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STUDIES AS A BASIS FOR A POSSIBLE INTRODUCTION OF NEWBORN SCREENING FOR CYSTIC FIBROSIS IN SWEDEN

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To all CF patients

Lungan min
andas ut och in,
lungor två
andas på.

Madeleine Olsson 9 år

ABSTRACT

Background: Cystic fibrosis (CF) is the most common life-shortening autosomal recessive disease in Caucasians. It is a multi-organ disease that results from mutations in the gene that encodes the CF transmembrane conductance regulator (CFTR) protein, which regulates ion transport at epithelial surfaces. The main clinical characteristics are progressive pulmonary disease and pancreatic insufficiency. Not long ago most affected patients died in childhood. The outlook for patients with CF has improved steadily over the years, as a result of more aggressive therapy and care in specialised centres. Current understanding of the molecular-biological defect that underlies CF will lead to new treatments. To benefit from these new treatments, patients should have as few irreversible lesions as possible. An early CF diagnosis by newborn screening is therefore highly desired. The overall aim of this thesis was to provide a basis for discussion of whether CF should be included in the national newborn screening programme in Sweden.

Paper I is a multi-centre study in which we analysed the *CFTR* mutation in 75% of the Swedish CF population (331 patients). The three most common *CFTR* mutations were deltaF508 68.3%, 394delTT 8.5% and 3659delC 7.9%, comprising 84.6% of the CF alleles in the material. This result shows that newborn screening for CF with an IRT/DNA approach is possible in Sweden.

Paper II is a questionnaire study of parental attitudes towards newborn screening for CF in which the parents of 143 CF patients and parents of children in two age-matched control groups participated. A majority of the parents, 70-86% in the different groups of parents, supported screening for CF. The parental attitude of CF parents was independent of the age of the child, the delay of diagnosis as experienced by the parents, and the well-being of the CF child at the time of diagnosis.

Paper III is a questionnaire study to the parents of the same 143 CF patients as in Paper II to investigate their experiences after the clinical CF diagnosis of their child, and to find out the effects of the diagnosis on the family. The parental experiences on receiving a clinical CF diagnosis were intense and emotional. The parents stated that the CF diagnosis greatly influenced other family members, such as siblings and grandparents. A majority of the CF families had not experienced any change in relationships within the family, or in the social life of the family, one year after the diagnosis.

Paper IV is a register study of 119 CF patients born between 1974 and 2001 in which we studied disease progression over time for lung, liver, nutritional and overall morbidity with Kaplan-Meier curves and proportional hazards regressions. The median age at diagnosis of the patients was 5 months. The patients with overall morbidity at diagnosis showed a slow decline of symptoms, with half of the patients becoming free of overall morbidity after 4.8 years; however, the patients who were older than 24 months at diagnosis had a lower probability of becoming free of morbidity (crude hazards ratio 0.14 [95% confidence interval 0.04, 0.45]) than those with an earlier diagnosis, at the age of 2-12 months ($p < 0.01$).

Paper V is a register study of all patients in the Stockholm CF Centre register with the diagnosis of CF and two verified *CFTR* mutations, in total 220 patients. *CFTR* mutations or larger deletions were found in all patients with classic CF. There was no statistical difference in lung function using the mixed model analysis for the different mutation groups studied. Patients born after 1985 had better lung function (FEV_1 and FVC) than those born earlier.

Conclusion: Our studies show that the conditions for a newborn screening programme for CF in Sweden are good and that a majority of parents in Sweden support the inclusion of CF in the newborn screening programme.

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LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerials (I-V):

I. Schaedel C, Hjelte L, de MONESTROL I, Johannesson M, Kollberg H, Kornfält R, Holmberg L. Three common CFTR mutations should be included in a neonatal screening programme for cystic fibrosis in Sweden.
Clinical Genetics 1999;56:318-322

II. de MONESTROL I, Bergsten Brucefors A, Sjöberg B, Hjelte L.
Parental support for newborn screening for cystic fibrosis.
Acta Paediatrica 2010; 100:209-215

III. de MONESTROL I, Bergsten Brucefors A, Sjöberg B, Hjelte L.
Parental experiences of a clinical cystic fibrosis diagnosis and impact on siblings and relatives.
Manuscript.

IV. de MONESTROL I, Klint Å, Sparén P, Hjelte L.
Age at diagnosis and disease progression of cystic fibrosis in an area without newborn screening.
Paediatric and Perinatal Epidemiology, in press.

V. de MONESTROL, Berg E, Cuppens H, Hjelte L.
Extensive genetic analyses find cystic fibrosis mutations and make longitudinal mixed models of lung function according to *CFTR* classes possible.
Manuscript.

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ABBREVIATIONS

ABPA	Allergic BronchoPulmonary Aspergillosis
AAV	Adeno-Associated Viruses
cAMP	cyclic Adenosine MonoPhosphate
CBAVD	Congenital Bilateral Absence of Vas Deferens
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CFRD	CF-related Diabetes Mellitus
DNA	DeoxyriboNucleic Acid
DIOS	Distal Intestinal Obstruction Syndrome
ENaC	Epithelial sodium (Na^+) channel
ER	Endoplasmic Reticulum
FEV_1	Forced Expiratory Volume in one second
FVC	Forced Vital Capacity
ICM	Intestinal Current Measurement
IgG	Immunoglobulin G
IRT	ImmunoReactive Trypsinogen
kb	kilo base pairs
kDa	kilo Dalton
NBD	Nucleotide-Binding Domains
NPD	Nasal Potential Difference
OLA	Oligonucleotide Ligation Assay
PAP	Pancreatitis-Associated Protein
RCT	Randomised Controlled Trial
SSCP	Single-Strand Conformation Polymorphism
TM	TransMembrane segments
WHO	World Health Organisation

1 INTRODUCTION

1.1 History of cystic fibrosis

”The child will soon die whose brow tastes salty when kissed” was a well-known quotation dating back to the eighteen century. Cystic fibrosis (CF) was first described independently by the Swiss paediatrician Guido Fanconi in 1936 [1] and the American pathologist Dorothy Andersen in 1938 [2]. The thick, sticky mucus clogging the ducts and mucus glands throughout the body also gave rise to the alternative name ”mucoviscoidosis” [3]. Andersen recognized CF to be a genetic disease transmitted in an autosomal recessive pattern [2]. The disease became known as a ”generalized exocrinopathy” because many exocrine glands were affected [4].

A critical discovery was made during the 1948 heat wave in New York by the paediatrician Paul di Sant’Agnese, who noticed that many of the infants presenting with heat prostration had CF. He demonstrated an excess of sodium and chloride in the sweat of patients with CF, which persisted in these patients after the heat wave subsided [5]. Elevated sweat chloride concentration offered a convenient diagnostic test. The pilocarpine iontophoresis technique of Gibson and Cooke, established in 1959, is still in use because of its outstanding discriminating power [6]. Sweat ducts were used to identify chloride transport as the basic defect in CF by Paul Quinton in 1983 [7]. Increased sodium reabsorption in the airways was identified as a regular feature of CF [8].

1.2 Sweat test

CF was initially a pathological diagnosis. The sweat test allowed patients without pancreatic insufficiency to be identified. Nearly every patient with a clinical diagnosis of CF has an elevated sweat chloride concentration. There are some conditions (sometimes single case reports) that have been reported to have an elevated sweat chloride concentration greater than 60 mmol/l. These conditions include adrenal insufficiency, stress, anorexia nervosa, ectodermal dysplasia, eczema, oedema, hypoparathyroidism, hypothyroidism, malnutrition/dehydration and nephrosis. Fewer conditions give a false negative sweat test: examples are oedema, malnutrition and treatment with systemic steroids. Sweat electrolytes are not affected by the intake of flucloxacillin or diuretics or by the administration of intravenous fluids [9]. A few patients with CF have normal or borderline sweat chloride concentrations, this is especially true for patients with the 3849+10kbC-T mutation [10].

The standard sweat test requires experienced technicians using standardised methodology [11, 12]. International standards suggest that a laboratory should be undertaking at least 50 tests per year. Sweating is stimulated by the iontophoresis of pilocarpine into the skin on the forearms, or the back of babies. Sweat is then collected onto preweighed filter paper or gauze. Sweat should be collected for 20-30 minutes with a sweat volume of 50-100 ml (a minimum sweat rate of 1 g/m^2 body surface area/min is required). The sweat test can be reliably performed on infants older than 2 weeks and weighing more than 3 kg, who are normally hydrated and without significant system illness [9].

During the first 24 hours after birth in full-term infants, sweat electrolyte values may be transiently elevated [13]. A decline in the levels occurs after the first week. Sweat chloride levels greater than 60 mmol/l are considered positive. Sweat chloride levels greater than 150 mmol/l are not physiological and should be questioned. Borderline levels between 30 and 60 mmol/l may be associated with CF and require further analyses and examinations [14]. Most of the studies exploring persons with equivocal sweat tests have focused on the chloride range 40-60 mmol/l, because 40 mmol/l corresponds to three standard deviations above the mean in the heterozygous carrier group [15]. Later studies have shown that CF patients occur with a similar frequency in the 30-40 mmol/l range as in the 40-60 mmol/l range [16]. Sweat chloride concentrations of 30-60 mmol/l were seen in only about 4% of sweat tests and 23%

of these patients were subsequently found to have two *CFTR* mutations.

Normal adults tend to have higher sweat chloride levels, and borderline values in this age group may not reflect CF. Sweat sodium concentration can be measured, but should never be used alone. In persons with CF, the sweat chloride level is usually higher than the sweat sodium level, but the converse is true in normal persons. The osmolarity of sweat can also be measured, but the reference ranges are much wider. There are other sweat tests than the standard pilocarpine iontophoresis, developed for easier sweat collecting or measuring. Most of them should be thought of as a ”screening sweat test” to help in deciding whether or not the patient should be sent to a hospital for the standard sweat test. One of the other tests is the Nanoduct,® measuring conductivity, which represents a non-selective measurement of ions. A good correlation between sweat chloride concentrations and sweat conductivity has been shown in a large study; the best conductivity cut-off value to diagnose CF was 90 mmol/l and the best conductivity cut-off value to exclude CF was 75 mmol/l [17]. A positive sweat conductivity result should be followed up with a formal measurement of sweat chloride concentrations.

Persons with borderline values may require repeated testing over a longer period of time.

1.3 Nasal potential difference

Respiratory epithelia generate a transepithelial electrical potential difference through the active transport of ions. In 1981, Knowles reported that nasal potential difference (NPD) was abnormal in patients with CF, with elevated Na^+ reabsorption and reduced Cl^- secretion in response to cAMP [18]. In Knowles' original studies, the NPD catheter was placed under the inferior turbinate and the technique was difficult to use. Alton showed some years later that a simpler technique with measurement from the floor of the nose (not exactly under the inferior turbinate) discriminated equally well between patients with CF and patients with bronchiectasis and Young's syndrome [19]. Both techniques discriminate well between CF and controls, which is confirmed [20].

NPD measurement has progressed from a research tool to a clinical diagnostic test [21], but it still requires a skilled operator and an experienced interpreter. There are no exact reference values, but there are standard procedures for how NPD is measured between the nasal epithelia and a reference electrode on the skin on the forearm [22-25].

The specific features seen in the NPD curve of a person with CF are:

1) The basal NPD results in a more negative baseline, which reflects the increased Na^+ transport via the amiloride sensitive ENaC channel.

2) A less negative potential after infusion with the ENaC channel inhibitor amiloride (due to a greater inhibition of the accelerated Na^+ transport through the ENaC channel).

3) Little or no change in the NPD in response to perfusion with a Cl^--free solution in conjunction with isoproterenol, which reflects an absence of the CFTR-mediated Cl^- secretion [18].

The presence of inflamed epithelia or nasal polyps alters bioelectrical properties and may give a false negative result [23].

In conclusion, the NPD of patients with classic CF is remarkably different from controls, even though exact reference values are not available. In atypical CF, the NPD may be borderline and there is no consensus about how to interpret these results.

1.4 Intestinal current measurement (ICM)

The abnormalities in epithelial ion transport can also be measured throughout the intestinal tract. The duodenum can be used [26], but the most common measurements are performed on rectal suction biopsies [27-29]. There is a clear

difference between ICM measurement in patients with classic CF and normal controls, and data are now emerging about the use of ICM as a clinical diagnostic tool [28, 30].

1.5 Diagnostic criteria for CF

Since the sweat test came into general use, diagnosis has been made on the basis of two positive sweat tests plus either a sibling or first cousin with CF, or pancreatic insufficiency, or lung disease [31]. After the discovery of the CF gene and analysis of mutations came into practice, diagnosis is not always clear cut. More than 1800 mutations have been identified, not all of which result in CF [32]. The vast majority of patients with CF have classical signs and symptoms. The European terminology proposed by the Diagnostic Working Group of the European Cystic Fibrosis Society in 2005 [14] is a revision of the 1998 statement from the Cystic Fibrosis Foundation Consensus Conference in the USA [33].

According to the European terminology, classic (or typical) CF is when patients have one or more phenotypic characteristics of CF and sweat chloride concentrations greater than 60 mmol/l. The phenotypic characteristics are: chronic sinopulmonary disease, specific or characteristic gastro-intestinal or nutritional abnormalities, salt loss syndromes, and male genital abnormalities resulting in obstructive azoospermia [33].

Non-classic (or atypical) CF is described as CF phenotype in at least one organ system and a normal (<30 mmol/l) or borderline (30-60 mmol/l) sweat chloride level. Confirmation of the diagnosis of CF in such patients requires detection of one disease-causing mutation on each *CFTR* gene *or* direct quantification of CFTR dysfunction by nasal potential difference measurement (NPD) [14, 33, 34]. Most of these patients have exocrine pancreatic sufficiency and mild lung disease. When two mutations are detected, at least one of them is classified as ”mild”. Some patients with single organ involvement with one or two *CFTR* mutations may be given an alternative ”diagnostic label” as recommended by a joint meeting with the World Health Organisation (WHO) in 2000; isolated obstructive azoospermia, chronic pancreatitis, ABPA, disseminated bronchiectasis, diffuse panbronchiolitis, sclerosing cholangitis and neonatal hypertrypsinogenemia [35]. These diagnoses are also called ”CFTR-related disorders”.

It is advised that inconclusive (equivocal) diagnosis and alternative diagnosis associated with CF according to the WHO list are followed up at a CF centre, at least for some time [14]. In a newborn screening programme or in a sibling of a patient with CF, the diagnosis of CF may come before the child has shown any clear symptoms. However, experienced CF doctors may recognise steatorrhea, failure to thrive or increased respiratory rate in those babies with classic CF already when they come for the confirmatory sweat test. (Personal communication Dr Renner, ERS online course The management of infants identified with CF following newborn screening, year 2010)

In conclusion, it is important to remember that a diagnosis of CF should be based upon clinical symptoms, not CF mutations. The sweat test remains the gold standard for CF diagnosis. The diagnosis of CF should never be based on a single positive sweat test result: the diagnosis should always be confirmed with a second sweat test, mutations analysis, or NPD.

1.6 Genetics

CF is a monogenic autosomal recessive disease. A recessive disease needs two disease-causing mutations residing on separate alleles (separate chromosomes). The chromosome constitutes two alleles, one inherited from the mother and the other from the father. So CF is an inherited disease in which one mutation comes from the mother and one from the father. The parents are carriers of the mutation, and carriers are asymptomatic (with some exceptions, which will be mentioned later). Recessive diseases

affect both sexes equally: there is an increased incidence associated with parental consanguinity and, after the birth of one affected child, there is a 25% risk of each subsequent child having the disease.

1.6.1 The CF gene and the protein produced

The CF gene was discovered in 1989 in cooperation between three research groups, those of Lap-Chee Tsui and Jack Riordan in Toronto and Francis Collins in Michigan [36-38].The CF gene was first mapped in family studies by the demonstration of linkage to a set of polymorphic DNA markers on chromosome 7. The region of the chromosome was further narrowed by physical cloning and analysis of the DNA for candidate genes. The gene was finally identified on the basis of predicted biochemical properties and the documentation of a 3-base-pair deletion that was associated exclusively with the disease in CF families. Knowledge of the functional properties of the gene was obtained through transfection studies showing that cDNA can reverse the defect of chloride conductance in epithelial cell cultures derived from CF patients. It was the first gene identified by positional cloning whose function was entirely unknown, and the most common mutation deltaF508 was found.

The gene is located on chromosome 7 (7q31.2), comprises 27 coding exons, is 250 kb in length and produces a transcript of length 6.5 kb. It encodes a protein of 1480 amino acids with a molecular weight of 170 kDa. The protein comprises five domains: two membrane-spanning domains (MSD1 and MSD2), each composed of six transmembrane segments (TM1 to TM12) that form the channel, two nucleotide-binding domains (NBD1 and NBD2) capable of ATP hydrolysis, and an intracellular regulatory domain (R), which contains numerous phosphorylation sites. This protein structure is similar to other members of the ATP-binding cassette (ABC) superfamily of membrane transporters, although the R domain is unique for this protein.

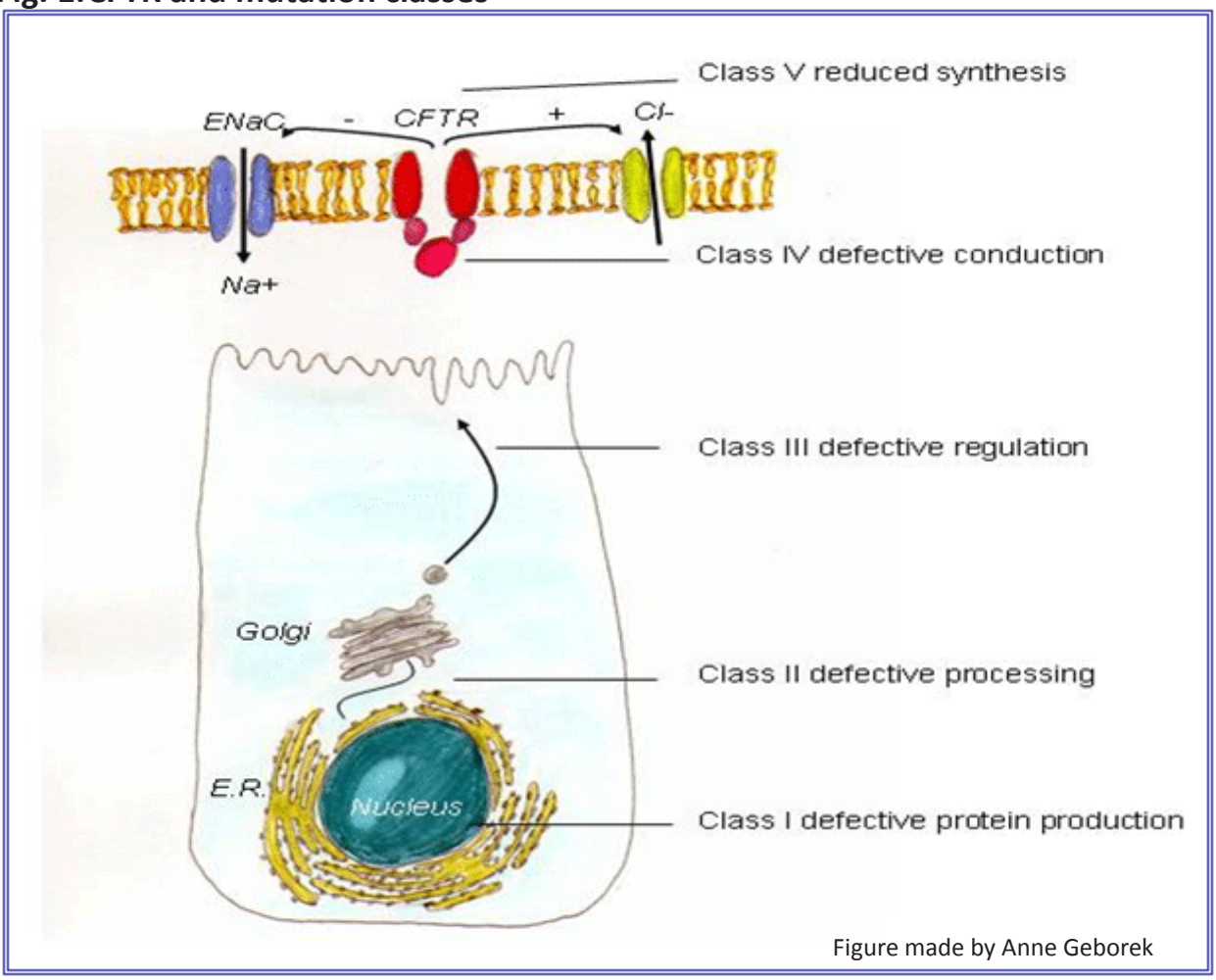
The gene and the protein were named ”Cystic Fibrosis Transmembrane conductance Regulator” (CFTR), because of its functional properties. The gene is always written in italics, *CFTR*, to distinguish it from the protein, which is written with an upright font. The CFTR protein is a cAMP-regulated chloride channel that secretes chloride ions from the cell. The activation of CFTR requires both phosphorylation of the R domain and hydrolysis of ATP at the NBDs [39]. CFTR is expressed on the apical membrane of epithelial cells lining the airways, sweat ducts, pancreatic ducts, intestines, biliary tree and vas deferens [40]. The pathophysiology of CF is complex and cannot solely be explained by the loss of the CFTR function as a chloride channel. In addition to being a chloride channel, CFTR regulates other apical membrane channels that transport ions directly or indirectly[41]. The CFTR has an inhibitory effect on the amiloride-sensitive epithelial Na^+ channel (ENaC). This inhibition is abolished in CF, which causes the increased epithelial Na^+ reabsorption found in the airways of CF patients [42]. CFTR has an activating effect on the outwardly rectifying Cl^- channel (ORCC). When CFTR does not function properly, Cl^- secretion by the cAMP pathway is diminished [43]. CFTR is involved in many other processes, such as intracellular membrane transport and the regulation of ion transporters on the basolateral membrane [41, 44, 45].

The discovery of the CF gene was important for refined diagnosis, for better insight into clinical problems, for looking at the impact of partial deficiency of the CFTR protein, and for improving the possibility to screen for drugs to correct the gene function. The discovery of the CF gene, however, has also revealed the complexity of the disease.

1.6.2 *CFTR* mutation classes

Increased knowledge about the molecular mechanisms of the different mutations and how they cause the symptoms of CF led to a classification into five major classes [46-48]. The mutation classes are based on the

Fig. 1.CFTR and mutation classes



mutation's effect on the structure and function of the CFTR protein. The five classes originally described are:

Class I: Defective protein production. No full-length CFTR protein is synthesised due to premature stop. No CFTR protein will reach the apical membrane.

Class II: Defective cellular processing and maturation. The protein will not be properly processed to a mature glycosylated form and transported to the apical membrane. The incorrectly folded protein will be recognised as abnormal by cellular "quality control", which leads to premature degradation in, for example, the endoplasmic reticulum (ER) or the Golgi complex. The mislocalisation of the deltaF508 protein is temperature-dependent and reversible in subphysiological temperatures (23-30^{o} C), under which conditions it can escape from the ER and reach the cell membrane[49].

Interestingly, patients homozygous for deltaF508 mutations may have residual CFTR activity, which would include them in another mutation class [50, 51].

Class III: Defective regulation. The protein reaches the apical cell membrane in normal amounts but is non-functional. Most of these mutations affect the nucleotide-binding domains (NBD1, NBD2), and this effect prevents normal response to stimulation by ATP and thus suppression of chloride channel activity. The CFTR regulation of other channels may also be affected [52, 53].

Class IV: Defective conductance. CFTR protein is found in normal amounts at the apical cell membrane, but the amount of current passed is sometimes reduced because the amount of time the channel is open is reduced. These mutations often affect the membrane-spanning domains that contribute to the channel pore.

Class V: Reduced synthesis or function. A reduced amount of CFTR or functional CFTR is found at the apical membrane, due to abnormalities of transcrip-tion and translation.

The classification was later expanded with a sixth class to accommodate more data:

Class VI: Decreased stability affecting the regulatory functions. Functional but unstable CFTR protein is present at the apical membrane. Class VI mutations are usually associated with truncation of the C terminus of CFTR, leading to instability of an otherwise fully processed and functional protein [41, 54, 55].

Some mutations disrupt the normal functioning of the CFTR protein in more than one way, and can belong to more than one mutation class. DeltaF508, for example, described above, prevents maturation (Class II) and it can be present at the apical membrane with residual, but down-regulated, activity of the chloride channel (Class III and Class IV).

Mutation classes I-III give complete loss of chloride channel activity and are called severe mutations. Classes IV-V retain some chloride channel activity and are therefore called mild mutations. Class VI is associated with severe CF presentation.

1.6.3 *CFTR* mutations

The most common *CFTR* mutation, deltaF508, comprises 66% of mutations worldwide, followed by G451X and G551D at 2.4% and 1.6% respectively [32]. The mutation names used in this thesis are now called ”legacy names” in the Cystic Fibrosis Mutation Database. The prevalence of mutations varies among CF populations and deltaF508 is not always the most common. There is a variation within countries as well, depending on the ethnic origin. The disease has a northwest to southeast gradient in Europe, being more common in the northwest and less common in the southeast. The highest occurrence of deltaF508 in a population is in Denmark, where almost 90% of all CF alleles are deltaF508 [56]. It has been suggested that the deltaF508 mutation occurred more than 52,000 years ago, in a population genetically distinct from any present European group, and spread throughout Europe in chronologically distinct expansions [57]. Other mutations have other geographical distributions. W1282X, for example, is common in Israel (especially among the Ashkenazi Jews), G542X in the Balearic Islands and N1303K in Tunisia [58].

The chromosome region where the CFTR gene is located is a genetically stable region of DNA [59]. There must have been a survival advantage for heterozygotes, which has led to natural selection and explains why this mutation is so common in Caucasian populations. One explanation suggested is that heterozygotes are protected against diarrhoeal diseases. In mice, *Salmonella enterica* (serovar typhi) utilizes CFTR to enter epithelial cells in the gut and there is a survival advantage for CF heterozygotes [60]. There is also a typhoid hypothesis, since the deltaF508 frequency increased after typhoid waves. But the *Vibrio cholera* toxin has at least three different ways of entering the cell and it is unclear if any of them is CFTR-dependent [58].

1.6.4 Genotype-phenotype correlations

CF has variability in phenotype (clinical picture, disease expression), ranging from the severe classical picture with pancreatic insufficiency and early lung involvement to much milder forms that present later in life. As there is also a wide variability in genotype (different types of mutations, giving different molecular mechanisms and functions), there is a rationale for looking at genotype-phenotype correlations. The variability of the phenotype is different in different organs; low for the sweat glands and male genital tract, higher for pancrease and highest for the respiratory system [55].

The genotype-phenotype correlation (genotype of *CFTR* gene mutation according to functional mutation class and phenotype) in CF is high

for pancreatic function [61, 62]. In general, patients with pancreatic insufficiency are homozygous or compound homozygous, (having different mutations) of two severe mutations (Class I-III or VI), whereas patients with pancreatic sufficiency have at least one mild mutation (Class IV or V). If an individual is compound heterozygous, the milder mutation is dominant. Patients with at least one mild CF mutation retain some chloride channel activity, so pancreatic function may be sufficient for digestion. Furthermore, lung disease is less severe, and the sweat chloride concentration may be in the normal range [62, 63].

The genotype-phenotype correlation has been more difficult to show in the lung. Some rare mutations, such as A455E, are linked to a better pulmonary outcome [62, 64]. To link deltaF508 and most other common mutations with the severity of the lung disease has been unsuccessful for many years. However, some studies have indicated such an association [65, 66].

Co-existent polymorphisms (DNA variations observed in about 1% of normal individuals) in the *CFTR* gene can also affect the gene and the protein produced. A well-described example is the association between the mild mutation R117H and the thymidine (T) sequence in intron 8, where the 5T allele leads to a substantial reduction in the amount of functional protein, while the 7T allele gives intermediate reduction and the 9T allele gives a normally functioning protein [67].

The genotype alone cannot explain the phenotype of CF. Modifying genes elsewhere in the genome significantly affect the CFTR protein. A modifier gene for the risk of being born with meconium ileus has been identified [68]. Other genes and their proteins influence the biochemistry and immunity of the lungs [69]. Environmental factors and the passage of time also influence the clinical course of each patient.

1.7 Incidence of CF

The incidence (or birth prevalence) of CF is higher in Caucasians populations. CF also occurs at high frequencies in countries to which European populations have migrated, such as the US. Within Europe it generally increases upon moving westwards. It ranges from below 1:2,000 in Ireland [70] to 1:25,000 in Finland [71]. The latest study on the Swedish CF population, in 1999, calculated an incidence of 1:5,600 and thus a carrier frequency of 1:37 [72]. In some populations the incidence is much higher, for example in Ohio Amish and the population of Saguenay-Lac St Jean area in Quebec, because of the founder effect. The incidence in non-Caucasians is lower, for example between 1:14,000 and 1:20,000 in black people in the US [9].

1.8 Clinical aspects of CF

1.8.1 Lung disease

The major cause of morbidity and mortality in CF is lung disease. Pathological studies indicate that the CF lung is almost normal at birth, but with submucosal glands impacted with mucus [73]. It allows hope that with early postnatal therapy directed against the basic defect in the lung, fatal disease can be prevented. Later studies have, however, shown increased inflammatory markers in the lower airways as early as at age 4 weeks, without evidence of bacteria [74]. In CF airway epithelium there is reduced chloride secretion and increased sodium reabsorption, which leads to reduced water content of the secretions. The periciliary liquid of the secretions is isotonic and of an osmolarity that allows water to reabsorb with Na^+ from the lumen (less osmotic active ions in the secretions result in less water in the secretions) [75, 76]. This results in reduced depth and increased viscosity of the periciliary liquid, which leads to slower clearance and

the trapping of inhaled bacteria in the viscous secretions [77]. Some studies also suggest that CF airway secretions have a lower capacity to kill bacteria, although the mechanism of this is unclear. There is a vicious circle of inflammation, infection, and increased levels of cytokines, neutrophils and bacterial products that, in the end, destroy the airway wall [78, 79].

There is also an increase in the volume of glands and secretory cells in the epithelium, giving more secretion. The result of mucus plugging is bronchiectasis. With the growth of blood vessels that accompanies it, haemoptysis can occur. Bronchial cysts, emphysema and atelectasis can develop, especially in the upper lobes, predisposing to pneumothorax [80].

Patients with CF develop bacterial infections early in life, which initially can be cleared with antibiotic therapy. They do not have more frequent viral infections, but the viral infections are more likely to be symptomatic [81]. The patients are treated with antibiotics during viral infection to prevent bacterial growth and lung exacerbations (periods of deterioration). Early in life, the pathogens are *Staphylococcus aureus* and *Haemophilus influenzae*. Eventually, *Pseudomonas aeruginosa* colonises and acquires a mucoid phenotype after some years in the CF lung, forming a biofilm [82, 83]. The growth of *Pseudomonas aeruginosa* in the airways of CF patients has several causes: perfect hypoxic microenvironments within mucous plaques, increased bacterial binding to the epithelium, and decreased bacterial clearance via innate immune mechanisms [84]. Antibiotic-resistant bacteria such as *Stenotrophomonas maltophilia* and *Burkholderia cepacia* (a complex of at least nine different species) can also colonise the CF lung. In the early 1980s in Toronto, Canada, *B. cepacia* colonised patients with CF and caused ”cepacia syndrome”, with death within a year in one-third of infected patients, which is to be compared with only 8% of patients matched for age and sex who did not acquire this bacterium [85]. It was concluded that transmission was person-to-person [86] and since then every patient with CF that cultures *B. cepacia* in sputum is separated from other CF patients.

Atypical mycobacteria (especially *Mycobacterium avium* complex and *Mycobacterium abscessus*), yeast and fungi are also common. *Aspergillus fumigatus* is often cultured from sputum and may give rise to an allergic bronchopulmonary aspergillosis (ABPA) in 2-16% of patients with CF [87].

CF lung disease is a progressive obstructive disease with chronic inflammation and infections, giving frequent lung exacerbations and tissue destruction. The end-stage of lung disease is respiratory failure, with severe hypoxia leading to pulmonary hypertension and secondary cardiac complications, which is responsible for at least 80% of CF-related deaths [84].

1.8.2 Gastrointestinal disease

In contrast to the lungs, the pancreas is often damaged at birth giving pancreatic insufficiency; present in 85-90% of patients with CF. The disease is characterised by malabsorption of fat and protein, steatorrhea (frequent, bulky, foul smelling, oily stools) and failure to thrive (growth failure). This is the most common presentation of CF in infancy. Excessive flatus may also be present. The child often has good appetite and might manage initially to grow normally, especially when breast-fed often, thanks to lipase in breast milk that will break down fat and make the malabsorption less pronounced. Even a malnourished CF child is often a ”happy” child and the medical records before the diagnosis is set can say ”the child is in good general health”. Pancreatic insufficiency may develop later during childhood.

Around 15% of newborns with CF can present with meconium ileus or meconium plug syndrome. Meconium ileus is an obstruction

of the distal ileum or proximal colon with inspissated meconium caused by the high protein concentration in the meconium. Genuine meconium ileus is said to be diagnostic of CF, but the diagnosis should always be confirmed.

Some infants with CF present with prolonged jaundice, which is presumably secondary to obstruction of intrahepatic bile ducts by thick bile. CFTR has been localised to the apical membrane in the intrahepatic bile ducts cells, but there is no evidence of CFTR in the hepatocytes. Liver disease develops in about one third of patients with CF, the characteristics are periportal fibrosis and focal biliary cirrhosis that presents before 18 years of age [88]. This may lead to portal hypertension and end-stage liver disease, needing liver transplantation. In a Swedish study, advanced fibrosis or cirrhosis was confirmed in about 10% of patients with CF, and 4% had cirrhosis with clinical liver disease (defined as multilobular cirrhosis, and including oesophageal varices or signs of hypersplenism) [89]. Oral therapy with the bile acid, ursodeoxycholic acid, aiming at improving bile viscosity is the only treatment currently available for CF-associated liver disease. Liver end-stage disease is the single most important non-pulmonary cause of death in CF, accounting for about 2.5% of overall CF mortality [88].

Rectal prolapse may be caused by CF. The malnutrition gives poor tissue quality and inadequate support for the rectum which, in combination with abnormal stools, may lead to rectal prolapse. The equivalent of meconium ileus in older patients, now called ”distal intestinal obstruction syndrome (DIOS)”, in which the terminal ileum or right colon becomes obstructed by faecal material, is a common problem at all ages. Pancreatitis in pancreatic sufficient patients is due to the blocking of enzymes (when blocking of pancreatic ducts prevents enzymes from entering the gut), which may start an inflammatory response and autodigest the gland [87]. Gall bladder abnormalities, such as gallstones and microgallbladder, can also occur.

1.8.3 Other manifestations and complications

Other presenting symptoms of CF include: hyponatremic or hypochloremic dehydration caused by the increased concentration of electrolytes in the sweat, hypokalemic metabolic alkalosis and pseudo-Barters syndrome secondary to chronic salt loss, hypoproteinemia with or without oedema and anaemia, manifestations of deficiency of the fat-soluble vitamins A, D, E and K (acrodermatitis, anaemia, neutropathy, night blindness, osteoporosis and bleeding disorders) and manifestations of a deficiency of zinc (mimicking dermal diseases).

Nasal polyps occur frequently and at early ages. Pansinusitis is often present, but often without symptoms. Obstructive azoospermia and infertility due to congenital bilateral aplasia of the vas deferens (CBAVD) is present in almost all postpubertal males with CF, but sperm production in the testicles is normal and CF men can become fathers with *in vitro* fertilisation. CBAVD may also be present without other clinical signs of CF. Women with CF have a reduced infertility, probably due to the thick cervical mucus that fails to undergo the usual midcycle thinning. A Swedish study has shown that puberty is delayed, even in patients with good clinical status, by approximately 2 years in females [90].

CF-related diabetes mellitus (CFRD) occurs in about 30% of patients (only in those with pancreatic insufficiency), with a peak onset occurring in young adulthood [91]. Progressive pancreatic disease and scarring of the pancreatic islets may give diabetes. CFRD has characteristics in between type I and type II diabetes mellitus, with an insulin insufficiency but without the risk of developing ketoacidosis because of the concomitant glucagon deficiency. Other factors unique to CF that affect glucose metabolism are raised energy expenditure and decreased intestinal transit time [92, 93]. Hyperosmolar complications and secondary late organ system complications, such as retinopathy or neuropathy, are observed. There is an association

between CFRD and more severe pulmonary disease [94], and female CF patients with diabetes have a poorer survival than male patients [95].

Other diseases or complications more frequent in patients with CF are: coeliac disease [96], gastroesophageal reflux, arthropathy, kidney stones, reduced bone mineral density, osteoporosis [97] and gastrointestinal cancer.

1.8.4 Treatments

CF centres were started in the US in the mid-1950s. In Cleveland, a treatment regimen that attacked complications aggressively gave dramatic results [87]. Three pillars of treatment were established: better nutrition, relief of airway obstruction and antibiotic therapy of the lung infection. CF centres followed in Europe and the centre-care, with specialised high-quality and evidence-based treatments, is fundamental for achieving better survival [98-100]. The European Cystic Fibrosis Society has published a consensus on ”Standards of care for patients with CF”, including the definition of, and the members of, a multidisciplinary CF team [101]. The details have changed since the 1950s, but ‘aggressive’ treatment still remains the foundation of care.

Better nutrition: In the late 1980s, pancreatic enzyme supplements were reformulated as enteric-coated microspheres to survive gastric acid, dissolve in the intestines and give better absorption [102, 103]. Releasing large doses of active enzymes lower in the gut, however, could predispose to fibrosing colonopathy [104]. Large doses of pancreatic enzymes do not fully correct malabsorption. Because both pancreatic and intestinal bicarbonate secretion are impaired in CF, the pH of the intestines is sometimes not sufficiently alkalinised to reach the optimal pH for the enzymes, and blockers of gastric acid secretion may have to be administered [105].

Fat malabsorption leads to less absorption of the fat-soluble vitamins A, D, E and K, which must be supplemented. Patients with CF also have disturbances in lipid metabolism, especially of essential fatty acids. These disturbances are linked to CFTR, but the connecting link has not been identified [106].

Patients with CF also have abnormal enterohepatic circulation of bile, increased metabolism and increased caloric demand due to severe lung disease. Nutritional supplementation is often necessary, both oral supplements and enteral feeding. Underweight is associated with poor pulmonary function and provides the rationale for vigorous nutritional treatment [107].

Physiotherapy and relief of airway obstruction: Clearance of the thick and sticky secretions of the airways has been an important therapy from early in the history of CF treatment. In London, postural drainage with ”tipping the patient so that the gravity will assist in drainage, clapping and pressure vibrations during expiration” was a form of mechanical stimulus to eliminate secretion widely used in the 1950s [87]. This method of physiotherapy spread around the world and was recommended for patients with CF and bronchiectasis for a long time, as is still the case in some parts of the world [108].

More active clearance methods became available in the 1980s. Swedish CF centres abandoned postural drainage with percussion and vibration after two studies in the 1980s that showed that physical exercise mixed with ”huffing” (a sort of active exhalation) was equally good [109-111]. Hand-held devices, into which the patient blows vibrate the airways to shake loose adherent mucus, are now used. These provide back pressure to maintain open airways and prevent their collapse, and have proven to be successful [112]. Many airway clearance techniques have been developed during the past 30 years. In Sweden, the treatment is highly individualized. The choice of technique depends on many factors, such as tradition, age, degree of lung disease, airway instability and hyper-reactivity, surroundings and the patient’s ability to learn. The most common airwayclearance techniques used in Europe are [111]:

1) Active cycles of breathing techniques, which may be combined with postural drainage with an assistant, or ”huffing”.

2) Autogenic drainage (AD) performed while sitting, without any devices.

3) Positive expiratory pressure (PEP), performed with the help of a PEP mask® while sitting.

4) High positive expiratory pressure (HiPEP), performed with the help of a PEP mask® while sitting.

5) Physical exercise combined with ”huffing”.

Humidification by mist tent was part of the initial care programme, now abandoned by many but still in use at the Stockholm CF centre. The treatment uses a plastic tent covering the bed into which an aerosol of sterile water is introduced during the night when the child is asleep, and adding water loosens the mucus.

Inhalation therapy with mucolytics, in combination with bronchodilators to avoid the bronchoconstrictive effect of mucolytics, is used more or less routinely before physiotherapy to loosen the mucus. However, children who sleep in a mist tent do not need this inhalation before morning physiotherapy. The number of sessions of physiotherapy per day may vary with degree of lung disease and whether exacerbation is present or not, but the standard physiotherapy regimen for a standard patient is twice a day. Inhalation of the mucolytic agent acetylcysteine has been used for a long time in Sweden.

A drug to improve sputum clearance has been developed especially for patients with CF. The sticky properties of the mucus are determined by several components, including free DNA. Cleaving free DNA into smaller pieces reduces the viscosity of the sputum. Recombinant DNA technology has made it possible to produce human DNase, which gives minimal adverse effects, improvement in pulmonary function and a reduction in the number of exacerbations [113]. Treatment with nebulised hypertonic saline is a very cheap treatment that results in a modest increase in pulmonary function and a reduction in exacerbations, presumably by temporarily drawing water into the airway to dislodge the mucus [114]. The airways also show oedema, smooth muscle hypertrophy and bronchoconstriction in CF. Bronchodilators are routinely administered. Inhaled steroids have not been proven to be effective in CF in controlled trials, but may be used in some patients.

Treatment of airway infection: Culture-specific antibiotic therapy has been important since the days of Andersen. Eradication of bacteria is a goal until chronic colonisation occurs. Most antibiotics for *Pseudomonas aeruginosa* are administered intravenously, an exception being drugs of the quinolone family. The most common intravenous combination given is an aminoglycoside and a beta-lactam, such as tobramycin (Nebcina) and ceftazidim (Fortum). This combination gives a synergic effect for killing bacteria and a slow development of resistance [115]. Home intravenous antibiotic treatment has been practised in Sweden since 1985 [116].

Formulation of antibiotics for aerosol use allows the patient to achieve high antibiotic concentrations in the lower airways while minimizing adverse systemic effects. However, patients with severe lung damage have ineffective ventilation of the most damaged and infected parts of the lung, and may therefore not benefit from inhaled antibiotics.

It has been long debated whether to use suppressive therapy in the absence of symptoms of exacerbation. In Denmark, patients colonised with *Pseudomonas aeruginosa* are given regular antibiotic treatment every 3rd month, and this treatment strategy has been in use since the beginning of the 1980s [117]. The results from a randomised trial of tobramycin specially formulated for aerosol use in alternate months over a 6-month period was published in 1993. They demonstrated that improved pulmonary function was achieved, with fewer exacerbations [118].

In conclusion, patients with CF should often be treated with antibiotics to prevent further bacterial growth and lung exacerbations. In Sweden, we most often start antibiotic therapy at signs of low grade of infection, also called ”on demand” treatment.

Suppression of inflammation: The intense inflammation in the CF lung was long considered to be an appropriate response to infection. The observations that patients with very low IgG levels were remarkably healthy, and were not vulnerable to infections, led to the thinking that a vigorous host response might be harmful in CF [119]. Suppression of inflammation with steroids or high-dose ibuprofen is, unfortunately, not acceptable because of the adverse effects [120, 121]. Continuous oral azithromycin therapy benefits patients colonised with *Pseudomonas aeruginosa* due to its anti-inflammatory effect [122].

Lung transplantation: When respiratory failure looms, life can be extended only by lung transplantation, the first of which was performed in 1983. Patients with CF are considered for double lung transplantation when they have an estimated life expectancy of less than 2 years, despite maximum medical therapy. Adults are generally referred when FEV_1 reaches a plateau around 30% of the predicted value [123, 124]. However, age, sex, the degree of lung infection and colonisation, and the rate of decline in FEV_1 also affect the decision. The supply of organs limits this option and patients die on the waiting list. Lung transplantation is a high-risk procedure with poorer survival than other organ transplantations. In adults with CF, 50% are alive 9.4 years after transplantation [125].

1.8.5 Future treatments

Refinements of symptomatic therapy will continue. The most interesting development for the future is, however, therapies that target the basic genetic defect. Three approaches have been proposed [87]. One is to circumvent the CF-related ion transport defects pharmacologically, by increasing chloride secretion through other channels and inhibiting excess sodium reabsorption through epithelial sodium channels (using, for example, Moli1901 and denufosol). The second approach is using mutation-specific therapies to correct the specific defect in mutant *CFTR*. The third approach is gene therapy.

High-throughput screening of drugs has identified small molecules that can restore the activity to a mutant CFTR. Mutation-specific therapy against Class I nonsense mutations (which lead to a premature stop) is now in Phase 3 of clinical trials. The molecule PTC-124 allows ”read-through” of premature stop codons in the DNA, without disrupting the normal termination signal. Oral intake of PTC-124 resulted in a normal NPD for 13 of 23 patients in the first Israeli study [126]. The most recent study with treatment for 12 weeks showed similar results in NPD, accompanied by trends toward improvements in pulmonary function and CF-related coughing [127]. PTC-124, or Ataluren, is also well-tolerated in children and ameliorated the NPD in half of the patients [128].

Compounds that increase the function of CFTR that is correctly located at the membrane (Class III, IV, V and VI mutations) are called ”potentiators”. The compound VX-770 can potentiate CFTR and has demonstrated good results in clinical trials, with dose-dependent improvement of sweat chloride levels, NPD and lung function in patients with the G551D mutation [129]. VX-770 has also demonstrated an effect for several CFTR mutation classes in the laboratory [130].

Compounds that correct the localization of deltaF508 from the endoplasmic reticulum to the cell membrane are called ”correctors”. Small molecules that function as chaperones are under investigation. The compound VX-809 showed promising results initially, but no effect on NPD or lung function was seen in Phase 2 trials [131]. Several other compounds are being investigated, as is co-administration of multiple compounds. An updated chart of new drugs can be seen on the ”Drug Development Pipeline”

Table 1. Wilson and Jungner criteria for appraising the validity of a screening programme

1.	The condition being screened for should be an important health problem.
2	The natural history of the condition should be well understood.
3	There should be a detectable early stage.
4	Treatment at an early stage should be of more benefit than at a later stage.
5.	A suitable test should be devised for the early stage.
6	The test should be acceptable.
7.	Intervals for repeating the test should be determined.
8.	Adequate health service provision should be made for the extra clinical workload resulting from screening.
9	The risks, both physical and psychological, should be less than the benefits.
10.	The cost should be balanced against the benefits.

of the Cystic Fibrosis Foundation (http://www.cff.org/treatments/Pipeline/).

Gene therapy, to insert one copy of normally functioning DNA into affected cells, should be possible but has proved to be very difficult in CF. Initial gene therapy trials that used adenovirus as vector resulted in too high activation of the immune system and too low efficiency. New trials are using adeno-associated viruses (AAV) and lipids as vectors. A gene therapy trial in the US showed physiological correction of chloride movement in nasal epithelial cells with AAV-*CFTR* gene therapy. Unfortunately, a Phase 2b trial did not result in significant improvement in spirometric values [132]. The UK Cystic Fibrosis Gene Therapy Consortium is developing non-viral vectors; the best so far is a cationic lipid vector. In its 2006 review, the consortium anticipates that repeat doses of non-viral vectors and the use of new plasmids, with new methods of delivering these vectors, will need several years to develop [84, 133]. So, it is still a hope that next-generation vectors will lead to effective gene transfer and cure of the lung disease in CF.

1.9 History of newborn screening

The word ”screening” means mass examination of the population to detect the existence of a particular disease. Newborn screening is a successful public health strategy around the world that has resulted in the reduction of mortality, and reductions in mental retardation and other serious disabilities in children since the introduction of screening for phenylketonuria (PKU) in the 1960s. Guthrie described a simple blood-screening test for PKU in newborns, a blood sample dried on filter paper, which was suitable for safe and stable transport to central laboratories and could be processed cheaply [134]. The dried blood sample, now called ”the Guthrie card”, could be used for other testing, and in 1975 screening for hypothyroidism was described [135]. This is the most important disorder that is currently tested for, and identifies the greatest number of patients.

A classic set of criteria for the principles of screening for a disease was set by Wilson and Jungner in 1968 (Table 1) [136]. These principles are still considered when deciding about the expansion of programmes. However, there is difference in opinion as to how to weigh

the various criteria against each other. It is important, however, to stress that both benefits and potential harms must be considered.

Newborn screening is evolving as new technologies develop. It has been shown that the newborn screening dried blood spot can be used for DNA extraction and analysis [137]. Tandem mass spectrometry could be modified to make it suitable for newborn screening in the 1990s [138]. It is now possible to detect about 40 disorders of amino acid, organic acid and fatty acid metabolism simultaneously using one single assay [139]. The new technologies make the future possibilities of newborn screening seem almost unlimited.

The newborn screening programme in Sweden started with screening for PKU in 1965. Screening programmes for galactosaemia, congenital hypothyroidism, congenital adrenal hyperplasia and biotinidase deficiency were added later. With about 100,000 newborns per year in Sweden, approximately 50 babies were found to have one of the five disorders screened for. Screening with tandem mass spectrometry has recently been introduced in Sweden (November 2010). Newborn screening is now carried out for 24 disorders. With this new screening the estimation is that 10 to 15 more sick babies will be found per year in Sweden. (Personal communication Ulrika von Döbeln, head of the Swedish Newborn Screening Laboratory, Karolinska University Hospital.)

1.10 Newborn screening for CF

Studies in the 1970s suggested that early diagnosis of CF with early initiation of therapy was beneficial. One study identified 16 sibling pairs in which the older sibling in each pair was diagnosed after 1 year of age, while the younger sibling was diagnosed at less than 1 year. Despite receiving similar treatment regimens after diagnosis, the younger sibling had significantly better chest radiography, total clinical scores, better lung function measurements, and fewer hospitalisations at 7 years of age [140]. At this time, no acceptable screening method for CF was available. The albumin level in meconium was suggested after reports of higher protein content in meconium of babies with meconium ileus. Several newborn screening studies with albumin in meconium were initiated also in Sweden [141-143]. However, this method could not be used, as there were numerous false-positive results from bloody meconium, prematurity, intrauterine infection, and some form of neonatal intestinal obstruction and false negative results due to pancreatic sufficiency [144].

There were other early tests, such as meconium lactase and faecal trypsin. Apart from the undeniable high false-positive and false-negative rates, there were drawbacks to the meconium tests, including problems of collecting, storing cold, and resampling not being possible [145].

Crossley et al. proposed another screening method, originally applied to stool [146], and further modified for serum [147]. This method measured immunoreactive trypsinogen (IRT) in dried blood spots. IRT is reduced in older patients with CF but is significantly elevated in the newborn period. The test detects most babies with CF, both pancreatic insufficient and pancreatic sufficient. Initially the elevation was thought to be due to trypsinogen leakage into plasma, resulting from on-going pancreatic destruction, however then only CF babies who were already pancreatic insufficient at birth would be detected. It is more likely that the CFTR abnormality in the pancreatic ducts results in elevated trypsinogen levels in the blood [148]. The method of Crossley was time-consuming and alternative approaches have been developed. There are commercially available assays, even fully automated, which all provide a similar distribution of results with a decline in IRT with age, separating babies with CF from healthy babies [145, 148]. However, different assays may give different results. The results from different kits may not be comparable. CF babies born with meconium ileus have, as a group, elevated IRT levels above that of normal newborns, but half of the babies with meconium ileus have an IRT

level below cut-off [145]. Thus babies with meconium ileus should always be examined to confirm or exclude the CF diagnosis, whatever IRT level they have. Many premature babies have a falsely elevated level of IRT [149].

Using the standardised "Guthrie" card made the population screening of newborns for CF possible. Newborn screening programmes were started at the beginning of the 1980s in Australia (New South Wales), USA (Colorado), and several European regions[150-152]. Concerns were, however, raised related to the screening protocol used, uncertainties regarding the lack of proven benefits, and regarding possible risks associated with screening [148]. Two randomised controlled trials (RCT) were planned, one by Farrell and Fost in Wisconsin, USA[153], and one in Wales and the West Midlands, UK [154]. Both trials started in 1985.

The discovery of the CFTR gene in 1989 allowed the inclusion of DNA testing into screening protocols. Most screening protocols use analysis of a panel of CF mutations on samples with raised IRT, a so-called IRT/DNA approach [155]. Homozygotes and compound heterozygotes can be assessed promptly and following a confirmatory sweat test, CF treatment is initiated often in the four first weeks of life. Babies carrying only one identified mutation usually proceed to a sweat test, in order to distinguish a CF baby from a carrier. DNA-testing must always be linked to genetic counselling.

The level of another protein, pancreatitis-associated protein (PAP), is increased in babies with CF and has proven to be a better serum marker of pancreatic alteration than exocrine enzymes [156]. Measurement of PAP level has been used in combination with IRT in newborn screening studies and the combination looks promising, with good specificity and sensitivity [157, 158]. PAP/IRT approach may be used in screening programmes where an avoidance of genetic test is desirable [159]. Limited renal function can, however, cause false positive newborn screening for CF using IRT and PAP [160].

2 AIMS OF THE THESIS

The overall aim of this thesis was to provide a basis for discussion of whether CF should be included in the national newborn screening programme in Sweden. Specific aims were:

Paper I: To assess the most common *CFTR* mutations in Sweden for a possible pilot study of newborn screening.

Paper II: To describe the attitudes among parents towards including CF in the newborn screening programme in Sweden and towards obtaining knowledge of their own carrier status.

Paper III: To investigate the experiences of the parents after their child's clinical CF diagnosis, and to find out the effects of the diagnosis on the family.

Paper IV: To study age at diagnosis and the disease progression of CF in an area without newborn screening.

Paper V: To describe the genotype of the patients at the Stockholm CF Centre, and to study genotype-phenotype associations regarding longitudinal lung function according to *CFTR* mutation classes.

3 MATERIALS AND METHODS

3.1 Study design

Paper I: descriptive multi-centre study with CFTR mutation analyses on DNA samples.

Paper II: case-control questionnaire study.

Paper III: questionnaire study.

Paper IV: single-centre register study with register data prospectively collected and retrospectively analysed. Lung, liver and nutritional outcomes were analysed with strict epidemiological methods. For definitions of lung, liver and nutritional morbidity, and of overall morbidity, see Table 2.

Table 2. Definitions of morbidities in Paper IV

Nutritional morbidity	Age < 18 years:	Weight below -0.5 SD from the birth weight or height below -0.5 SD from the birth height, both according to Swedish growth chart
	Age ≥ 18 years:	BMI < 18.5
Lung morbidity	Age < 7 years:	Pathologic scintigraphy of the lungs or pulmonary X-ray with pathological changes other than peribronchial thickening
	Age ≥ 7 years:	Spirometry with FEV_1 < 85% predicted or pulmonary X-ray with pathological changes other than peribronchial thickening
Liver morbidity		Pathological ultrasonography, for example heterogeneous echo texture of the liver parenchyma or GGT or ALT above upper reference levels
Overall morbidity		If lung morbidity, liver morbodity or nutritional morbidity occurs.

BMI = body mass index
FEV_1 = forced expiratory volume in the first second
GGT = gamma glutamyltransferase
ALT = alanine transaminase

At each annual review the patients were defined as ”healthy” or ”having symptoms of morbidity” for every area of morbidity. The patients were followed from: (i) healthy at diagnosis to endpoint morbidity, (ii) diagnosis with symptoms of morbidity to endpoint free of morbidity, (iii) free of morbidity to endpoint relapse of morbidity.

Paper V: single-centre register study with register data prospectively collected and retrospectively analysed.

3.2 Study populations

Paper I: About 75% of the Swedish CF population at the end of the 1990s was included: 331 patients attending the centres in Stockholm (177 patients), Lund (124 patients) and Uppsala (30 patients). Only one Swedish CF centre did not participate, the West Swedish CF Centre in Gothenburg.

Paper II: Parents of 143 CF patients and parents of two control groups. To each CF patient the aim was to match three control patients with diabetes mellitus and three healthy controls from the general population. The CF patients were born between 1968 and 2000, and were registered at the Stockholm CF Centre on December 31, 2000. Six CF patients from the register were excluded because of being adopted, born outside the Nordic countries or given the diagnosis as an adult. The control children were taken from the Swedish Childhood Diabetes Registry and the Total Population Registry at Statistics Sweden, in total 402 diabetes controls (because of too few young diabetes patients) and 428 population controls (one ineligible after randomisation because of emigration or death). Matching was made for sex, year of birth and birth region (countryside, town or one of the three biggest Swedish cities).

Paper III: The parents of the same 143 CF patients as in Study II.

Paper IV: 119 CF patients born between 1974 and 2001 with a first visit to the Stockholm CF Centre within 6 months of diagnosis and a minimum of two annual reviews. In May 2004, 172 CF patients were registered at the Stockholm CF Centre register and 120 met the inclusion criteria; one patient was excluded because of a diagnosis through a pilot newborn screening (made in the 1970s).

Paper V: All patients in the Stockholm CF Centre register with the diagnosis of CF, annual reviews performed after 1985, and two verified *CFTR* mutations were included, in total 220. Those older than 7 years, 182 patients, were included in the mixed model analysis of longitudinal lung function (after exclusion of one patient with a CF diagnosis at 60 years of age). Fifty-one patients were never included in the study – 18 of these had an equivocal diagnosis where two mutations were not found, one patient had three mutations (one double mutant allele) which is difficult to evaluate, and the remaining 32 with classic CF had either died or did not attend the centre any longer so mutation analyses could not be done.

3.3 The Stockholm CF Centre

The Huddinge Hospital (now called the Karolinska Huddinge) was opened in 1974 and CF children living in Stockholm came under the care of paediatrician Birgitta Strandvik. During the following years, patients from other parts of the country were consecutively referred to the hospital. In cooperation with the Swedish Cystic Fibrosis Association, the patients' association, funds for a multi-disciplinary CF centre according to international standards were obtained in 1985. From that year on a multi-disciplinary team was established consisting of CF physicians (paediatricians together with a pulmonologist), CF nurses, CF physiotherapist, CF psychologist, CF social worker, CF dietician and (later) a technical assistant. The Stockholm CF Centre today has one multidisciplinary team for children and one for adults, the majority of the patients being adults.

3.4 The CF Register

The CF register at the Stockholm CF centre is the basis for this thesis. Physicians at the Stockholm, Lund and Uppsala CF centres started in 1992 to work together to create a patient database. The aim was to enter demographic and clinical data from the annual reviews, and to be able to give a summary of the annual review to the patient. Thus the aim was twofold: to create a quality register and a research register. The software is an Access database, which makes the output of data easy. The CF register has the same structure at all five CF centres in Sweden; each centre is in possession of their own data. Swedish data legislation is, naturally, followed. Informed consent is obtained from the patient at the first annual review at the Stockholm CF Centre. Data have been entered by the same CF nurse since 1993. In Stockholm, quality controls that examine outliers and random samples have been done by a CF physician. A web-based continuation of the CF register will soon be available.

3.5 Care at the Stockholm CF Centre

Patients attending the Stockholm CF Centre are given annual reviews in order not to miss a slight aggravation of the CF disease. If an annual review shows severe symptoms of deterioration a new control is carried out after only six months. If the patient cancels an annual review because of lung exacerbation, a new one is planned. The time between two annual reviews has rarely been as long as two years. Weight, height, and blood parameters are recorded, pulmonary function is tested, and pulmonary radiology and a clinical examination are carried out at each annual review. Ultrasound investigations of the liver started in 1989. Lung physiology of children (younger than 7 years) was investigated by scintigraphy during the years 1978-2002. The treatment policies have been reasonably constant since the start of the centre in 1974 [161].

Patients have been recommended a diet high in energy and polyunsaturated fatty acids. They have been supplemented with fatsoluble vitamins, retinol and alfa-tocopherol; the dosages of which are monitored by measuring their serum concentrations. Supplementation with polyunsaturated fatty acids intravenously (Intralipid^{R}) is standard when the patient is receiving intravenous antibiotic treatment and monthly during such conditions as poor pubertal growth. The liver has been treated by ursodeoxycholics from 1983 when it shows signs of irregular echogenicity of the parenchyma with ultrasonography, or when the blood liver parameters are elevated, and after confirmation of fibrosis with liver biopsy. Patients at the Stockholm CF Centre are always put onto bromhexin as a mucolytic directly after receiving the CF diagnosis, later adding acetylcysteine. Lung treatment is composed of aggressive antibiotic treatment, physiotherapy and physical activity. Antibiotics are given at the slightest clinical deterioration such as weight loss, elevated mucus production or deteriorated spirometry. Antibiotics are given at viral infections, at cultivations of *Hemophilus influenzea* and *Moraxella catarralis*, at cultivation of *Staphylococcus aureus* together with symptoms of the airways, or when raised antibodies for the *Staphylococcus aureus* are detected. The aim of physiotherapy has been to teach the patients self-treatment by combining inhalation with mucus clearance. Physical activity has been a major treatment since the 1980s in order to improve the general physical condition of the patients and to clear the lungs from mucus.

3.5 Assessments

The CF register was used for the extraction of study populations, demographics, diagnostic data, clinical data and the results of examinations from annual reviews during exacerbationfree intervals.

Diagnostic criteria. The CF diagnosis used in Papers I-IV was based on old diagnostic criteria with clinical symptoms and two positive sweat tests. Sweat tests were performed as described by Gibson and Cook [6]. At the time of Paper I, higher sweat chloride reference values were used for adults (80 mmol/l for adults and 60 mmol/l for children) than those subsequently used. Sweat chloride reference values of 60 mmol/l were used for both adults and children in Papers II-IV. New European diagnostic criteria were used in Paper V [14], and *CFTR* mutation analysis were used to confirm the diagnosis for patients with intermediate sweat chloride values (30-60 mmol/l).

Anthropometric parameters (weight and height) were measured yearly (Papers IV and V).

Biochemical parameters (blood concentrations of gamma glutamyltransferase (GGT) and alanine transaminase (ALT)) were determined yearly by routine laboratory methods. Reference values defined by the hospital laboratory were used (Paper IV).

Pancreatic insufficiency was determined by a pathological direct exocrine pancreatic function test with duodenal intubation and stimulation of pancreatic secretion by a test meal using the method developed by Lundh [162]. Levels of faecal elastase-1 were also used to establish pancreatic insufficiency, and were determined by routine laboratory methods after 2004, with a reference value of 200 µg/g [163]. Pancreatic insufficiency was determined on clinical grounds for some patients (fewer than 5) when pancreatic function tests showed borderline results and pancreatic supplementation was needed to establish normal growth (Papers IV and V).

Ultrasonography of the liver was performed according to a routine laboratory protocol from 1989 at every annual review up to age 15. It continued to be used at every annual review thereafter only if CF-associated liver disease was present, otherwise every second annual review thereafter if CF-associated liver disease had not been diagnosed. Results from ultrasound investigations were described as normal or pathological (Paper IV).

Pulmonary radiology was performed according to a routine laboratory protocol every annual review, and the result described as normal, minor changes (peribronchial thickening) or major changes (bronchiectasis) (Paper IV).

Pulmonary CT or HRCT was performed according to a routine laboratory protocol after 1991 every second annual review from 4 years of age, unless it was clinically needed every annual review. The results were described as normal, minor changes (peribronchial thickening), or major changes (bronchiectasis) (Papers IV and V).

Pulmonary scintigraphy was performed using the Xenon^{133} isotope on children younger than 7 years between 1978 and 2002 and the result was described as normal or pathological (Paper IV).

A pulmonary function test was performed every annual review, and measured forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV_1) separately. The highest value from the first two technically satisfactory forced expirations was used. In Papers IV and V, for the patients 7-18 years of age, the percentage of the predicted Solymar reference given in the report from the Laboratory of Clinical Physiology at the hospital was used [164]. In Paper IV, for the patients older than 18 years, the percentage of the predicted Hedenström reference given in the report from the Laboratory of Clinical Physiology was used [165, 166]. In Paper V, for the patients older than 18 years, the percentage of the predicted ECSC was calculated from the absolute values in litres [167].

Chronic colonisation with *Staphylococcus aureus* was defined as having positive antibodies against staphylococcus alpha toxin or teichoic acid [168, 169].

Chronic colonisation with *Pseudomonas aeruginosa* or *Burkholderia cepacia* was defined as positive bacterial cultures every month during six months or, in the case of *Pseudomonas* colonization, positive antibodies against pseudomonas aeruginosa exotoxin [170].

Diabetes mellitus was defined as having plasma venous glucose greater than 11.1 mmol/L in a 2-hour oral glucose tolerance test (OGTT) with 75 g glucose, performed using routine laboratory methods (Paper V).

3.6 Genetic analyses

After the CF gene was discovered in 1989, analysis of the *CFTR* mutation for clinical purposes has been done at the Laboratory of Clinical Genetics at the Karolinska Hospital, Stockholm, Sweden. Initially, only deltaF508 was analysed. Several further mutations were later added to the analysis [171] and, when available, commercial kits have been used according to the manufacturer's instructions. During recent years the Oligonucleotide Ligation Assay (OLA) analysis Cystic Fibrosis V.3.0 from Abbott Laboratories, IL, USA, screening for the most common 33 mutations in the *CFTR* gene has been used.

For Papers I & V genetic analyses were done, at the end of the 1990s, at the research laboratory of Professor Lars Holmberg at the Department of Paediatrics, University Hospital, in Lund, on the DNA of patients with one or two unknown mutations (after the above analysis at the Karolinska Hospital). For Paper V genetic analyses were done, between 2002 and 2010, at the research laboratory of Professor Harry Cuppens at the Center for Human Genetics, Katholieke Universiteit Leuven (KULeuven), Belgium, on the DNA of patients with still one or two unknown mutations.

Analyses in Lund: *CFTR* mutations were examined with DNA extraction, amplification, and sequencing according to standard methods. DNA was also extracted and amplified from Guthrie cards. SSCP (Single Strand Conformation Polymorphism) analysis was used to visualise deltaF508, 394delTT and 3659delC on the same gel. In this technique, a mixture of 95% formamide and the PCR products was heated and the products were then separated by polyacrylamide gel

electrophoresis. The electrophoretic mobility of a single-stranded PCR product depends on its size and on its specific conformation. A small fraction of the PCR products remains in a double-stranded state and can be seen as homoduplex and heteroduplex bands on the gel.

Analyses in Leuwen: The complete coding regions, and exon/intron junctions (30 intronic nucleotides at each junction), of the two *CFTR* genes of the DNA sample were sequenced with a cycle sequencing protocol on an ABI 3130xl Genetic Analyser (Applied Biosystems, Foster City, CA, USA). Sequences were verified against wild-type sequences published by Zielenski in 1991, using the SeqScape v2.6 software (Applied Biosystems, Foster City, CA, USA). The nucleotide and amino acid number assignment was used as published by Riordan in 1989. If two CF-causing mutations could still not be identified on both *CFTR* genes, the presence of large deletions, or duplications, involving at least one complete *CFTR* exon was searched for by the Multiplex Ligation-dependent Probe Amplification (MLPA) assay according to the instructions of the manufacturer (MRC-Holland, Amsterdam, The Netherlands).

3.7 The questionnaire (pp 21-24)

The questionnaire used in Papers II and III was part of a validated questionnaire on health and lipids constructed to describe the quality of life in patients with familial hypercholesterolaemia and their unaffected relatives. It had previously been used at the University of Linköping, Sweden [172, 173]. The original questionnaire was derived from literature and from three senior consultants in internal medicine. Items were of Likert-type scale with three to five response categories or open-ended items. The original questionnaire was tested for validity and factor analysis was performed.

Our questions were discussed and the language simplified with the help of statisticians and experts on questionnaires at the Research and Development Department, Statistics Sweden. Our questionnaire and information leaflet were tested on ten randomly selected parents of healthy children and on five parents of CF children at the West Swedish CF Centre in Göteborg. Parents understood the questions correctly after having read the information leaflet. When answering the questionnaire, the control parents were asked to try to think of how they would have reacted and felt if their child had received the diagnosis of CF.

3.8 Statistical analyses

Descriptive statistics were used in all papers.

Papers II and III: Comparisons between groups were made with statistical tests for independent groups: Pearson Chi^2 test (or Fischer's exact test when expected values were less than five) for categorical variables and Kruskal-Wallis ANOVA by rank for continuous variables. Analysis within groups was performed with Friedman's test. In Paper II, the effects of age, group and interaction between age and group, with respect to the answers to the questionnaire, were analysed with multiple logistic regression; odds ratios and 95% confidence intervals were estimated.

Paper IV: The probability of experiencing a specific scenario (the event) as a function of time was measured with Kaplan-Meier survival curves. The effects of sex, birth cohort, age at diagnosis and length of time span between start of symptoms and diagnosis were analysed with the Cox proportional hazards regression model; crude hazard ratios (expressed as relative risks) and 95% confidence intervals were estimated.

Paper V: A mixed model analysis, with a random coefficient model, was used. The advantage of a mixed model analysis is that random variation between the slopes of individual patients, and variable length of follow-up and time of observations in individual patients, are allowed in the same model [174]. In comparisons between mutation groups, Kruskal-Wallis ANOVA by rank was used for continuous variables and the Chi^2

Information leaflet about the questionnaire, ”Parental attitudes towards analysis of newborn babies for the disease cystic fibrosis” (translation from Swedish).

Your child/One of your children has been randomly selected by Statistics Sweden to match a child with the disease, cystic fibrosis (CF). The year of birth of your matched child is written on the questionnaire. We ask you as a parent to answer this questionnaire. The questions are about your parental attitudes ***if you had had a child with the disease, cystic fibrosis.*** We want to know how you would have liked to have had the diagnosis of CF of your child determined. We also want to know about your attitudes towards an analysis of newborn babies for CF. Such an analysis is also called newborn screening. The corresponding questionnaire will be filled in by parents of children with CF and by parents of children with diabetes mellitus. The results will help us to decide whether or not to start a newborn screening programme for CF in Sweden. Your address and the year of birth of your child have been collected from the Registry of the Total Population at Statistics Sweden. The Registry receives information from the National Census and there might be some errors. If you have been contacted incorrectly, (if, for example, you do not have a child born the year stated), please contact Mr X at Statistics Sweden.

What is CF? CF is one of the most common severe inherited diseases in the Caucasian population. In Sweden approximately one in 5,500 children each year is born with CF. Both parents must have the disease trait (a faulty gene), and for these parents the odds in every pregnancy is one in four of having a sick child. The persons having one disease trait (one faulty gene) are healthy. A child that inherits two disease traits (two faulty genes), one from each parent, will have the disease.

What are the symptoms of CF? If you have CF you have saltier sweat than normal and thick, sticky mucus. The latter will lead to problems especially in the lungs and the gastro-intestinal tract. Bacteria will thrive in the sticky mucus of the lungs, which will lead to pneumonias that, in the long run, will cause damage to the lungs. The sticky mucus will obstruct the pancreatic juice from reaching the intestine where it should help to digest the food, and the child will not thrive as it should.

Treatment. There is as yet no cure for the disease. The treatment of the lungs is aimed at reducing the viscosity of the mucus and clearing the lungs by expectorating the mucus, as well as treating the bacteria. Patients with CF will therefore take expectorants every day and do physiotherapy for the lungs twice a day. At the slightest sign of deterioration, they will be given antibiotics that might be given as a ”drip”, i.e. directly into a blood vessel. Nowadays this is most often given at home. Digestive problems will be ameliorated by pancreatic enzymes given at every meal in capsules.

Prognosis. In the 1960’s many CF patients died as children. In Sweden we now count on most persons with CF reaching adulthood, the oldest today being about 60 years old. Nowadays patients live longer due to centralized care and more intensive treatment. In order for the treatment to be as effective as possible, the diagnosis of CF should be given before there is irreparable damage to the lungs. The hope for the future for patients with CF is some kind of curative treatment (for example. gene therapy). In order to benefit the most from a curative treatment, one should detect the disease early.

Methods to detect the disease. There are, in principle, two ways of detecting the disease. One is to investigate the child as soon as it shows signs of disease. The other way is to analyse all newborn babies with a blood test (newborn screening). If the screening indicates CF, an elaborate investigation will accompany the first test. A blood test to detect some diseases is taken already on the maternity ward (the PKU test). Previously the tests for the disease, CF, have not been sufficiently accurate. They have now become very reliable but the question is if they should be used. To analyse all newborn babies for CF may, for example, imply that all sick children will not yet have shown signs of the disease. One will also find some children being healthy carriers of the disease trait, as is the case for the parents of sick children.

As **your child has been randomly selected to match a child with CF,** it is very important that you answer this questionnaire but it is, of course, voluntary. The questionnaires are numbered so that a possible reminder could be sent out. Only Statistics Sweden will have this code. All the work will be done without us knowing who has answered what. Feel free to contact any of us if you have any questions.

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TRANSLATION OF THE QUESTIONNAIRE

The CF parents were asked all questions.
The control parents, with diabetes children or children from the population register, were asked the questions in bold.

Parental attitudes towards newborn screening for cystic fibrosis 2001-04-30

Please fill in one alternative and write on the lines. Put the completed form in the postage-paid envelope and post it.

1.The questionnaire has been completed by:

__Mother
__Father
__Mother and father

2:1 The child's parents are:

__Living together. Go to question number 2:3
__Not living together

2:2 The child lives with:

__Mainly with mother
__Mainly with father
__Equal amount of time with mother and father

2:3 We the parents at the time of the diagnosis were:

__Living together
__Not living together

3:1 Was any prenatal analysis of the foetus other than routine prenatal ultrasound carried out?

__Yes
__No. Go to question number 4:1

3:2 Why was the prenatal analysis of the foetus carried out?

__Because of high maternal age (above 35 years)
__Because of worry
__Because of inherited disease in the family
__Because of something else

4:1 How was the well-being of your child at time of the CF diagnosis?

__Good
__Rather good
__Neither good nor bad
__Rather bad
__Very bad

4:2 How was the timing of the CF diagnosis?

__Too early: we had not, for example, seen any symptoms
__Good, at the right time for us and the baby
__Too late, we went to the doctor late
__Too late, it took a long time for the doctors to diagnose
__Other time, write with your own words……
……..

4:3 How was the well-being of your child one year after the diagnosis?

__Good
__Rather good
__Neither good nor bad
__Rather bad
__Very bad

4:4 How is the well-being of your child today?

__Good
__Rather good
__Neither good nor bad
__Rather bad
__Very bad

5:1 What was your first reaction for yourself after your child received the diagnosis of CF?

5. What would your first reaction for yourself have been if your child had received a diagnosis of CF?

__Relief to know it's a treatable disease
__Relief to get an explanation to symptoms of the child
__Neither relief nor anxiety
__Anxiety because the disease is not curable
__Anxiety because the disease is thought to be lethal

5:2 How was your well-being before the CF diagnosis of your child?

__Good
__Rather good
__Neither good nor bad
__Rather bad
__Very bad

5:3 2 How was your well-being after the CF diagnosis of your child?

__Good
__Rather good
__Neither good nor bad
__Rather bad
__Very bad

5:4 Describe in your own words why you felt as you did after the CF diagnosis of your child?

.....
.....
.....

6. Have you, as parents, had your CF carrier status genetically determined?

__Yes
__No
__Don't know/Don't remember

7. Would you have wanted to know if you were a CF carrier before your child was born?

__Yes
__No
__Don't know

8. If newborn screening had been possible when your child was born, would you have wanted it to be performed?

__Yes
__No
__Don't know

9. Had relationships within the nuclear family changed <u>one year</u> after the diagnosis?

__The relationships were better
__The relationships were unchanged
__The relationships were worse

10. Had the social life of the family, for example meeting friends and relatives, changed <u>one year</u> after the CF diagnosis?

__Social life was better
__Social life was unchanged
__Social life was worse

11.1 How was the rest of the family/extended family affected by the CF diagnosis?

__Not at all. Question 11.2 does not have to be answered.
__To a low degree
__To a moderate degree
__To a high degree
__To a very high degree

11.2. Who was/were affected by the diagnosis and how?

__Husband/wife/partner. *How?*.....
.....
__Older sibling of CF child. *How?*.....
.....
__Younger sibling of CF child. *How?*.....
.....
__Grandparents. *How?*.....
.....
__Other relatives. *How?*.....
.....

Thank you for your participation!

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test or Fisher's exact test was used for categorical variables.

The *p*-values were adjusted using the Bonferroni procedure when pairwise comparisons between groups were made (Papers II and V). A *p*-value below 0.05 was considered statistically significant in all papers.

3.9 Ethical considerations

Informed oral consent was obtained in Paper I, which was the standard practice in 1998 and had been orally agreed upon by the Ethical Committee in Lund (where the first author worked).

Approvals were obtained from the local Ethics Committee at the former Huddinge University Hospital (now the Karolinska University Hospital Huddinge) for Papers II-V.

The Board of the Swedish Childhood Diabetes Registry approved the use of diabetes patients as controls in Paper II. Furthermore, the study was advertised in the journal of the Swedish Association of Diabetes. In this way, patients and parents were informed about the study and were given the opportunity to decline a possible matching procedure.

Statistics Sweden did the matching procedure and distributed the questionnaires to all control parents for blinding purposes.

The ethical consideration that required most careful handling in this research project arose in Paper II. The questions posed to CF parents might upset and stress them. The questions posed to control parents about how they would have reacted if their child had received the diagnosis of CF might also upset and stress them. We cannot fully exclude that parents felt anxiety and stress when reading and answering the questionnaire. Parents were free to phone if they had any questions, but no parent phoned. We know, however, that we upset one control parent who returned an unfilled questionnaire with the remark that she had given birth to a baby whom she had given away for adoption directly after birth.

4 RESULTS

Paper I

The allele frequencies of the three most common CFTR mutations (deltaF508, 394delTT and 3659delC) were 68.3%, 8.5% and 7.9% respectively, in total 84.6%, of the CF alleles in the material. Thirty-five other mutations were found, but none with a frequency higher than 1.2% in the whole material.

As many as 88% of the patients had the deltaF508 in either homozygous (47%) or heterozygous (41%) form. About 73% of the patients were either homozygous for deltaF508, 394delTT or 3659delC or compound heterozygous for two of these mutations. Assuming Hardy-Weinberg equilibrium, 97.6% of the CF patients will be expected to carry at least one copy of these mutations. In 13 patients (3.9%), none of the three most common mutations were found in any of the two alleles and most of them had non-Swedish ancestry.

The 394delTT mutation was equally distributed in the three different CF centres; Uppsala 6.7%, Stockholm 8.2% and Lund 9.3% of alleles.

The 3659delC mutation was most frequent in the northern part of Sweden and was the second most common at the CF centres in Stockholm and Uppsala (10.5% and 13.3% respectively), whereas the allele frequency in the south of Sweden (Lund) was 2.8%.

Paper II

A majority of parents in all three groups was in favour of a newborn screening programme for CF; 86% of the CF parents, 70% of the diabetes

parents and 77% of the parents from the Population Registry (Fig. 2).The response rates were 85%, 74% and 70%, respectively. The parental attitude was independent of the age of the child, as well as delay of diagnosis and well-being of the child at the time of diagnosis.

Almost half of the CF parents reported having genetically examined their *CFTR* gene, while none of the control parents had (Fig. 3).

Almost half of the parents in all three groups indicated that they wanted to know their CF carrier status before having a child (Fig. 4). One out of four did not want this knowledge. The parents of younger children had half the odds of wanting to know their CF carrier status before giving birth (OR = 0.64, 95% CI = 0.47-0.87, $p > 0.01$).

Sixty percent of all the CF parents experienced the diagnosis as delayed; 62% of the parents of the patients younger than 18 years and 47% of parents of adult patients reported a doctor's delay when their child received the CF diagnosis (Fig. 5).

Half of the CF parents felt bad before and after the CF diagnosis of their child, while a quarter experienced a good level of well-being at both times. Worst levels of well-being were reported by the parents of CF children below the age of 18 years before the diagnosis of the child.

According to the CF parents a majority of the CF children were in a poor clinical condition when receiving their CF diagnosis. However, one year later, and at the time of answering the questionnaire, a majority were in a good clinical condition (Fig. 6).

Fig. 2 Answer to questionnaire in Paper II

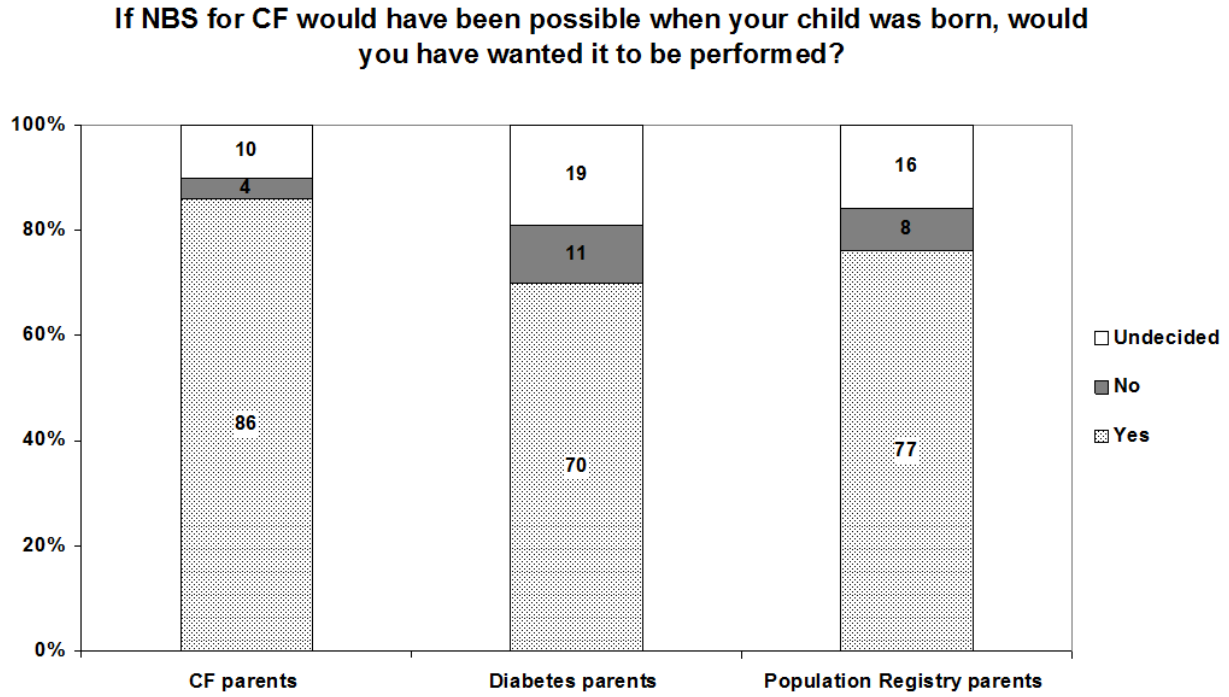


Fig. 3 Answer to questionnaire in Paper II

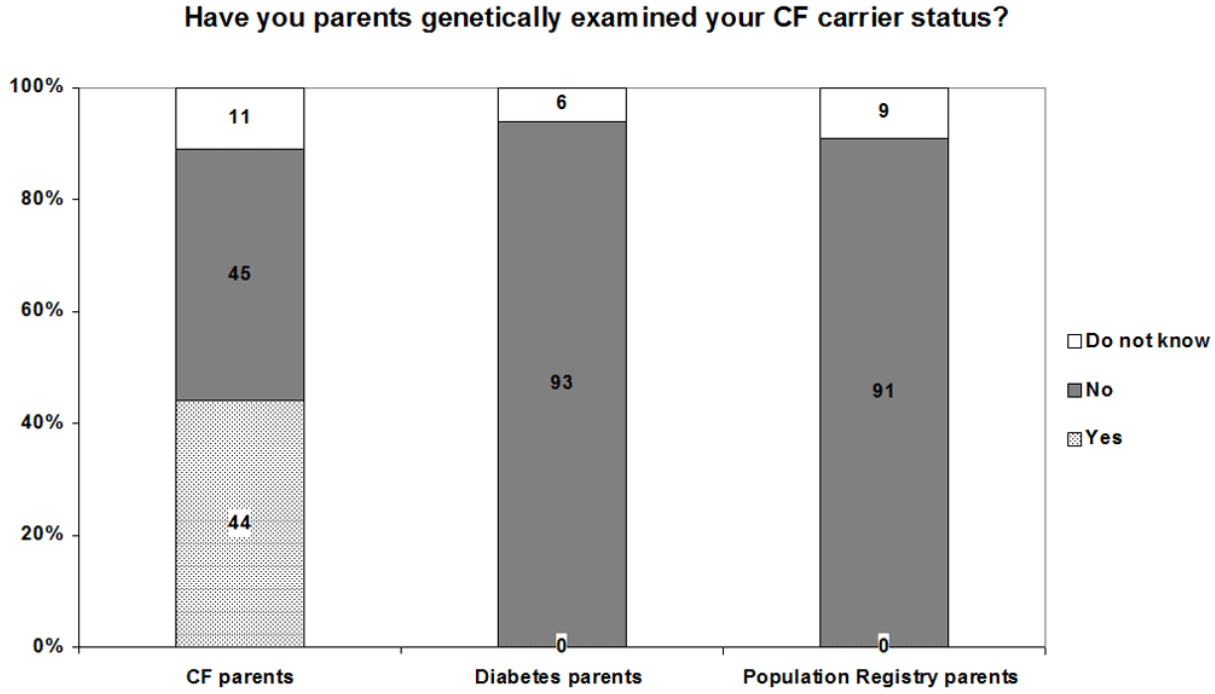


Fig. 4 Answer to questionnaire in Paper II

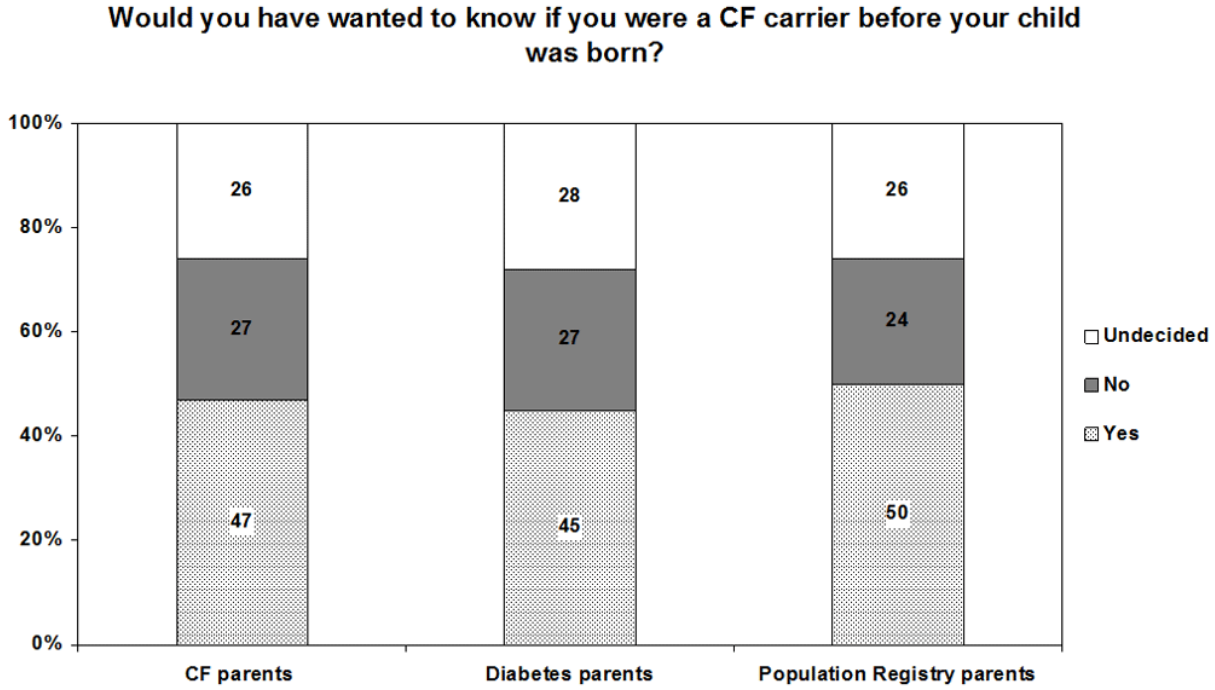


Fig. 5 Answer to questionnaire in Paper II

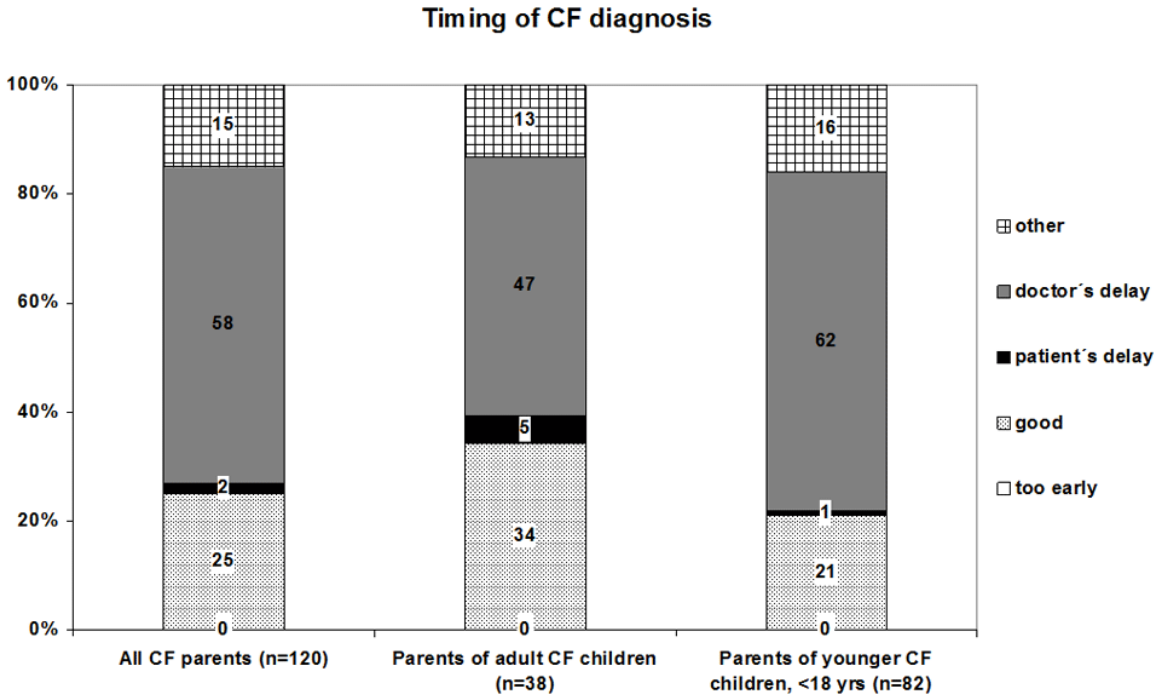
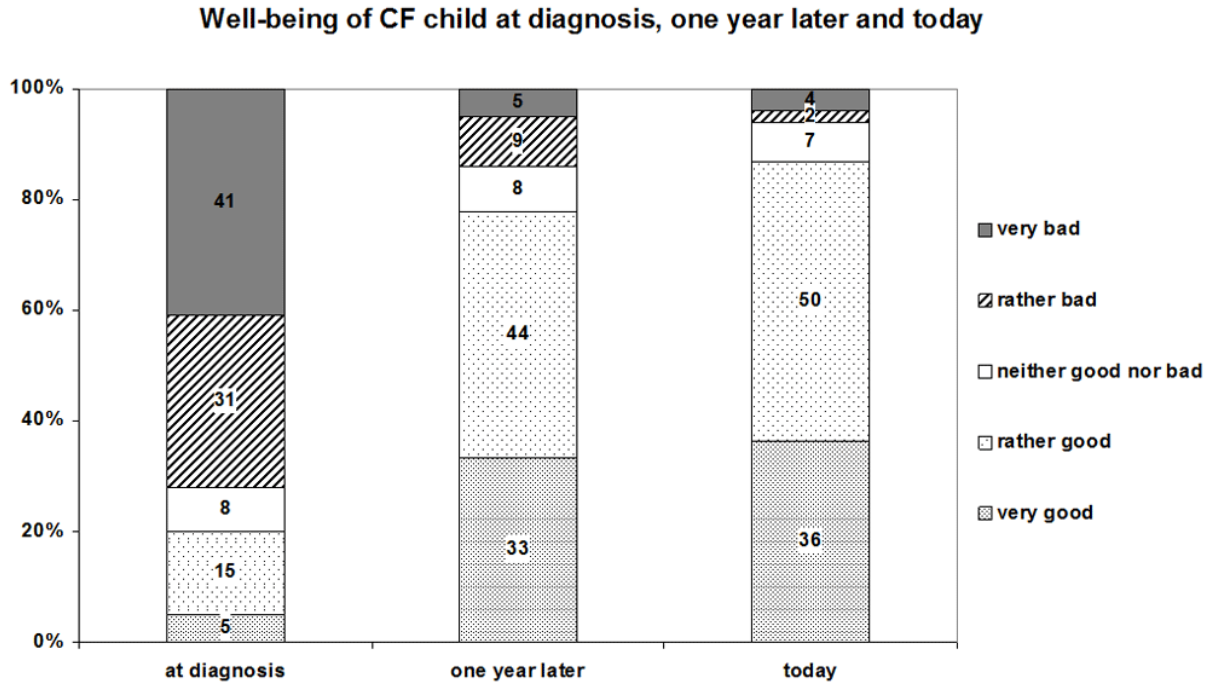


Fig. 6 Answer to questionnaire in Paper II



Paper III

The parental experiences of receiving the CF diagnosis of their child were intense and emotional: 60% solely negative, 30% mixed and 10% solely positive. The parents most often experienced anxiety or fear (24%) (Table 5). Other common categories were: crisis, shock, grief, lack of knowledge or understanding, and that life started focusing on treatment and care. The parents of adult CF patients often mentioned that their child had been given a specific life expectancy ($p < 0.001$).

According to the CF parents 89% of the relatives were affected by the CF diagnosis: siblings received less attention and time (52%); grandparents and other relatives showed ”anxiety, worry, fear or sadness” (67% and 40%, respectively), as well as ”lack of knowledge or understanding” (17% and 30%, respectively) (Table 6).

A majority of the CF families had not experienced any change in the relationships within the family or in the social life of the family one year after the CF diagnosis (Fig. 7).

Table 5. The parental experiences after their child's CF diagnosis. Paper III

		Parents of children ≥18 yrs (*n*=32)	Parents of children <18 yrs (*n*=76)	All parents (*n*=108)
Categories	- with examples	*n* (%)	*n* (%)	*n*(%)
Anxiety or fear		9 (28%)	16 (21%)	25 (23%)
	- about the future *- about not having any knowledge about the disease* *- about the death of the child* *- about not being able to do enough*			
Relief in receiving a diagnosis		4 (12%)	10 (13%)	14 (13%)
	- after a long time with symptoms *- in the knowledge that it was because of a disease and not because we as parents were too anxious or inexperienced* *- giving the possibility to be able to start the right treatment*			
Both anxiety and relief		3 (9%)	14 (18%)	17 (16%)
	- it was a relief to be able to start the treatment because I saw that she became better but still there was, and still is, an anxiety about the future			
Life expectancy mentioned*		10 (31%)	4 (4%)	14 (13%)
	- not live longer than 15 years *- not reach adulthood* *- we would only have the child on loan for 7 years*			
Incurable or deadly disease mentioned		4 (12%)	10 (13%)	14 (13%)
	- we felt bad because CF is an incurable, deadly disease			
Mentioned it was good starting medication		2 (6%)	4 (5%)	6 (6%)
	- for the child it was good to start medication as soon as possible, even though it was a hard time for us parents			

Crisis, shock, grief	3 (9%)	16 (21%)	19 (18%)
- a shock/crisis to know there was no natural explanation for the symptoms but a serious disease			
- a grief that my child has an incurable disease			
Anger, injustice, guilt	0	4 (5%)	4 (4%)
- we felt a vast guilt and loneliness – why our child?			
- we were irritated and upset because we had to make the diagnosis ourselves despite seeing physicians for 1.5 years			
- it was unfair that I had a second ill child			
Lack of knowledge or understanding	7 (22%)	9 (12%)	16 (15%)
- we were anxious because we did not know anything about CF			
- we understood it was a severe disease but could not understand what it would mean for us			
- it took years to accept it			
Hard life focused on treatment and care	1 (3%)	12 (16%)	13 (12%)
- life after the diagnosis was totally focused on learning the treatment and looking at the child to see when extra treatment was necessary			
- because the father worked twice as much			
- there was no time for reflection			
Adulthood neglected	1 (3%)	4 (5%)	5 (5%)
- our own lives were totally set aside, adulthood was neglected			
Criticism of information and care	5 (16%)	6 (8%)	11 (10%)
- we received too little information about the disease from incompetent hospital staff without CF knowledge and we had to find facts about the disease ourselves			
Good information and care	2 (6%)	6 (8%)	8 (7%)
- after the operation we received good information and a "positive" view of CF. Our daughter was well and had a good chance of being given the diagnosis early			
First criticism, later good information and care	2 (6%)	3 (4%)	5 (5%)
- the initial information, given by paediatricians that had never seen a child with CF, was very poor but the subsequent information, by the CF paediatrician, was much better and clearer			
Other	1 (3%)	11 (14%)	12 (11%)
- our child was so ill that it was uncertain if she would survive			
- I was all alone with my child			
- I got depressed			
- there was nobody who could help us with the siblings			

Answers to the open questions were categorized as above. Several categories were possible from one parent's answer.

*Life expectancy more often mentioned by parents of children $\geq$18 yrs (Chi^2 test, $p<0.001$). No other significant differences between the two groups of parents were found.

Table 6. The CF parents' descriptions of how relatives were influenced by the CF diagnosis. Paper III

Categories - with examples	Siblings (*n*=65) *n* (% of siblings)	Grandparents (*n*=72) *n* (% of grandparents)	Other relatives *(n*=43) *n* (% of other relatives)
Anxiety, worry, fear or sadness	18 (28%) *-she was anxious about her ill sister* *-worried about getting a disease himsel*	48 (67%) *- anxiety for the future* *- fear of contact and transmitting infections*	17 (40%) *- worries about their own family planning*
Receiving less attention and time	34 (52%) *-the sibling often had to be with grandparents during the first year when the CF child and we the parents were at the hospital*	0	0
Jealousy	8 (12%)	0	0
All siblings have CF	8 (12%)	0	0
Having made sweat test/CFTR analysis	3 (5%)	2 (3%)	3 (7%)
Denial	0	8 (11%) *- the grandparents pretend the child does not have a disease, they deny it*	0
Guilt, shame	0	6 (8%) *- they felt guilty for not knowing about the in herited disease trait* *- they felt shame about having a sick grandchild*	0
Grief	0	4 (6%)	0
Overprotection	0	2 (3%)	0
Lack of knowledge or understanding	0	12 (17%) *- they do not understand* *-they feel sorry for our child*	12 (30%) *- they do not understand a CF child's sensitivity to infections andthat a CF child has to eat a lot and special food*
Giving constructive help and care	0	8 (11%) *- have learnt the physio-therapy and help us*	10 (23%) *- try to support us* *- do not come to family gatherings if their children have a cold*
Not giving help and care	0	4 (6%) *- are afraid of baby-sitting*	2 (5%) *- did not dare to be alone with the CF child*
Pity	0	0	2 (5%)
Effect on relatives family planning	0	0	4 (9%) *- worries about their own family planning*
Other	10 (15%) *- they wonder if they are CF carriers*	4 (6%) *- they only saw the disease and not the child*	5 (12%) *- they gave the CF child more presents*

Answers to the open questions were categorized as above. Several categories were possible from one parent's answer.

Fig. 7. Answer to questionnaire in Paper III

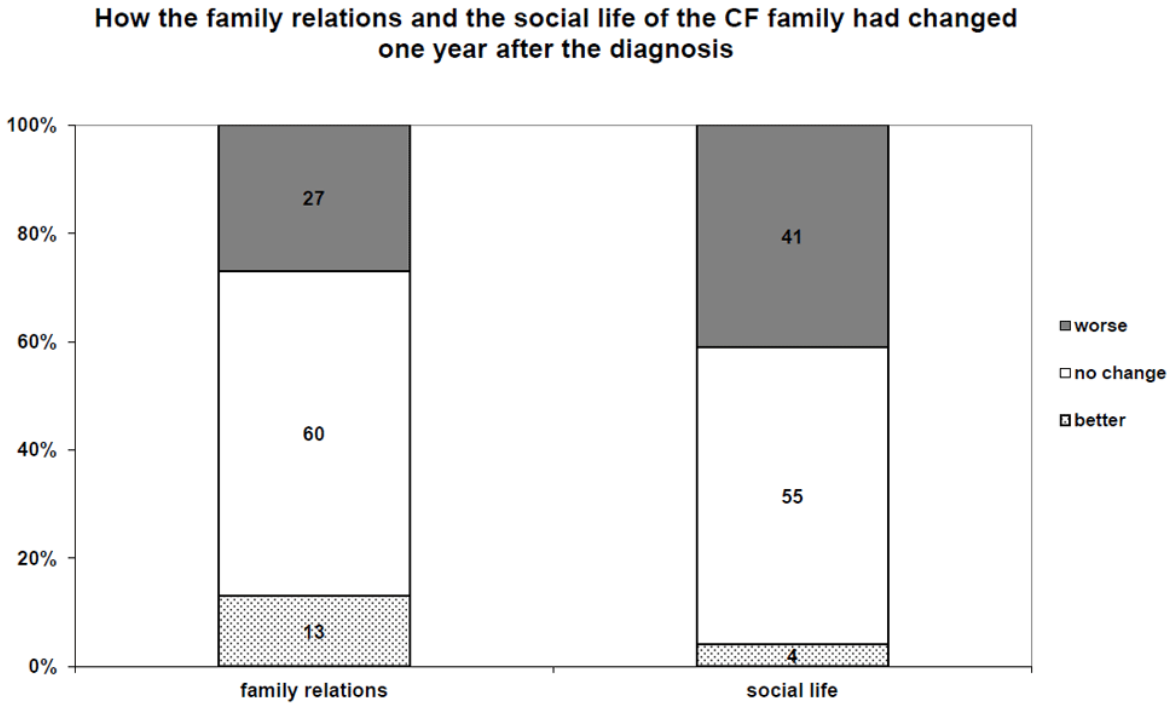
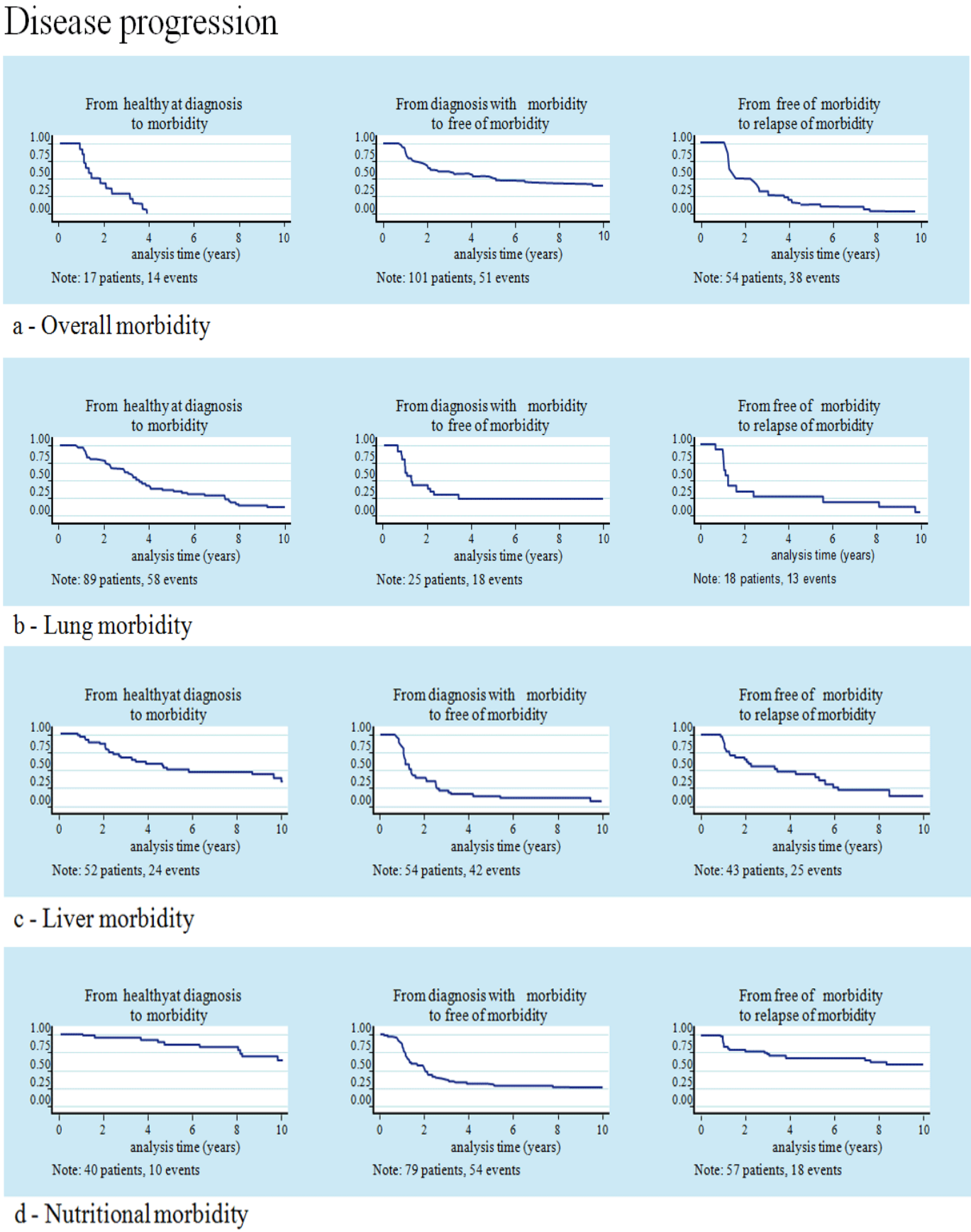


Table 7. Age at diagnosis, in months, for 5-year cohorts for patients in Paper IV.

Birth cohort	N Observations	Lower quartile	Median	Upper quartile
1975-1979	10	2.0	12.0	32.0
1980-1984	15	2.0	5.0	89.0
1985-1989	23	4.0	11.0	22.0
1990-1994	28	0.5	3.0	13.5
1995-1999	27	2.0	8.0	24.0
2000-2004	12	2.0	3.5	7.0

Fig. 8. Disease progression in Paper IV



Paper IV

The median age at diagnosis in 5 year cohorts was 3 to 12 months, with a median age of 5 months (Table 7). There was no trend in change during the past 30 years. The median age at start of CF symptoms, as reported by the parents, was 1.0 months. Incidences of morbidity at diagnosis, using our definitions, were: nutritional morbidity 66%, lung morbidity 21% and liver morbidity 45%. Symptoms of morbidity were absent at diagnosis in 14% of the patients.

The patients were followed from: (i) ”healthy” at diagnosis to endpoint ”morbidity”, (ii) diagnosis with symptoms of morbidity to endpoint free of morbidity, (iii) free of morbidity to endpoint relapse of morbidity. The patients were followed for ten years. The progression of the different morbidities can be seen in the Kaplan-Meier curves (Fig. 8).

The patients healthy at diagnosis showed a rapid decline, with half of the patients experiencing overall morbidity (symptoms in at least one of the morbidities) after 1.4 years. However, they had a slower decline to the endpoint of the specific morbidities; 50% experienced lung morbidity after 3.4 years and liver morbidity after 4.8 years, while 50% never reached nutritional morbidity during the 10 years of follow-up.

The patients with overall morbidity at diagnosis showed a slow decline of symptoms, with half of the patients becoming free of overall morbidity after 4.8 years; however the patients over the age of 24 months at diagnosis had a lower probability of becoming free of morbidity (crude hazard ratio 0.14 [95 % confidence interval 0.04, 0.45]) than those with an earlier diagnosis, at the age of 2-12 months ($p < 0.01$).

The relapse in overall morbidity came rapidly despite CF treatment; half of the patients relapsed in overall morbidity after 1.2 years.

In conclusion: The Kaplan-Meier curves showed a better course of events for the patients without symptoms of morbidity at diagnosis. There were no associations found between morbidity and age at diagnosis, or time interval between start of symptoms to diagnosis. The most important finding was that patients with overall morbidity above the age of 2 years at diagnosis had an increased risk of continued CF morbidity – the initiation of CF treatment did not result in any healthy period.

Paper V

Four large deletions were found by MLPA. *CFTR* mutations, or large deletions, were found in all patients with classic CF. The mutations found could be classified according to the five *CFTR* classes in only 95% of patients (347/364), since not all mutations have been functionally characterised.

The patients were divided in five groups according to the mutation classes. Mutations with unknown classification were grouped according to pancreatic function (Table 1 in paper V). Longitudinal pulmonary function data of FVC and FEV_1 as percentage of the predicted values were analysed for 182 patients older than 7 years (1931 observations).

Patient characteristics (for those older than 7 years) showed a significant difference in age between the groups ($p = 0.001$), mean age being lower in the groups with Class I and Class II mutations, group A-C, than in Group E (multiple comparisons between the groups showed $p = 0.001$, $p = 0.001$ and $p = 0.001$ respectively, with p-values adjusted according to Bonferroni).

The proportion of patients having bronchiectasis did not differ between the groups ($p = 0.28$). Notably, the frequency of patients with bronchiectasis was high also in the groups with milder mutations, and with equal frequency in these two latter groups regardless of pancreatic status.

Patients in all groups were most often chronically colonised with *Pseudomonas aeruginosa* in their lower airways. The proportion of patients with *Pseudomonas aeruginosa* differed significantly between the groups (p = 0.04), Group B differing significantly from Groups D and E (p = 0.02 and p = 0.01, respectively, with p-values adjusted according to Bonferroni). There was no significant difference between the groups regarding chronic colonisation of *Staphylococcus aureus* or *Burkholderia cepacia* (p = 0.13 and p = 0.89, respectively).

The mixed model of FVC and FEV_1 did not show any statistical significance in lung function against age, calculated from the mixed model analysis for the five studied mutation groups; FVC p = 0.74, however FEV_1 showed a tendency of significance with p = 0.13. Fig 5-9 show the individual and population regression lines of FEV_1 for the five studied groups. The linear regressions are only significant in FEV_1 for mutation Groups A-C (groups including only Class I and Class II mutations). The slope of decline of FEV_1 per year was -1.16% for Group A, -0.86% for Group B and -0.94% for Group C, with no significant difference between the groups.

To investigate the possible effect of modern CF treatment with improved pancreatic enzymes, nutrition, antibiotics and physiotherapy starting in 1985, we divided the patients in two groups: those born before 1985 and those born after, followed to age 25. The patients born 1985-2009 had significantly better lung function, FVC as well as FEV_1, measured as the percentage of predicted ($p < 0.001$ and p = 0.004 respectively).

5 GENERAL DISCUSSIONS

When I started working at the Stockholm CF Centre at end of the 1990s, I encountered a couple of CF children with delayed clinical diagnosis who were in very poor clinical condition. Newborn screening for CF was at that time in use in Australia, New Zealand, parts of Italy, and parts of the US, and was being discussed in many more countries. The rationale for newborn screening was that early diagnosis provides an opportunity to improve disease control and prevent early complications. Professor Phil Farrell published the first results from the randomised controlled trial (RCT) of newborn screening for CF in Wisconsin, USA, and this showed improved growth and nutrition, and probably also advantages in terms of slower progression of the lung disease, in screened infants compared to non-screened infants [175]. Long term benefits at the age of 10 years were also shown in an Australian study in children screened for CF compared to a historical cohort of children diagnosed before the screening was introduced [176].

The most common *CFTR* mutation in Sweden

To be able to perform newborn screening for CF with an IRT/DNA approach, the spectrum of *CFTR* mutations in each country must be known when deciding which mutations should be included in the screening panel. Collaboration with Charlotta Schaedel and Lars Holmberg in Lund, performing genetic analyses, resulted in Paper I, which presents the three most common *CFTR* mutations in the Swedish population. DeltaF508 was the most common mutation, with a gene frequency of 68.3%, comparable to that in most populations [32].

DeltaF508 was more common in the south of Sweden, as expected. This is close to Denmark, where the highest frequency of deltaF508 in Europe has been reported [56]. The second most common mutation, 394delTT, first found by Claustres et al. in a single French allele [177], has been described as an old Nordic mutation [178]. The frequency of 394delTT in our study was high, 8.5%, with an equal distribution among the three Swedish CF centres. The frequency, however, is much higher in Finland, between 30-35% [71, 179]. One of the Finnish studies showed that the 394delTT was clustered and enriched in a rural location, consistent with a local founder effect. There is an indication that the 394delTT mutation has been introduced into the Nordic countries by a route different from that of the deltaF508 mutation [178], presumably by the route from the south-east to the north-east of Europe [180]. The mutation may, thus, have been introduced into Sweden through Finland.

The 3659delC mutation, first described by Kerem et al., had previously mostly been found in single cases [181]. The frequency of 3659delC was 7.9% in Paper I and most patients with this mutation were from the northern part of Sweden, which suggests a founder effect. The 3659delC mutation has a frequency of 5.9% of Finnish CF alleles, scattered throughout the country, indicating a Swedish influence in Finland [179]. The West Swedish CF Centre in Göteborg, which was the only CF centre not participating in Paper I, later published its mutation frequencies [182]. The frequencies of the three most common mutations in the country were about the same in their material; deltaF508 62.4%, 394delTT 5.0% and 3659delC 1.9%.

Paper I developed a simple and effective method of analysing the three mutations from Guthrie cards as well as visualizing them distinctly with SSCP on the same gel. A newborn screening programme was, however, never initiated.

One disadvantage with the IRT/DNA protocol in newborn screening for CF is the high rate of carrier detection. Several studies had shown that this approach detects a greater number of carriers than the number expected from the prevalence of carriers in the general population [183, 184]. Carrier detection is, from the child's perspective, not of immediate benefit and can be considered as a violation of the "right-not-to-know" principle. CF carriers (heterozygotes for *CFTR* mutation) may differ in phenotype from those of persons without any *CFTR* mutation. CF heterozygotes are associated with chronic pancreatitis [185, 186], disseminated bronchiectasis [187] and chronic rhinosinusitis [188].

A Health Technology Assessment document on "Screening for CF" was published in the UK in 1999. The authors concluded that antenatal genetic screening should be offered routinely, pre-conceptional genetic screening should be made available, and health authorities should consider introducing neonatal screening. The detailed experiences of 20 programmes of neonatal screening had been reviewed, and showed that more than 5 million babies had been screened with a low false-positive rate (0.5 per 1000), acceptable detection rate (90%), and favourable positive predicted value (33%) [189]. When the first Cochrane Report was published in 2000, the authors concluded that there was insufficient evidence of either harm or benefit from early diagnosis of CF through newborn screening [190].

Psychosocial aspects of newborn screening for CF

One of the potential psychosocial risks of a newborn screening programme is negative influence on family relationships, such as mother-child bonding. Temporary rejection of their babies during the period of uncertainty or following the procedures of diagnosis was reported in four mothers out of 29 in the Wales and the West Midlands newborn screening trial [191]. However, there were many draw-backs with this study, for example a long delay of between 2-35 days for the confirmatory test, and a study design that did not identify the group to which the mothers belonged (screened

or controls).There is not much evidence from the literature that the mother-child relationship is significantly affected. The opposite has been reported. Helton et al. concluded that an early diagnosis from newborn screening did not interfere with the mother-baby relationship [192]. On the contrary, a delayed diagnosis of a CF child whilst symptomatic may lead to negative psychosocial effect, which is discussed later.

From the Wisconsin trial, anxiety and feelings of depression have been reported among parents with a falsepositive screening result while awaiting definitive diagnostic assessment, and anxiety could persist for a year in a minority of cases despite a negative sweat test [193, 194]. Since then the importance of minimal delay between informing the family of the possibility of CF and the definitive diagnostic assessment has been emphasised by many. Tailoring information to the local healthcare setting is likely to best serve parents. The communication with the parents before and after screening is of utmost importance, because all parents need information. An effective communication between healthcare providers and parents is central to the success of newborn screening programmes for CF [195, 196]. There are good recommendations for communication in the European consensus document of bestpractise guidelines for cystic fibrosis newborn screening [155].

The reported psychosocial benefits of newborn screening for CF include the avoidance of stress and anxiety related to delayed diagnosis [192, 197, 198], and the possibility for early genetic counselling to obtain information about their carrier status and to be able to make choices about future pregnancies [196]. These psychological benefits outweigh the psychological disadvantages.

Parental attitudes towards newborn screening for CF

Parents who had a child identified as a CF carrier in the Wisconsin study completed a questionnaire. The majority responded that they were glad to be aware of their child's CF carrier status and did not report feelings of confusion or guilt [195]. Eighty-five percent of the parents in the survey answered "Yes" to the question "Do you think newborn screening for CF should be done?". The big problem with this study was that only 28% (138 of 483 possible participants) returned a complete questionnaire. Other studies have also shown support for newborn screening for CF [191, 192, 198]. Only parents of children with CF or parents of children identified as carriers participated in these studies.

We wanted to find out the attitudes of Swedish parents of children with CF and of parents of healthy children towards introducing newborn screening for CF. In our discussion with the Ethics Committee we were advised to ask a group that could understand the information given, the questions asked and the difficulties associated with receiving a disease diagnosis. We decided to include a group of parents of children with diabetes, but we argued for keeping the group of parents of healthy children. A vast majority of all the parents in Paper I supported newborn screening. The parents of CF children had the highest degree of support, 86%. Interestingly this support for newborn screening did not correlate with the well-being, or rather the poor clinical condition, of most of the children at time of the CF diagnosis.

An article asking mothers of healthy babies about their attitudes towards newborn screening and their knowledge about newborn screening for CF and sickle cell disease was published in the US before Paper 1 was published [199]. This study showed results similar to ours, 83% the responding mothers being in favour of newborn screening.

The group of parents of children with diabetes in our study reported the lowest support for newborn screening for CF, 70%. There was a significant difference between the proportions of support between the CF parents and the diabetes parents. We believe that this may be caused by a greater ability of the diabetes parents to understand the difficulties associated

with a false-positive screening result, and other psychosocial risks. This ability arises as a consequence of their own experiences of receiving a diagnosis of diabetes for their child.

Fewer, almost half, of the parents were positive towards obtaining knowledge of their own carrier status than were positive towards a newborn screening programme for CF. The question about their own CF carrier status may be more delicate and raise difficult questions of how to act if aware of a CF carrier status before giving birth. The parents of younger children (< 18 years) more seldom wanted to know their CF carrier status before giving birth. This could be attributed to the difficulty for the parents of younger children to think about how life would be without their child, or that younger children are less affected by the disease.

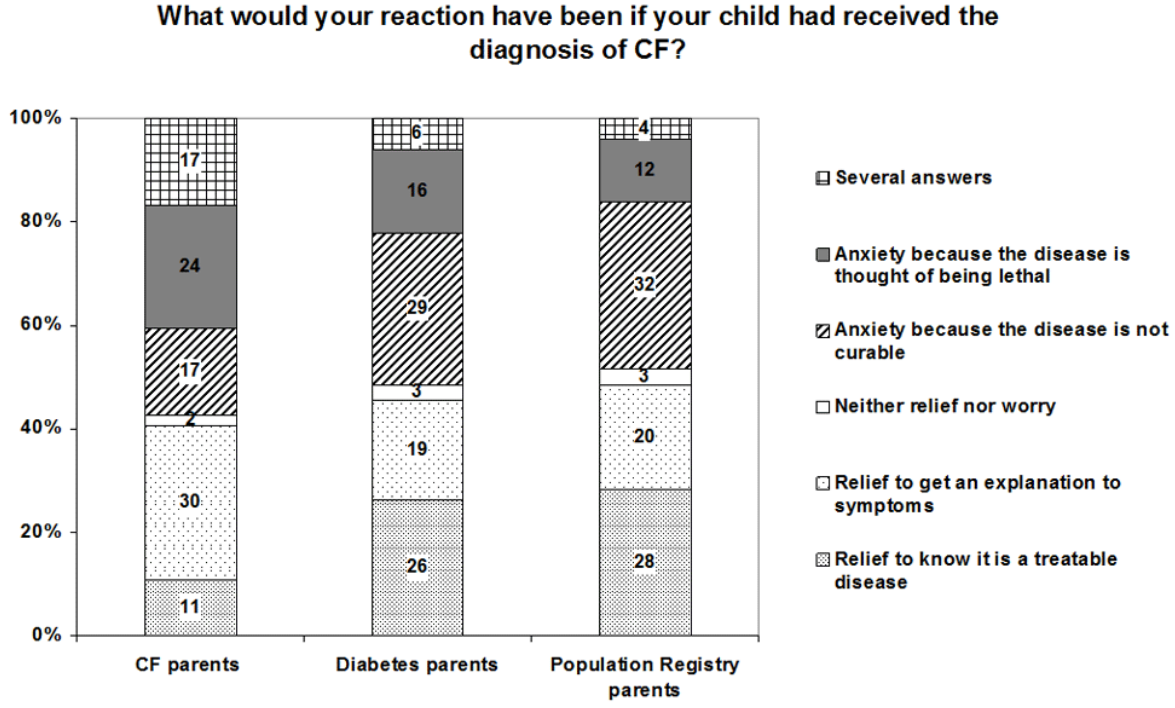
Information is crucial in a screening scenario and in a study like ours. A weakness of our study was that it was necessary that parents understand the information given, including the genetics of CF. In the information leaflet, we tried to give neutral information about CF and newborn screening in a concise way, including the possible identification of clinically healthy CF babies, the potential psychosocial risks to those with false-positive results or the possible detection of a carrier status of the child. It can also be argued that parents do not have detailed knowledge, and that they answer from a general point of view.

Response rates of questionnaires studies in Sweden are high in international comparison. The response rates in our Paper II were between 70% and 85% in the different groups. This high response rate, together with the matching procedure, is one of the strengths of our study.

Parental reactions to a diagnosis

The questionnaire included a question ”What would your first reaction for yourself have been if your child had received a diagnosis

Fig. 9 Answer to questionnaire, discussion p. 39



of CF?" The parents tended to report either relief or anxiety about the future of their child when answering the question about what it first of all would imply for them if their child had received the diagnosis of CF in a NBS programme (Fig. 9). There were almost as many answers of "relief" as of "anxiety" in all groups, but the CF parents answered differently from the parents of the two control groups. Among the answers with "relief", the CF parents mostly answered "relief from obtaining an explanation of symptoms" (30%), while the control parents mostly answered "a relief in knowing that it is a treatable disease" (26% of the diabetes parents and 28% of the parents from the population registry). Among the answers with "anxiety", the CF parents mostly answered "anxiety because the disease is thought of as being lethal" (24%), while the control parents mostly answered "anxiety because the disease isn't curable" (29% of the diabetes parents and 32% of the parents from the population registry). Very few of the parents gave a neutral answer. Many of the parents, especially the CF parents, gave several answers even though they had been asked to give only one answer. An effect of interaction between group and age was found (logistic regression analysis, $p < 0.01$). The CF parents of younger children showed more "relief in a given CF diagnosis" compared to the CF parents of adult patients (OR = 2.80, 95% CI = 1.30-6.04, $p < 0.05$), whereas the parents of younger population registry controls showed less relief and more anxiety compared to the parents of adult population registry controls (OR = 0.53, 95% CI = 0.33-0.85, $p < 0.05$). The CF parents of adult patients showed less relief and more anxiety compared to the parents of adult population registry controls (OR = 0.28, 95% CI = 0.13-0.60, $p < 0.01$).

Later consideration of this question raised the possibility that it had been too hypothetical. The control parents may have had difficulties in appreciating the implications of having a sick child, while the CF parents often delivered several answers. We decided not to include this in the manuscript. We describe and discuss this question here because the answers may be useful in formulating hypotheses.

Prental reactions to and delayed diagnosis

When newborn screening is not conducted, a delayed diagnosis is often seen and may result in negative psychosocial effects on the family, for example causing mothers considerable personal stress [197]. A delayed diagnosis also results in less confidence in medical caregivers. A Dutch study explored retrospectively the relationship between the period preceding diagnosis and the way parents of 45 children with CF experienced and handled the disease of their child [198]. Most parents with a delayed diagnosis remember the period of uncertainty and anxiety preceding the diagnosis and would have preferred an earlier diagnosis.

To describe the impact of delayed diagnosis on US families, forty diagnostic stories were condensed into that of a single family and a qualitative description was made [200]. Families were frustrated and stressed by diagnostic incompetence. Ineffectual care led to long-term anxiety, emotional trauma, and self-doubt. Relationships with one another and with doctors were seriously affected. CF parents consistently expressed that newborn screening would help others avoid the pain they had suffered by not knowing the diagnosis earlier.

When asking the parents of the CF patients at the Stockholm CF Centre in Paper II, 60% reported a delayed diagnosis. The vast majority described it as doctor's delay. Some parents, 15%, chose the alternative "Other", and supplied an explanation with their own words. Many of these parents stated that the diagnosis was delayed, but, for example, "It was understandable", or "It could have been earlier, but it was not too late". Up to 75% may have experienced the diagnosis as delayed. Among the 82 parents of CF children younger than 18 years, as many as 63% experienced a delayed diagnosis. Thus, there was no less delay over time. No parent thought the CF diagnosis was given too early. The avoidance of a delayed diagnosis is one of the main rationales for newborn screening for CF, at least for the family of a baby with CF.

Parental experiences of a clinical cystic fibrosis diagnosis

It has been suggested that ”Prior to the implementation of a large population screening program it is essential to study potential difficulties in the parental emotional response” [192]. We wanted to study the psychosocial implications of the diagnosis on families of affected CF children. The questionnaire sent out to the parents of CF patients therefore contained more questions, for example about the social life of the family, how the family member in the nuclear family, the grandparents, and other relatives were influenced by the CF diagnosis. Most of these questions were open-ended to let the parents write whatever they wanted. Only CF parents were included, since our aim was to collect information about parental experiences at time of the CF diagnosis. A control group of parents with experiences of another diagnosis was not considered to be of importance. Furthermore, experiences are disease specific [201]. Our aim was to provide the basisfor generating hypotheses and for an evaluation of the psychosocial impact of a possible future newborn screening programme for CF in Sweden. We wanted to hear the views of the parents without comparing the parents' perception with what actually happened.

Qualitative research can be explained as having an aim to ”study things in their natural setting”, not to express everything in numbers [202]. Qualitative research begins with an intention to explore a particular area, collect ”data” (observations and interviews), and generate ideas and hypotheses from these data, defining preliminary questions that can then be addressed in quantitative studies. However, analysing qualitative data should be done using more than one research method (triangulation). We have not done a quantitative study, but Paper III does use a ”qualitative approach” in which data is collected openly and ideas are generated by the results.

Parents have reported many strong, frank and open-hearted stories. A reflection while reading the responses was that many parents still had very intense and emotional experiences that they remembered vividly. We could confirm the reactions of shock and relief, which were the first reactions of the diagnostic phase already mentioned in the psychological study (thesis) by Cecilia Falkman, who studied 52 Swedish CF children and their families in 1977 [203].

Response to a chronic illness is a complex process. Parents are overwhelmed by the CF diagnosis during the year following the diagnosis [204]. This could be compared to reactions after severe trauma; posttraumatic stress syndrome. Posttraumatic stress disorder has been observed among parents of children with cancer, indicating the importance of offering appropriate psychological interventions [205, 206]. Myra Bluebond-Langner has described issues and strategies that CF families adopt to preserve a normal life for as long as possible [204]. In our study, we recognized all the issues and the strategies described by Bluebond-Langner: the tasks of care, information about the disease and the child's condition, reminders of the disease and its consequences, the child's difference from other children, competing needs and priorities within the family, and the ill child's future. The parents of adult CF patients more often mentioned that their child had been given a specific life expectancy, and the life expectancy mentioned was always death during childhood years. This mirrors the low survival rate that CF patients used to have. Co-author Agneta Bergsten-Brucefors, who has been working as a psychologist on the adult CF team for over 15 years, has experiences of adult CF patients who were constantly aware of having a restricted life-expectancy and having difficulties in planning their life. There was a significant difference between the parents with older and younger children: the parents of children younger than 18 years seldom wrote about having been given a specific life expectancy. This may be explained by current knowledge of the disadvantages of giving a specific life expectancy and that, due to gradual improvements in survival rates, health staff are more optimistic regarding the prognosis [72].

However, the authors of a review about quality of life and rare diseases describe several studies that show that, although some rare diseases do not affect life expectancy, the majority of diseases lead to physical, emotional and/or psychosocial limitations with a wide range of disabilities [207]. Translating this to a CF context, one may hypothesise that even though patients with CF do not have as low a life expectancy as before, they still have physical, emotional and/or psychosocial limitations with a wide range of disabilities. One of the limitations we should be aware of is the burden of care we put on the patients and their parents. The parents of younger CF children (< 18 years) often revealed that life focused on treatment and care. This has been described before, by Eiser et al., who found that difficulties with regard to illness routines, such as physiotherapy, caused stress in mothers caring for a child with CF [208]. Health professionals need to support parents, who nowadays have the major responsibility for the daily management of their child's condition. Nursing practice can help and support these families [209]. The responsibilities of family members to assist or support the CF individual during CF treatment continue even in adulthood [210].

The family members, in our study, were also affected by the diagnosis. The parents reported that almost 9 out of 10 relatives were affected by the diagnosis. Siblings often received less attention and time, and showed anxiety (Table 6). Older siblings were reported to show anxiety to a greater extent than younger siblings. Jealousy was sometimes mentioned for both older and younger siblings, according to the parents. Siblings of CF patients might be at risk of developing emotional, behavioural, and social problems, as are siblings of paediatric cancer patients [211]. The parental reports of how CF siblings and relatives are affected are interesting, since there are not very many publications on this issue.

The parents in our study reported negative as well as positive influences of the CF diagnosis on grandparents. An interesting finding was that the grandparents were the only relatives reported to show denial (11%), disgrace and guilt (8%), grief (6%) and overprotection (3%) (Table 6). This might be due to a lack of knowledge or understanding, as reported in 17% of grandparents.

In our study 10% of parents were critical of the information and care given during the diagnostic phase (Table 5). Correct information and tools for adapting to the diagnosis are important for parents.

A positive finding in our study was that relationships and social life were intact in a large number of families. Some parents reported that life had become better after the diagnosis of their child. We have only sought the views of the parents in Paper III. A future challenge will be how to help relatives to become as good a support as possible to the parents. To obtain this we have initiated special information sessions in the evenings for relatives, with special invitations going out to the grandparents.

Age at diagnosis

In Paper IV we used the register data to look for age at diagnosis and age at first symptom as described by the parents. Cystic fibrosis has different phenotypes, which are all included in the same register, although the vast majority of cases show classic severe CF. Age at first symptom and age at diagnosis will differ between different phenotypes, and the distribution of those variables will be skewed. It is interesting to compare age at first symptom and age at diagnosis. As is shown in Table 1 in Paper IV, the median age at first symptom is low, between 0.5 and 1.0 months, although the range is wide (due to patients with a milder atypical phenotype). The median age at diagnosis is between 5 and 6 months (also with a wide range) in our material (Table 1, Paper IV). This is close to the median age of 4 months reported for "active" CF patients in the UK and in France, both countries that at that time had some screened patients [212].

The Swedish demographic study in 1999 showed a similar median age at first symptom

of 1 month but a median age of diagnosis of 9 months (also with a wide range) [72]. Tha patients in our study is only one cohort of the Swedish CF patients, but it does include about one third of all Swedish CF patients. The cohort is complete, as all CF patients who attended the Stockholm CF Centre since its start in 1974 are included in the register. There is a variation in the number of children with CF born every year, and in the number of adults who receive the diagnosis every year. We divided the age at diagnosis into 5-year cohorts, and the age showed a wide variation, between 3 and 12 months, in the different cohorts. The age at diagnosis is higher in Sweden than it would be if a screening programme were in use. Every study of the influence of newborn screening on clinical outcomes has confirmed that diagnosis occurs at a significantly younger age [98, 154, 175, 213-218]. Some Australians physicians write: ”this alone is sufficient to justify newborn screening for CF” [219]. In a newborn screening programme, most babies with CF are diagnosed within 1 month.

The most important finding in Paper IV was that patients above 24 months of age at diagnosis and having symptoms of overall morbidity have a lower probability of ever becoming free of morbidity than patients diagnosed at a younger age. This speaks in favour of diagnosing patients with symptoms as early as possible. We were, however, not able to confirm the finding of a worse outcome for the patients diagnosed after 2 months of age, as Sims et al. reported from the UK [220].

Disease progression

The Kaplan-Meier curves in Paper IV give a good insight into the disease progression of CF. We created a scenario that allowed the patients to move from ”morbidity” to ”healthy” and *vice versa*. ”Healthy” represented a normal clinical status whatever medication or treatment burden the patient had. Patients with a good clinical status at diagnosis were defined as healthy by our definitions. There were patients defined as healthy for overall disease at diagnosis. Their decline towards endpoint morbidity was, however, rapid.

The nutritional morbidity differed from the other morbidities in the study. During the 10-year follow-up, half of the patients did not reach morbidity or relapse. The nutritional study of the Scandinavian CF Study Consortium has shown that the growth of Swedish CF children is as good as that of healthy children [221].

The largest benefits of newborn screening for CF are the nutritional benefits, with significantly better height and weight for children diagnosed by screening than for children with a clinical diagnosis [176, 215, 220, 222-224]. Deficiencies of proteins and fat-soluble vitamins, such as vitamin E, can also be avoided by newborn screening [218, 225]. Nutritional status in CF has a direct effect on lung function.

Lung disease is the main cause for morbidity and mortality in CF patients. For that reason, many physicians have demanded evidence for a positive influence of newborn screening on lung disease before starting screening, despite other evidence in favour of screening. Unfortunately, the Wisconsin RCT did not show the probable advantages with respect to lung disease for screened CF infants discussed in the preliminary report. Fewer CF changes on chest radiographs in the screened group was apparent only at the time of diagnosis, not as the children grew older [226]. This might, however, relate to the fact that an increased number of children in the screened group were infected with *Pseudomonas aeruginosa*. In several studies better lung function were found in the screened groups [98, 218, 227].

The UK screening programme in Wales and the West Midlands has not reported anything on pulmonary outcome, and there were few reports of clinical benefits. The main benefit reported was reduced time in hospital for the screened group [154].

The lung morbidity we show in Paper IV is not very encouraging (Fig 8). For the patients with lung morbidity at diagnosis, the symptoms were rapidly treated; half of them were free of lung

morbidity after 1.3 years. However, the relapse was rapid despite CF treatment; half of them had relapsed into lung morbidity after 1.2 years. There is an advantage for the patients without lung morbidity at diagnosis. They showed a steady decline, with half of the patients experiencing lung morbidity after 3.4 years. We could not find any associations between the investigated factors and the outcome of lung morbidity.

The CF child with lung morbidity at diagnosis relapsed rapidly in lung morbidity, and might therefore have an increased risk of needing more lung therapy. Maybe oral antibiotics will not help and the child will have to be put onto an intravenous antibiotic treatment with its associated burden of care for the parents, as well as the psychological burdens for child and parents. Data from the UK CF register has shown that children diagnosed by screening require fewer long term therapies and fewer high intensity therapies than clinically diagnosed [220].

The aim of a newborn screening programme for CF is to find only the patients with severe classic CF. We studied lung function with mixed model analysis according to mutation classes in Paper V. The patients with atypical CF often have Mutation Classes IV and V on one allele. The contrary, however, does not always apply; one of those mutations does not mean that the patient cannot have a classic form of CF. We could not show a significant difference in lung function, expressed as FVC or FEV_1 of percent predicted, according to the mutation groups in our study. The younger cohort in our study (born 1985-2009) did have a significantly better lung function, in FVC as well as FEV_1 ($p < 0.001$ and $p = 0.004$, respectively). This is as expected, and is probably due to the modern CF treatment.

In a study comprising the majority of Swedish CF patients in 1998, it was shown that severity of pulmonary disease was predicted to some extent by *CFTR* genotype [228]. The group of patients with one or two missense mutations (being milder mutations) had a slower decline of FEV_1 compared with the other genotypes in the study ($p = 0.01$ when compared with homozygous deltaF508). The patients with missense mutation did not have a statistically significant decline of FEV_1, the decline was not different from zero. A more rapid deterioration was seen in patients with concomitant diabetes mellitus, pancreatic insufficiency and chronic *Pseudomonas aeruginosa* infection.

In 2005, de Gracia et al showed that patients homozygous or compound homozygous for mutations of Classes I and II had significantly lower spirometric values, greater loss of pulmonary function, a higher proportion of end-stage lung disease, a higher risk of suffering from moderate to severe lung disease and a lower probability of survival than patients compound heterozygous for Mutation Classes III-V [229]. The hypothesis of the study of de Gracia et al was that the severity of the lung disease is related to whether or not the CFTR protein reaches the epithelial cell surface.

Research into mutation classes and their possible effect on lung function is interesting. Genotype-phenotype correlations are difficult regarding pulmonary phenotype. The new project called CFTR2, the Clinical and Functional Translation of CFTR, is an international initiative that seeks to provide complete, advanced and expert-reviewed functional and clinical information on CFTR that will help to obtain genotype-phenotype information as it gathers information from patient registries around the world. This genotype-phenotype information will help those screening protocols that use DNA sequencing and find rarer mutations.

In Paper V we report all known genotypes of the patients at the Stockholm CF Centre (Table 1 in Paper V). There is a wide range of different mutations. One drawback of using screening with a IRT/DNA approach in a multi-ethnic community, for example in large cities, is that it will not identify patients with mutations specific to other ethnic origins, and ethnic discrimination may occur. The British and French screening programmes try to compensate for this with a second IRT sample in babies with very high first IRT sample in whom no CF mutations are found [230, 231].

Conclusion about newborn screening for CF

Support for introducing a newborn screening programme for CF has increased during the past 10 years. Screening has occurred for more than 25 years in some regions. There are results not only from observational studies but also from RCTs (in particular, the Wisconsin study). There are clear benefits of newborn screening regarding earlier diagnosis, less parental distress by avoiding delayed diagnosis, nutritional benefits and health economics. Cost analyses estimates from the Wisconsin trial suggest diagnosis through screening could be less expensive [232]. Regarding costs there is also a retrospective cohort study showing that newborn screening for CF reduces the treatment cost of people with CF, and this means that screening is cost-effective [233]. Several studies show that the potential of better survival is higher [98, 215, 227, 234, 235].

Early diagnosis by screening allows genetic counselling for families of CF babies and for carrier parents, and cascade family testing [236]. So even if carrier detection is not desirable, it can benefit the family of the carrier baby. As two authors from Australia put it: ”When the parenting stress associated with the detection of unaffected carriers is balanced against the impact of a delayed CF diagnosis which the families of an affected child must endure, it seems obvious that newborn screening for CF remains desirable” [219].

One drawback of newborn screening is the occurrence of inconclusive screening results. Occasionally, babies are identified by newborn screening in whom a CF diagnosis can neither be confirmed nor excluded. They have raised IRT, borderline sweat test results, and *CFTR* mutations with unknown pathogenic potential. Although these children show no or little signs of disease, the long-term outcomes for these patients are unknown. The families of these infants must be given clear information about this inconclusive screening result. The child may over time develop CF or CFTR-related disorder and should be followed at a CF Centre. Inconclusive screening results have been studied in Italy and it appears to be understood and associated with lower anxiety levels than CF diagnosis [237].

The Cochrane Report from 2009 concluded that nutritional benefits are clear, that screening seems less expensive than a traditional clinical diagnosis, and that screening provides potential for better pulmonary outcome [223]. The Centers for Disease Control and Prevention in the US, stated as early as 2004 that: ”on the basis of evidence of moderate benefits and low risk of harm, CDC believes that newborn screening for CF is justified” [238]. In 2010, 14 European countries perform newborn screening for CF (nationally or as a regional service), and all states in the US have started. It should now also be time for Sweden to start a newborn screening programme for CF [239].

6 CONCLUSIONS

The conditions for a newborn screening programme for CF in Sweden are good. This study has shown that three common mutations, deltaF508, 394delTT and 3659delC, together account for 84.6% of all CF alleles. Including these three mutations in the second tier of an IRT/DNA approach would give a low risk of false negative results in Swedish persons.

A majority of parents in Sweden support the inclusion of CF in the newborn screening programme. The parental attitude is independent of the age of the child, as well as delay of diagnosis and well-being of the CF child at the time of diagnosis.

Nearly half of all the parents want to know their CF carrier status before having a child. Fewer of the parents of children younger than 18 years want this knowledge.

Parental experiences on receiving a clinical CF diagnosis are intense and emotional; mostly negative, some mixed and few only positive experiences. The parents most often experience anxiety or fear.

Parents state that the CF diagnosis greatly influences other family members. Half of the

siblings receive less attention and time. Both the grandparents and the other relatives show anxiety, worry, fear, sadness as well as lack of knowledge or understanding.

A majority of the CF families do not experience any change in relationships within the family, or in the family's social life, one year after the diagnosis.

The median age at diagnosis of the patients studied here was 5 months, and this age has not decreased during the last 30 years.

The patients with symptoms from the lungs, the liver or nutritional symptoms at CF diagnosis can become free of symptoms after beginning CF treatment, except for patients who are older than 2 years at diagnosis. These patients have a higher probability of never becoming free of symptoms than other patients.

The patients in a good clinical condition at diagnosis can remain in a good condition, half of them for almost one and a half years after the diagnosis.

All patients with classical CF in our study have *CFTR* mutations or larger deletions.

The patients homozygous for Class I mutations have as severe a clinical picture as the patients homozygous for Class II mutations.

There was no statistical difference in lung function for the different mutation groups studied.

Patients born after 1985 have better lung function (measured by FEV_1 and FVC) than those born earlier.

My overall conclusion is that it is now time for Sweden to plan for a pilot study of newborn screening with the IRT/DNA approach including the three severe *CFTR* mutations: deltaF508, 394delTT and 3659delC.

7 FUTURE PERSPECTIVES

The major challenge now is to start a pilot study of newborn screening for CF in Sweden. This needs to be carried out in cooperation with many healthcare services around the country. It is not currently lawful to carry out genetic screening. Is it possible to make an exception for CF? Should the screening result not reveal whether mutations have been found, only give the result as low, moderate or high suspicion of CF. (This is how the result of the Swiss newborn screening for CF is formulated.) (Personal communication Toni Torresani, director of the Swiss Neonatal Screening Laboratory, University Children's Hospital, Zurich, Switzerland). Or should Sweden embark on the IRT/PAP approach?

The effect of the CF diagnosis and CF care on the extended family, such as siblings and grandparents, should be further studied. This is an area where little has been done. It would be interesting to follow-up our questionnaire with interviews with siblings and grandparents.

The Swedish CF Register should continue and I would like to carry out more register research in cooperation with the other Swedish CF Centres and the rest of Europe.

There is also a need for more functional studies regarding several *CFTR* mutations, especially rare mutations, in order to classify them. We are planning to continue with functional analyses, such as chloride efflux measurements on respiratory epithelial cells from nasal brushings, nasal potential measurements and X-ray microanalysis of the electrolyte composition of airway surface liquid (ASL) in rare *CFTR* mutations.

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