expiratory volume in 1 second; FVC, forced vital capacity; PEF, peak expiratory flow; and CRP, C-reactive protein.
†Odds ratio per unit of clinical variable.
‡Variables included in multivariate logistic regression model.

Table 2. Independent predictors of sinonasal polyposis in total population.*

	Odds ratio (95% CI)	Regression coefficient
Rhinorrhea	2.6 (1.2-5.9)	0.95
FVC ≥70% predicted	3.1 (1.1-8.6)	1.13
ROC area	0.66	…

CI indicates confidence interval; FVC, forced vital capacity; and ROC, receiver operating characteristic curve.
*Odds ratios and regression coefficients are adjusted for overoptimism by bootstrapping.

After stepwise multivariate analysis of these parameters, independent predictors for the presence of sinonasal polyposis were symptoms of rhinorrhea and FVC values 70% or greater of the predicted value (**Table 2**). This predictive model was not reliable (goodness of fit, P<0.004), and the discriminatory power of the model was moderate (ROC area, 0.66). Therefore, this predictive model would not be useful in clinical practice.

Because generally in current practice only patients with ORL symptoms are referred to an ORL physician, the predictive model was restricted to symptomatic children. Significant predictors (P<0.15) for the presence of sinonasal polyps in patients with ORL symptoms were male sex, older age, symptoms of rhinorrhea and postnasal drip, and high FVC values. After stepwise multivariate analysis of these parameters, independent predictors were male sex, age 10 years or older, rhinorrhea, and FVC values 70% or greater of the predicted value (**Table 3**). The Hosmer-Lemeshow goodness of fit was not significant for this model (P=0.53), and the ROC area was 0.77, which indicates good discriminatory capacity.

Based on the regression coefficients obtained from the multivariate regression model, a risk score was derived (fourth column in **Table 3**). By assigning points for each variable present, a total score was calculated for each patient using the following equation:

score = 1 for male sex + 1 for age 10 years or older + 1 for presence of rhinorrhea + 2 for FVC 70% or greater of predicted value.

Table 3. Independent predictors of sinonasal polyposis in symptomatic population.*

	Odds ratio (95% CI)	Regression coefficient	Contribution to score
Male sex	2.9 (1.1-7.5)	1.1	1
Age ≥10 y	2.6 (1.0-6.6)	0.9	1
Rhinorrhea	2.7 (1.1-7.0)	1.0	1
FVC ≥70% predicted	7.2 (1.8-29.1)	2.0	2
ROC area	0.77	…	…

CI indicates confidence interval; FVC, forced vital capacity; and ROC, receiver operating characteristic curve.
*Odds ratios and regression coefficients are adjusted for overoptimism by bootstrapping.

Table 4. Number of symptomatic patients with and without sinonasal polyposis across categories of risk score.*

Risk score	N†	Polyposis	No polyposis
1	1	0	1
2	15	5	10
3	15	4	11
4	24	20	4
5	5	5	0
Total	60	34	26

*Risk score was obtained from the following rule: 1 (male sex) + 1 (age ≥10 years) + 1 (rhinorrhea) + 2 (forced expiratory volume ≥70%).
†Number of subjects per score category.

In our population the score ranged from 1 to 5 (**Table 4**). With a threshold score of 4 or higher, the positive predictive value of this scoring rule was 86%, and the negative predictive value was 71% (sensitivity, 74%; specificity, 85%).

DISCUSSION

This cross-sectional analysis of an unselected population of children with CF demonstrated a high prevalence of sinonasal polyposis (50%). Significant univariate predictors for the presence of sinonasal polyposis were male sex, older age, symptomatic rhinorrhea, good pulmonary function, and low CRP levels. Although in the unselected study population the presence or absence of sinonasal polyps could not be accurately predicted by a combination of these univariate predictors, in the symptomatic population, male sex, age 10 years or older, rhinorrhea, and FVC values 70% or greater of those predicted were significant predictors in a multivariate regression model.

Our study is the only series to include children only. Recent studies in children and adults with CF have reported prevalences of nasal polyposis ranging from 32% to 45% [3-6], similar to the prevalence of nasal polyposis found in our population (40%). Also, the prevalence of sinonasal polyposis (nasal polyps and/or bulging of the lateral nasal wall) of 48% and 57% found in other studies [3,4] was similar to the prevalence in our population (50%).

Only 41 (59%) of the 70 patients with sinonasal polyposis reported one or more ORL symptoms (**Table 1**). This is consistent with other studies, suggesting that children with CF underreport their symptoms [7,15]. This could be explained by the congenital nature of their disease, the lack of a healthy baseline status for comparison, and adaptation to their symptoms [15].

According to the literature, the most frequent symptoms in children with polyposis are rhinorrhea and nasal obstruction [4-6,16]. In our population, the most commonly encountered symptoms in patients with sinonasal polyposis were rhinorrhea (36%),

postnasal drip (29%), and nasal obstruction (27%). Of these symptoms, only rhinorrhea was a significant independent predictor for the presence of sinonasal polyposis. This might be explained by the fact that rhinorrhea is the most objective symptom and therefore more easily recognized by parents than, for example, nasal obstruction. Our findings stress the importance of detailed history taking in children with CF and of focusing on objective symptoms (*e.g.*, mouth breathing instead of nasal obstruction).

Several authors have reported that patients with CF and nasal polyps have better pulmonary function [10,11] and greater weight and height [11], are less frequently colonized by *Staphylococcus aureus* [10], more frequently colonized by *Pseudomonas aeruginosa* [11], and are more frequently male [9] than children without nasal polyps. However, others could not confirm the correlation of these clinical characteristics with the presence of nasal polyps [2,5]. In our total population, significant univariate predictors for the presence of sinonasal polyposis were male sex, good pulmonary function, and low CRP levels; in the symptomatic population, older age was also a significant predictor.

No medical treatment has proven effective in preventing progression of rhinologic complications in children with CF. Recently, the first randomized controlled trial of topical steroid treatment for nasal polyps in adult patients with CF has been performed, showing a statistically significant reduction in polyp size compared with placebo [17]. However, no significant reduction in symptoms in the steroid-treated group could be demonstrated. Also, the therapeutic effectiveness of functional endoscopic sinus surgery has not been established in a randomized controlled trial, but various studies report a statistically significant reduction of symptoms after surgery [8,18].

When evidence becomes available that medical and/ or surgical treatment is effective in preventing progression of sinonasal polyposis in children with CF, there might be a good indication to involve the ORL physician routinely in the follow-up of these children. For now, our study showed that selection of "high-risk" patients for ORL referral on the basis of symptoms and clinical characteristics is of little value.

We demonstrated that in the children with ORL symptoms, using a scoring rule including the predictors male sex, age 10 years or older, symptoms of rhinorrhea, and FVC values 70% or greater of those predicted, the presence of sinonasal polyposis could be reasonably predicted (positive predictive value, 86%; negative predictive value, 71%). By using this simple scoring rule, referral to an ORL physician could become more effective. Although internal validation of the model by bootstrapping techniques demonstrated that the model is robust, the actual performance needs further external validation.

In conclusion, sinonasal polyps are present in half of the children with CF, but only 59% of those with polyps are symptomatic. As children with CF underreport their ORL symptoms, a detailed ORL history of each patient should be obtained regularly and should focus on objective symptoms. In an unselected population of children with CF, no combination of ORL symptoms and clinical characteristics could accurately predict the presence of sinonasal polyposis. In patients with CF and ORL

symptoms, the presence of sinonasal polyposis could be reasonably predicted using a predictive model that includes male sex, age 10 years or older, symptoms of rhinorrhea, and FVC values 70% or greater of those predicted. A scoring rule including these independent predictors could reasonably classify children with CF and nasal symptoms into a category with increased risk for sinonasal polyposis, thus facilitating the decision on ORL referral.

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Chapter 7

Infection and inflammation influence aerobic capacity in children with cystic fibrosis

Submitted

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ABSTRACT

Rationale: Several studies have shown that pulmonary function and nutritional status are important determinants of aerobic exercise performance in patients with cystic fibrosis (CF). Other determinants, such as genetic polymorphisms, infection and inflammation, have never been investigated.
Objectives: To identify independent predictors of aerobic capacity in children and adolescents with CF.
Methods: A retrospective cohort study involving pediatric patients of a large Dutch CF center was performed. The effects of CFTR genotype, ACE I/D polymorphism, annual lung function, nutritional status, inflammation (IgG), and *P. aeruginosa* colonization on peak oxygen uptake (VO_2peak) were analyzed longitudinally, using a random effects model.
Results: Data for 870 maximal exercise tests in 202 children (50.5% males) with CF were analyzed. In a multivariate model, lung function and nutritional status were positively associated with VO_2peak. Age, *Pseudomonas* colonization, and total IgG levels were negatively associated with VO_2peak; CFTR genotype and ACE I/D polymorphism were not associated with VO_2peak.
Conclusions: Beside lung function and nutritional status, inflammatory status and *Pseudomonas* colonization independently affect aerobic capacity in children and adolescents with CF.

INTRODUCTION

Aerobic capacity (peak oxygen uptake; VO_2peak) is not only a determinant of well-being but also of survival in patients with cystic fibrosis (CF) [1,2]. Several studies have shown that VO_2peak in patients with CF is associated with lung function [3-7] and nutritional status [3,5,6], but the effects of genetics, inflammation, and infection on VO_2peak are less well established.

Mutations in the cystic fibrosis transmembrane regulator (CFTR) gene have been suggested to affect exercise tolerance [8]. While the severity of the CFTR mutation (mild versus severe) is associated with pancreatic insufficiency, lung function, and survival [9,10], data on the effect of CFTR genotype on aerobic capacity are conflicting [11,12]. Moreover, so-called modifier genes might also influence aerobic capacity in patients with CF. A polymorphism (I/D) in the gene encoding angiotensin-converting enzyme (ACE) is associated with better endurance in elite distance runners, rowers, and mountaineers [13]; however, the results of two studies on the effect of this polymorphism on pulmonary function in CF are conflicting [14,15], and the effect of ACE I/D polymorphisms on exercise capacity in patients with CF has never been investigated.

In addition to genetic variability, differences in the severity of inflammation might account for differences in exercise capacity. Recently, the severity of systemic inflammation was found to be associated with impaired exercise capacity, irrespective of FEV_1, in chronic inflammatory diseases, such as COPD [16]. Inflammation in CF is often triggered by *P. aeruginosa* (PA), which is associated with a decline in lung function [17,18]. The individual effects of inflammation and PA colonization on aerobic capacity in CF have never been studied.

Thus the objective of this longitudinal, single-center study was to identify independent determinants of aerobic capacity in CF. Potential determinants included lung function, nutritional status, CFTR genotype, ACE I/D polymorphism, total serum IgG levels, and PA colonization.

METHODS

Patients

The CF Center of the University Medical Center Utrecht (Utrecht, the Netherlands) treats about 250 children with CF. All patients older than 6 years undergo a routine yearly multidisciplinary examination, including exercise testing. The study was approved by the Medical Ethics Committee of the University Medical Center Utrecht, and all participants and/or their parents gave informed consent.

Exercise Testing

The standard exercise protocol for the annual examination was used. Children up to 12 years of age performed a treadmill test according to the modified Bruce protocol [19], and older children used an electronically braked cycle ergometer (Lode Exam-

iner; Lode; Groningen, the Netherlands). Workload increased 15 Watts per minute. Subjects were asked to maintain a pedaling rate at 60 revolutions per minute. During the tests, patients were encouraged to perform to the best of their ability. Both tests were continued to voluntary exhaustion.
Continuous respiratory gas analysis and volume measurements were performed breath-by-breath with a triple V valveless mouthpiece and stored in a computerized exercise system (Oxycon Champion; Viasys GmbH; Hoechberg, Germany).
Maximal workload (Wpeak), oxygen uptake (VO_2), carbon dioxide production (VCO_2), and respiratory exchange ratio (RER, =VCO_2/VO_2) were measured. The mean VO_2 achieved during the last 30 seconds of exercise was taken as VO_2peak [7]. Maximum effort was defined as the presence of clinical signs of intense effort, with subjects being unable to maintain walking/cycling speed [20], plus the presence of heart rate > 180 beats/min and/or peak RER > 1.0 [21]. VO_2peak and Wpeak are expressed as a percentage of predicted (VO_2peak% and Wpeak%, respectively), based on data for an age- and sex-matched Dutch reference population [22].

Other clinical measurements

PA colonization was considered to be present if more than 50% of the PA cultures were positive for at least 3 consecutive years [23]. Information on the nutritional status was based on standard deviation scores (SDS) for body mass index (BMI) obtained from growth diagrams for the Dutch population [24]. Forced expiratory volume in 1 second (FEV_1) was obtained from maximal expiratory flow-volume curves (Masterscreen; Viasys GmbH; Hoechberg, Germany). Values are expressed as percentage of predicted values [25].

ACE genotyping

Genomic DNA was isolated from EDTA blood using Proprietary M-PVA Magnetic Bead Technology (Chemagen Biopolymer-Technologie AG; Baesweiler, Germany).
ACE I/D polymorphisms were determined by real-time PCR using fluorescent hybridization probes and a LightCycler (Roche Diagnostics; Basel, Switzerland) as described earlier with some slight modifications [26-28].
Briefly, the reaction volume was 20 µl, containing 1 µl of DNA (40–80 ng), 0.2 µM forward primer and 0.8 µM reversed primer as reported by Rigat *et al.* [26], 2 µl of 10x reaction buffer (LightCycler DNA master hybridization probes, Roche Diagnostics; Basel, Switzerland), 1.6 µl of 25 mM MgCl2 stock solution and 0.1 µM of each of the probes. The detection probes were the same as described by Somogyvári *et al.* [27]. The PCR conditions were as follows: denaturation at 95 °C for 60 s, followed by 50 cycles denaturation (95 °C for 10 s), annealing (first 10 cycles: 67 °C for 20 s, followed by 0.5 °C stepwise decrease per cycle to 61 °C) and extension (72 °C for 30 s). Melting curve analysis consisted of heating to 95 °C for 5 s, 45 °C for 60 s, followed by an increase of the temperature to 75 °C at 0.2 °C/s. To exclude mistyping of I/D heterozygotes as D/D homozygotes, a second PCR reaction was performed under the same conditions except for using the primer pair described earlier [27,28]. Verifi-

cation of the real-time PCR results with those of electrophoresis and using SSP-PCR revealed no mistyping.
Allele frequencies of the ACE I/D polymorphism were compared with those in 200 healthy controls [29].

Statistics

Normally distributed data were summarized using means and standard deviations (SD). In order to test whether continuous variables were normally distributed, a Kolmogorov-Smirnov test was performed. To analyze the effect of the potential predictors on VO_2peak, a random effects model for repeated measurements was used (SAS version 9.1, PROC MIXED). A linear rate of decline was assumed. Potential predictors were entered as covariates in the model. Intercept and age were specified as random effects to allow for individual differences [30]. Potential predictors included maximal exercise testing method (treadmill or cycle ergometer), sex, age, CFTR and ACE genotype, PA colonization, lung function (FEV_1), nutritional status (BMI), and total IgG levels. Predictors that did not contribute to the univariable model (P>0.15) were dropped from the full model.

RESULTS

Results were available for 202 children and adolescents (50.5% males). In total 870 valid maximal exercise tests were performed (400 on treadmill, 470 on cycle ergometer), an average of 4.3 per person per year. Eighty-three children became 12 during the study period and switched from treadmill to cycle ergometer testing. **Table 1** summarizes the characteristics of the participants.
The frequency of the DD, DI, and II genotypes of the ACE gene in CF patients (27.5, 50.0, and 22.5%, respectively) was not significantly different from that in controls (25.0, 53.5, and 21.5%, respectively). Moreover, the frequency conformed to the Hardy-Weinberg equilibrium, indicating that there was no sampling bias in this population, or problems with genotyping of the stored samples.
The average annual decline in VO_2peak/kg and VO_2peak% (SE) was 0.83 (0.12) mL/min/kg and 1.24 (0.26) %, respectively. VO_2peak/kg was significantly associated with the method of exercise testing and with sex, even after correction for age. Therefore, further analyses were performed using VO_2peak% as outcome measure. VO_2peak% was significantly lower with cycle ergometer testing than with treadmill testing (**Table 2**), but this difference disappeared after correction for age. VO_2peak% was not significantly different between males and females. BMI and FEV_1 were positively associated with VO_2peak% (**Table 2**), and age, PA colonization, and IgG were negatively associated with VO_2peak%. Because of the potential correlation between IgG and PA colonization, we added an interaction term for these two determinants to the model. The effect of the interaction term was not significant (P=0.42). VO_2peak was not associated with CFTR or ACE genotype.

Table 1. Patient (N=202) characteristics at first and last visits. Data are presented as means (SD), unless otherwise specified.

Variable	First visit	Last visit
Age (yr)	9.2 (3.6)	13.1 (4.1)
Sex (% male)	50.5	
CFTR genotype:		
Homozygous ΔF508 (%)	63.9	
Heterozygous ΔF508 (%)	30.7	
Other (%)	5.4	
CFTR genotype classes:		
Class I-III (%)	85.6	
Class IV-V (%)	6.9	
Unknown (%)	7.4	
ACE genotype (N=200):		
DD (%)	27.5	
DI (%)	50.0	
II (%)	22.5	
Height (SD score for age)	-0.68 (1.04)	-0.64 (1.08)
Weight (SD score for height)	-0.28 (0.85)	-0.38 (0.90)
BMI (SD score for age)	-0.38 (0.82)	-0.43 (0.88)
FEV_1 (% predicted)	87.7 (20.8)	77.9 (21.8)
P. aeruginosa colonization (%)	31.7	50.0
VO_2peak (L/min)	1.28 (0.54)	1.72 (0.66)
VO_2peak/kg (mL /min/kg)	44.3 (8.6)	41.2 (9.1)
VO_2peak% (% of predicted)	88.9 (21.0)	85.3 (20.2)

SD indicates standard deviation; CFTR, cystic fibrosis transmembrane regulator; ACE, angiotensin converting enzyme; FEV_1, forced expiratory volume in one second; BMI, body mass index; IgG, Immunoglobulin G; and VO_2peak%, peak oxygen uptake.

DISCUSSION

Many studies have shown that aerobic capacity is important for well-being and prognosis in patients with CF [1,2]. Our large longitudinal study of determinants of aerobic capacity in children and adolescents with CF confirmed the association between lung function and nutritional status and VO_2peak reported previously [3-7]. Moreover we identified IgG and PA colonization as independent determinants of VO_2peak, which supports the concept that CF is a systemic inflammatory disease.
The observed inverse association between total IgG levels and aerobic capacity was independent of lung function, nutritional status, and PA colonization. Elevated IgG

Table 2. The effects of clinical parameters on VO_2peak% in 202 children with CF. The effects were calculated using a random effects model for repeated measurements. Intercept and age were specified as random effects to allow for individual differences.

	Univariable			Multivariable		
Determinant	**ΔVO_2peak%**	**SE**	**P**	**ΔVO_2peak%**	**SE**	**P**
Method (Bicycle vs. treadmill)	-5.9	1.4	<0.0001			
Sex (male vs. female)	-2.6	2.5	0.3			
Age (year)	-1.24	0.26	<0.0001	-0.57	0.25	0.02
ΔF508/ΔF508 vs. other	-0.1	2.8	1.0			
CFTR genotype (severe vs. mild)	-4.5	6.1	0.5			
ACE genotype			0.8			
DD vs. II	0.3	3.6				
DI vs. II	-1.2	3.1				
P. aeruginosa colonization	-5.2	1.7	0.003	-3.3	1.7	0.05
FEV_1 (% predicted)	0.29	0.04	<0.0001	0.18	0.04	<0.0001
BMI (SD score)	6.67	0.86	<0.0001	3.71	0.90	<0.0001
IgG (g/l)	-1.14	0.20	<0.0001	-0.51	0.21	0.01

VO_2peak% indicates peak oxygen uptake (% predicted); SE, standard error; CFTR, cystic fibrosis transmembrane regulator; ACE, angiotensin converting enzyme; FEV_1, forced expiratory volume in one second; BMI, body mass index; SD, standard deviation; and IgG, Immunoglobulin G.

levels may represent a hyperimmune response that is ineffective against infection and possibly destructive to the airway [31]. Indeed, due to hyperinflammation, CF patients with low IgG antibody levels have a better prognosis than patients with high IgG antibody levels [31-33]. Peripheral muscle force is decreased in children with CF, even in those with minimal changes in lung function [34,35], but aerobic capacity can be improved by training without there being significant changes in lung function or nutritional status [36,37]. These findings suggest that systemic inflammatory processes have a direct detrimental effect on peripheral muscle efficiency, reducing aerobic capacity [38,39]. The negative effect of increased systemic inflammation on aerobic capacity is not limited to CF, but also seems to be present in patients with COPD [16,40,41].

PA colonization was also associated with a low aerobic capacity, irrespective of the total serum IgG level. After PA infection, not only IgG but also other mediators are involved in the ensuing inflammatory response. This may explain why PA colonization still affected aerobic capacity even after correction for IgG level. PA colonization may also have a direct effect on aerobic capacity, because sustained PA lung infection in mice was found to preferentially weaken the diaphragm, an effect not directly correlated with the degree of pulmonary inflammation induced under these conditions [42].

The association between lung function and aerobic capacity is in accordance with two other longitudinal studies showing FEV_1 to be the main predictor of VO_2peak [4,5] and in accordance with the findings of cross-sectional studies involving both children and adults with CF [3,6,7]. In the longitudinal study by Klijn *et al.*, VO_2peak was also associated with fat free mass (FFM), but to lesser degree than with FEV_1 [5]. Moreover, nutritional status (weight adjusted for height, BMI, or lean body mass) was found to be associated with VO_2peak in adults with CF [3,6,43]. Our study confirms this association. However, Pianosi *et al.* found no association between VO_2peak and weight adjusted for height, possibly because the study subjects were well-nourished (mean weight 103% of predicted, with no patient weighing less than 85% of predicted) [4].

Potential genetic determinants of VO_2peak include CFTR genotype and modifier genes such as ACE. There are five classes of CFTR mutations, based on their functional effects [8]. Class I, II, and III mutations result in more severe CF than class IV and V mutations [8]. In contrast to Selvadurai *et al.* [11], who found the highest aerobic capacity in patients with class IV and V mutations and the lowest in patients with class I and II mutations in their small cross-sectional study, we did not find CFTR mutation class (severe versus mild) to be associated with VO_2peak. However, we had too few patients with class III, IV, and V mutations to enable separate analysis per CFTR class. Kaplan *et al.* also found no differences in exercise capacity between patients who were homozygous and heterozygous for the ΔF508 gene mutation [12]. The effect of CFTR mutation on aerobic capacity needs further investigation.

We also did not find an effect of the ACE I/D polymorphism on aerobic capacity. Although best known for its function in regulation of blood pressure, ACE also has a role in diverse cellular processes, including cell growth and survival of nonvascular tissues [44]. Local renin-angiotensin systems (RAS) have been described in several tissues, including skeletal muscle, and are suggested to influence the metabolism of these tissues [45]. The ACE I/D polymorphism is associated with enhanced endurance, especially in specific groups of athletes (elite distance runners, rowers, and mountaineers), perhaps secondary to enhanced muscle efficiency [13]. Although an association between the ACE I allele and a higher VO_2peak has been reported in patients with congestive heart failure and in elderly individuals, most other studies failed to find such an association [46-49]. In one study in patients with CF, the DD genotype was associated with earlier pulmonary dysfunction and earlier colonization with PA compared with the II genotype [14]. However, in another large study there was no significant difference in distribution of ACE I/D polymorphisms between CF patients with a mild and severe pulmonary phenotype [15].

Our study was limited by the fact that we did not analyze all potential determinants of aerobic capacity in CF, for instance effort and habitual physical activity. Effort is hard to quantify, but maximal exercise tests were considered valid only if subjects showed clinical signs of intense effort and were unable to maintain speed, and heart rate and/or RER were above established cut-off points. It has been shown that habitual physical activity is a significant predictor of VO_2peak in CF, but the effect is relatively weak [50]. Another limitation is that we did not analyze the effect of

interleukins and CRP on aerobic capacity. However, interleukins, *e.g.* IL-6, are not routinely measured, and low-sensitivity CRP, rather than high-sensitivity CRP, is measured in most patients.

In conclusion, this large longitudinal study confirmed that aerobic capacity in children and adolescents with CF is determined by lung function and nutritional status. Furthermore, this is the first study showing an independent association between high total serum IgG or PA colonization and low aerobic capacity. Genetic variation in the CFTR and ACE gene did not seem to be important determinants of VO_2peak in CF.

The results of our study not only stress the importance of optimizing nutritional status in underweight CF patients, but also indicate that prevention and aggressive treatment of inflammation and PA infections may increase aerobic capacity in these patients. Randomized controlled trials are needed to investigate the potential positive effect of anti-inflammatory and anti-pseudomonal agents on aerobic capacity, and consequently well-being and prognosis in patients with CF.

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CHAPTER 8

Disease modifying genes in cystic fibrosis (review)

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ABSTRACT

The variation in cystic fibrosis (CF) lung disease and development of CF-related complications correlates poorly with the genotype of the CF transmembrane regulator (CFTR) and with environmental factors. Increasing evidence suggests that phenotypic variation in CF can be attributed to genetic variation in genes other than the CFTR gene, so-called modifier genes.

In recent years, multiple candidate modifier genes have been investigated in CF, especially genes that are involved in the control of infection, immunity and inflammation. Some of these genes have been rather conclusively identified as modifiers of the CF phenotype, whereas associations found in other genes have not been confirmed or are conflicting.

Identification of genetic variation in modifier genes, obtained by genotype–phenotype studies in well-defined patient populations, may be used as an aid to prognosis and may provide the possibility of new therapeutic interventions.

1. INTRODUCTION

The clinical course of cystic fibrosis (CF) varies widely. Whereas some individuals die early in childhood, others live well in adulthood suffering from with only mild lung disease [1]. Before the identification of the CF gene in 1989, it was assumed that variation in the severity of disease was due to allelic variation in the gene defective in CF, the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene [2]. CFTR is a multi-domain protein with a complex regulation. It is a small chloride channel that is found at the apical border of epithelial cells lining most exocrine glands [2]. More than 1350 mutations in the CFTR gene have been identified so far [3].

2. CFTR AND CF PHENOTYPE

Mutations in the CFTR gene can disrupt CFTR function by different mechanisms depending on their nature and on the domain in which these alterations occur [2]. Based on these observations, CFTR mutations are subdivided into five classes [4,5]. Class I mutations are mutations causing premature termination signals, typically resulting in little or no protein production. Class II mutations, such as ΔF508, result in the production of an abnormally folded CFTR that is not trafficked normally to the apical cell membrane. Class III mutations produce proteins that are inserted in the apical cell membrane but have a defective regulation, mostly because of mutations in the nucleotide-binding domains. Class IV mutations affect amino acids located in the pore of the channel, which will give rise to a chloride channel with a normal regulation, but with reduced single channel currents. Class V mutations affect splicing of CFTR transcripts and result in significantly decreased production of normal CFTR.

Recently, the number of classes was extended to six [2]. Class VI mutations harbor nucleotide alterations that affect the regulatory properties of the CFTR protein (*e.g.*, towards other ion channels like ENaC or ORCC) [2]. Lastly, some mutations will disrupt the function of the CFTR in more than one way, and will therefore have to be categorized in different mutation classes [2].

A clear correlation is observed between CFTR genotype and pancreatic exocrine insufficiency: CFTR mutation classes I, II, and III are associated with significant declines in CFTR expression or function and are invariably associated with pancreatic exocrine insufficiency [6]. CFTR mutation classes IV and V are associated with some residual epithelial cell CFTR function and are often associated with pancreatic exocrine sufficiency [7,8]. A recent retrospective cohort study on more than 10,000 patients enrolled in the US CF Foundation National Registry demonstrated a significantly lower mortality rate in patients with class IV and V mutation compared with class II [8]. Patients with class IV and V mutations also showed a higher forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC), a higher height and weight and less *P. aeruginosa* colonization. Patients with class I,

II and III mutations all showed similar clinical characteristics. However, although patients with class IV and V mutations showed a milder clinical phenotype and significantly lower mortality rates, variation in the severity of disease was found to be only partially related with allelic variation in CFTR. There is substantial phenotypic variability within patients having the same CFTR mutation class and even identical mutations. Thus, other factors modify the CF phenotype.

3. OTHER FACTORS ASSOCIATED WITH CF PHENOTYPE

A great number of environmental factors such as *P. aeruginosa* [9], *B. cepacia* [10], nutritional support [11], early (antibiotic) treatment [12,13], tobacco use [14] and socioeconomic status [15] have been shown to be associated with the phenotype of CF. However, these factors can insufficiently account for the degree of variability observed in individuals with identical CFTR phenotypes. Even siblings and twins with identical CFTR genotypes and sharing mainly the same environment show different disease severity [16]. Monozygous twins have a significantly higher concordance of nutritional status and severity of lung disease than dizygous twins, suggesting that CF disease severity is modulated by secondary genetic factors besides the CFTR gene itself [1,16]. These secondary genetic factors are called modifier genes or gene modifiers [17,18].

4. MODIFIER GENES

Modifier genes in monogenic diseases are genes other than the single mutated gene, which affect disease expression. Modifier genes can affect disease expression via several ways. They can be involved in intracellular processes in the cell where the causal or primary gene (*e.g.*, CFTR gene) is expressed [17]. Products of modifier genes can affect splicing, transcription, translation, protein trafficking, glycosylation or other posttranslational processes, but also protein expression, degradation or secretion [17].
Alternatively, modifier genes may exert its action in cells in which no expression of the primary gene takes place [17]. The expression of modifier genes may regulate responses like inflammation, fibrosis, host defense, ion transport, or virtually any other response to a disturbance of homeostasis [17].
Nowadays, one of the most common methods to study associations between genetic variation in 'candidate' modifier genes and clinical phenotype is the identification of single nucleotide polymorphisms (SNPs) [19]. SNPs are single base pair positions in the genome at which variation in nucleotides gives rise to alternative alleles in normal individuals and populations [20]. In contrast to gene mutations occurring in less than 1% of the individuals in a population, SNPs are present in more than 1% of the individuals and comprise common genetic variation in normal individuals. Evidence of linkage between a SNP and disease phenotype indicates that the SNP is

located in a modifier gene or that the SNP is in linkage with a modifier gene. Functional SNPs altering disease phenotype are, for instance, an SNP in the promoter region of a modifier gene affecting the transcription rate, an SNP in an exon affecting protein structure or function, and an SNP within an intron interfering with splicing mechanisms [20].

5. MODIFIER GENES IN CF

Modifier genes may influence the severity of CF phenotypes through a variety of mechanisms. They may modulate the phenotype by acting on the basic molecular level, such as providing alternative chloride conductance, and regulating splicing and expression of the CFTR gene. They may also modulate susceptibility to infection and inflammatory response. Furthermore, CF pulmonary phenotype may be modified by genes associated with mucociliary clearance and epithelial tissue damage and repair. Finally, gastrointestinal injury (liver, pancreas, intestine) in CF may be influenced by genes modulating proteolysis and fibrosis.
Several genes investigated as potential modifier genes of CF phenotype will be summarized below. In **Table 1** and **Table 2** potential modifiers of pulmonary and gastrointestinal phenotype are displayed.

5.1. Mannose-binding lectin (MBL)

MBL is a serum protein participating in innate immune defense [21,22]. MBL activates the complement system by MBL-associated serine proteases and may interact with receptors on phagocytes [21]. The ligands for MBL, mannose and N-acetylglucosamine oligosaccharides, are present on a wide variety of microorganisms. Human MBL is derived from a single gene on chromosome 10, MBL2. In exon 1 of the MBL2 gene, 3 single-base substitutions independently cause low serum levels of MBL [21,22]. The normal (wild-type) allele has been designated A, whereas the common designation for the variant alleles (B, C, D) is 0. Even in heterozygotes, each of the 3 variants reduces the amount of functional MBL subunits individuals 5- to 10-fold. Besides the exon 1 alleles, several nucleotide substitutions in the promoter region of the MBL2 gene affect the MBL serum level. Particularly, a polymorphism in codon − 221 (X/Y type) has a significant down-regulating effect on serum MBL serum concentrations [21].
It has been shown that MBL variant alleles resulting in low functional MBL serum levels are associated with an increased risk of different types of infections that occur primarily in children, but also in adults. Moreover, MBL deficiency has also been shown to play a role in autoimmune diseases [21]. In all 4 published studies concerning CF patients, a significantly reduced lung function was found in carriers of MBL 0 alleles when compared with AA homozygotes [21,23-25]. In one study, carriers of variant alleles were found to be more frequently colonized with *B. cepacia* [21], whereas in another study, patients homozygous for variant alleles had a nearly significantly higher frequency of colonization by *P. aeruginosa* [24]. This, however,

Table 1. Potential pulmonary modifier genes investigated in CF.

Gene	Author	Ref	N pat	Polymorphism	FEV_1	CXR	PA	BC	Mortality
MBL	Garred *et al.*	[21]	149	A/0 + X/Y	−		0	++	++
	Davies *et al.*	[23]	558	A/0 + X/Y	−		0	0	
	Gabolde *et al.*	[24]	164	A/0	−		0		
	Yarden *et al.*	[25]	179	A/0 + X/Y	−		0		
NOS1	Grasemann *et al.*	[32]	75	AATn ≥ 12	0		++		
	Grasemann *et al.*	[31]	40	AATn ≥ 12	0		++		
	Texereau *et al.*	[33]	59	GTn > 27	+		0	0	
NOS3	Grasemann *et al.*	[28]	70	894G/T	0		+[a]		
AAT	Doring *et al.*	[35]	215	M/S,Z	0		++		
	Meyer *et al.*	[36]	269	M/S,Z			0		
	Mahadeva *et al.*	[34]	157	M/S,Z	+	0	0	0	
	Mahadeva *et al.*	[60]	79	M/S,Z					0
	Frangolias *et al.*	[38]	716	M/S,Z	0		+	0	0
			716	1237G/A	0		0	0	0
	Henry *et al.*	[37]	124	1237G/A	+	++	−		
HLA-II	Aron *et al.*	[40]	98	DR4−/+	0		−		
			98	DR7−/+	0		+		
TNF-α	Hull and Thomson	[45]	53	− 308G/A	−	0	0		
GST M1	Hull and Thomson	[45]	53	A,B/0	0	−	0		
GST M3	Flamant *et al.*	[46]	146	A/B	+		0		
TGF β_1	Arkwright *et al.*	[48]	171	869T/C	+		0	0	0
ACE	Arkwright *et al.*	[49]	261	D/I	+		0	0	0
β_2AR	Buscher *et al.*	[50]	126	16R/G	−		0		
CLC-2	Blaisdell *et al.*	[55]	31	Various	0				

N pat indicates number of patients; FEV_1, forced expiratory volume in one second; CXR, chest X-ray; PA, *P. aeruginosa* colonization; BC, *B. cepacia* colonization; MBL, mannose-binding lectin; NOS, nitric oxide synthase; AAT, α1-antitrypsin; HLA, human leukocyte antigen gene II; TNF-α, tumor necrosis factor-α; GST, gluthatione S-transferase; TGF-β_1, transforming growth factor-β_1; ACE, angiotensin 1 converting enzyme; β_2AR, β_2 adrenoreceptor; and CLC-2, chloride channel-2.

Symbols: ++, strongly positive association ($p < 0.01$); +, positive association ($p < 0.05$); 0, no significant association; −, negative association ($p < 0.05$); − −, strongly negative association.

[a] In females only.

Table 2. Potential gastro-intestinal modifier genes investigated in CF.

Gene	Author	Ref	N pat	Polymorphism	LD	Diabetes	MI	Weight
MBL	Gabolde *et al.*	[26]	216	A/0	++			
AAT	Mahadeva *et al.*	[34]	157	M/S,Z	0			
HLA-II	Carrington *et al.*	[41]	67	DQB1 Asp57−/+		−		
	Lanng *et al.*	[42]	57	DR3, DR4		0		
	Duthie *et al.*	[43]	274	DQ6−/+	+			
TNF-α	Hull and Thompson	[45]	53	− 308G/A				−
GST M1	Hull and Thompson	[45]	53	A,B/0				0
GST M3	Flamant *et al.*	[46]	146	A/B				0
GST P1	Henrion-Caude *et al.*	[47]	106	105I/V	− −			
ACE	Arkwright *et al.*	[49]	261	D/I	−	0		
HFE	Rohlfs *et al.*	[51]	89	C282Y			0	
	Devaney *et al.*	[52]	122	C282Y			0	
ATB^0	Larriba *et al.*	[54]	48	Various			0	

N pat indicates number of patients; LD, liver disease; MI, meconium ileus; MBL, mannose-binding lectin; AAT, α1-antitrypsin; HLA, human leukocyte antigen gene II; TNF-α, tumor necrosis factor-α; GST, gluthatione S-transferase; TGF-β1, transforming growth factor-β1; ACE, angiotensin 1 converting enzyme; HFE, putative hereditary hemochromatosis gene; and ATB^0, neutral amino acid transporter B^0.
Symbols: ++, strongly positive association ($p < 0.01$); +, positive association ($p < 0.05$); 0, no significant association; −, negative association ($p < 0.05$); − −, strongly negative association.

was not confirmed in 2 other studies [23,25]. Finally, associations between allelic variants of MBL and the presence of liver cirrhosis and survival have been described [21,26]. In conclusion, the MBL gene appears to be a well validated modifier of CF phenotype.

5.2. Nitric oxide synthase (NOS)

Nitric oxide (NO) has been shown to be important in a variety of regulatory processes in the lung, including host defense, inflammation, and bronchomotor control [27]. Three isoforms of NO synthases (NOSs) exist: neuronal NOS (NOS1), inducible NOS (NOS2), and endothelial NOS (NOS3). NOS1 and NOS3 are constitutively expressed in various cell types, whereas NOS2 is regulated by cytokines primarily at the level of gene transcription [28].

Exhaled NO levels are elevated in individuals with inflammatory lung diseases, such as asthma and bronchiolitis compared with healthy controls [27,29]. In contrast, despite the inflammatory nature of CF lung disease, NO levels in exhaled air

from CF patients are lower than those from healthy controls and individuals with other inflammatory lung diseases [27,29], as described in [30].
Recently, Grasemann demonstrated the number of AAT repeats in the NOS1 gene to be negatively associated with nasal and expired NO in CF patients [31,32]. Furthermore, CF patients with a high number of AAT repeats were more frequently colonized with *P. aeruginosa* and *A. fumigatus*. In another population, an association between the number of GT repeats in the promoter region of the NOS1 gene and higher exhaled NO levels and less decline in lung function was observed [33]. Grasemann's group also found an association between a common polymorphism (G847T) in the NOS3 gene, higher exhaled NO levels and a decreased risk for colonization with *P. aeruginosa* in females [28]. This finding was not found in males and the total population. In conclusion, at least the NOS1 gene seems to be a significant modifier of pulmonary phenotype in CF.

5.3. α1-Antitrypsin (α1-AT)

A major cause of the chronic pulmonary disease in CF is a persistent imbalance between neutrophil proteinases, most importantly elastase, and anti-proteinases, in particular α1-AT [34]. α1-AT is an acute-phase glycoprotein and is the most important proteinase inhibitor in the lung. The most clinically important variants are the common S (Glu264Val) and Z (Glu342Lys) alleles which are found in approximately 12% of the population. They result in plasma α1-AT levels in the homozygote state of 60% and 10%, respectively, when compared with the normal MM homozygote [34]. Another polymorphism in the 3' non-coding region enhancer-binding element of the α1-AT gene (G1237A) may reduce the rise in the level of α1-antitrypsin during the acute phase response, whilst leaving baseline levels unaffected [34].
Doring *et al.* found an earlier age of chronic *P. aeruginosa* acquisition in CF patients who carried α1-AT deficiency alleles [35], but this could not be confirmed by Meyer *et al.* [36]. Doring *et al.* did not find an association between deficiency and lung function [35], but in another study, in which was adjusted for age, a significantly better lung function was found in patients with a deficient genotype (M, S, Z) compared with patients with a normal phenotype [34]. In the latter study, no association between the G1237A polymorphism and lung function was found. On the other hand, in a study by Henry *et al.*, this polymorphism was associated with a better lung function and less change in X-ray score and fewer infective exacerbations [37]. Finally, in a large recent Canadian study (N = 716), one of the few that adjusted for confounders, none of the associations between deficiency Z and S genotypes or G1237A polymorphisms, and lung function or age of *P. aeruginosa* colonization could be confirmed [38]. It thus can be concluded that, despite the considerable amount of data, the impact of α1-AT on CF phenotype is still unclear.

5.4. Human leukocyte antigen (HLA) system

The HLA system is the human version of the major histocompatibility complex (MHC). It controls quantitative and qualitative aspects of immune responses. Certain HLA alleles have shown to be associated with increased susceptibility to in-

fectious diseases, higher risk of autoimmune diseases and development of cancer [39]. One study in CF patients demonstrated that colonization with *P. aeruginosa* was significantly less likely among HLA DR4 carriers, whereas carriers of the DR7 allele were more likely colonized with *P. aeruginosa* and showed higher IgE levels [40]. No association with lung function or nasal polyposis was found. Associations with HLA genotype have also been described for CF-related diabetes (DQB1 Asp57) and liver disease (DQ6) [41-43].

5.5. Tumor necrosis factor-α (TNF-α)

TNF-α is a pro-inflammatory cytokine found in high concentration in the lungs of patients with CF [44]. A TNF-α promoter gene polymorphism (G-308A) is associated with higher constitutive and inducible levels of transcription of TNF-α. CF patients carrying a G-308A polymorphism were demonstrated to have a significantly lower lung function and body weight compared with patients without this polymorphism [45].

5.6. Glutathione S-transferases (GST)

Glutathione is the major local pulmonary anti-oxidant and is present in high concentrations in the epithelial lining fluid. GSTs detoxify harmful organic hydroperoxides which are generated as a result of exposure to oxidant stress, such as that found in the lungs of CF patients. GSTs conjugate the hydroperoxides with glutathione, thereby preventing further pulmonary damage [45]. One gene in this anti-oxidant family, GST M1, has a common non-functioning allele (GST M1-0). CF patients homozygous for this allele have worse chest radiographic scores and worse Shwachman scores of disease severity [45]. Another association has been found between a variant genotype consisting of a three-base pair deletion for GST M3 and better lung function [46]. Finally, a polymorphism in GST P1 (Ile105Val) was shown to be associated with liver disease [47].

5.7. Transforming growth factor-β_1 (TGF-β_1)

TGF-β_1 is a cytokine with both pro-inflammatory and anti-inflammatory properties that promotes the proliferation of fibroblasts and the deposition of collagen. A polymorphism (T869C) resulting in lower production of TGF-β_1 was shown to result in a slower deterioration in lung function in CF patients [48].

5.8. Angiotensin I converting enzyme (ACE)

ACE is another pro-inflammatory molecule, activated by TGF-β_1, and may have a role in the development of more severe organ damage. A polymorphism in ACE, consisting of the presence (I) or absence (D) of a 250-bp DNA fragment, is associated with serum ACE level. A high-producer ACE genotype (DD) in CF patients was found to be associated with patient's age at which FEV_1 became lower than 50% of predicted and with the development of liver cirrhosis [49].

5.9. Other modifier genes

Apart from modifier genes involved in host defense and inflammation, also a number of other modifier genes have been described. β2-Adrenoreceptors (β2AR) are important mediators of cyclic AMP in the airway. They may modify the CFTR activity in CF patients with mutations that have residual function of CFTR (*e.g.*, ΔF508). For instance, one β2AR polymorphism (R16G) was shown to be related with reduced lung function and a greater decline in lung function [50].

In two studies, CF patients, meconium ileus (MI) was observed more frequently in carriers of a polymorphism (C282Y) in the gene for hemochromatosis (HFE) compared with patients without MI (19% vs. 10% and 31% vs. 13%, respectively). In both studies, this finding did not reach statistical significance [51,52].

A very strong association was found between a locus on chromosome 19q13, called CFM1, and meconium ileus (MI) [53]. The human CFM1 locus is in closest linkage with a gene coding for a Na+-dependent neutral amino acid transporter called ATB0, also named SLC1A5 [54]. However, no association between MI and a particular allele or haplotype in ATB0 could to be demonstrated [54].

Finally, genes coding for chloride channels besides the CFTR are potential modifier genes. CLC-2 is one candidate alternative chloride channel, which is located in respiratory epithelia. However, polymorphisms in the CLC-2 gene do not seem to be correlated with severity of lung disease [55].

6. INTERPRETATION OF RESULTS OF MODIFIER GENE STUDIES

It can be noticed that the majority of studies on modifier genes in CF are on rather small numbers of patients, most of the time not repeated and if so, often having conflicting results. When interpreting results of modifier genes studies, the following needs to be taken into account.

Firstly, in modifier gene studies, a good definition and ascertainment of the phenotype are crucial. Without careful definition of the phenotype, both false negative and false positive results may occur [17]. For example, outcomes of modifier gene studies on associations with *P. aeruginosa* may differ because of differences in the frequency and the type of culture. Furthermore, definitions of CF-related complications like diabetes or liver cirrhosis are not consistent. Also, many outcomes for lung function are used (cross-sectional vs. longitudinal decline, dichotomization, 'severe' vs. 'mild') [17]. Many studies are unable to confirm the results of previous studies due to these inconsistent phenotype definitions.

Secondly, modifier gene studies will provide associations but chance associations will certainly occur because of the number of comparisons that will be possible [56]. Modifier gene studies provide an association between a genetic polymorphism and disease phenotype, However, they do not demonstrate a causal (*i.e.*, functional or physiological) relationship between a SNP and a phenotype. It seems therefore mandatory to establish the functional relationship between SNP and phenotype [17,56].

Thirdly, each modifier gene should be tested simultaneously with other potential modifier genes [17]. Interaction can occur between candidate modifier genes, for example, when they act in the same biologic pathway [1]. A valid association between a modifier gene and a certain phenotype should hold up under simultaneous testing of genes [17,49]. However, the possibility of testing of interactions is often limited by study size.
Fourthly, each modifier gene should be adjusted for potential confounders, including age, sex, age of CF diagnosis, CFTR genotype, pancreatic sufficiency status, body mass index and infection with *P. aeruginosa* [38]. Lack of adjustment for these confounders may result in spurious associations.
Finally, reported associations should be replicated in large independent populations. Large nationwide multi-center studies on modifier genes in CF are now performed, for example, by the Canadian Consortium for CF Modifier Gene Studies which has already enrolled over 1300 Canadian CF families [57] and the US Gene Modifier Study Group which has enrolled over 800 subjects [58]. These large studies will have enough statistical power to establish valid associations between potential modifier genes and CF phenotype found in previous smaller studies.

7. APPLICATIONS AND PERSPECTIVES OF MODIFIER GENE STUDIES

Using a multivariate model including clinical patient characteristics and a number of modifier genes, the clinical course of pulmonary disease or the development of CF-related complications like diabetes and liver cirrhosis may be predicted. Thus, one of the potential applications of the results of modifier gene studies is an aid to prognosis. Patients found to be at high risk for a complication can be more often and more intensively screened and treatment of this complication can be started before symptoms have developed.
Furthermore, modifier gene studies will lead to a better understanding of CF pathophysiology and provide the possibility of new therapeutic interventions [1]. The identification of genes that influence CF phenotype illuminates therapeutic targets, either the protein product of the gene or the entire pathway in which it acts. Therapeutic interventions can be developed to minimize the consequences and enhance the benefits of modifier genes of CF phenotype [18,56]. A case report of such a successful intervention has already been published: infusion of MBL in a CF patient known to carry MBL-deficient alleles [59]. In the future, similar therapies based on modifier gene genotyping in individuals with CF may become an important part of CF treatment regimens [1].

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Chapter 9

An age-dependent effect of mannose-binding lectin on disease severity in cystic fibrosis

Submitted

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ABSTRACT

Background: Genetic polymorphisms in the gene encoding mannose-binding lectin (MBL), MBL2, have been associated with poorer outcome in cystic fibrosis (CF); however, results of recent studies are conflicting.
Aims: To determine the effect of genetic polymorphisms in the MBL2 gene on lung function and *P. aeruginosa* (PA) colonization in a cohort of 306 children and adults with CF.
Methods: The association between the MBL2 genotype and lung function and PA colonization was analyzed longitudinally using random effects models and Kaplan-Meier analysis, respectively.
Results: Up to 18 years of age, patients with MBL2 low-producer genotypes showed a significantly better lung function compared with those with wildtype (FEV_1 89.8 and 79.6% of predicted, respectively, P=0.02); this association was not found after 18 years (FEV_1 52.3 and 64.5%, respectively, P=0.2). Similarly, under 18 years but not thereafter, those with low-producer haplotypes had a smaller decline in FEV_1 compared with high-producer genotypes. MBL2 genotype was not associated with PA colonization.
Conclusions: In this group of children MBL low-producer genotypes are not associated with poor outcome, but rather with better lung function and a smaller decline in lung function. In adult CF patients no such association was found.

INTRODUCTION

There is considerable variation in the clinical course of disease in cystic fibrosis (CF), even among patients who are homozygous for the most common CFTR mutation, ΔF508 [1]. Environmental influences seem to only partially contribute to the heterogeneity of CF phenotype [2]. This suggests that genetic variation in genes other than CFTR influence CF phenotype, the so-called modifier genes [3-5].

One of these potential modifier genes is the MBL2 gene, encoding mannose-binding lectin (MBL). MBL is a serum protein participating in innate immune defence. In its functional oligomeric form, the protein binds to carbohydrates on the surface of microorganisms, initiating complement-mediated lysis and acting as an opsonin to facilitate phagocytosis [6]. MBL also influences the inflammatory response, but this mechanism is not yet fully elucidated [6].

In exon 1 of the MBL2 gene, 3 single-base substitutions at codon 54 (glycine with aspartic acid, allele B), at codon 57 (glycine with glutamic acid, allele C), and at codon 52 (arginine with cysteine, allele D) independently cause low serum levels of MBL [7]. These structural polymorphisms are commonly designated 0, as opposed to the wildtype allele named A, and reduce the amount of functional MBL subunits in heterozygous individuals 5- to 10-fold [7]. Furthermore, one of the nucleotide substitutions in the promoter region of the MBL2 gene, the G to C substitution at codon –221 (Y/X type), also has a significant downregulating effect on the MBL serum concentration [7,8].

In 1999, Garred *et al.* demonstrated an association between MBL2 low-producer genotypes and decreased lung function in CF [7], and consequently suggested MBL supplementation in MBL-deficient CF patients [9]. Subsequently, various studies reported conflicting data on the effects of MBL on lung function: in some, the association was only observed in subgroups [10,11], in others no associations were found [12,13], and in recent studies even the opposite was reported [14,15] as compared to the results of Garred *et al.* However, most of these studies were limited by the small numbers of subjects and their cross-sectional design.

Importantly, both the age of the patients and the time frame in which the study is performed may be relevant, since adult CF patients living nowadays have been treated differently during childhood than the current pediatric population. This may influence lung function and PA colonization, and may cause different effects of a modifier gene in both children and adults [14,15]. This might be avoided by analysis of the effects of a modifier gene by age and by time frame.

The aim of this study was to analyze the association between MBL2 genotypes and lung function and *P. aeruginosa* (PA) colonization in a large single-center population of patients with CF. Data were analyzed cross-sectionally and longitudinally, and effects were compared between children (<18 years) and adults (>18 years).

METHODS

Study subjects

The Cystic Fibrosis Center Utrecht of the University Medical Center Utrecht currently treats about 250 children (age under 18 years) and 100 adults (age 18 years or older) with CF (living in the central region of the Netherlands). All patients undergo a routine yearly multidisciplinary examination. Since 2000, all clinical and laboratory data are recorded in an electronic database.

Measurements

Standard deviation scores for height and weight were calculated using standard growth diagrams for the Dutch population [16]. The pulmonary function [forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC)] were measured by a pneumotachograph and converted to percentage of predicted values [17,18]. Sputum cultures were performed at least once a year in all patients. PA colonization was considered to be present when more than 50% of the PA cultures in the preceding 3 years were positive [19].

Based on their functional effects, CFTR mutations were divided in 5 classes [1]. Class I, II and III mutations result in a more severe CF phenotype compared with class IV and V [1].

MBL2 allele frequencies were compared with 281 unselected healthy adult controls [20]. All participants and/or their parents gave informed consent and the Medical Ethics Committee of the University Medical Center Utrecht approved the study.

Genotyping

Biallelic SNPs of the MBL2 gene were determined using sequence-specific primers (SSP) and polymerase chain reaction (PCR). The identification numbers of the SNP loci and the sequences of SNP-specific primers with their complementary consensus primers are shown in **Supplementary Table 9A**. The PCR conditions were as previously described [21].

Statistical analysis

Unpaired Mann-Whitney U and Kruskal-Wallis tests were used to compare not normally distributed continuous data, and unpaired T test and ANOVA to compare normally distributed continuous data. Chi-square and Fisher's exact tests were used to compare dichotomous variables.

To estimate the difference between the genotypes in rate of decline of FEV_1 we used a random effects model for repeated measurements (SAS version 9.1, PROC MIXED). A linear rate of decline was assumed. Genotype, age, an interaction term for these, gender, CFTR class (severe versus mild) and PA colonization were entered as covariates in the model. The intercept was specified as random effect to allow for individual differences [22].

Survival analysis with Kaplan-Meier log-rank statistics was used to compare the effects of genotype on age at PA colonization.

Haplotypes for the 4 SNP positions (A/BCD and X/Y) were estimated from unphased genotype data using the Baysian statistical method in PHASE 2.1 (http://www.stat.washington.edu/stephens/software.html) [23,24]. MBL haplotypes were defined as high, intermediate and low-producer haplotypes according to previous studies [7,15,25].

RESULTS

A total of 306 patients with available genomic DNA were included, which is about 90% of the total population of the CF Center Utrecht (**Table 1**); of these, 298 patients underwent pulmonary function testing. Of these 298 patients, a total of 1651 annual lung function recordings were available (mean of 5.5 recordings per patient). Longitudinal PA colonization data were available from 1982 onwards.
The frequencies of the A/A, A/0 and 0/0 genotypes were 61.8%, 33% and 5.2%, respectively. The frequencies of the X/X, X/Y and Y/Y genotypes were 61.1%, 34.0% and 4.9%, respectively. The frequency of MBL2 variant alleles at inclusion did not differ between CF patients and healthy controls (**Table 2**). The frequency of the

Table 1. Characteristics of 306 patients with CF as measured at the last visit to the outpatient clinic. Data are presented as mean (SD), unless otherwise specified.

Variable	All patients (N=306)	Homozygous ΔF508 (N=192)
Age (yr)		
Range	2.1 - 63.7	2.5 - 50.1
Median	15.0	14.6
Sex (% male)	52.6	52.1
Genotype:		
Homozygous ΔF508 (%)	62.7	100
Heterozygous ΔF508 (%)	32.1	-
Other (%)	5.2	-
Genotype classes:		
Class I-III (%)	81.4	100
Class IV-V (%)	10.5	-
Unknown (%)	8.2	-
FEV_1 (% of predicted value)	74.6 (26.2)	74.1 (25.7)
Height (SD score for age)	-0.73 (1.04)	-0.70 (1.05)
Weight (SD score for age)	-0.31 (1.02)	-0.43 (0.99)
P. aeruginosa colonization (%)	53.6	55.2

Table 2. Frequencies of MBL2 genotypes at inclusion in 306 CF patients and 280 healthy controls.

		CF patients		Controls	
		N	(%)	N	(%)
MBL2 A/0	A/A	189	61.8	166	59.3
	A/0	101	33.0	94	33.6
	0/0	16	5.2	20	7.1
MBL2 Y/X	Y/Y	187	61.1	169	60.3
	Y/X	104	34.0	98	35.0
	X/X	15	4.9	13	4.6

There was no significant difference in genotype distributions between patients and controls.

0/0 genotype was not significantly different between children (N=202) and adults (N=104) (4.5 and 6.7%, respectively).

First, the effect of the structural MBL2 polymorphisms on lung function was analyzed cross-sectionally. Overall, FEV_1 as measured at the last visit to the outpatient clinic did not differ significantly between the different MBL2 A/0 genotypes (**Table 3**). However, in those aged < 18 years FEV_1 was higher in patients with the MBL2 A/0 or 0/0 genotype compared with the A/A genotype (89.8% and 79.6%, respectively, P=0.02; **Table 4**). This difference was not observed at 18 years of age or older (52.3% vs. 64.5%, respectively, P=0.2). Also, in a subpopulation of ΔF508 homozygous patients, MBL2 was significantly associated with FEV_1 in children (A/0 or 0/0 genotype vs AA genotype: 89.9% vs. 80.0%, P=0.03), but not in adults (56.6% vs. 57.7%, P=0.3).

We next investigated the association of haplotypes consisting of the structural (A/0) and the X/Y promoter alleles together on lung function. When using the most common definition of high and low MBL producers [7], median FEV_1 in those with MBL low-producer haplotypes (A/A, YA/0) was 7.4% higher under the age of 18 years but 7.0% lower after 18 years of age, compared with high-producer haplotypes (XA/0, 0/0); however, these differences were not significant (P=0.79 and P=0.87, respectively).

Table 3. Median age, median FEV_1 and percentage PA colonization in the different MBL2 structural genotypes in 306 patients with CF.

Determinant	N	A/A	N	A/0	N	0/0	P-value
Age (years)	189	15.2	101	13.9	16	17.1	0.19
FEV_1 (% predicted)	184	75.8	98	80.6	16	87.3	0.29
P. aeruginosa colonization (%)	189	50.3	101	59.4	16	50.0	0.32

Table 4. Differences in median FEV_1 (% predicted) between MBL2 structural genotypes.

	A/A		A/0 and 0/0		
Population	**N**	**FEV_1 (%)**	**N**	**FEV_1 (%)**	**P-value**
All	184	75.8	114	82.5	0.17
Children	117	79.6	77	89.8	0.02
Adults	67	64.5	37	52.3	0.21
ΔF508/ ΔF508	116	75.5	70	83.8	0.11
No ΔF508/ ΔF508	68	76.8	44	80.0	0.81
Severe genotype	154	74.6	92	81.3	0.21
Mild genotype	19	77.1	13	87.7	0.29

When FEV_1 data were analyzed longitudinally in the total population of children and adults, patients with low-producer haplotypes [7] had a lower annual decline (0.99%) in FEV_1 compared with high-producer haplotypes (1.64%, (P=0.005). When only patients under 18 years were considered, the annual decline in FEV_1 was significantly less in patients with low producer haplotypes than in patients with high-producer haplotypes (1.15% and 2.15%, respectively, P=0.02). This difference was not observed in patients older than 18 years of age (-0.88% and 1.03%, respectively, P=0.7). When alternative definitions of MBL low and high producers [15,25] were used, similar associations between MBL2 haplotypes and FEV_1 were found as those obtained by using the definition of Garred *et al.* [7].

MBL2 A/0 genotypes were not associated with PA colonization at the time of the last visit. There was also no significant difference in the PA free survival period between the various MBL2 genotypes and haplotypes (*e.g.*, Cox proportional hazard of A/0 or 0/0 vs. AA=1.35, P=0.12). Also, patients with the A/0 or 0/0 genotype were not more frequently infected with *B. cepacia* (2.6% vs 2.1%) compared with the A/A genotype.

DISCUSSION

The present study in a large population of both children and adult CF patients suggests that, during childhood, MBL2 low-producer genotypes and low-producer haplotypes are associated with better lung function and a lower decline in lung function compared with high- producer genotypes and haplotypes, whereas this association is not seen at adult age.

The age-dependent effect of MBL on lung function could also explain some of the differences in outcome compared with earlier studies [7,26]. In the first two reports on MBL2 (in which children and adults were studied together), a lower lung function in patients with MBL2 low- producer genotypes and haplotypes was found [7,26]. Davies *et al.* analyzed children and adults separately and found a significant negative effect of the 0/0 genotype on lung function solely in adult patients [11]. Choi *et al.* investigated only adults, and found a lower lung function in patients both

with the A/0 or 0/0 genotype, but this proved to be significant only in a subgroup of patients homozygous for ΔF508 [27]. A more recent study on MBL serum concentrations seemed to confirm that MBL-deficient adult CF patients had a lower lung function, but this difference was not significant [14]. In the present study we also found a lower lung function in adult patients with low-producer genotypes, but (as in other studies) this was not significant.

In contrast with the observations in adults, but in line with a recent report by Olesen *et al.* [15], we found that pediatric patients with low-producer haplotypes had better lung function and less decline in lung function than patients with high-producer haplotypes. This also concurs with Muhlebach *et al.*, who described better lung function in pediatric patients with low MBL serum levels [14], and with Davies *et al.*, who found slightly higher FEV_1 values in children homozygous for structural MBL2 mutant alleles [11]. Two other studies in which children were analyzed separately found an opposite effect, but the results were not convincing [7,25]; in the study by Yarden *et al.* the association was only significant when using a cut-off value for FEV_1 of 90% [25] and in the study by Garred *et al.* the effect was significant at 16 years of age but not at 8 years of age [7]. Considering these latter studies and the present study, we postulate that MBL variant alleles seem to have a positive effect on lung function in children with current therapy.

Although MBL is supposed to protect against pathogens and to promote clearance of apoptotic cell debris, high concentrations of MBL may cause tissue inflammation through higher complement activation, as was suggested in the context of diabetes [28], primary biliary cirrhosis [29], and rheumatic heart disease [30]. One may hypothesize that children with high levels of MBL experience more severe lung inflammation after infection leading to a more rapid decline in FEV_1 values. MBL-insufficient children are, however, suggested to be more susceptible to infections and over time recurrent or prolonged infections may increasingly manifest as impaired lung function. This could result in poorer lung function in adulthood in MBL-insufficient patients

The time frame of the study population may also play a role. For example, the effect of MBL on lung function in adults observed in most later studies was not as strong as that found in that by Garred *et al.*, in which the median age was 16.2 (7.2-40.7) years at inclusion in 1989 [7,8,12,13,15]. This means that these latter patients have received a very different treatment from the current ones, at least during their childhood. Patients are now receiving improved treatment, living longer, and reaching more severe stages of disease at an older age. This means that historical data (*e.g.*, from the childhood of current adults) may not be safely compared or pooled with contemporary data (*e.g.*, of current children) [31]. Furthermore, one could hypothesize that with improved treatment strategies, the benefits of MBL (protection against different infections) may not outweigh the potential greater inflammation caused by high MBL levels [14]. Although MBL replacement therapy with plasma-derived MBL has become available and was once safely given to an MBL-insufficient CF patient [9], our data do not support the routine use of MBL replacement therapy in CF patients.

Most studies, including ours, did not find a significant association between MBL2 variants and PA colonization. However, one study found more PA colonization in MBL2 high-producers, whilst another small study found a later age at PA colonization in MBL2 high producers, but no difference in current colonization [8,10]. The apparent lack of effect of MBL2 variation on PA colonization could be explained by the fact that PA does not seem to bind to MBL [32], and deterioration of lung function may be independent of PA colonization.

Surprisingly, Garred *et al.* demonstrated significantly higher FEV_1 in A/A patients with PA colonization compared with those carrying variant alleles, but no significant difference was observed between the 2 genotypes in patients without PA colonization [7]. The authors postulate that MBL might play a role in the clearance or neutralization of PA-derived LPS or other toxic substances released from bacteria or that MBL may have a protective role against viral infections suggested to precede PA colonization and exacerbation. This may have become less relevant nowadays with modern anti-pseudomonal drugs.

Importantly, the MBL2 genotype does not seem to be a major determinant of survival, since in our population no significant difference was found in genotype distributions between the children and adults. Preferentially, we would have performed a Kaplan Meier analysis to analyze differences in survival between the MBL genotypes, but the follow-up time was too short. Two other studies that analyzed the effect of MBL2 polymorphisms on survival reported that variation in MBL2 is associated with reduced survival [7,13]. However, the older study found decreased survival in patients with both one and two variant alleles, whilst in the more recent study only the less common 0/0 MBL2 genotype appeared to influence survival. The question is whether these findings are relevant for current pediatric CF patients, since the clinical status of CF patients has fortunately improved considerably in recent years.

The present study has several limitations. First, although the number of patients was large for a single-center study, a multi-center study could have enabled a much larger study population and consequently more power. On the other hand, a single-center study reduces the influence of the center (*e.g.*, different treatment regimes) and increases data quantity and quality on each individual [31].

Second, we did not determine MBL serum levels. However, the effect of MBL2 genotypes and haplotypes on MBL serum levels in CF is well established [6,8,15]. The question is whether MBL2 genotype or serum levels should be used in association studies. MBL level may vary 10-fold within identical genotypes [7], making serum concentration more suitable. On the other hand, MBL is known as a mild acute phase protein, resulting in varying concentrations over time [33].

Third, in the present study not all genetic variants in the MBL2 gene (*e.g.*, H/L and P/Q) were determined. However, previous analyses of the 'secretor haplotypes' have detected that the X/Y promoter polymorphism in combination with the exon 1 polymorphisms (A/0) has the most significant impact on the MBL serum level [34]. Extending the number of haplotypes would result in a smaller number of patients per haplotype and consequently lower statistical power.

A Cochrane meta-analysis of all data available from the different studies would be desirable, but is currently not realistic in view of the different populations, determinants and outcomes. For reliable results the future gold standard should be carefully designed, with prospective studies, recruiting children at diagnosis and following them up for their lifetime with regular monitoring of well-defined outcome measures. Such studies are logistically complex and will yield results only in the medium to longer term [31].

In conclusion: many studies on variation in MBL on CF pulmonary phenotype have been published but with conflicting results, mainly due to differences in the age of patients, timing of the study, patient populations, definitions of MBL2 haplotypes, follow-up and definitions of the outcomes.

Based on our own results and previous reports, we conclude that low MBL seems to be associated with worse lung function in older cohorts of CF patients and in adult CF patients. In contrast, low MBL in children currently alive seems to have a positive effect on CF phenotype, since our study confirmed other recent studies demonstrating that children with low MBL have a somewhat better lung function. However, these findings do not support the use of MBL substitution therapy [9].

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Chapter 10

Lung function and *Pseudomonas* colonization in cystic fibrosis; The role of genetic variation in the Toll pathway

Submitted

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ABSTRACT

Background: Variation in cystic fibrosis (CF) lung disease correlates poorly with the genotype of the CF transmembrane regulator and with environmental factors, suggesting an important role for so-called modifier genes. One class of potential modifiers include toll-like receptors (TLRs). TLRs recognize molecular patterns on a variety of micro-organisms including *P. aeruginosa* (PA), and play a prominent role in the activation of innate immunity and promotion of inflammation.
Aims: To analyze the association between polymorphisms in TLR genes and CF lung disease in a large single-center population of CF patients.
Methods: A retrospective cohort study was performed in 249 patients with a severe CFTR genotype (53.4% males, median age 11.9 years, range 2.2-50.1 years). Two single nucleotide polymorphisms (SNPs) in the TLR4 gene, four in the TLR2 gene and two in the CD14 gene were determined. Annual lung function and PA colonization data were available from 2000 and 1992 onwards, respectively. Outcomes were analyzed both cross-sectionally and longitudinally.
Results: Patients with the TLR4 8551 AG (D299G) genotype had a 13.2% higher FEV_1 (95% CI: 2.4-24.1%) and tended to have less PA colonization (odds ratio 0.50, 95% CI 0.22-1.11) than patients with the AA genotype. None of the TLR2 and CD14 polymorphisms were significantly associated with lung function or PA colonization.
Conclusions: Variant alleles at position 8551 of the TLR4 gene have a protective effect on lung function in CF, possibly due to decreased inflammation. TLR2 and CD14 SNPs do not seem to influence lung function and PA colonization in patients with CF.

INTRODUCTION

There is considerable variation in the clinical course of disease in patients with cystic fibrosis (CF). Some of this heterogeneity can be attributed to differences in mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, but even CF patients who are homozygous for the most common CFTR mutation, ΔF508, show great variability in the clinical course of CF [1]. Environmental influences, like socioeconomic status, drug compliance and nutritional status, only partially contribute to the heterogeneity of CF phenotype, suggesting that genetic variation in genes other than CFTR contribute to the clinical course [2]. The total contribution of these so-called modifier genes to variation in CF lung disease severity, independent of CFTR genotype, was estimated to range from 54 to100% [3].

The lethal lung pathology in CF is caused by recurrent infections, primarily with *P. aeruginosa* (PA). Many studies have shown that infections in CF result in inflammatory responses which occur early in life, and are severe and sustained [4], leading to progressive destruction of pulmonary tissues. Any genetic variability that interferes with the infection-inflammation cascade might therefore influence lung disease progression in CF.

One class of potential modifiers of disease progression in CF includes genes encoding toll-like receptors (TLRs). TLRs recognize different pathogen associated molecular patterns (PAMPs) and have prominent roles in the activation of innate and adaptive immune responses to infection [5]. TLR4 is the main receptor for lipopolysaccharide (LPS) from Gram-negative bacteria such as *P. aeruginosa* (PA), whereas TLR2 interacts with a series of other bacterial ligands, including lipopeptides, peptidoglycan, and lipoteichoic acid of Gram-positive bacteria [6,7]. One of the co-receptors of the TLR4 receptor complex, CD14, is also a high-affinity LPS-binding protein [6,7].

In non-CF populations, associations have been described between TLR4 [8-10], TLR2 [11,12], and CD14 [13,14] single nucleotide polymorphism (SNPs) and susceptibility to bacterial infections, as well as inflammatory entities like asthma and atherosclerosis.

In CF patients, one relatively small study recently demonstrated an association between a SNP in the CD14 gene and age at PA colonization [15], while another study could not demonstrate an association between a SNP in TLR4 and lung function and PA colonization in CF [16]; however, extensive evaluation of the role of TLR polymorphisms is lacking so far.

We speculate that variant alleles in TLR can result in damping of the inflammatory cascade in CF, which may have a beneficial effect on disease progression. Therefore, we studied the effects of polymorphisms on PA colonization and longitudinal development of lung function in a large single-center population of CF patients.

METHODS

Study subjects

The Cystic Fibrosis Center Utrecht of the University Medical Center Utrecht (Utrecht, the Netherlands) treats about 250 children and 100 adults with CF living in the central region of the Netherlands.

CFTR mutations were determined in all patients and divided in 5 classes, based on their functional effects [1]; Class I, II and III mutations result in a more severe CF phenotype compared with class IV and V [1]. In order to include a large but homogenous population, only patients homozygous or compound heterozygous for class I, II and III mutations were included in the present study.

SNP allele frequencies were compared with a control population, consisting of 281 healthy participants from a Dutch birth cohort study [17]. All participants and/or their parents gave informed consent and the Medical Ethics Committee of the University Medical Center Utrecht approved the study.

Outcome measures

Since 1998, all patients undergo a routine yearly multidisciplinary examination, including PA culture and lung function measurements. The pulmonary function (forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC)) were measured by a pneumotachograph system and converted to percentage of predicted values [18,19]. PA colonization was determined by sputum culture and considered to be present when more than 50% of the PA sputum cultures in the preceding 3 years were positive [20].

Standard deviation scores for height and weight were calculated using standard growth diagrams for the Dutch population [21].

Genotyping assays

Biallelic SNPs were determined using sequence-specific primers (SSP) and polymerase chain reaction (PCR). The identification numbers of the SNP loci and the sequences of SNP-specific primers with their complementary consensus primers are shown in **Supplementary Table 10A**. The PCR conditions were as previously described [22]. Two SNPs in the TLR4 gene were determined, four in the TLR2 gene, and two in the CD14 gene.

Because of difficulties in primer design for SSP-PCR of the TLR4 8551A/G SNP (rs4986790, D299G), we performed melting curve analysis to detect this polymorphism using a pair of fluorescence resonance energy transfer (FRET) probes and the LightCycler (Roche Diagnostics, Basel, Switzerland) [23]. Oligonucleotide sequences used for PCR amplification of rs4986790 were F- AAGAAATTAGGCTTCATAAGC and R-CCAAGAAGTTTGAACTCATGGTAA. The sensor probe was ACTACCTC-GATGGTATTATTGACTTATT-FL and the anchor probe was LC640-AATTGTTT-GACAAATGTTTCTTCATTTTCC-ph.

Statistical analysis

Unpaired Mann-Whitney U and Kruskal-Wallis tests were used to compare not normally distributed continuous data and the unpaired T test and ANOVA to compare normally distributed continuous data. In order to test if continuous variables were normally distributed, a Kolmogorov-Smirnov test was performed. Chi^2 and Fisher's exact tests were used to compare dichotomous variables.

The Hardy-Weinberg equilibrium was evaluated using the chi^2 test.

SNPs that were univariately associated with lung function or PA colonization ($P<0.15$) were included in a multiple linear regression model and multivariate logistic regression, respectively, to adjust for potential confounders such as age, sex and CFTR mutation class.

To analyze the effect of the SNPs on FEV_1 longitudinally, we used a random effects model for repeated measurements (SAS version 9.1, PROC MIXED). A linear rate of decline was assumed. SNP, age, an interaction term for these, gender, CFTR class (severe versus mild) and PA colonization were entered as covariates in the model. The intercept was specified as a random effect to allow for individual differences [24].

Survival analysis with Kaplan-Meier log-rank statistics was used to compare the effects of the polymorphisms on age at PA colonization.

RESULTS

Patient population

In total, 306 six patients (age 2.1 - 63.7 years, 53.4% males) were enrolled in the study.

CFTR genotype class was classified as severe (I-III), mild (IV-V) and unknown in 249, 32 and 25 patients, respectively. Patients with a severe genotype were significantly younger at the time of the last visit to the outpatient clinic compared with patients with a mild genotype (median age 14.4 and 15.5 years, respectively, $P=0.0001$). In order to include a large but homogenous population, only the 249 patients with a severe CFTR genotype were further analyzed.

The patient characteristics of the 249 patients with severe CFTR genotype are summarized in **Table 1**. The observed allele frequencies of the TLR4, TLR2 and CD14 SNPs in patients and healthy controls are given in **Table 2**. None of the allelic distributions differed significantly between patients and controls, and all allelic distributions were in Hardy-Weinberg equilibrium. Furthermore, allelic distributions of TLR2, TLR4 and CD14 SNPs did not differ between patients with mild and severe CFTR genotype classes (data not shown).

TLR4

There was a strong linkage between TLR4 8551 A/G (D299G) and 8851 C/T (T399I) (98.3%). Since the 8551 A/G mutation, but not the 8851 C/T mutation, was shown to

Table 1. Characteristics at last visit of 249 patients with CF having a severe CFTR genotype (homozygous or compound heterozygous class I, II or III mutation).

Variable	
Median age in yrs (range)	11.9 (2.2 – 50.1)
Sex (% male)	53.4
CFTR genotype:	
ΔF508/ ΔF508 (%)	77.1
ΔF508/ other (%)	20.9
Other/ other (%)	2.0
CFTR mutation class:	
Class I (%)	20.5
Class II (%)	77.1
Class III (%)	2.4
FEV_1 (% of predicted value)	73.6 ± 26.3
Height (SD score for age)	-0.75 ± 1.05
Weight (SD score for height)	-0.40 ± 0.95
P. aeruginosa colonization (%)	54.5

Data are presented as mean (SD), unless otherwise specified.

Table 2. Genotype frequencies of TLR4, TLR2 and CD14 in 249 CF patients with a severe CFTR genotype and 281 healthy controls.

Gene	Variant	Amino Acid Change	Reference SNP	Genotype	Patients (%)	Controls (%)	Genotype	Patients (%)	Controls (%)	Genotype	Patients (%)	Controls (%)
TLR4	8551 A/G	D299G	rs4986790*	AA	88.4	87.6	AG	11.2	12.2	GG	0.4	0.2
	8851 C/T	T399I	rs4986791	CC	88.0	89.3	CT	11.6	10.0	TT	0.4	0.7
TLR2	-16934 A/T		rs4696480	AA	20.5	29.9	AT	54.2	48.0	TT	25.3	22.1
	1892 C/A	P631H	rs5743704	CC	91.6	92.5	CA	8.4	7.1	AA	0.0	0.4
	2258 G/A	R753Q	rs5743708	GG	92.4	95.0	GA	7.6	5.0	AA	0.0	0.0
CD14	-550 C/T		rs5744455	CC	66.3	66.9	CT	28.9	29.9	TT	4.8	3.2
	-159 C/T		rs2569190	CC	24.1	26.9	CT	51.0	51.9	TT	24.9	21.2

There was no significantly different distribution of allelic frequencies between patients and controls.
* Determined in 236 patients.

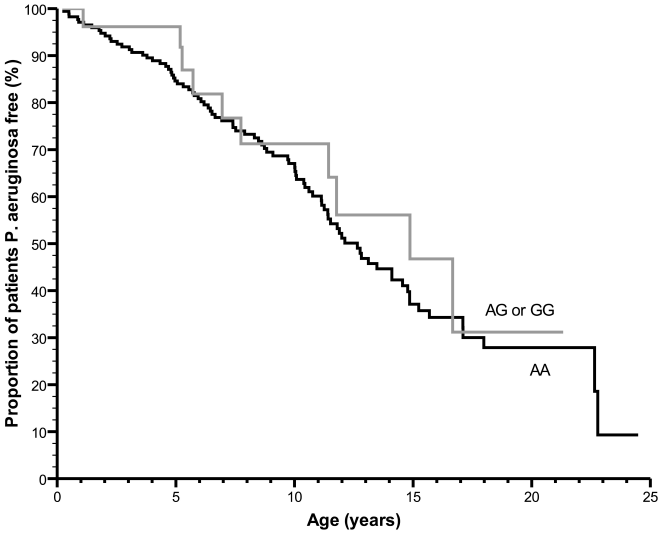

Figure 1. Differences in cumulative *P. aeruginosa* (PA)-free survival between CF patients with a severe CFTR genotype born after 1982 with the TLR4 8551 AA and AG/GG genotype. Cox proportional hazard P=0.49.

interrupt TLR4 mediated signaling in vitro [8], and because of the strong linkage between the two SNPs, further analyzes were performed on 8551 A/G.
At their last visit to our outpatient clinic, patients with the TLR4 8551 AG genotype had a 13.2% higher FEV_1 (95% confidence interval (CI): 2.4-24.1%) than patients with the AA genotype (**Table 3**). This association remained significant after correction for age and gender (difference in FEV_1 9.5%, 95% CI 0.8-18.1%). Also, when lung function data were analyzed longitudinally, patients with a variant allele at position 8551 had a 9.0% higher FEV_1 compared with a wildtype genotype (95% CI 1.6-16.3%).
Patients with one or two variant alleles at position 8551 tended to have less PA colonization compared with patients with a wildtype genotype (OR 0.50, 95% CI 0.22-1.11) (**Table 4**). However, this association was not significant after correction for age and gender (odds ratio (OR) 0.59, 95% CI 0.24-1.46). Also, the age at first PA colonization did not differ between patients with the AA and with the AG or GG genotype (**Figure 1**).

TLR2

The TLR2 -16934 A/T polymorphism appeared to be significantly associated with lung function (**Table 3**). However, this association was not very plausible since the heterozygotes had the highest FEV_1. The association was not significant after correction for age and gender. Furthermore, the difference in lung function between patients with the AA and TT genotype was not significant. None of the other investigated TLR2 polymorphisms were associated with lung function (**Table 4**). Also,

Table 3. FEV_1 (% predicted) at last visit for different TLR4, TLR2 and CD14 genetic polymorphisms in 242 CF patients with a severe CFTR genotype.

Gene	Variant	Genotype	FEV_1 (%)	Genotype	FEV_1 (%)	Genotype	FEV_1 (%)	P-value[a]
TLR4	8551 A/G[b]	AA	72.5	AG	85.7	GG	[c]	0.02
	8851 C/T	CC	72.7	CT	80.9	TT	[c]	0.13
TLR2	-16934 A/T	AA	68.4	AT	77.7	TT	69.1	0.03
	1892 C/A	CC	73.8	CA	71.3			0.7
	2258 G/A	GG	73.3	GA	77.2			0.5
CD14	-550 C/T	CC	73.1	CT	75.5	TT	68.5	0.7
	-159 C/T	CC	74.4	CT	74.6	TT	70.7	0.6

[a] Student's t-test or ANOVA.
[b] Determined in 236 patients.
[c] No patient with two variant alleles underwent lung function testing.

Table 4. Colonization with *P. aeruginosa* at last visit for different TLR4, TL2 and CD14 genetic polymorphisms in 249 CF patients with a severe CFTR genotype.

Gene	Variant	Genotype	PA (%)	Genotype	PA (%)	Genotype	PA (%)	P-value[a]
TLR4	8551 A/G	AA	56.5	AG	39.3	GG	[b]	0.08
	8851 C/T	CC	56.6	CT	40.0	TT	[b]	0.09
TLR2	-16934 A/T	AA	52.9	AT	53.3	TT	58.7	0.7
	1892 C/A	CC	55.3	CA	47.6			0.5
	2258 G/A	GG	55.2	GA	47.4			0.5
CD14	-550 C/T	CC	55.2	CT	51.4	TT	66.7	0.6
	-159 C/T	CC	50.0	CT	54.3	TT	59.7	0.6

[a] Chi-square test
[b] Because only one patient had two variant alleles, data on patients with one and two variant alleles were analyzed together.

none of the investigated TLR2 polymorphisms were associated with PA colonization at last clinical visit or age at first colonization.

CD14

Neither of the investigated CD14 polymorphisms were found to be associated with lung function or lung function decline (**Table 3**). In contrast to a previous study [15], CD14 159C/T was not associated with PA colonization at last visit (**Table 4**) or age at first PA colonization (Cox proportional hazard: P=0.38).

DISCUSSION

This study is the largest study so far on the effect of polymorphisms in genes of the toll pathway on lung function and PA colonization in patients with CF. We used a large, clinically well-defined, single-center patient population with a severe CFTR genotype and performed both cross-sectional and longitudinal analyzes.
We demonstrated that the TLR4 8551A/G (D299G) polymorphism was associated with lung function independent of PA colonization. No associations with lung function or PA could be demonstrated for the other investigated TLR2 and CD14 SNPs.
Our results are in contrast with a recent cross-sectional study in CF that could not demonstrate an association between the TLR4 8551A/G polymorphism and lung function; however, this latter study was relatively small (N=100), and patients with a mild CFTR genotype were not excluded [16].
The beneficial effect of the TLR4 8551A/G polymorphism on lung function could be explained by previous functional studies in humans, which showed an effect of the TLR4 8551A/G polymorphism on the cytokine response to endotoxins [8,9]. Thus, carriage of the TLR4 8551G allele may lead to a blunted response to LPS [8,9]. In patients with infections, this would result in dampened cytokine activation and a lower subsequent inflammatory response, leading to less parenchymal lung damage.
A beneficial effect of variation in genes encoding proteins involved in the innate immunity in CF has already been described for MBL [25,26]. Although MBL protects against pathogens and promotes clearance of apoptotic cell debris, high concentrations of MBL may enhance tissue inflammation through higher complement activation [26]. This is supported by studies that found a better lung function in CF patients who were MBL deficient [25,26]. The authors hypothesized that patients with high levels of MBL (*i.e.*, not MBL deficient) experience more severe lung inflammation leading to a more rapid decline in FEV_1 values. In parallel, high TLR4 expression may result in more severe lung inflammation and consequently poorer lung function.
PA is considered to be one of the most important determinants for deterioration of lung function [27,28]. We could not detect significant differences in PA colonization between patients with variant alleles at position 8551 compared with patients with wildtype genotypes, but the percentage of PA colonization in the variant group was slightly less with 39.3% of the patients being colonized compared with 56.5% in the wildtype group. However, it not unlikely that the increased PA colonization is caused by, rather than the cause of, increased lung disease. This mutual influence of poor lung function and PA colonization has been described frequently [4], but the origin and trigger points of this vicious circle remains to be resolved.

In our population none of the three investigated TLR2 polymorphisms was associated with lung function or PA colonization. In an in vitro study, PA appeared to be signaled through TLR2 in airway cells, despite the presence of TLR4 in these cells [29]. Contrary to TLR4, TLR2 is available on the apical surface of respiratory epithelial cells [29]. However, a recent study demonstrated unimpaired TNF-α re-

sponses of TLR2-deficient bone marrow cells to PA compared with wildtype cells [6]. Also in vivo, TLR2-deficient mice had preserved bacterial killing of PA [6]. This may indicate that TLR2 does not have an important role in host defense against PA colonization.

Also in our study, the two investigated CD14 SNPs were not associated with lung function. Contrary to a recent Australian study, we did not detect an association between the CD14 -159C/T polymorphism and PA-free survival [15]. This could be explained by the fact that the Australian study population was small (N=45) and young (mean age 3 years), and patients with a mild CFTR genotype were not excluded. However, when we included all CFTR genotypes and used a maximal follow-up time of 6 years, similar to the Australian study, no significant differences between the CD14 alleles were found (data not shown).
In other studies, the -159C/T was found to be associated with enhanced transcriptional activity and increased soluble CD14 levels [13,14]. High membrane-bound and soluble CD14 levels are positively associated with the magnitude of airway neutrophil response to LPS [30]. However, in most epidemiological studies in non-CF populations, the CD14 -159C/T does not substantially increase the risk of Gram-negative infections [31,32]. Based on our data, we cannot confirm earlier data that reported CD14 to play a significant role in CF phenotype.

The present study has several limitations. First, although the number of patients was large for a single-center study, a multi-center study could have resulted in a much larger study population and consequently more power. On the other hand, a single-center study reduces 'center' influences (*e.g.* treatment regimes) and increases data quantity and quality for each individual in terms of comparability between patients [33].
Second, we included all patients homozygous or compound heterozygous for class I, II or III mutations and not only patients homozygous for the ΔF508 mutation. Although this made our population more genetically heterogeneous, the results did not significantly differ when analyzes were performed for the ΔF508 homozygous patients only (data not shown). Perhaps it might have been better to study only patients with class IV and V mutations for modifier gene effects, since a TLR gene might have more impact on severity in patients with mild CFTR mutations; however, the number of patients (N=32) was too small to perform these analyzes.
Third, we did not include all known SNPs and all TLR genes possibly involved in modifying the course of CF lung disease. For instance, the flagella of PA is also known to be a virulence factor and is recognized by TLR5, rather than TLR4 [34]. In vivo and ex vivo studies showed an important role for TLR5-TLR4 cooperation in the recognition and immune response to PA [34]. Whether correction for genetic variation in the TLR5 gene would result in a significant effect of TLR4 on PA colonization, remains to be elucidated.

In conclusion, this large single-center study in CF patients has shown a significant association between the presence of one or two variant alleles at position 8551 of the TLR4 gene and better lung function, but not with PA colonization. It is hypothesized that the endotoxin hyporesponsiveness caused by the SNP results in dampened cytokine activation and less subsequent parenchymal lung damage. Currently investigated TLR2 and CD14 genes do not seem to influence lung function and PA colonization in patients with CF.

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Chapter 11

Interleukin-6 gene promoter polymorphism affects lung function and Pseudomonas colonization in cystic fibrosis

Submitted

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ABSTRACT

Background: Airway disease in cystic fibrosis (CF) is characterized by chronic bacterial lung infections, increased inflammation, and dysregulated interleukin (IL) production. Single nucleotide polymorphisms (SNPs) may influence the transcription of the IL genes, and may affect anti-bacterial defense and inflammatory responses.
Aims: To analyze the association between polymorphisms in IL genes and CF lung disease in a large single-center population of CF patients.
Methods: A retrospective cohort study was performed in 306 patients with CF (52.6% males, median age 15.0 years, range 2.2-63.7 years). SNPs in the IL-6, IL-8, CXCR-1 and CXCR-2 and IL-10 genes were determined. Annual lung function and *P. aeruginosa* (PA) colonization data were available from 1997 and 1982 onwards, respectively. Outcomes were analyzed both cross-sectionally and longitudinally.
Results: Patients with the IL-6 -174 GC and CC genotype had a 6.3% and 9.9% higher FEV_1 than patients with the GG genotype, respectively (P=0.04). These variant alleles were associated with better longitudinal lung function measurements, lower age at PA colonization, and lower serum total IgG levels. None of the investigated IL-8, CXCR-1 and CXCR-2 and IL-10 polymorphisms were found to be associated with lung function, PA colonization or IgG levels.
Conclusions: The IL-6 -174 G/C promoter polymorphism seems to affect pulmonary outcome and PA colonization in CF. The more intense inflammatory response caused by this SNP may result in more damage to the airway surface, which would favor colonization by PA, and lead to poorer lung function. SNPs in IL8 (receptor) and IL-10 genes do not seem to be major determinants of lung function and PA colonization in CF.

INTRODUCTION

Airway disease in cystic fibrosis (CF) is characterized by persistent bacterial infections and a neutrophil dominated inflammatory response [1]. Chronic infection with *P. aeruginosa* (PA) causes ongoing inflammation of lung tissue with destruction of the airways and decline of lung function [2]. CF is also known to fail to eradicate PA colonization in spite of a high antibody response to PA, both locally in the lungs and systemically [3]. Besides the failing adaptive immunity, components of the innate immunity may play a role in both this failure of anti-pseudomonal eradication, as well as the dysregulation of inflammatory responses [3].
In CF patients, many cytokines may be involved in the inflammation and dysregulated responses; concentrations of pro-inflammatory cytokines such as interleukin-6 (IL-6) and IL-8 were found to be elevated in the sputum and bronchoalveolar lavage fluid (BALF) of patients with CF compared to healthy controls [4]. On a genetic level, the synthesis of IL-6 and IL-8 is controlled by the transcription factor nuclear factor-κB (NF-κB) [5]. Anti-inflammatory cytokines, including IL-10, were observed to be relatively downregulated in CF airway cells [6]. The principle action of IL-10 is to increase the synthesis of I-κB, the inhibitor of NF-κB. Downregulation of IL-10 leads to increased pro-inflammatory cytokines due to less inhibition of NF-κB actions [5].
The total contribution of genetic variations influencing the course of CF disease, independent of CFTR genotype, was estimated to be relatively high and to range from 54-100% [7]. These so-called modifier genes may modify anti-bacterial defense and inflammatory responses in the lung, leading to variation in CF lung disease severity, despite a similar CFTR genotype. Single nucleotide polymorphisms (SNPs) have been demonstrated to regulate the transcription rate of a number of IL genes, and IL genes could therefore act as CF modifier genes.
In the current study, we investigated whether SNPs in IL genes might result in differences in PA colonization or in differences in lung damage after PA colonization. Therefore, the most frequently published functional SNPs in IL-6, IL-8, 2 IL-8 receptors (CXCR-1 and CXCR-2) and IL-10 genes were studied in relation to lung function and PA colonization in CF patients. Over 300 patients from a single center were included and data were analyzed both cross-sectionally and longitudinally.

METHODS

Patients

The Cystic Fibrosis Center Utrecht of the University Medical Center Utrecht (Utrecht, the Netherlands) currently provides clinical care for about 250 children and 100 adults (age 18 years or older) with CF living in the central region of The Netherlands. All patients undergo a standardized yearly multidisciplinary examination.
Standard deviation scores for height and weight were calculated using standard growth diagrams for the Dutch population [8]. The pulmonary function (forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC)) were measured

by a pneumotachograph and converted to percentage of predicted values [9,10]. PA colonization was considered to be present when more than 50% of the PA cultures in the preceding 3 years were positive [11]. Sputum cultures were performed at least once a year. In all pediatric patients, total serum IgG levels were determined annually.
Based on their functional effects, CFTR mutations were divided in 5 classes [12]. Class I, II and III mutations result in a more severe CF phenotype compared with class IV and V [12].
In total, 306 patients with available genomic DNA were included and 298 patients underwent pulmonary function testing. Of these patients, a total of 1,651 annual lung function recordings were available (mean of 5.5 recordings per patient) from 1997 onwards. Longitudinal PA colonization data were available from 1982 onwards (235 patients).
All participants and/or their parents gave informed consent and the Medical Ethics Committee of the University Medical Center Utrecht approved the study.

Genotyping

Per gene the most frequently published SNP was chosen. Biallelic SNPs were determined using sequence-specific primers (SSP) and polymerase chain reaction (PCR). The identification numbers of the SNP loci and the sequences of SNP-specific primers with their complementary consensus primers are shown in **Supplementary Table 11A**. The PCR conditions were as previously described [13]. Allele frequencies of the IL gene polymorphisms were compared with those of a previously published control population [14].

Statistical methods

Unpaired Mann-Whitney U and Kruskal-Wallis tests were used to compare not normally distributed continuous data, and unpaired T test and ANOVA to compare normally distributed continuous data. Chi-square and Fisher's exact tests were used to compare dichotomous variables. Genotypes were tested for Hardy–Weinberg equilibrium (HWE) by using the chi-square test.
To analyze the difference in FEV_1 between the genotypes longitudinally we used a random effects model for repeated measurements (SAS version 9.1, PROC MIXED). A linear rate of decline was assumed. Genotype, age, an interaction term for genotype and age, gender, CFTR class (severe versus mild) and PA colonization were entered as covariates in the model. The intercept was specified as random effect to allow for individual differences [15].
Survival analysis with Kaplan-Meier log-rank statistics was used to compare the effect of genotype on age at first PA colonization.
Haplotypes were estimated from unphased genotype data using the Bayesian statistical method in PHASE 2.1 (http://www.stat.washington.edu/stephens/software.html) [16,17].

RESULTS

A total of 306 patients were included, which is about 90% of the total population of the CF Center Utrecht (**Table 1**). The allele frequencies of the tested IL SNPs are given in **Table 2**. All alleles were in Hardy-Weinberg equilibrium and did not differ from the control population [14].
In the cross-sectional analysis, the IL-6 -174 G/C promoter polymorphism was found to be significantly associated with FEV_1 at last visit (**Table 3**). Patients with the GC or CC genotype had a 6.3% and 9.9% higher FEV_1 than patients with the GG genotype, respectively (P=0.04). Also, after correction for age and gender, an additive effect of the C allele on FEV_1 was found (ΔFEV_1 (SD) per C allele: +4.0% (1.9%), P=0.03). This association was even more outspoken in the longitudinal analysis, evaluating 1651 lung function measurements. Correcting for age and gender, patients with the GC and CC genotype had a 3.5% (3.1%) and 9.7% (2.4%) higher FEV_1 (SE), respectively, than patients with the GG genotype (P=0.008).
The presence of one or two C alleles was also associated with a lower degree of PA colonization at last visit (**Table 4**). Furthermore, PA-free survival in patients born af-

Table 1. Characteristics of 306 patients with CF participating in the study as measured at the last visit to the outpatient clinic.

Variable	
Age (yr)	
Range	2.1 - 63.7
Median	15.0
Sex (% male)	52.6
Genotype:	
Homozygous ΔF508 (%)	62.7
Heterozygous ΔF508 (%)	32.1
Other (%)	5.2
Genotype classes:	
Class I-III (%)	81.4
Class IV-V (%)	10.5
Unknown (%)	8.2
FEV_1 (% of predicted value)	74.6 (26.2)
Height (SD score for age)	-0.73 (1.04)
Weight (SD score for age)	-0.31 (1.02)
P. aeruginosa colonization (%)	163 (53.3)
ABPA (%)	33 (10.8)

Data are presented as mean (SD), unless otherwise specified.
FEV_1 indicates forced expiratory volume in one second; and ABPA, allergic bronchopulmonary aspergillosis.

Table 2. Genotype frequencies of IL genetic polymorphisms in 306 CF patients.

Gene	Variant	Position	Reference SNP	N	Genotype	Frequency (%)	Genotype	Frequency (%)	Genotype	Frequency (%)
IL-6	Promoter	-174 G/C	rs1800795	306	GG	109 (35.6)	GC	142 (46.4)	CC	55 (18.0)
IL-8	Intron	678 C/T	rs2227306	306	CC	112 (36.6)	CT	137 (44.8)	TT	57 (18.6)
CXCR-1	S276T	2607 G/C	rs2234671	306	GG	271 (88.6)	GC	34 (11.1)	CC	1 (0.3)
CXCR-2	Exon	1208 T/C	rs1126579	306	TT	79 (25.8)	CT	150 (49.0)	CC	77 (25.2)
IL-10	Promoter	-1082 A/G	rs1800896	306	AA	95 (31.0)	AG	142 (46.4)	GG	69 (22.5)

All alleles were in Hardy-Weinberg equilibrium

Table 3. FEV_1 (% predicted) at last clinical visit for different interleukin genetic polymorphisms in 306 CF patients.

Gene	Position	Genotype	FEV_1	Genotype	FEV_1	Genotype	FEV_1	P (ANOVA)	P (LR)
IL-6	-174 G/C	GG	69.6	GC	76.3	CC	79.8	0.04	0.01
IL-8	678 C/T	CC	74.4	CT	74.2	TT	75.6	0.94	0.82
CXCR-1	2607 G/C	GG	75.0	GC	71.0	CC	-*	0.39	0.39
CXCR-2	1208 T/C	TT	78.4	TC	73.5	CC	72.6	0.31	0.17
IL-10	-1082 A/G	AA	74.7	AG	74.1	GG	75.3	0.96	0.92

ANOVA indicates analysis of variance (no model assumed); and LR, linear regression (additive effect assumed).
*Because only one patient had two variant alleles, lung function data of patients with one and two variant alleles were pooled.

ter 1982 was also significantly higher in patients with the CC and GC genotype compared with the GG genotype (**Figure 1**). Finally, patients with the CC and GC had 1.42 (0.64) and 1.21 (0.50) g/L lower total serum IgG levels, respectively, compared with the IgG level of 12.19 (4.44) found in patients with the GG genotype (P=0.02). FEV_1, PA colonization and IgG levels were highly correlated (Pearson's correlation coefficient for all correlations: P<0.001).

With respect to the other SNPs in the IL-8, IL-8 receptors or IL-10 genes, no single polymorphism was found to be significantly associated with FEV_1 and PA colonization. This holds true for both the cross-sectional as well as longitudinal analyses. Furthermore, none of these SNPs was associated with total serum IgG levels.

Table 4. Colonization with *P. aeruginosa* (PA) at last clinical visit for different interleukin genetic polymorphisms in 306 CF patients.

Gene	Position	Genotype	PA positive (%)	Genotype	PA positive (%)	Genotype	PA positive (%)	P (χ2)	P (CA)
IL-6	-174 G/C	GG	67 (61.5)	GC	70 (49.3)	CC	26 (47.3)	0.10	0.05
IL-8	678 C/T	CC	26 (45.6)	CT	75 (54.7)	TT	62 (55.4)	0.44	0.29
CXCR-1	2607 G/C	GG	144 (53.1)	GC	19 (54.3)	CC	-*	0.90	
CXCR-2	1208 T/C	TT	40 (50.6)	TC	79 (52.7)	CC	44 (57.1)	0.70	0.42
IL-10	-1082 A/G	AA	50 (52.6)	AG	81 (57.0)	GG	32 (46.4)	0.34	0.51

x^2 indicates chi-square test (no model assumed); FDR, false discovery rate; and CA, Cochran Armitage trend test (additive effect assumed).

*Because only one patient had two variant alleles, PA colonization data in patients with one and two variant alleles were pooled.

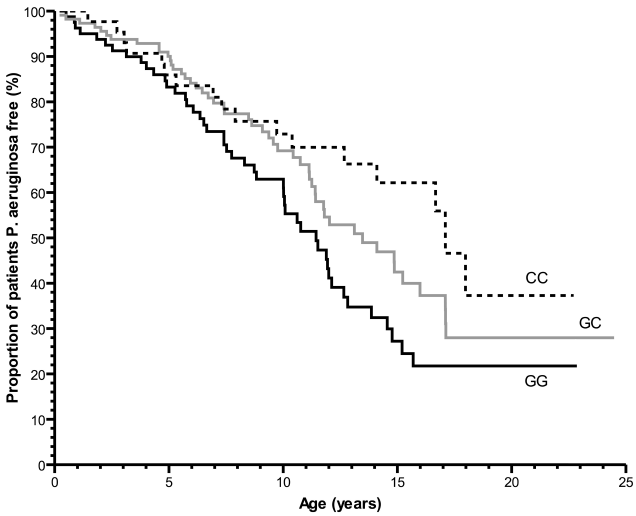

Figure 1. Effect of IL-6 -174 G/C polymorphism on PA free survival in CF patients born after 1982. Cox proportional hazard P=0.03.

DISCUSSION

This large single-center study demonstrated that the IL-6 -174 G/C polymorphism is associated with better lung function, less colonization with PA and lower total serum IgG levels. In contrast, the investigated SNPs in the IL-8, IL-8 receptors or IL-10 genes do not seem to be associated with lung function, PA colonization and IgG levels in CF patients.

Although we are aware that multiple testing could have resulted in false-positive associations, the IL-6 -174 G/C promoter polymorphism was, however, associated with all outcome parameters investigated (lung function, *Pseudomonas* colonization and IgG levels) in an additive fashion, when analyzed both cross-sectionally and longitudinally.

The number of patients was large for a single-center study, but a multi-center study could have resulted in a much larger study population and consequently more power. On the other hand, a single-center study reduces the influence of 'center' (*e.g.* treatment regimes) and increases data quantity and quality for each individual in terms of comparability between patients [18]. Therefore, we believe that our conclusion that IL-6 promoter polymorphism is associated with the degree of lung disease in CF patients is justified.

IL-6 plays an important role in the initiation of the acute phase and other systemic responses [19]. Locally, IL-6 is also considered to exert an effect by influencing inflammatory, immune, and coagulation processes [20]. Circulating levels of IL-6 are largely regulated at the level of expression, due to the rapid plasma clearance of this cytokine [21] The expression of IL-6 mRNA involves synergistic interaction of a number of transcription factors with the IL-6 promoter, including NF-κB and NF-IL-6. Binding sites for these factors are present in close proximity to nucleotide -174 in the IL-6 promoter [22]. Polymorphisms, such as the -174 G/C SNP (which is located adjacent to the negative regulatory domain and in close proximity to an NF-IL-6 binding motif) cause inter-individual variation in IL-6 transcription and expression [22]. Several studies have shown that DNA-protein interaction with G-containing oligonucleotides is greater than with C-containing oligonucleotides [21-23] and suggests that increased DNA-protein interaction in this region is associated with increased IL-6 cytokine production. Thus, the -174G allele is associated with a higher IL-6 level [21].

Furthermore, several studies have found elevated concentrations of IL-6 in sputum, bronchoalveolar lavage fluid (BALF) and serum in CF patients compared with healthy controls [4,24]. Adherence of PA and/or products of these organisms and neutrophil elastase can induce bronchial epithelial cell to secrete IL-6, which likely contributes directly to the pulmonary pathologic condition [25]. IL-6 stimulates B cells, contributing to increased antibody production, hypergammaglobulinemia, and local immune complex deposition [25]. We did not measure IL-6 levels, but determined total serum IgG levels as a marker of ongoing inflammation. We found a significant association between serum IgG levels and the -174 G allele.

We hypothesize that the higher IL-6 production caused by the -174 G allele may lead to a more intense inflammatory response, resulting in damage to the airway surface, which would favor infection and bacterial colonization, *e.g.* by PA, and lead to poorer lung function. Functional studies are needed to confirm this hypothesis. Other clinical studies have shown that that the altered IL-6 response that is caused by the -174G/C SNP is associated with the risk and outcome from a variety of conditions ranging from juvenile rheumatoid arthritis and severe sepsis to insulin sensitivity and carotid artery intimal thickening [23,26-28].

Apart from IL-6, well known functional SNPs in the IL-8 and IL-8 receptors (CXCR-1 and CXCR-2) were not associated with lung function or PA colonization in our CF patients. IL-8 is a potent chemoattractant for neutrophils [4]. In different studies increased IL-8 concentrations in sputum were associated with decreased pulmonary function in patients with CF [29,30]; whereas, similar to our study, these associations were not confirmed by other studies on CF [31,32].

However, different SNPs in the IL-8, CXCR-1 and CXCR-2 have been identified and associations have been described between IL-8, CXCR-1, and CXCR-2 SNPs and respiratory syncytial virus bronchiolitis, COPD/asthma and systemic sclerosis, respectively [33-35]. Again, not all studies could confirm these associations. Our study indicates that the analyzed IL-8, CXCR-1, and CXCR-2 SNPs are not a major contributor to lung function and PA colonization in CF.

IL-10 was also investigated as an anti-inflammatory cytokine, the principle action of which is to stimulate the synthesis of I-κB, the inhibitor of NF-κB. Studies in CF patients have shown that IL-10 controls A. fumigatus-specific and PA-specific T-cell responses [36].

The presence of a G allele at position -1082 is associated with increased IL-10 production [37]. A recent study suggested an association between the -1082GG genotype with an increased occurrence of allergic bronchopulmonary aspergillosis in CF [38]. However, in accordance with our study, no association has been found between the IL-10 -1082 A/G polymorphism and pulmonary function [39] or PA colonization [38].

In conclusion, out of several SNPs in regulatory elements of IL genes, the IL-6 -174 G/C promoter polymorphism seems to be associated with improved pulmonary outcome and less infection and inflammation. SNPs in IL8 (receptor) and IL-10 genes do not seem to be major determinants of lung function and PA colonization in CF.

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Chapter 12

Addendum:

Prognosis in cystic fibrosis; an integrated study

INTRODUCTION

In this thesis on prognostic factors for the course of cystic fibrosis (CF), several clinical and genetic parameters were suggested to have a significant effect on CF lung disease. The issue is whether these associations are clinically relevant. Furthermore, the contribution of a single predictor to CF lung disease needs further evaluation in the context of the other predictors.
We performed a multivariate analysis with the predictors identified in the work presented in this thesis. These included gender, current age, age at diagnosis, pancreatic insufficiency, CFTR genotype class, diagnosis order (with respect to having a younger or older sibling with CF), nutritional status (height/weight), *P. aeruginosa* (PA) colonization, the presence of CF-related liver disease (CFRLD), the presence of nasal polyps, MBL2 haplotype (high versus low producer), the TLR4 Gly299Asp polymorphism, and the IL-6 -237G/C polymorphism. Since IgG levels, as surrogate marker for ongoing inflammation, were not measured in adults, this predictor was not included in the multivariate analysis.

METHODS

Patients

All patients aged ≥4 years treated in the Cystic Fibrosis Center Utrecht of the University Medical Center Utrecht (Utrecht, the Netherlands) between 2000 and 2007 were included. All patients underwent a routine yearly multidisciplinary examination.

Measurements

Patients were classified as pancreatic-sufficient or pancreatic-insufficient on the basis of their need for pancreatic enzyme replacement. Standard deviation scores for height and weight were calculated using standard growth charts for the Dutch population [1]. The pulmonary function (forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC)) were measured by a pneumotachograph and converted to percentage of predicted values [2]. PA colonization was considered to be present when more than 50% of the PA cultures in three consecutive years were positive [3]. Sputum cultures were performed at least once a year.
CF patients were considered to have developed CFRLD if at least two of the following conditions were present on at least two consecutive examinations spanning a 1-year period [4]: (1) clinical hepatomegaly (increase in liver span and consistency, with liver edge palpable more than 2 cm below the costal margin on the mid-clavicular line), confirmed by ultrasonography; (2) abnormal serum liver enzyme levels, consisting of elevation above the upper normal limits of two of the following: aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyltransferase (GGT); (3) ultrasound abnormalities other than hepatomegaly (*i.e.*, increased, heterogeneous echogenicity, nodularity, irregular margins, splenomegaly). Ultrasonographic pattern of steatosis did not represent a diagnostic criterion.

Based on their functional effects, CFTR mutations were divided in five classes. Class I, II and III mutations result in absence of or non-functional CFTR, whereas class IV and V mutations (allowing some function of CFTR) usually result in less severe disease manifestations [5].
In all patients nasal endoscopy was carried out using a 2.2-mm flexible endoscope; the presence of nasal polyps and bulging of the lateral nasal wall were noted. Since bulging is a sign of sinusal polyposis in infants and children [6], in the present study nasal polyposis and bulging of the lateral nasal wall were analysed as a single entity.

Genotyping

Biallelic single nucleotide polymorphisms (SNPs) of the MBL2 and IL-6 gene were determined using sequence-specific primers (SSP) and polymerase chain reaction (PCR). The identification numbers of the SNP loci and the sequences of SNP-specific primers with their complementary consensus primers are shown in **Supplementary Table 12A**. The PCR conditions were as previously described [7].
Because of difficulties in primer design for SSP-PCR of the TLR4 8551A>G SNP (rs4986790, D299G), we performed melting curve analysis to detect this polymorphism using a pair of fluorescence resonance energy transfer (FRET) probes and the LightCycler (Roche Diagnostics; Basel, Switzerland) [8]. Oligonucleotide sequences used for PCR amplification of rs4986790 were F- AAGAAATTAGGCTTCATAAGC and R-CCAAGAAGTTTGAACTCATGGTAA. The sensor probe was ACTACCTC-GATGGTATTATTGACTTATT-FL and the anchor probe was LC640-AATTGTTT-GACAAATGTTTCTTCATTTTCC-ph.

Statistical analysis

In order to test if continuous variables were normally distributed, a Kolmogorov-Smirnov test was performed. If variables were not normally distributed, a logarithmic transformation was performed. Subsequently, we first used linear regression models with FEV_1 (% of predicted) as dependent and each potential predictor as independent variable, together with age and gender. Subsequently, we aimed to find out which of these predictors were mutually independent. Therefore, all variables which showed a univariable association ($P<0.20$) were entered in a multivariable linear regression model, again with FEV_1 as the outcome variable. In that model we used stepwise backward selection such that variables with the lowest predictive value were deleted until all variables showed a significant association with FEV_1 ($P<0.10$).
Prior to the multivariable analysis we used single imputation techniques for missing genetics values (CFTR 12.0%, SNPs 21.7%). Statistical analyses were performed using SPSS Inc., 2006, Chicago USA, version 15.0.

RESULTS AND DISCUSSION

Of the 398 patients treated at the CF Center Utrecht between 2000 and 2007, 374 met the inclusion criteria. Patient characteristics are presented in **Table 1**.
All investigated variables, apart from the CF sibling status and MBL2 haplotype, were significantly associated with FEV_1, and included in the multivariable analysis (**Table 2**). Independent predictors of FEV_1 were CFTR mutation (severe vs. mild), height (SD score for age), weight (SD score for weight), PA colonization, nasal polyps and IL-6 -237G/C polymorphism. Age at diagnosis, pancreatic insufficiency, CFRLD

Table 1. Characteristics of 374 CF patients at their multidisciplinary evaluation in the CF Center Utrecht.

Variable	
Gender (% male)	200 (53.5)
Age at diagnosis in yrs, median (range)	0.5 (0.0-45.5)
Pancreatic insufficiency (%)	351 (93.3)
Genotype:	
Homozygous ΔF508 (%)	215 (57.5)
Heterozygous ΔF508 (%)	115 (30.7)
Other (%)	24 (6.4)
Not determined	20 (5.3)
Genotype classes:	
Class I-III (%)	39 (10.4)
Class IV-V (%)	290 (77.5)
Unknown (%)	25 (6.7)
Not determined (%)	20 (5.3)
Sibling with CF	
No (%)	277 (74.1)
Yes, first within family (%)	47 (12.6)
Yes, second within family (%)	46 (12.3)
Yes, third within family (%)	4 (1.0)
Current age in yrs, median (range)	15.8 (4.0-64.8)
FEV_1 (% of predicted value)	74.7 (27.4)
Height (SD score for age)	-0.83 (1.10)
Weight (SD score for weight)	-0.28 (0.79)
P. aeruginosa colonization (%)	206 (55.1)
CF-related liver disease (%)	93 (24.9)
Nasal polyps (%)	195 (52.1)

Table 2. Univariable and multivariable predictors of FEV_1 (% of predicted) in 374 CF patients.

Variable	Univariable[1]	P-value	Multivariable[1]	P-value
Age at diagnosis (log, yrs)	2.76 (1.56)[2]	0.08		
Pancreatic insufficiency (%)	-18.1 (4.9)	0.0002		
Genotype classes (I-III vs. IV-V)	-17.4 (3.7)	<0.0001	-10.0 (3.4)	0.003
Sibling with CF		0.42		
Sib 1 vs. no sib	-2.8 (3.6)			
Sib 2 or 3 vs. no sib	3.2 (3.4)			
Height (SD score for age)	2.78 (1.04)[2]	0.008	1.89 (0.92)[2]	0.04
Weight (SD score for weight)	9.35 (1.11)[2]	<0.0001	7.94 (1.07)[2]	<0.0001
Pseudomonas colonization	-13.7 (2.4)	<0.0001	-10.3 (2.2)	<0.0001
CF-related liver disease	-5.30 (2.68)	0.05		
Nasal polyps	4.85 (2.40)	0.04	3.75 (2.09)	0.07
MBL (high versus low producer)[3]	-1.90 (3.70)	0.61		
TLR4 Gly299Asp	7.8 (4.14)[4]	0.09		
IL-6 -237G/C	4.33 (1.79)[5]	0.02	2.97 (1.45)	0.044

[1] Adjusted for age and gender.
[2] Change in FEV_1 per unit of variable.
[3] According to Garred *et al*. [26].
[4] Gly/Asp and Asp/Asp vs. Gly/Gly.
[5] Additive model assumed. Change in FEV_1 per variant allele (*i.e*. change in FEV_1 for CC genotype is 2x4.33).

and the TLR4 Gly299Asp polymorphism were not associated with FEV_1 after correction for the other predictors.

Our observation that severe CFTR mutations are associated with poorer lung function is in accordance with the study of Kerem *et al*., who analyzed the effect of CFTR genotype on phenotype in 17,853 patients with CF [9]. Numerous other studies also found this association, including the European Epidemiologic Registry of CF (ERCF) [10].

The univariate association between age at diagnosis and lung function was probably influenced by confounding by CFTR class: patients with a severe CFTR mutation have a significantly earlier age at diagnosis compared with patients with a mild mutation [9]. Other studies have described a beneficial effect of early diagnosis on lung function, but this was in asymptomatic children diagnosed through newborn screening [11-14].

There is a strong association between pancreatic status and CF lung disease [10]. Also in our study pancreatic insufficiency was univariately associated with lung function, but not in a multivariate model. Several studies have demonstrated a strong correlation between CFTR genotype and pancreatic insufficiency [9,10]. Our results suggest that organizing the CFTR genotype into five classes, on the basis of their effect on protein production, and dichotomizing them into severe (class I-III) and mild (class

IV and V) is a predictor of lung function rather than pancreatic status. However, pancreatic insufficiency may be overestimated from use of a surrogate marker—pancreatic enzyme supplementation. Since overestimation of pancreatic insufficiency is probably greater in the genotypes that have higher rates of pancreatic sufficiency, this misclassification should result in a bias towards the null hypothesis.

The study in **chapter 4** of this thesis showed that within families with two or more siblings with CF, the second CF sibling has a better lung function at the age of 20 years than the first sibling. This beneficial effect was probably caused by an earlier age at diagnosis. However, the multivariate analysis performed in **chapter 4** shows that having a sibling with CF is not an important contributor to prognosis at the population level.

In accordance with several other studies, nutritional status (height and weight) was independently associated with lung function [15-17]. These results stress the importance of optimizing nutritional status in underweight CF patients.

The significant independent effect of PA colonization on lung function also corresponds with several earlier studies [18-20]. Prevention (*e.g.*, by segregation) and aggressive antibiotic treatment of PA could be a major contributor to improvement of prognosis in CF.

Children with CFRLD were recently found to have a significantly lower lung function, but no worse chest radiography and clinical scores, compared with children without CFRLD [21]. Also in our study CFRLD was univariately associated with poorer lung function, but not after correction for potential confounders. Again, the univariate association was probably influenced by confounding by CFTR: the severity of CFTR genotype class is associated with both CFRLD and lung function [4,9]. The study in **chapter 5** of this thesis shows a slightly better lung function 5 years after diagnosis compared with matched controls without CFRLD. However, this potential beneficial effect of CFRLD on lung function does not seem to play an important role at the population level.

In accordance with both earlier [22,23] and later studies [24], we found an independent positive effect of the presence of nasal polyps on lung function in patients with CF. So far, the cause of the positive association between polyps and lung function has not been elucidated. Increased heterozygosity for ΔF508 was suggested in patients with nasal polyps [24], but in **chapter 5** the association between polyps and lung function remained after correction for CFTR genotype.

In **chapter 9** we showed that low MBL in younger children with CF seemed to have a positive effect on lung function. This was no longer observed in older CF patients and adults. Since the effect of a modifier can differ between children and adults (as shown in **chapter 9**), and children and adults were not analyzed separately in this study, no association between MBL2 haplotype and lung function was found.

Chapter 10 seemed to indicate that the presence of one or two variant alleles at position 8551 of the TLR4 gene resulted in better lung function in patients with CF. In this chapter, the TLR4 polymorphism was univariately associated with lung function. However, after multivariate analysis the effect of this polymorphism was no longer significant. Therefore, the role of the TLR4 polymorphism seems limited compared

to the other factors analyzed here, such as the IL6 -237G/C promoter polymorphism. Further research is needed to investigate the interplay between these two genes.
Chapter 11 demonstrated that the IL-6 -174 G/C polymorphism is associated with better lung function, less colonization with PA and lower total serum IgG levels. We hypothesized that the higher IL-6 production caused by the -174 G allele [25] might lead to a more intense inflammatory response, resulting in damage to the airway surface, which would favor infection and bacterial colonization, *e.g.* by PA. Also the multivariate analysis in this chapter showed a significant independent effect of the IL-6 -174G/C SNP on lung function. However, functional studies are needed to elucidate this association.

In conclusion, the multivariate analysis in this chapter showed that, although clinical and genetic characteristics may have a strong univariate association with lung function, multivariate analyses are needed to correct for potential confounding. The strongest predictors of poor lung function in CF were severe CFTR genotype class, low weight and the presence of PA colonization. Other significant independent predictors for worse CF course of lung disease were low height, the absence of nasal polyps and IL6 -174 wildtype genotype (GG). Finally, although several significant strong independent predictors have been identified an exact prediction of prognosis will not be possible for individual patients, especially since medical therapies and consequently the prognosis continue to improve.

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Chapter 13

General Discussion:
Investigating prognosis in CF

Of the numerous studies on the prevalence and prognosis of CF, most show a large variability in results due to important differences in study design and outcome measures. In this chapter we discuss the most important methodological aspects which are relevant in prognostic research on CF.

INVESTIGATION OF TRENDS

Aspects regarding design

Cross-sectional vs. cohort studies into survival

In 2000, Fogarty *et al*. [1] reported median age at death from CF in 10 different countries and found substantial differences in survival. Median age at death was significantly higher in the United States compared with the Netherlands. In this thesis (**chapter 3**) we compared CF birth cohort survival data between the United States and the Netherlands. Although we found a better survival in the American patients in the 1980-to-1984 cohort, this difference disappeared over the subsequent cohorts in more recent years, indicating an improvement in Dutch CF care.

Our data seem to indicate that this improvement of CF care in the Netherlands could not be captured by a cross-sectional study like the one conducted by Fogarty *et al*. [1]. Median age at death is an indirect marker of current mortality experience. In a disease such as CF (for which life expectancy is consistently increasing), median age at death will invariably underestimate median survival [2]. These findings stress the necessity of longitudinal survival studies in diseases such as CF.

A limitation of analyzing cohort survival is that this method takes time. Moreover, estimates of median survival are unlikely to be relevant to individual newly-born cases because of improvements in treatment that hopefully lead to a longer lifespan [3]. An alternative is the current survival method, which only requires observations over a one-year period [3]. All current cases alive in one year have to be identified and the deaths in that year noted. For each age, a mortality rate is calculated. It is then assumed that the calculated age-specific mortality rates will apply to the current cohort over their future lifespan. Applying these mortality rates to the current cohort gives an estimate of their future survival [3]. These data are the most up-to-date available, and represent a useful summary of the current age-specific mortality rates. Again, caution is required when using such data to predict future survival, particularly for the CF population which has seen regular improvements in survival over the past 30 years. Furthermore, this method may be of limited value for populations like the Dutch population, because of relatively low numbers of annual CF deaths.

Cross-sectional vs. cohort studies on CF-related complications

Many CF-related complications are age-dependent. CF-related liver disease (CFRLD) develops progressively over time, but most studies until now have been cross-sectional. A better method to describe the natural history of CFRLD is estimation of

cumulative proportions of patients developing CFRLD using the Kaplan-Meier approach. Using Kaplan-Meier analyses, (**chapter 5**), we demonstrated that CFRLD occurs early in the course of the disease, mainly in the prepubertal period and involved 37.5% of patients by 18 years of age. This has clinical implications which would otherwise remain unnoticed: adults do not need to be regularly screened for CFRLD.
Furthermore, the Kaplan-Meier analysis provides a more robust method to identify risk factors for CF-related complications, because the age at development of this complication can be taken into account. This is not only relevant for CFRLD but also for infection and colonization with micro-organisms such as *Pseudomonas aeruginosa* (PA). This was illustrated in **chapter 4**: younger sibs were more frequently colonized with PA in the first decade of life than their older counterparts, but this difference disappeared in the second decade of life. The difference would not have been observed when analyzing differences in prevalence of PA colonization at the last clinical visit.

INVESTIGATION OF PREDICTORS

Aspects regarding design

Single-center vs. multicenter studies

All analyses in this thesis were performed at the CF Center Utrecht only, or in collaboration with the CF Center of the Haga Teaching Hospital. The advantage of single-center studies is that they may generate qualitatively superior data, and thus may allow a better definition of phenotype. Furthermore, patients within a single center receive similar treatment and this will reduce the influence of environmental differences, including those related to the level and type of care received [4]. However, these studies may be underpowered for rare polymorphisms or subgroup analysis. In contrast, large groups will increase power, but pose limitations to the quality of the clinical data collected. For the largest of such studies, often the data may originate from several centers, which could lead to a lack of consistency. As a possible example, patients at a center treating PA aggressively may fail to show an effect of a modifier which has a significant impact in the context of less aggressive treatment. Large studies from many centers are less likely to be able to consider environmental issues (including adherence to treatment, pollution, smoking, etc.), which the investigators involved in smaller, single-site studies could take into account.

Power

The power of any modifier gene study will be determined by population size, the allele frequency, and the degree of clinical effect of the gene polymorphism that is studied. The latter determinant complicates power calculations, especially for continuous outcome measures such as lung function. For example, for the estimated effect of modifier genes on FEV_1 %predicted, assumptions need to be made on the

mode of effect of modifier genes (recessive, dominant, additive), the estimated difference in FEV_1, and standard deviation.
To study the effects of multiple different genes within a defined study group, where the allele frequency and the degree of clinical effect will differ for each gene, power calculations are even more difficult. Therefore, in our work we did not make power calculations, which also implies that some of the studies in this thesis could be underpowered.

Aspects regarding study-population

Age of the subjects

One challenge of modifier gene studies relates to the possibility of modifiers having differential effects (beneficial and adverse), depending on the age of the patients and clinical status of the lung disease [5]. This was demonstrated in **chapter 9**. Genotypes associated with low MBL production do not seem to influence already decreased lung function in older cohorts of CF patients and in adult CF patients. In contrast, low MBL in children living now seems to have a beneficial effect on CF lung function. Thus, the null variant may be beneficial early in life, or before chronic infection with *P. aeruginosa*, but not later in life, because of the already chronic infection with *P. aeruginosa*. These confounding variables suggest that it may be difficult to define the magnitude of the effect of MBL variants in overall outcome (survival) [5].

Aspects regarding prognostic determinants

Single SNPs vs. haplotypes

Analysis of multiple SNPs can suffer from problems that are associated with many predictors, some of which are highly correlated. A popular strategy, suggested by the block-like structure of the human genome, is to use haplotypes to try to capture the correlation structure of SNPs in regions of little recombination [6]. Using haplotype analysis increases power and, more importantly, haplotypes can capture the combined effects of tightly linked cis-acting causal variants. In this thesis we used haplotypes for the SNPs in codon -221 and in exon 1 of the MBL2 gene, known to have cis-acting effects (**chapter 9**).
However, there are several problems with haplotype-based analyses, such as whether or not to include rare haplotypes [6]. Another problem with defining haplotypes is that block boundaries can vary according to the population sampled, the sample size, the SNP density and the block definition [6]. Although haplotype analysis seems to be a natural approach, it might ultimately confer little or no advantage over analyses of multipoint SNP genotypes [6]. Furthermore, the widespread adoption of tagging strategies - facilitated by knowledge of LD that is obtained from the HapMap project and other sources (see below) - diminishes the potential utility of

haplotype analyses. Nevertheless, haplotypes form a basic unit of inheritance and therefore have an interpretability advantage (*e.g.* regarding evolution).

SNPs vs. more modern techniques

More and more focus is beginning to switch from SNPs studies, like the ones described in this thesis, to more modern techniques, such as microarray analysis and genome wide screening.

Microarray analysis was already used by Wright *et al*. [7]. This investigation used high-density oligonucleotide microarray analysis of nasal respiratory epithelium to investigate the molecular basis of phenotypic differences in severity of CF lung disease. However, one important limitation of this technique is the difficulty in distinguishing between those differences in gene expression that contribute to differences in phenotype and those that are the result of differences in phenotype. This is particularly true in CF for genes involved in inflammatory and immune responses [8]. Furthermore, microarray analysis only provides hypothetical leads: identification of functional polymorphisms in the genes and subsequent population-based studies to determine segregation by phenotype are still the only method to demonstrate association.

Whole genome association studies have the potential to be significantly more sensitive for identifying novel modifiers than standard family linkage studies [8]. The International HapMap Consortium recently completed genotyping over 3.8 million SNPs in three different populations and found that certain SNPs tend to be transmitted together from generation to generation in haplotype blocks [9]. By genotyping specific SNPs that characterize these blocks (tagSNPs) [10,11], large stretches of chromosomes will be simultaneously evaluated for linkage disequilibrium between CF groups with contrasting severity of lung disease. This genotyping will be challenging and expensive, however, because segments of linkage disequilibrium are only tens of thousands of bases in length. This will require genotyping hundreds of thousands of tagSNPs in a very large group of patients to scan the whole genome [12]. Study design and statistical interpretation of results will require accounting for both the large-scale multiple-hypothesis testing inherent in the technique and the potential influence of population stratification [12]. When specific chromosomal regions are identified that consistently segregate with severity of lung disease, further evaluation within these regions will lead to identification of previously unsuspected genes responsible for modifying CF phenotype. Although results are not yet available, whole genome approaches to identify novel modifiers of CF are underway [13].

Aspects regarding outcome measures

Different lung function measures

Lung function is mostly used as primary outcome measure in CF. Although FEV_1 % predicted is commonly accepted as reflecting severity of CF lung disease, it is essen-

tial that the FEV_1 % predicted be age-adjusted to allow comparison between groups. Even this correction can be problematic, because the typical % predicted correction is based on decline in lung volume with age in non-CF control subjects. Different approaches to correcting lung function for age in CF have been developed, including a correction based on expected decline in all individuals with CF [14] and another based on lung function in ΔF508 homozygotes [15]. These corrections were obtained in US populations, and it is unclear whether these corrections could be applied to non-US populations. Therefore, European/ Dutch CF lung function reference data are needed.

Another problem is the analysis of FEV_1: lung function is often dichotomized using an arbitrarily chosen cut-off value or sometimes classified into mild 'severe' and 'mild' lung disease [16]. This can result in loss of information and introduce spurious associations.

Furthermore, since the progression of lung disease is not linear, it is not possible to quantitate disease severity across a broad age range in cystic fibrosis on the basis of a few measures. Longitudinal analyses of multiple measures of lung function (spirometry, FEV_1) provide better definition of pulmonary status and the rate of decline, and these approaches will enhance the quality of the study [5]. Strong effects on lung function are earlier detected by longitudinal analyses but, if strong enough, will in most cases also cause significant associations in cross-sectional analyses. In this thesis we performed most analyses both cross-sectionally and longitudinally using random effect models (**chapters 4, 7, 9, 10 and 11**). However, no major differences were found between the longitudinal and cross-sectional analyses.

Lung function vs. other measures

The cornerstone of epidemiological studies, including modifier gene studies, is accurately defining phenotype. Although survival age is the ultimate measure of CF severity, the improving ages of death mean that mortality is not a useful endpoint to evaluate the progress of patients with CF or to analyze potential predictors. The strong correlation of longitudinal measures of FEV_1 with age at death has established FEV_1 as the prime surrogate for lung disease severity in CF. However, there is growing evidence that the critical events in lung pathology occur at a very early age and, therefore, standard lung function testing is not optimal to analyze predictors for the earliest stages of CF lung disease [17]. Furthermore, the improvements in lung function among patients with CF over the past decade have rendered these measurements less useful as clinical trial endpoints, since the average rate of decline has slowed and normal lung function is, on average, preserved until later ages [18]. As a result, these endpoints are of relatively poor sensitivity and precision, and large numbers of study subjects are required.

Alternatives for lung function measurements include pulmonary exacerbation rates, quality of life measures, growth, respiratory cultures, inflammatory markers, chest radiographs, chest computed tomography (CT), and newer imaging modalities, but each of the alternatives had disadvantages [19].

The ideal endpoint needs to be accurate, precise, and reliable [19]. Surrogate endpoints, *e.g.* for survival, must be biologically plausible, reflect clinical severity, and correlate with true outcomes [19]. They should also be responsive (such that the measurement changes in the anticipated direction either with disease progression or with an intervention), and, ideally, the endpoint would also be measured with minimal risk and be inexpensive and easy to perform. Since FEV_1 fulfills most of these criteria, and was shown to be the best predictor of survival [20,21], this parameter was used as primary outcome measure in our studies.
One of the secondary outcome measures in this thesis was PA colonization. However, also this measurement has several limitations including definition of colonization, age-of-diagnosis bias (as described in **chapter 4**), and the frequency and type of culture. In expectorating patients with CF, sputum cultures accurately reflect lower respiratory organisms [22,23]. In contrast, although oropharyngeal cultures are widely used as a noninvasive surrogate for lower airway cultures in nonexpectorating patients with CF, they have poor sensitivity and positive predictive value for lower airway infection [24], and oropharyngeal isolates of PA can have different genotypes than lower airway isolates from the same patient [22]. Thus, in preexpectorating infants and nonexpectorating patients, flexible bronchoscopy with bronchoalveolar lavage (BAL) is the only means to directly sample the lower respiratory tract for detection of bacteria and inflammation. However, BAL is often not feasible on a routine basis, it is invasive and not without risks. Therefore, in this thesis sputum cultures were used and in nonexpectorating patients oropharyngeal cultures.

Aspects regarding analysis

Single gene vs. multi gene analysis

Most modifier gene studies performed so far have analyzed one, or only a small number of SNPs. Testing for a number of potential modifier genes and not just one may increase the reliability of positive associations. However, in studies testing a large number of SNPs the multiple comparison problem plays a role, increasing the likelihood of positive correlations appearing by random chance (α error). [6]. A Bonferroni correction could be applied, but this correction is too conservative for tightly linked SNPs [6]. Other corrections include the computationally demanding permutation procedure and the false discovery rate (FDR) [25], but these methods are not often used. One way around this is to have a second cohort in which hypotheses generated in the first cohort can be tested. With large enough populations, this may be achievable by splitting the group randomly prior to initial data analysis, although detrimental impacts on power caused by lower numbers must of course be considered [4].
Another statistical problem is interaction between genes. However, testing for interaction between the different genes is statistically difficult. No good statistical techniques are available to account for polymorphisms in multiple genes simultaneously.

Because of the small number of tightly linked SNPs analyzed in this thesis we decided not to correct for multiple testing. Ideally, results are tested in a second cohort.

Univariate vs. multivariate analysis

A large number of predictors was analyzed in this thesis. Although most of the associations were significant, the question is whether these associations are also clinically relevant. Furthermore, the question remains: what is the contribution of the identified predictors to the prediction of CF lung disease compared to the other analyzed predictors. For this reason, we performed a multivariate analysis using the predictors identified in **chapters 4 to 7** and in **chapters 9 to 11**. Since IgG levels were not measured in adults, this predictor was not included in the multivariate analysis. The results are reported in **chapter 14**. All investigated variables, apart from the CF sibling status and MBL haplotype, were significantly associated with FEV_1, and included in the multivariable analysis. The significant differences in lung function between older and younger sibs (**chapter 4**), as well as the nonsignificant contribution of having a sibling, to CF lung function (**chapter 14**) illustrates that predictors that are significant are not always clinically relevant.

Other predictors do have a strong univariate association with lung function, such as pancreatic status, but not after correction for other predictors, such as CFTR class. CFTR class and pancreatic status are highly correlated. The multivariate analysis showed that CFTR is a better predictor of lung function than pancreatic status. Also, the IL-6 polymorphism seems to be a stronger predictor of lung function than the TLR4 polymorphism. Therefore, the effect of potential predictors should preferentially be analyzed in a multivariate model with well-defined parameters.

Another important issue is that some predictors, including modifier genes, may only have an effect in a subpopulation. As an example, certain genes may modify severity only in patients of one gender, with so-called 'mild' CFTR mutations, or at certain ages or stages of disease. This requires a multivariable analysis, subgroup analysis, or population stratification.

CONCLUSIONS

Assessment of prognostic indicators in CF is important as it may influence the timing of lung transplantation and may provide indications of response to new or more intensive medical regimes. It is also important to be able to inform patients about their prognosis.

A large number of prognostic studies have been performed in CF patients, but there is an enormous variability in results due to important differences in study design, study population, outcome measures, and statistical analysis.

Large single-center or well-defined multicenter patient populations are needed to ensure sufficient power and to allow subgroup and multivariate analyses. FEV_1 is still the most commonly used prognostic end-point, but universally accepted CF reference values are needed. Although statistically more demanding, data should

be analyzed longitudinally to provide better definition of pulmonary status and the rate of decline. Using universally accepted study designs and outcomes will allow meta-analyses. Nevertheless, predicting prognosis on an individual basis will still remain almost impossible because of the discovery of new medical therapies and of improving survival.

The approach to identifying genetic modifiers of CF phenotype is evolving. Small association studies are giving way to large multicenter association studies. The clinical specimens available from the large studies combined with emerging whole genome technologies will permit identification of novel modifiers of CF phenotype in the near future. Identification of these modifiers will provide better insights into pathophysiologic mechanisms for lung disease in cystic fibrosis, and spawn an array of biologic and clinical research activities to better characterize mechanisms of disease activity. These studies, in turn, will result in increased ability to predict severity of CF lung disease in individuals and, most important, provide targets for future therapeutic intervention.

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Chapter 14

Summary

Samenvatting

SUMMARY

The work presented in this thesis has addressed three questions concerning prognosis of cystic fibrosis (CF):

1. What are the actual birth prevalence and survival (trends) of CF patients?
2. What are clinical predictors of CF lung disease and CF-related complications?
3. What are genetic predictors of CF lung disease?

Trends

Until recently, the birth prevalence of CF in the Netherlands was considered to be 1 in 3,600. This birth prevalence was obtained between 1961 and 1965 and has never been measured thereafter. In **chapter 2** we showed that the average birth prevalence of CF in the Netherlands between 1974 and 1994 was 1 in 4,750 live births. Importantly, we also found that survival for Dutch birth cohorts of CF patients has improved dramatically over the past decades. All successive 5-year birth cohorts between 1950 and 1994 demonstrate a higher survival rate than the previous 5-year cohort. Finally, it has been demonstrated that there was a better survival among American patients compared to Dutch patients in the 1980-to-1984 cohort; however, this difference has disappeared over the subsequent cohorts. Also, the difference in survival between the United Kingdom and the Netherlands found in the 1980-to-1982 cohort appears to have disappeared for later cohorts.

Respiratory failure is the leading cause of death among CF patients. Unfortunately, the prognosis for CF patients requiring invasive mechanical ventilation (IMV) for acute respiratory failure (ARF) has been unfavorable. In **chapter 3** it was shown that ARF requiring assisted ventilation in adults with CF is still associated with high mortality (73%). Acute or chronic respiratory failure is a significant risk factor for poor outcome. In contrast, however, in the two Dutch CF centers investigated, none of the children requiring assisted ventilation for ARF died. Furthermore, although their number was small, the long-term outcome of these children was similar to that of a non-ventilated CF control group.

Clinical predictors

In **chapter 4** we analyzed the effect of birth order in families with two or more siblings with CF. Siblings with CF share the same CFTR mutations and are generally exposed to the same environment. We showed that, in the first decade of life, younger siblings tend to be more frequently colonized with *P. aeruginosa* (PA), but do not have a worse lung function than their older counterparts, probably due to an earlier age at diagnosis. In the second decade of life, however, no difference in PA colonization was observed and younger sibs actually had a better lung function than their older counterparts. This underscores the importance of early diagnosis in the prevention of long-term deleterious effects on lung function.

CF-related liver disease (CFRLD) is a common CF-related complication. Our study in **chapter 5** showed that CFRLD occurs mainly in the prepubertal period. The cumulative incidence of CFRLD was 16.8%, 33.4%, and 37.5% at the age of 10, 15 and 18 years, respectively. There appeared to be a strong association between the CFTR genotype and CFRLD. None of the patients with a mild CFTR genotype developed CFRLD. This study also showed a trend towards an increased incidence of CFRLD in male patients and in patients who were pancreatic insufficient. In our cohort, meconium ileus and age at diagnosis were not important risk factors for CFRLD. Finally, we showed that lung function in CF patients was not negatively influenced by the presence of CFRLD. Moreover, our patients with CFRLD tended to have better lung function 5 years after diagnosis compared with controls.
Another common CF-related complication discussed in **chapter 6** is nasal polyposis. To select those children who might benefit from treatment by the otorhinolaryngology (ORL) physician, it is important to know whether symptoms and clinical characteristics can predict the presence of nasal polyposis in patients with ORL symptoms. We demonstrated that nasal polyps were present in half of the children with CF, but only 59% of those with polyps were symptomatic. In children with CF, the presence of nasal polyps was significantly associated with high FEV_1, FVC, and PEF values. This finding is in accordance with earlier studies, but the cause of the positive association between polyps and lung function has not yet been elucidated. Other significant predictors of polyps were male gender, symptoms of rhinorrhea and postnasal drip, and low CRP levels. In current practice only CF patients with ORL symptoms are referred to an ORL physician. In these patients, the presence of sinonasal polyposis could be reasonably predicted using a predictive model that includes male gender, age 10 years or older, symptoms of rhinorrhea, and FVC values 70% or greater of those predicted.
Apart from lung function, also aerobic capacity (peak oxygen uptake; VO_2peak) is a determinant of well-being and survival in patients with CF. In **chapter 7**, a large longitudinal study confirmed that aerobic capacity in children and adolescents with CF was determined by lung function and nutritional status. Furthermore, this was the first study showing an independent association between high total serum IgG or PA colonization and low aerobic capacity. These results not only stress the potential importance of optimizing nutritional status in underweight CF patients, but may also indicate that prevention and aggressive treatment of inflammation and PA infections may increase aerobic capacity in these patients. Finally, this study showed that genetic variations in the CFTR and ACE gene are not important determinants of aerobic capacity in CF.

Genetic predictors

Increasing evidence suggests that phenotypic variation in CF can be attributed to genetic variation in genes other than the CFTR gene, the so-called modifier genes. **Chapter 8** gives an overview of multiple candidate modifier genes that have recently been investigated in CF. Most of them are genes that are involved in the control of infection, immunity and inflammation. The majority of studies on modifier genes in

CF have included rather small numbers of patients. Furthermore, they are mainly cross-sectional, mostly not replicated, and often show conflicting results.
We performed a large single-center study in a well-defined population consisting of over 300 CF patients and analyzed data both cross-sectionally and longitudinally.

One potential modifier gene investigated in **chapter 9** was the MBL2 gene, encoding mannose-binding lectin (MBL). MBL is a serum protein participating in innate immune defense. Three single-base substitutions in exon 1 and a promoter polymorphism in codon -221 of the MBL2 gene, independently cause low serum levels of MBL. Based on our own results an previous reports, we showed that MBL2 low producer genotypes are associated with worse lung function in older cohorts of CF patients and in adult CF patients. In contrast, low MBL in children living now seemed to have a positive effect on CF phenotype. Our study confirmed several other recent studies showing that children with low MBL have rather better lung function. These findings do not support the use of MBL substitution therapy.
Chapter 10 analyzed the effect of another potential class of CF modifier genes: the toll-like receptors (TLRs). TLRs recognize different pathogen associated molecular patterns and have prominent roles in the activation of innate and adaptive immune responses to infection. The amino-acid change of aspartic acid to glycine (D299G) in the TLR4 gene results in a blunted response to lipopolysaccharide (LPS), which is present on Gram-negative bacteria such as PA. **Chapter 10** showed that this polymorphism is associated with better lung function and less lung function decline in patients with CF. It was hypothesized that the endotoxin hyporesponsiveness caused by the SNP results in dampened cytokine activation and less subsequent parenchymal lung damage, thus leading to better lung function in patients with this polymorphism. The TLR2 and CD14 SNPs investigated in **chapter 10** did not seem to influence lung function and PA colonization in patients with CF.
In **chapter 11** the effects of genetic variation in interleukin (IL) genes are described. Defects in the adaptive immunity can play a role both in the failure of anti-pseudomonal defense and in the dysregulation of the subsequent inflammatory responses. Our study demonstrated the IL-6 -174 G/C polymorphism to be associated with better lung function, less colonization with PA and lower total serum IgG levels. This IL-6 promoter polymorphism is associated with increased IL-6 cytokine production. We hypothesize that the higher IL-6 production caused by the -174 G allele leads to a more intense inflammatory response (IgG) with the production of factors that could damage the airway surface (poorer lung function), and so favor infection and bacterial colonization, *e.g.* by PA. Our study did not demonstrate a significant contribution of genetic variation in the IL-8, IL-8 receptors and IL-10 genes on CF phenotype.

In **chapter 12** we performed a multivariable analysis of the predictors of FEV_1 identified in this thesis. The multivariable analysis showed that, although clinical and genetic characteristics may have a strong univariable association with lung function, multivariable analyses are needed to correct for potential confounding. The stron-

gest predictors of poor lung function in CF were severe CFTR genotype class, low weight and the presence of PA colonization. Other significant independent predictors for worse CF course of lung disease were low height, the absence of nasal polyps and IL6 -174 wildtype genotype (GG). Age at diagnosis, pancreatic insufficiency, CFRLD and the MBL2 and TLR4 polymorphisms were not associated with FEV_1 after correction for the other predictors.

The most important findings of this thesis were:

1. The actual birth prevalence of CF in the Netherlands is clearly lower than it was 30 years ago (**chapter 2**). Survival in CF has dramatically improved. Young children with CF who have acute respiratory failure (ARF) have a good prognosis, but ARF in adult CF patients is associated with high mortality (**chapter 3**).
2. In families with 2 or more siblings with CF, younger siblings have a better lung function than their older counterparts, probably due to an earlier age at diagnosis (**chapter 4**). The presence of CF-related liver disease or sinonasal polyps does not negatively influence pulmonary disease in children with CF, and nasal polyps are even associated with better lung function (**chapter 5 and 6**). Beside lung function and nutritional status, inflammatory status and *P. aeruginosa* colonization independently affect aerobic capacity in children and adolescents with CF (**chapter 7**).
3. Polymorphisms in genes encoding mannose-binding lectin, toll-like receptor 4 and interleukin-6 are associated with CF lung disease (**chapter 9, 10 and 11**).

SAMENVATTING

In dit proefschrift beantwoordden we 3 vragen over de prognose van cystic fibrosis (CF):

1. Wat zijn de huidige geboorteprevalentie en overleving (trends) van CF patiënten?
2. Wat zijn klinische voorspellers van het pulmonale ziektebeloop bij CF en het optreden van CF-gerelateerde complicaties?
3. Wat zijn genetische voorspellers van het pulmonale ziektebeloop bij CF?

Trends

De geboorteprevalentie van CF in Nederland werd tot kort geleden geschat op 1 op 3.600. Deze geboorteprevalentie was verkregen tussen 1961 en 1965 en was daarna nooit meer gemeten. In **hoofdstuk 2** lieten we zien dat de geboorteprevalentie van CF in Nederland tussen 1974 en 1994 1 op 4.750 levendgeborenen was. Bovendien vonden wij dat de levensverwachting van Nederlandse geboortecohorten van CF patiënten de laatste decennia drastisch is verbeterd. Alle opeenvolgende 5-jaars geboortecohorten tussen 1950 en 1994 lieten een betere overleving zien dan het voorafgaande cohort. Tenslotte werd aangetoond dat voor het geboortecohort 1980-1984 er een betere overleving was voor patiënten uit de VS dan voor patiënten uit Nederland; echter, dit verschil verdween voor de daaropvolgende cohorten. Ook het gevonden verschil in overleving tussen het Verenigd Koninkrijk en Nederland voor het cohort 1980-1982 bleek verdwenen voor latere cohorten.
Respiratoir falen is de belangrijkste oorzaak van overlijden van CF patiënten. Helaas was de prognose van CF patiënten die invasieve mechanische beademing voor acuut respiratoir falen behoeven ongunstig. In **hoofdstuk 3** lieten we zien dat acuut respiratoir falen met beademingsbehoefte bij volwassenen nog steeds geassocieerd is met hoge sterfte (73%). Acuut op chronisch respiratoir falen is een significante risicofactor voor een slechte uitkomst. Daarentegen overleed in de twee onderzochte Nederlandse CF centra geen van de kinderen met beademingsbehoefte voor acuut respiratoir falen. Tevens was de langetermijn uitkomst van deze kinderen, hoewel hun aantal klein was, gelijk aan die van de niet-beademde CF controlegroep.

Klinische voorspellers

In **hoofdstuk 4** analyseerden we het effect van de geboortevolgorde in gezinnen met 2 of meer kinderen met CF. Broers en zusters met CF delen dezelfde CFTR mutaties en zijn meestal blootgesteld aan dezelfde omgevingsfactoren. We toonden aan dat jongere broers en zussen in het eerste decennium van het leven vaak eerder gekoloniseerd worden met *P. aeruginosa* (PA), maar geen slechtere longfunctie hebben dan hun oudere broers of zussen, waarschijnlijk tengevolge van een eerdere leeftijd bij diagnose. In het tweede decennium van het leven kon geen verschil in PA kolonisatie meer worden waargenomen en hadden jongere broers en zussen een betere longfunctie dan hun oudere tegenhangers. Dit onderstreept het belang van

een vroegtijdige diagnose van CF bij het voorkomen van schadelijke effecten op de longfunctie op de lange termijn.
CF-gerelateerde leverziekte (CF-LZ) is een veelvoorkomend CF-gerelateerde complicatie. Onze studie in **hoofdstuk 5** liet zien dat CF-LZ voornamelijk in de prepuberale periode optreedt. De cumulatieve incidentie van CF-LZ was 16,8%, 33,4% en 37,5% op de leeftijd van respectievelijk 10, 15 en 18 jaar. Er bleek een sterke associatie te zijn tussen CFTR genotype en CF-LZ. Geen van de patiënten met een mild CFTR genotype ontwikkelde CF-LZ. Onze studie toonde ook een trend tot een hogere incidentie van CF-LZ bij mannelijke patiënten en bij patiënten met pancreasinsufficiëntie aan. In ons cohort waren meconium ileus en leeftijd bij de diagnose geen belangrijke risicofactoren voor CF-LZ. Tenslotte toonden we aan dat de longfunctie van CF patiënten niet negatief werd beïnvloed door de aanwezigheid van CF-LZ. Bovendien neigden onze patiënten met CF-LZ vaak tot een betere longfunctie 5 jaar na diagnose in vergelijking met controles.
Een ander veelvoorkomende CF-gerelateerde complicatie besproken in **hoofdstuk 6** is neuspoliepen. Om kinderen te selecteren die baat kunnen hebben van behandeling door de KNO-arts, is het belangrijk om te weten of symptomen en klinische kenmerken de aanwezigheid van neuspoliepen kunnen voorspellen bij patiënten met KNO-klachten. We toonden aan dat neuspoliepen aanwezig waren bij de helft van de kinderen met CF, maar slechts 59% van de personen met poliepen was symptomatisch. Bij kinderen met CF was de aanwezigheid van neuspoliepen significant geassocieerd met hoge FEV_1, FVC en PEF waarden. Deze bevinding komt overeen met andere studies, maar tot op heden is de oorzaak van de positieve associatie tussen neuspoliepen en longfunctie niet opgehelderd. Andere belangrijke voorspellers van poliepen waren mannelijk geslacht, klachten van rhinorrhoe en postnasal drip, en lage CRP spiegels. In de huidige praktijk worden alleen CF patiënten met KNO-klachten verwezen naar de KNO-arts. Bij deze patiënten kon de aanwezigheid van neuspoliepen redelijk voorspeld worden met behulp van een voorspellend model bestaande uit mannelijke geslacht, leeftijd 10 jaar of ouder, klachten van rhinorrhoe, en een FVC groter of gelijk aan 70% van voorspeld.
Naast longfunctie is ook aerobe capaciteit (piek zuurstofopname; VO_2peak) een bepalende factor voor het welzijn en de overleving van patiënten met CF. In **hoofdstuk 7** bevestigde onze grote longitudinale studie dat aerobe capaciteit bij kinderen en adolescenten met CF beïnvloed wordt door longfunctie en de voedingstoestand. Bovendien was dit de eerste studie die een onafhankelijke associatie tussen hoog totaal serum IgG en PA kolonisatie en lage aerobe capaciteit liet zien. Deze resultaten onderstrepen niet alleen het potentiële belang van het optimaliseren van de voedingstoestand bij CF patiënten met ondergewicht, maar geven ook aan dat preventie en agressieve behandeling van ontsteking en PA infecties de aerobe capaciteit bij deze patiënten zou kunnen verbeteren. Tenslotte is uit dit onderzoek gebleken dat genetische variaties in de CFTR en ACE genen geen belangrijke determinanten van aerobe capaciteit bij CF zijn.

Genetische voorspellers

Er is toenemend bewijs dat fenotypische variatie van CF kan worden toegeschreven aan genetische variatie in genen anders dan het CFTR gen, de zogenaamde modifier genes. **Hoofdstuk 8** geeft een overzicht van verschillende kandidaat modifier genes die onlangs zijn onderzocht in CF. De meeste hiervan zijn genen die betrokken zijn bij de controle van de infectie, afweer en ontsteking. Het merendeel van de studies naar modifier genes bij CF is uitgevoerd bij relatief kleine aantallen patiënten. Verder zijn ze voornamelijk cross-sectioneel, meestal niet herhaald, en laten vaak tegenstrijdige resultaten zien.

Wij hebben een grote single center studie uitgevoerd bij een goed gedefinieerde populatie bestaat uit meer dan 300 CF patiënten en hebben gegevens zowel cross-sectioneel als longitudinaal geanalyseerd.

Een mogelijke modifier gene onderzocht in **hoofdstuk 9** was het MBL2 gen, dat codeert voor mannose bindend lectine (MBL). MBL is een serum eiwit dat deelneemt in de aangeboren immuunrespons. 3 enkele basepaarveranderingen in exon 1 en een promoter polymorfisme in codon -221 van het MBL2 gen veroorzaken onafhankelijk van elkaar lage serumwaarden van MBL. Mede op basis van eerdere studies lieten we zien dat MBL2 laag producerende genotypen geassocieerd zijn met een slechtere longfunctie in oudere leeftijdsgroepen van CF patiënten en bij volwassen CF patienten. Daarentegen bleek laag MBL bij de huidige generatie kinderen een positief effect op het CF fenotype te hebben. Onze studie bevestigde verschillende andere recente studies die lieten zien dat kinderen met een laag MBL een betere longfunctie hebben. Deze bevindingen ondersteunen niet het gebruik van MBL substitutie therapie.

In **hoofdstuk 10** analyseerden we het effect van een andere potentiële klasse van CF modifier genes: de toll-like receptoren (TLRs). TLRs herkennen verschillende pathogeen geassocieerde moleculaire patronen en hebben een prominente rol in de activatie van de aangeboren en adaptieve immuunrespons tegen infecties. De aminozuurverandering van aspartaat naar glycine (D299G) in het TLR4 gen resulteert in een afgezwakte response op lipopolysaccharide (LPS), dat aanwezig is op Gram-negatieve bacteriën zoals PA. Uit **hoofdstuk 10** blijkt dat dit polymorfisme is geassocieerd met een betere longfunctie en minder achteruitgang van de longfunctie bij patiënten met CF. We hypothetiseren dat de verlaagde response op endotoxinen veroorzaakt door de SNP resulteert in verminderde activatie van cytokines en minder latere parenchymale longschade, hetgeen leidt tot een betere longfunctie bij patiënten met dit polymorfisme. De TLR2 en CD14 SNPs onderzocht in **hoofdstuk 10** lijken longfunctie en PA kolonisatie bij patiënten met CF niet te beïnvloeden.

In **hoofdstuk 11** werden de effecten van genetische variatie in interleukine (IL) genen beschreven. Defecten in de adaptieve afweer kunnen een rol spelen, zowel in het mislukken van de verdediging tegen PA en in de disregulatie van de latere inflammatoire responsen. Onze studie toonde aan dat het IL-6 -174 G / C polymorfisme geassocieerd is met een betere longfunctie, minder kolonisatie met de PA en lagere totale serum IgG spiegels. Dit IL-6 promoter polymorfisme is geassocieerd met een verhoogde cytokine IL-6 productie. We hypothetiseren dat de hogere IL-6 productie

veroorzaakt door het -174 G-allel leidt tot een meer intense inflammatoire respons (IgG) met de productie van factoren die het luchtwegoppervlak beschadigen (slechtere longfunctie), en zo bacteriële infectie en kolonisatie , o.a. door PA, kunnen bevorderen. Onze studie liet geen significante bijdrage van genetische variatie in de IL-8, IL-8 receptoren en IL-10 genen aan het CF fenotype zien.
In **hoofdstuk 12** hebben wij een multivariabele analyse uitgevoerd op de voorspellers van FEV_1 die beschreven zijn in dit proefschrift. De multivariabele analyse liet zien dat, hoewel klinische en genetische kenmerken een sterke univariabele associatie met de longfunctie kunnen hebben, multivariabele analyses nodig zijn om te corrigeren voor mogelijke confounders. De sterkste voorspellers van een slechte longfunctie bij CF waren ernstige CFTR genotype klasse, een laag gewicht en de aanwezigheid van PA kolonisatie. Andere belangrijke onafhankelijke voorspellers voor een ernstiger pulmonaal ziektebeloop bij CF waren kleine lengte, de afwezigheid van neuspoliepen en het IL6 -174 wildtype genotype (GG). Leeftijd bij diagnose, pancreasinsufficiëntie, CF-LZ en de MBL2 en TLR4 polymorfismes waren niet geassocieerd met FEV_1 na correctie voor de andere voorspellers.

De belangrijkste bevindingen van dit proefschrift waren:

1. De huidige geboorteprevalentie van CF in Nederland is duidelijk lager dan die van 30 jaar geleden. De levensverwachting van CF is drastisch verbeterd (**hoofdstuk 2**). Jonge kinderen met CF met acuut respiratoir falen (ARF) hebben een goede prognose, maar ARF bij volwassen CF patiënten is geassocieerd met een hoge mortaliteit (**hoofdstuk 3**).
2. In gezinnen met 2 of meer broers of zussen met CF hebben jongere broers en zussen een betere longfunctie dan hun oudere gezinsleden, waarschijnlijk ten gevolge van een eerdere leeftijd bij diagnose (**hoofdstuk 4**). De aanwezigheid van CF-gerelateerde leverziekte of neuspoliepen heeft geen negatieve invloed op het pulmonale ziektebeloop bij kinderen met CF, en neuspoliepen zijn zelfs geassocieerd met een betere longfunctie (**hoofdstuk 5 en 6**). Naast longfunctie en voedingsstatus hebben inflammatiestatus en *P. aeruginosa* kolonisatie een onafhankelijk effect op aerobe capaciteit bij kinderen en adolescenten met CF (**hoofdstuk 7**).
3. Polymorfismen in genen die coderen voor mannose bindend lectine, toll-like receptor 4 en interleukine-6 zijn geassocieerd met het pulmonale ziektebeloop bij CF (**hoofdstuk 9, 10 en 11**).

Chapter 15

Appendices

SUPPLEMENTARY TABLES

Supplementary Table 3A. Indications of admissions of cystic fibrosis patients to the intensive care unit

Indication	Number of admissions		
	Males (45 patients)	Females (38 patients)	All (83 patients)
Acute respiratory failure	20	13	33
Lung transplantation	10	7	17
Postoperative monitoring	13	11	24
Haemoptysis	6	1	7
Pneumothorax	4	0	4
Initiation of non-invasive home mechanical ventilation	2	3	5
Other	3	7	10
All indications*	58	42	100

*Because some patients had multiple admissions during the study period, the total number of admissions (100) is higher than the total number of patients admitted to the ICU (83).

Supplementary Table 3B. Characteristics of adult cystic fibrosis patients with acute respiratory failure requiring assisted ventilation.

Patient nr	Hospital	Sex	Age (y)	FEV_1	FVC	BMI	CF-related Complications	MO	Steroid Use	Acute on chronic	Invasive ventilation	LOS ICU (d)	Lung Tx	Survival
1	UMCU	M	29.1	20	28	16.7	PI, LD	PA	No	Yes	Yes	19	No	Yes
2	UMCU	M	24.9	28	34	18.5	PI, DM, ABPA	PA, AF	Yes	Yes	Yes	11	No	No
3	UMCU	F	32.1	24	54	20.1	PI, DM, LD, HEM	PA	No	Yes	Yes	41	No	No
4	UMCU	F	25.1	30	50	16.0	PI, LD, DM	PA	No	Yes	Yes	1	No	No
5	UMCU	F	37.7	21	31	19.5	PI, DM, HEM	PA, AF	Yes	Yes	No	7	No	Yes
6	UMCU	M	27.3	18	32	17.6	PI, PTX	PA	No	Yes	No	8	No	Yes
7	UMCU	M	23.8	27	31	15.2	PI, LD, PTX	SA, SP, HI	No	Yes	No	3	No	Yes
8	UMCU	M	20.1	20	31	13.6	PI, LD	PA	No	Yes	No	3	No	Yes
9	UMCU	M	23.8	20	31	14.8	PI, HEM	PA	No	Yes	Yes	9	Yes	Yes*
10	UMCU	F	26.8	19	33	14.0	PI, DM, PTX	PA, AF	No	Yes	Yes	4	No	No
11	UMCU	F	41.3	36	52	19.7	PI, LD, HEM	PA	Yes	Yes	Yes	5	No	No
12	UMCU	M	23.8	23	35	27.5	PI, LD	PA, AF	No	No	No	4	No	No
13	UMCU	M	23.6	21	45	18.3	PI, DM, HEM	PA	No	No	Yes	2	No	Yes
14	UMCU	F	18.9	33	51	12.8	PI	PA	No	Yes	Yes	6	No	No
15	UMCU	F	29.6	32	43	18.6	PI, DM, LD, HEM	PA	No	Yes	Yes	7	No	No
16	UMCU	M	23.5	20	27	19.6	PI, HEM, ABPA	PA, AF	Yes	Yes	Yes	10	No	No
17	UMCU	M	27.4	15	27	22.7	PI, DM, HEM	PA	No	Yes	No	2	No	No
18	UMCU	M	20.7	16	24	17.9	PI	PA	No	Yes	Yes	12	No	No
19	UMCU	M	23.6	23	37	21.7	PI, DM, HEM	PA	Yes	Yes	No	2	Yes	Yes*
20	UMCU	M	17.0	39	47	18.1	PI, PTX	PA, BC	No	No	Yes	4	No	No
21	UMCU	F	15.4	26	44	12.6	PI, DM, PTX	PA, SM, SH	No	Yes	Yes	33	No	No
22	HAGA	F	18.6	50	82	19.9	PI, DM	PA	Yes	No	Yes	9	No	Yes
23	HAGA	M	29.6	22	38	18.8	PI, DM, HEM, PTX	PA	No	Yes	Yes	4	No	No
24	HAGA	F	39.7	24	34	20.2	PI, DM, PTX	PA	Yes	Yes	Yes	1	No	No
25	HAGA	M	30.4	19	35	22.0	PI, HEM, PTX	PA	Yes	Yes	No	1	No	No
26	HAGA	M	22.3	22	42	19.2	PI, LD,PTX	PA, BC	Yes	Yes	No	2	No	No

UMCU indicates University Medical Center Utrecht; HAGA, Haga Teaching Hospital;F, Female; M, Male; FEV_1, Forced expiratory volume in one second (percentage of predicted); FVC, Forced expiratory volume (percentage of predicted); BMI, Body mass index; PI, Pancreatic insufficiency; DM, diabetes mellitus; LD, liver disease; HAEM, hemoptysis; PTX, pneumothorax; ABPA, Allergic Broncho Pulmonary Aspergillosis; MO, Micro-organism; PA, *Pseudomonas aeruginosa*; AF, *Aspergillus fumigatus*; BC, *Burkholderia Cepacia*; SA, *Staphylococcus aureus*; SH, *Streptococcus hemoliticus*; LOS ICU, Length of stay at intensive care unit; and LTX, lung transplantation.
*Survival after LTX while ventilated at ICU.

Supplementary Table 9A. SNPs determined using SSP-PCR.

dbSNP identifier	SNP*	Amino acid change	Name	Primer
rs7096206	−290 G/C	Promoter	Y/X	F-CATTTGTTCTCACTGCCAC**G/C**
				R-GATGCCAGAGAATGAGAGCTG
rs5030737	154 C/T	D52C	D-variant	R- T (C) CCTTGGTG (C) CATCAC**G**†
				R- CT (T) CCTTGGTG (C) CATCAC**G**†
				R- CT (C) CCTTGGTG (T) CATCAC**G**†
				R-TTCT (T) CCTTGGTG (T) CATCAC**G**†
				R- CT (C/T) CCTTGGTG (C/T) CATCAC**A**†
				F-ACGGTCCCATTTGTTCTCACT
rs1800450	161 G/A	G54D	B-variant	R-CCCCTTTTCT (C/T) CCTTGGTG**T**†
				R- CCCTTTTCT (C/T) CCTTGGTG**C**†
				F-ACGGTCCCATTTGTTCTCACT
rs1800451	170 G/A	G57E	C-variant	R-ACCTGGTTCCCCCTTTTCT**T**
				R- CCTGGTTCCCCCTTTTCT**C**
				F-ACGGTCCCATTTGTTCTCACT

* Base pair substitutions are defined based on the coding strand.
† Different primers were used in 1 run for overlapping sequences in exon 1.

Supplementary Table 10A. SNPs determined using SSP-PCR.

dbSNP identifier	Gene	SNP*	Amino acid change	Primer
rs4986791	TLR4	8851 C/T	T399I	F-TCTCAAAGTGATTTTGGGACAA**C**
				F-TTCTCAAAGTGATTTTGGGACAA**T**
				R-GAGAGAGGTCCAGGAAGGTC
rs4696480	TLR2	-16934 T/A		F-ATTGAAGGGCTGCATCTGG**T**
				F-ATTGAAGGGCTGCATCTGG**A**
				R-GTGTGCCCCAAAGCTCATG
rs5743704	TLR2	1891 C/A	P631H	F-CCAGGCCAAAAGGAAGC**C**
				F-TCCAGGCCAAAAGGAAGC**A**
				R-GGAAATGGGAGAAGTCCAGTT
rs5743708	TLR2	2257 A/G	R753Q	R-AGGTCTTGGTGTTCATTATCTTC**T**
				R-GGTCTTGGTGTTCATTATCTTC**C**
				F-ATGATGTGGGCCTGGCTC
rs5744455	CD14	-651 C/T		F-AAGGGGGAATTTTTCTTTAGAC**C**
				F-GAAGGGGGAATTTTTCTTTAGAC**T**
				R-CTGAGGTTCGGAGAAGTTGC
rs2569190	CD14	-159 C/T		F-CAGAATCCTTCCTGTTACGG**C**
				F-CAGAATCCTTCCTGTTACGG**T**
				R-CTGAGGTTCGGAGAAGTTGC

* Base pair substitutions are defined based on the coding strand.

Supplementary Table 11A. SNPs determined using SSP-PCR.

Gene	Variant	Position*	Reference SNP	Genotype
IL6	Promoter	-174 G/C	rs1800795	R-TGCAATGTGACGTCCTTTAGCAT**C/G**
				F- AAGTAACTGCACGAAATTTGAGGG/A†
IL-8	Intron	678 C/T	rs2227306	R-GTCATAACTGACAACATTGAAC**A/G**
				F-AGTTGAGCAAAAGGTAACTCAGA
CXCR-1	S276T	2607 G/C	rs2234671	F-CCCAGGTGATCCAGGAGA**G/C**
				R-TCAGAGGGTTGGAAGAGACATT
CXCR-2	Exon	1208 T/C	rs1126579	F-CCATTGTGGTCACAGGAAG**T/C**
				R-GTCTTGTGAATAAGCTGCTATGA
IL-10	Promoter	-1082 A/G	rs1800896	R-(CT)CTTACCTATCCCTACTTCCCC**T/C**
				F-AGGCCTCCTGCACCTAGGT

* Base pair substitutions are defined based on the coding strand.
† Different consensus primers were used in 1 run for overlapping sequences.

Supplementary Table 12A. SNPs determined using SSP-PCR.

Gene	dbSNP identifier	SNP*	Amino acid change	Name	Primer
MBL2	rs7096206	−290G>C	Promoter	Y/X	F-CATTTGTTCTCACTGCCAC**G**
					F-CATTTGTTCTCACTGCCAC**C**
					R-GATGCCAGAGAATGAGAGCTG
	rs5030737	154C>T	D52C	D-variant	R- T (C) CCTTGGTG (C) CATCAC**G**†
					R- CT (T) CCTTGGTG (C) CATCAC**G**†
					R- CT (C) CCTTGGTG (T) CATCAC**G**†
					R-TTCT (T) CCTTGGTG (T) CATCAC**G**†
					R- CT (C/T) CCTTGGTG (C/T) CATCAC**A**†
					F-ACGGTCCCATTTGTTCTCACT
	rs1800450	161G>A	G54D	B-variant	R-CCCCTTTTCT (C/T) CCTTGGTG**T**†
					R- CCCTTTTCT (C/T) CCTTGGTG**C**†
					F-ACGGTCCCATTTGTTCTCACT
	rs1800451	170G>A	G57E	C-variant	R-ACCTGGTTCCCCCTTTTCT**T**
					R- CCTGGTTCCCCCTTTTCT**C**
					F-ACGGTCCCATTTGTTCTCACT
IL6	rs1800795	-174 G/C	Promoter		R-TGCAATGTGACGTCCTTTAGCAT**C**
					R-TGCAATGTGACGTCCTTTAGCAT**G**
					F- AAGTAACTGCACGAAATTTGAGGA#
					F- AAGTAACTGCACGAAATTTGAGGG#

* Base pair substitutions are defined based on the coding strand.
† Different primers were used in 1 run for overlapping sequences in exon 1.
# Different consensus primers were used in 1 run for overlapping sequences.

LIST OF ABBREVIATIONS

ABPA	Allergic bronchopulmonary aspergillosis
ACE	Angiotensin converting enzyme
ARF	Acute respiratory failure
BALF	Bronchoalveolar lavage fluid
BC	*Burkholderia cepacia*
BMI	Body mass index
CF	Cystic fibrosis
CFRD	CF-related diabetes
CFRLD	CF-related liver disease
CFTR	Cystic fibrosis transmembrane regulator
CI	Confidence interval
CO_2	Carbon dioxide
CRP	C-reactive protein
ENT	Ear, nose and throat
FEV_1	Forced expiratory volume in 1 second
FFM	Fat free mass
FVC	Forced vital capacity
HWE	Hardy–Weinberg equilibrium
ICU	Intensive care unit
IFN-γ	Interferon γ
IgG	Immunoglobulin G
IL	Interleukin
IMV	Invasive mechanical ventilation
LPS	Lipopolysaccharide
LTX	Lung transplantation
MBL	Mannose-binding lectin
MI	Meconium ileus
NBS	Newborn screening
NIV	Non-invasive ventilation
OR	Odds ratio
ORL	Otorhinolaryngologic
PA	*Pseudomonas aeruginosa*
PAMP	Pathogen associated molecular pattern
PEF	Peak expiratory flow
PI	Pancreatic insufficiency
PS	Pancreatic sufficiency
RAS	Renin-angiotensin system
RER	Respiratory exchange ratio
ROC curve	Receiver operating characteristic curve
SA	*Staphylococcus aureus*
SD	Standard deviation

SDS	Standard deviation score
SE	Standard error
SNP	Single nucleotide polymorphism
SSP	Sequence-specific primers
TGF-β_1	Transforming growth factor β_1
RAS	Renin-angiotensin system
RER	Respiratory exchange ratio
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
UMCU	University Medical Center Utrecht
VO_2peak	Peak oxygen uptake
VO_2	Oxygen uptake
Wpeak	Maximal work load

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CURRICULUM VITAE

Martijn Slieker werd op 14 mei 1979 geboren te Rhenen. Hij genoot zijn middelbare school opleiding op het Christelijk Lyceum Veenendaal, waar hij in 1997 zijn VWO diploma behaalde. Datzelfde jaar begon hij aan de studie Geneeskunde aan de Universiteit Utrecht. Tijdens zijn studie was hij student-assistent Epidemiologie, lid van de Opleidingscommissie van de faculteit Geneeskunde en voorzitter van de Co-Raad. Zijn co-assistentschap Keel-, Neus- & Oorheelkunde (KNO) liep hij in de Massachusetts Eye & Ear Infirmary van de Harvard Medical School te Boston. Zijn keuzeco-assistentschap genoot hij op de Pediatrische Intensive Care Unit van het UMC Utrecht (supervisor prof. dr. A.J. van Vught).

Martijn verrichtte zijn wetenschappelijke stage op de afdelingen Kinderlongziekten en KNO Heelkunde van het UMC Utrecht, waar hij onderzoek deed naar longfunctiemetingen bij kinderen en naar KNO-complicaties bij cystic fibrosis (supervisoren dr. C.K. van der Ent en dr. A.G. Schilder). Dit laatste onderzoek vormde het begin van zijn latere promotietraject.

In 2003 behaalde Martijn summa cum laude zijn artsexamen en begon hij aansluitend als AGIKO Kindergeneeskunde. Sindsdien heeft hij zijn promotieonderzoek naar klinische en genetische voorspellers van het ziektebeloop bij cystic fibrosis (promotoren prof. dr. C.K. van der Ent en prof. dr. E.A.M. Sanders) afgewisseld met de klinische opleiding tot kinderarts (opleider prof. dr. J.L.L. Kimpen, thans dr. J. Frenkel). Tevens volgde hij tussen 2003 en 2005 de opleiding tot klinisch epidemioloog aan het Netherlands Institute for Health Sciences (NIHES) van de Erasmus Universiteit te Rotterdam. Daarnaast liep Martijn in 2004 een onderzoekstage aan het National Heart and Lung Institute van het Royal Brompton Hospital te Londen (supervisor prof. dr. K. Welsh).

Thans is Martijn voor het klinische deel van zijn opleiding werkzaam in het St. Antonius Ziekenhuis te Nieuwegein (opleider dr. J.A. Schipper). Daarnaast is hij bestuurslid van de Stichting FOK, de Juniorafdeling van de Nederlandse Vereniging voor Kindergeneeskunde (NVK) en de NVK Congrescommissie. Tevens is hij lid van de stuurgroep Landelijke CF Registratie van de Nederlandse Cystic Fibrosis Stichting (NCFS).

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