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CYSTIC FIBROSIS IN ESTONIA

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To my family
To CF patients

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LIST OF ORIGINAL PUBLICATIONS

I **Klaassen T**, Teder M, Viikmaa M, Metspalu A. Neonatal screening for the cystic fibrosis main mutation delta F508 in Estonia. *J Med Screen* 1998; 5: 16–9.

II Teder M, **Klaassen T**, Oitmaa E, Kaasik K, Metspalu A. Distribution of CFTR gene mutations in cystic fibrosis patients from Estonia. *J Med Genet* 2000; 37: E16:1–4.

III **Kahre T**, Teder M, Panov M, Metspalu A. Severe CF manifestation with anaemia and failure to thrive in a 394delTT homozygous patient. *J Cystic Fibrosis*. 2004; 3: 58–60.

ABBREVIATIONS

ABC transporters	ATP–binding cassette transporters
A1AT	α-1-antitrypsin gene
ADP	adenosine diphosphate
AMPK	AMP-activated protein kinase
ARMS	amplification refractory mutation system
ATP	adenosine triphosphate
cAMP	3', 5'-cyclic adenosine monophosphate
CaCC	Ca^{2+} sensitive chloride channel
CBAVD	congenital bilateral absence of the *vas deference*
CF	cystic fibrosis
CFGAC	Cystic Fibrosis Genetic Analysis Consortium
CFMDB	Cystic Fibrosis Mutation Database, available from: http://www.genet.sickkids.on.ca/cftr
CFTR	cystic fibrosis transmembrane conductance regulator gene
CI	confidence interval
DGGE	denaturing gradient gel electrophoresis
DIOS	distal intestinal obstruction syndrome
ENaC	epithelial amiloride sensitive Na^+ channel
EWGCFG	European Working Group on Cystic Fibrosis Genetics
FEV_1	forced expiratory volume in one second
FVC	forced vital capacity
GOR	gastrooesophageal reflux
GT	gene therapy
IRT	immunoreactive trypsine test
MI	meconium ileus
MR	mortality rate
NBD	nucleotide binding domain of CFTR protein
ORCC	outwardly rectifying chloride channel
PAAG	polyacrylamide gel
PCR	polymerase chain reaction
PI	pancreatic insufficiency
PKA	protein kinase A
PS	pancreatic sufficiency
R-domain	regulatory domain of CFTR protein
rhDNase	recombinant human DNase
SD	standard deviation
SSCP	single strand DNA conformational polymorphism
TGF-β	transforming growth factor β
TM	transmembrane segment of CFTR protein
TMD	transmembrane domain of CFTR protein

1. INTRODUCTION

Cystic fibrosis (CF) is the most common life-shortening autosomal recessively inherited disorder among Caucasians (1 in 2500), but is found in all racial and ethnic groups (Welsh *et al.,* 1995). The first comprehensive description of the symptoms of cystic fibrosis was provided in 1938 by Dorothy Andersen who called it "cystic fibrosis of the pancreas" (Andersen, 1938). Cloning of the cystic fibrosis transmembrane conductance regulator (CFTR) gene in 1989 (Rommens *et al.,* 1989; Riordan *et al.,* 1989) significantly advanced our understanding of the biology and pathophysiology of the CF cell thus enabling more precise genetic testing and novel treatment.

CF is still an important medical problem. Although the life span of CF patients has increased significantly, from around 5 years in the 1950s (Davis *et al.,* 1996) to about 31 years in 2002 (Cystic Fibrosis Foundation, 2003), due to its cost CF still has a major impact on the health care system. Patients with this disease need daily care and lifelong continuous medication, and despite aggressive treatment severe complications can occur, often requiring organ transplantations or treatment in intensive care units. To provide continuous best care over an extended period a multidisciplinary team working in specialized centrums is needed.

At the same time, CF can be viewed as a model for single gene disorders. CF is a classical monogenic disease and was one of the first genetic diseases to have its gene identified and characterized in detail. To date the CF gene and the protein as well as their structure, function and interactions with other proteins have been thoroughly investigated. However, investigations are still continuing, as the complexity of the CF phenotype has raised questions about the supplementary functions of the CFTR channel, genotype–phenotype correlations and the role of additional genetic factors or modifier genes.

In Estonia no DNA diagnostics existed before molecular studies of CF began in 1993. At that time the data about the incidence of CF, the distribution and frequency of CFTR mutations in Eastern European countries were limited. In countries bordering Estonia the incidences of the CF disease range over a large scale, from 1 in 5,600 in Sweden (Lannefors and Lindgren, 2002) to 1 in 25,000 in Finland (Kere *et al.,* 1994). The incidence of the CF disease in Estonia was not known before the present study. Based on the clinical data and sweat chloride concentration, the CF disease was clearly overdiagnosed, partly due to the problems of correctly testing the sweat chlorides. In 1993 more than 90 children were registered as having the CF, but after reconsidering the clinical data, retesting sweat chlorides concentration and performing a molecular analysis of the CFTR gene, only 17 of these patients were considered to have CF. There was also a wide variation in the frequency of the major mutation, F508del, among different populations and also in countries neighboring Estonia.

In Denmark the F508del mutation accounted for 88% of all CF mutations, but in Finland the proportion of the main mutation was only 45% (European Working Group on CF Genetics, 1990). To date more than 1,200 sequence changes have been found in the CFTR gene, as reported in the Cystic Fibrosis Mutation Database (CFMDB; Available from http://www.genet.sickkids.on.ca/cftr/), and great regional as well as population differences have been established (Bobadilla *et al.,* 2002), therefore the characterization of the mutational spectrum of Estonian CF patients was important in order to offer our clinicians a reliable diagnostic test, which would improve genetic counseling and make prenatal diagnostics of CF possible.

The present study was undertaken 1) to establish the incidence of CF in Estonia, 2) to determine the frequency of carriers of the main mutation, 3) to identify the spectrum of CFTR gene mutations in Estonian patients, 4) to investigate their phenotype and correlate it with the genotype, 5) to analyze demographic data, the survival and mortality of Estonian patients and to compare the results with other populations, 6) to introduce DNA testing for the most common mutations.

2. REVIEW OF THE LITERATURE

2.1. Clinical picture of cystic fibrosis

2.1.1 Definition and incidence of CF

Cystic fibrosis (CF) is classically described as a triad: chronic obstructive pulmonary disease, exocrine pancreatic insufficiency and elevation of chloride ion concentration in sweat (Taussig, 1984; Davis *et al.,* 1996). CF is a monogenic disease with a complex phenotype caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene on chromosome 7q31.2 (Rommens *et al.,* 1989). The CFTR gene encodes a protein of 1494 amino acids (http://www.ensembl.org; Riordan *et al.,* 1989) functioning as a cyclic adenosine monophosphate (cAMP)–regulated chloride ion channel (Rich *et al.,* 1990). The dysfunction of the channel results in defective chloride ion conductance and associates with aberrant sodium ion and water conductance across the apical membrane of epithelial cells in a variety of organs (Welsh *et al.,*1995).

CF is the most common fatal autosomal recessive disease, affecting mostly Caucasian populations, with an incidence of about 1 in 2500 (Welsh *et al.,* 1995). However, even in Europe its frequency varies between different ethnic and distinct geographic populations, with an incidence of 1 in 1700 in Northern Ireland and 1 in 25,000 in Finland (Welsh *et al.,* 1995; Kere *et al*., 1994).

The incidence of CF varies dramatically between different races. CF is a rare disorder among Africans, with an estimated incidence of 1 in 17,000 (Hamosh *et al.,* 1998) and even more uncommon among East Asians: 1 in 350,000 in Japan (Iwasa *et al.,* 2001). Higher incidences are observed in American populations of these ethnic groups (1 in 15,000 in African Americans and 1 in 31,000 in Asian Americans, respectively), suggesting Caucasian admixture (Hamosh *et al*., 1998).

Classical CF. CF is a highly variable disorder (Table 1). Patients are diagnosed with different modes of presentation from birth to adulthood, with considerable variability in the severity and rate of disease progression. Chronic obstructive pulmonary disease, pancreatic insufficiency and elevated sweat chloride (>60 mmol/l) constitute the classic diagnostic symptoms for CF (Davis *et al.,* 1996). In addition, failure to thrive due to malabsorption and increased energy consumption, hypoproteinemia and deficiency of fat-soluble vitamins are commonly found in early childhood (Rosenstein and Zeitlin, 1998; Cutting, 1997; Koch and Hoiby 2000). Almost all patients have chronic sinopulmonary disease, and almost all postpubertal males have obstructive azoospermia.

Although the disease markedly reduces the life expectancy of CF patients, survival rates have increased greatly over recent decades. In 1964 the average life expectancy of a child with CF stood at just 5 years (Davis *et al.,* 1996). With wider acceptance of more aggressive treatment, the median survival age had been raised to 18 years in 1976, and to almost 30 years in the mid 1990s (Davis *et al.,* 1996).

Table 1. Typical phenotypic features of cystic fibrosis (by Rosenstein and Zeilin, 1998**)**

Chronic sinopulmonary disease manifested by:
• Persistent colonisation/infection with typical CF pathogens: including *S. aureus,* non-typeable *H. influenzae*, mucoid and non-mucoid *P. aeruginosa,* and *B. cepacia*
• Chronic cough and sputum production
• Persistent chest radiograph abnormalities (e.g., bronchiectasis, atelectasis, infiltrates and hyperinflation)
• Lower airway obstruction manifested by wheezing and air trapping
• Nasal polyps; radiographic or computed tomography of abnormalities in paranasal sinuses
• Digital clubbing
Gastrointestinal and nutritional abnormalities, including:
• Intestinal: meconium ileus, distal intestinal obstruction syndrome, rectal prolapse
• Pancreatic: exocrine pancreatic insufficiency, endocrine pancreatic insufficiency leading to diabetes mellitus in older patients
• Hepatic: chronic hepatic disease manifested by clinical or histologic evidence of focal biliary cirrhosis or multilobular cirrhosis
• Nutritional: failure to thrive (protein — caloric malnutrition); hypoproteinaemia and oedema;complications secondary to fat-soluble vitamin deficiency
Salt loss syndromes:
• Acute salt depletion
• Chronic metabolic alkalosis
Male urogenital abnormalities resulting in obstructive azoospermia

In summary, the diagnosis of CF should be based on one or more typical clinical features (Table 1) or a history of CF in a sibling or a positive neonatal screening test, plus laboratory evidence of a CFTR abnormality as documented by elevated sweat chloride values, the identification of two CF mutations or *in vivo* demonstration of characteristic abnormalities in ion transport across the nasal epithelium (Rosenstein and Zeitlin, 1998).

Atypical presentation of CF. In about 2% of CF patients there is an "atypical" phenotype that consists of chronic sinopulmonary disease, pancreatic sufficiency, and borderline (40–60 mmol/l) or normal (<40 mmol/l) sweat chloride concentrations (Rosenstein and Zeitlin, 1998; Koch and Hoiby, 2000). Hence,

sweat testing has been the most widely used diagnostic test for CF, and elevated electrolytes in sweat should be diagnostic, but a negative sweat test does not exclude the possibility of CF (Wallis, 1997). Only a few other very rare conditions can create false positive sweat test results (hypotyroidism, mucopolysaccharidosis, adrenal insufficiency, pseudohypoaldosteronism, etc), but none of these disorders is easily confused with CF (Welsh *et al.,* 1995).

In addition, there are patients in whom a single clinical feature predominates: e.g. electrolyte abnormalities, pancreatitis, liver disease, sinusitis/nasal polyps, congenital bilateral absence of the *vas deferens*, bronchiectasis and diffuse panbronchiolitis (Rosenstein and Zeitlin, 1998). If two CF disease-causing mutations have been found by DNA analysis in these clinically atypical cases, CF is confirmed. If one or zero CF — causing mutations have been found after extensive screening for CFTR mutations, then CF is possible, but further analyses are needed (Dequeker *et al.,* 2000).

The diagnosis of CF can be difficult if the sweat test and routine mutation screening of the CFTR gene are inconclusive. Recently, the use of novel diagnostic methods such as the measurement of nasal potential difference (Ahrens *et al.,* 2002) and bioelectric measurement of rectal mucosa biopsies in Ussing chambers have been proposed as a complementary diagnostic tool (Mall *et al.,* 2000). Furthermore, CF patients usually demonstrate abnormal serum trypsinogen levels, increased faecal elastase or increased fat excretion in 72-hour stool (Cutting, 1997).

2.1.2. Clinical picture and pathophysiology of the respiratory disease

Lung disease is manifested in almost all CF patients by recurrent episodes of infection and inflammation that lead to tissue destruction, fibrosis and eventually respiratory failure. Pulmonary disease is the primary cause of death in about 90% of CF patients, but the severity of lung disease and the rate of decline in lung function are highly variable (Cutting, 1997; Kraemer *et al.,* 2000; Rantjen and Döring, 2003). At birth, the lungs of CF patients appear normal (Davis, 1996). The earliest manifestation of CF lung disease is generally a cough, being at first intermittent, but over time becoming daily and productive. CF patients may have only bronchitic symptoms for long periods occasionally interrupted by acute pulmonary exacerbations (Welsh *et al.,* 1995; Kraemer *et al.,* 2000).

The lungs of patients with CF develop adherent, viscous secretions that cause poor airways clearance, dilatation of acinar and duct lumens of submucosal glands, leading to mucuos obstruction of bronchioles accompanied by bronchiolar wall inflammation (Cutting, 1997). *In vivo* studies indicate that inflammation preceedes infection, and that infection is the consequence of abnormal mucus secretion (Khan *et al.,* 1995). The next phase of the disease is

local and generalized chronic bacterial infection with pathogens such as *Staphylococcus aureus*, *Haemophilus influenzae* and *Pseudomonas aeruginosa* (Cutting, 1997; Rantjen and Döring, 2003). Once established, eradication of resident microbes is virtually impossible (Koch and Hoiby, 2000). The altered adherence properties for bacteria to the respiratory epithelium, hyperabsorption of airway fluid, defective mucociliary clearance and also the abnormal salt concentration on airway surface liquid that reduces antimicrobial potency (Wine, 1999) and impairs the innate immune system (Travis *et al.,* 2001), all predispose the airways to infection (Boucher, 2002; Rantjen and Döring, 2003).

Chronic inflammation ensues, and recurrent cycles of infection, inflammation and tissue destruction lead to bronchiolectasis, bronchiectasis (a characteristic finding on chest radiographs of CF patients) and finally the development of cysts. Damaged lung tissue is replaced by fibrosis (Cutting, 1997). At the final stage, death may occur due to severe hypoxaemia, respiratory failure or cardiac complications (Welsh *et al*., 1995).

Upper airways. Most patients with CF also have complaints of chronic rhinitis caused by chronic sinusitis accompanied by increased volumes of secretions and moderate airflow obstruction. The prevalence of a complication of sinus disease or nasal polyposis has been reported in 10–30% of all CF patients and in 38–47% of CF adults (Hadfield *et al.,* 1999; Koch and Hoiby, 2000).

2.1.3. Pancreatic disease

Exocrine pancreatic insufficiency (PI) is present from birth in about 85–90% of CF patients (Davis *et al.,* 1996; Cutting, 1997). Similarly to that observed in the lung, abnormal mucus secretions cause obstruction of the pancreatic ducts, followed by dilatation of the secretory ducts and acini. The pancreatic enzymes continue to be produced, but cannot reach the gut, so the gland undergoes tissue destruction and fibrosis (Rantjen and Döring, 2003; Quinton, 1999). This process begins *in utero* and can continue over a period of many years, and the degree of destruction usually correlates with the age of the patient (Quinton, 1999).

Clinically, pancreatic enzyme deficiency leads to malabsorption of protein and fat and fat-soluble vitamins, producing a distended abdomen and bulky, loose, greasy, foul-smelling stools (Welsh *et al.,* 1995). Uncorrected maldigestion results in failure to thrive, hypoproteinemia, hypoalbuminemia. Due to the deficiency of fat-soluble vitamins A, D, E, K, a variety of disorders can be seen, such as anaemia, central nervous system and visual disturbances, rickets, bone demineralisation and coagulopathy (Wilfond *et al.,* 1994; Cutting, 1997; Welsh *et al.,* 1995; Haworth *et al.,* 1999; Campbell *et al.,* 1998).

A further important consequence of the severe pancreatic damage is deficiency of pancreatic bicarbonate, resulting in a diminished capacity to buffer influxes of gastric acid into the duodenum (Robinson *et al.,* 1990). This

reduces the efficacy of endogenous and exogenous pancreatic enzymes and favours the precipitation of bile salts, which thereafter are less effective in fat solubilisation, leading to impaired lipolysis (Walters *et al.,* 1998).

The endocrine function of the pancreas can also be impaired in CF disease. Insulin–dependent diabetes becomes prevalent with increasing age, and beyond the age of 10–15 years there is an almost linear decrease in the percentage of patients with a normal oral glucose tolerance test (Koch and Hoiby, 2000). About 9–12% of all CF patients require insulin therapy (Cystic Fibrosis Foundation, 2003; European Epidemiologic Registry of CF, 2000).

2.1.4. Gastrointestinal disease

Diseases of the gastrointestinal tract are usually less prominent in CF patients. However, about 10 to 15% of CF newborns suffer from obstruction of the small intestine, called meconium ileus (MI), due to reduced water secretion and sludging of intestinal contents (Davis *et al.,* 1996, Welsh *et al.,* 1995). The accumulation of undigested proteins (e.g albumin), when mixed with intestinal mucus, produces an impervious and hyperviscid meconium substance (Quinton, 1999). Dehydratation of the intestinal contents can already be detected *in utero* by ultrasonography as hyperechogenic fetal bowel (Scotet *et al.,* 2002).

Later in life, recurrent episodes of bowel obstruction called distal intestinal obstruction syndrome or the equivalent of MI are also characteristic for CF, and are encountered by about 20% of CF patients. Complete obstruction is associated with failure to pass stool, abdominal distension and vomiting, whereas partial obstruction is accompanied only by intermittent abdominal pain (Welsh *et al.,* 1995).

Tenacious intestinal residue may serve as a lead point for intussusception or cause recurrent rectal prolapse. In childhood, rectal prolapse in CF patients is a common complication (occurrence about 20%), but is an infrequent event for adults with cystic fibrosis (Cutting, 1997; Welsh *et al.,* 1995).

2.1.5. Hepatobiliary disease

Severe liver disease is the second leading cause of mortality in CF (Cutting, 1997). However, clinical evidence of severe liver disease with hepatic cirrhosis and portal hypertension occurs in only 2–7% of all CF patients (Wilschanski *et al.,* 1999; Colombo *et al.,* 2002), but focal biliary cirrhosis is present at autopsy in about 25% of patients (Cutting, 1997). The secretion of chloride ions and thereafter water into the biliary duct is necessary to keep bile acids and proteins soluble in the bile fluid. Abnormal inspissated mucus secretions in the bile ductules causes obstruction, dilatation, inflammation and focal biliary cirrhosis that over time progress to multilobular cirrhosis (Cutting, 1997). The early

pathogenesis of CF liver disease is incompletely understood, but is known to involve biliary dysfunction, hepatic stellate cell activation and a disruption of the homeostasis of the extracellular matrix, leading to portal hypertension and cirrhosis (Lewindon *et al.,* 2002).

Another symptom, cholelithiasis, has been diagnosed in 12% of CF patients, but gallbladder abnormalities can be found in about half of CF patients by radiography (Cutting, 1997).

2.1.6. Genitourinary abnormalities

As mean survival age is progressively increasing, issues of fertility and reproduction are more frequently raised in adult clinics. It is well known that the vast majority (95–98%) of men with CF are infertile due to the congenital bilateral absence of the *vas deferens* (CBAVD), with resultant obstructive azoospermia (Davis *et al.,* 1996; Edenborough, 2001). The absence of secretions from the seminal vesicles causes chemical abnormalities in the semen and azoospermia with low semen plasma volume (<1.5 ml) (Cutting, 1997). The prevalence of CBAVD among infertile males is about 1–2% (Okada *et al.,* 1999). As testicular biopsies have demonstrated spermatogenesis, male infertility in CF can be treated by performing artificial insemination (Okada *et al.,* 1999). About 2% to 3% of males with CF are fertile and have had offspring (Cutting, 1997).

Fertility in women with CF is also reduced. Reliable figures concerning fertility in female patients are difficult to determine, and are estimated to be in the range of 10% to 20% (Welsh *et al.,* 1995). In CF patients thick, tenacious cervical secretions have been observed, which together with menstrual irregularity due to chronic disease and low body mass index, contribute to conceiving difficulties (Edenborough, 2001; Welsh *et al.,* 1995). Only about 70–80% of pregnancies will result in a delivery (Edenborough, 2001). The Cystic Fibrosis Foundation Patient Registry (2003) reported live births in 1.9% of US female CF patients of reproductive age.

2.1.7. Changes in sweat glands

The most consistent functional alteration in CF has been elevated concentrations of chloride ion and sodium ion in sweat, which are the basis for the principal diagnostic test for the disease (Davis *et al.,* 1996). The number of sweat glands is normal and there are no structural abnormalities in the eccrine sweat glands in CF (Welsh *et al.,* 1995). Normal sweat chloride values in neonates range from 6 to 32 mmol/l (mean 16.9 mmol/l) (Castellani *et al.,* 1999).

The sweat gland is composed of two regions: the secretory coil and the resorptive duct, and the functions of both components are defective in CF

patients. The coil secretes an ultrafiltrate in response to cholinergic and β-adrenergic agonists that is almost isotonic with plasma. The β-adrenergic response of the secretory coil is consistently absent in CF (Sato and Sato, 1984). Normally, as the ultrafiltrate passes the water-impermeable duct, chloride ion and sodium ion are absorbed (Welsh *et al.,* 1995).

CFTR is the only channel in the sweat duct capable of reabsorption of chloride ion and if defective, electrolyte concentrations are up to 5 times higher (Cutting, 1997). In a sweat test, a chloride ion concentration of >60 mmol/l is found in approximately 98% of patients with CF (Koch and Hoiby, 2000). In patients with chloride concentration within the normal or borderline range and also bearing specific CFTR mutations e.g. 3849+10kbC→T, delayed diagnosis has been reported (Padoan *et al.,* 2002).

The major clinical manifestation of abnormal sweat gland function is excessive salt loss, which in young children can sometimes lead to hypochloremic, hyponatremic alkalosis and dehydratation. Because of the sweat electrolyte defect, young CF patients are more prone to heat prostration (Cutting, 1997; Davis *et al.,* 1996).

2.1.8. Screening for cystic fibrosis

There have been long debates about the usefulness, timing, population and cost-effectiveness of screening for CF (Dankert-Roelse *et al.,* 1997; Merelle *et al.,* 2001; Mastella *et al.,* 2001; Lee *et al.,* 2003). Neonatal screening for CF became feasible with the development of an immunoreactive trypsin test suitable for use on dried blood spots (Pollitt, 1998). Mostly two stage protocols based on a double immunoreactive trypsin test and subsequent DNA analyses have been developed (Pollitt, 1998; Scotet *et al.,* 2000; Farrell *et al.,* 2001).

Several studies have reported that the nutritional status of CF patients diagnosed earlier through the screening program was significantly better (Farrell *et al.,* 2001; Dankert-Roelse *et al.,* 1997). In other surveys reduced pulmonary inflammation, deterioration of lung function and longer survival were detected (Corey *et al.,* 1997; Kraemer *et al.,* 2000; Wagener *et al.,* 2003). It was also suggested that it is cheaper to diagnose CF by screening rather than by expressed clinical symptoms (Farrell *et al.,* 2001; Lee *et al.,* 2003).

The extensive Cochrane Library review of CF screening concludes that more studies are required to evaluate long-term pulmonary outcome, and the effectiveness of neonatal screening in reducing CF mortality and morbidity (Merelle *et al.,* 2001). However, screening for CF has already been linked to the neonatal screening programmes in Australia and at least partly in 8 countries in Europe (Italy, Austria, Poland, Belgium, the Netherlands, Spain, the UK and France) (Zabranski, 2001). Of patients with CF in the United States, 10% are identified through newborn screening (Wagener *et al.,* 2003).

2.2. Cystic fibrosis transmembrane conductance regulator gene

Cystic fibrosis is caused by sequence alterations in the CFTR gene identified in 1989 (Rommens *et al.,* 1989). The structure of the gene, exon sequences and the main mutation were described (Riordan *et al.,* 1989), and genetic analysis and allelic association were also performed (Kerem *et al.,* 1989). Although the disease phenotype associated with CF is usually very severe, the frequency of CF carriers in the Caucasian population is very high: 1 in 25 people carry one CFTR mutation (Welsh *et al.,* 1995).

The high frequency of CF carriers has always raised questions about the possible explanations of this phenomenon. Linkage analysis and the cloning of the CFTR gene eliminated the hypothesis of multiple CF loci (Rommens *et al.,* 1989, Riordan *et al.,* 1989). Studies of sex ratio and segregation distortion in favour of mutant CF alleles did not reveal confirmatory evidence (European Working Group on CF Genetics, 1995). Various forms of selective advantage have been proposed, including the higher fertility of CF carriers, which was confuted by Jorde and Lathrop (1988), and the protection of heterozygotes against another disease such as an increased resistance to *Vibrio cholera* infections (Gabriel *et al.,* 1994) and typhoid fever (Pier *et al.,* 1998). Still, evidence of increased resistance to secretory diarrheal diseases remained controversial. There are studies with mouse models heterozygous for CF secreting half of the normal intestinal fluid after exposure to cholera (Gabriel *et al.,* 1994). Other studies did not reveal differences in the intestinal responses to several secretagogues between heterozygotic and normal mice (Guthbert *et al.,* 1995) or CF carriers and normal subjects (Hogenauer *et al.,* 2000). Pier and coworkers (1998) showed that diminished levels of CFTR in heterozygotes results in the internalization of fewer *Salmonella typhi* into the gastrointestinal submucosa, and therefore decreases susceptibility to typhoid fever.

2.2.1 Structure of CFTR gene

The CF gene spans over 200 kb of genomic DNA; initially 24 exons were identified (Riordan *et al.,* 1989), and later 3 more were recognized: 6b, 14b, 17b (Zielenski *et al.,* 1991), bringing the current count to 27 exons (Figure 1). The encoded mRNA is 6128bp long and can be detected in a variety of tissues, mainly at the apical border of the epithelial cells that line most exocrine glands (Riordan *et al.,* 1989; Rowntree and Harris 2002).

DNA sequence analysis of the CFTR promoter region in humans and in rodents has revealed high GC content, typical to the so-called “house-keeping” genes, but lacks a TATA box, an RNA polymerase recognition site characte-

ristic of genes that exhibit tissue-specific expression (Yoshimura *et al.,* 1991). This region does, however, contain consensus sequences for binding sites of a variety of known transcription factors (Vuillaumier *et al.,* 1997). In humans multiple transcription start sites, including the major one, have been described (Yoshimura *et al.,* 1991; Koh *et al.,* 1993). Experiments with cell lines have identified the basal CFTR promoter as a 250bp fragment upstream of the ATG translation start codon (Koh *et al.,* 1993; Vuillaumier *et al.,* 1997).

It is proposed that the tissue-specific regulatory elements are located outside the basal promoter region and are associated with DNase I hypersensitivity sites in 5´UTR, in introns (1, 2, 3, 10, 16, 17a, 18, 20, 21) and in 3´UTR (+5.4, +7.4, +15.6kb) (Smith *et al.,* 2000; Rowntree and Harris, 2002). There is also evidence that the different tissues that express CFTR may have unique regulatory elements, as indicated by different sites of transcriptional initiation (+1, +60, +70, +100) (Koh *et al.,* 1993).

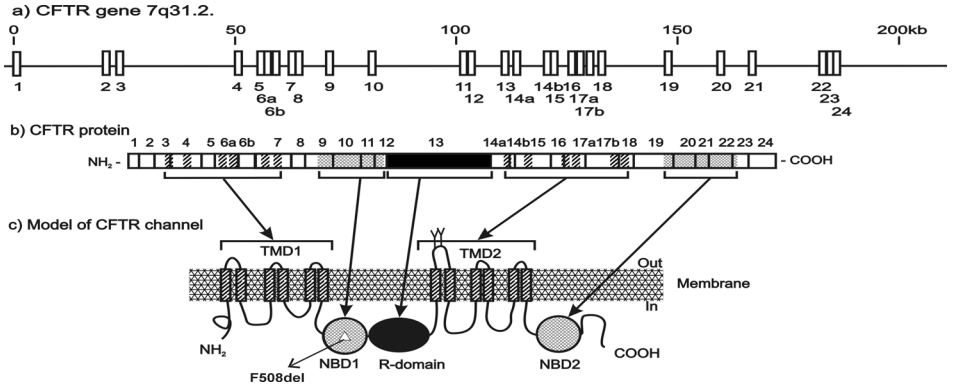

Figure 1 a) The structure of the CFTR gene; b) the CFTR protein; c) the schematic representation of the CFTR chloride channel. The different domains: transmembrane domain 1 and 2 (TMD1, TMD2); nucleotide binding domain 1 and 2 (NBD1, NBD2); the regulatory domain (R-domain); the N-terminal (NH_2) and C-terminal (COOH) ends; two glycosylation sites (YY) present on the fourth extracellular loop (N894 and N900) and the main CFTR mutation F508del (Δ) are indicated (modified from Welsh *et al.,* 1995).

2.2.2. Regulation of CFTR gene

Regulation of expression of the CFTR gene is complex. Both CFTR mRNA and protein are highly conserved within a wide range of vertebrate species (Trezise *et al.,* 1993; Tizzano *et al.,* 1994). They show patterns of expression that seem to be tightly regulated both developmentally (Trezise *et al.,* 1993; Tizzano *et al.,* 1994) and in a tissue-specific manner, but with differences depending on the species (Vuillaumier *et al.,* 1997). Expression of CFTR is mainly restricted to

epithelial cells in the lung, intestine, pancreas, liver, gall bladder, kidney, salivary and sweat glands, testis and uterus (Rich *et al.,* 1990; Anderson *et al.,* 1991). In each of these tissues only a subpopulation of specified cells is involved in CFTR expression.

In humans, the pancreas and intestinal tract are the locations of the most abundant CFTR expression (Crawford *et al.,* 1991; Tizzano *et al.,* 1995). In the human fetal lung the CFTR gene mRNA is seen throughout the respiratory epithelium (Trezise *et al.,* 1993). Postnatally, CFTR protein is expressed at very low levels in the surface epithelium of the airways, and only at higher levels in the serous portion of the submucosal glands (Crawford *et al.,* 1991; Tizzano *et al.,* 1995). CFTR is predominantly localized in the apical membrane of the epithelial cells (Gregory *et al.,* 1990), but may also be expressed in the baso-lateral membrane of the kidney tubules and sweat duct epithelia (Schwiebert *et al.,* 1998).

Alternative splicing. In addition to a complex regulatory process for CFTR transcription, the primary transcript can be influenced by tissue and time-specific alternative splicing. Different alternatively spliced forms of CFTR (e.g. lacking exon 4, 5, 9, 12 or the second half of CFTR) have been detected (Warth *et al.,* 1996; Devuyst *et al.,* 2002; Mak *et al.,* 1997; Vankeerberghen *et al.,* 2002).

CFTR mRNAs without exon 9 encode a protein that is misfolded and therefore nonfunctional and is shown to be present in a fraction of CFTR transcripts in nearly all individuals (Delaney *et al.,* 1993). Exon 9 skipping is found to be inversely correlated with the length of the polymorphic polythymidine tract (5T/7T/9T) upstream of the exon, as in transcripts derived from genes with 5T alleles, exon 9 is spliced out in approximately 95% of cases (Mak *et al.,* 1997). The percentage of non-functional exon 9^- CFTR may vary between tissues: it is higher in the *vas deferens* than in the nasal epithelium (Mak *et al.,* 1997). CFTR exon 9 skipping can also be modulated by promoter architecture and by atypical 5´splice sites flanking exon 9 (Pagani *et al.,* 2003; Hefferon *et al.,* 2002).

2.3 CFTR protein

2.3.1 Structure of CFTR protein

The CFTR gene encodes a 1494-amino acid long transmembrane protein with a molecular mass of approximately 170 kDa when fully maturated (Riordan *et al.,* 1989; Welsh and Smith, 1993; http://www.ensembl.org/). Protein has a symmetrical structure (Figure 1) with two transmembrane domains (TMD1,

TMD2) contributing to the channel pore, two nucleotide binding domains (NBD1, NBD2) for binding ATP, a large hydrophilic regulatory domain (R-domain) rich in protein kinase A (PKA) and protein kinase C phosphorylation sites (Riordan *et al.,* 1989; Welsh and Smith, 1993). These features are characteristic of a large family of ATP–binding cassette transporters (ABC transporters) found in eukaryotes and prokaryotes. However, the presence of the large hydrophilic R-domain after the NBD1 makes CFTR unusual among ABC transporters (Riordan *et al.,* 1989; Dean *et al.,* 2001). Being an ion channel makes CFTR unique in this gene superfamily, in which most other members are ATP-driven membrane transporters (Dean *et al.,* 2001).

There are 6 extracellular and 4 intracellular loops between transmembrane segments. It is proposed that 77% of the protein resides in the cytoplasm, 19% in the membrane-spanning segments, and 4% in the extracellular loops, which (except for first and fourth loops) are very short (Riordan *et al.,* 1989; Sheppard and Welsh, 1999). The fourth extracellular loop contains 2 *N*-linked glycosylation sites at amino acid asparagine N894 and N900 (Sheppard and Welsh, 1999; Akabas, 2000).

The NBDs of CFTR contain the sequences, highly conserved through the ABC transporter superfamily, called Walker A and Walker B and LSGGQ motifs (Dean, 2001). These sequences are responsible for the binding and hydrolysis of ATP and other nucleotide molecules, and therefore involved in chloride channel regulation (Anderson *et al.,* 1991; Gadsby and Nairn, 1994; Sheppard and Welsh, 1999).

2.3.2. Maturation of CFTR protein

The post-translational control of the maturation of CFTR through the endoplasmatic reticulum is also very important. Maturation of CFTR is a rather inefficient process. The majority (75%) of CFTR (half-life 7–16 hours) and nearly all of F508del-CFTR molecules (half-life 20–40 min) fail to mature and are rapidly degraded by cytosolic proteasomes and other ATP–dependent proteases (Ward and Kopito*,* 1994; Bebök *et al.,* 1998).

During co-translational transport, the CFTR polypeptide is integrated in the endoplasmatic reticulum membrane and is *N*-glycosylated through the addition of two core glycosylation groups on its fourth extracellular loop. In this way the molecular weight of the CFTR protein increases from 130 kDa to 150 kDa (Ward and Kopito, 1994; Lukacs *et al.,* 1997; Sheppard and Welsh, 1999). With the aid of chaperone molecules, such as calnexin (Pind *et al.,* 1994) and heat shock protein 70 (Yang *et al.,* 1993), the polypeptide is correctly folded, becomes protease resistant and is transported to the Golgi-stacks (Choo-Kang and Zeitlin, 2001). In this last compartment, the glycosylation groups are further modified to form a mature protein of 170 kDa and will be transported to the cell

membrane, where they can function as a chloride channel (Sheppard and Welsh, 1999). CFTR trafficking to the cell membrane is thought to take place by exocytosis (Weixel and Bradbury, 2000). From the cell surface CFTR is constitutively internalized via clathrin-coated vesicles. The mature CFTR passes through several cycles of endocytosis and exocytosis into the membrane, and this recycling process is regulated by cAMP concentration (Lukacs *et al.,* 1997; Kunzelman and Nitscke, 2000; Silvis *et al.,* 2003).

Discussions are ongoing as to whether CFTR exists in the cell membrane as a monomer (Marshall *et al.,* 1994; Chen *et al.,* 2002) or in a dimeric state, thereby increasing the complexity of chloride channel regulation (Wang *et al.,* 2000, Ramjeesingh *et al.,* 2001).

2.3.3. Function of CFTR protein as chloride channel

CFTR is an epithelial anionic channel stimulated by ATP and cyclic AMP-dependent PKA (Drumm *et al.,* 1990; Berger *et al.,* 1991). CFTR Cl^- channel activity may also be regulated by membrane-associated phosphatases in an inhibitory manner and by protein kinase C isoforms in an either stimulatory or inhibitory manner (Welsh and Smith, 1993; Jia *et al.,* 1997). CFTR channel is characterised by small conductance (6–10 pS) with an ability to transport passively 10^6 –10^7 ions/s (Bear *et al.,* 1992). The channel has a linear current voltage relationship in Cl^- solutions (Sheppard and Welsh, 1999). It is selective for anions over cations and exhibits the following anion permeability sequence: $Br^- \geq Cl^- > I^- > F^-$ (Anderson *et al.,* 1991; Bear *et al.,* 1992). These features are conferred on CFTR Cl^- channels by the function of the TMDs, the NBDs, and the R-domain. The TMD1 and TMD2 contribute to the formation of the Cl^- selective pore (Anderson *et al.,* 1991, Sheppard *et al.,* 1993). The NBDs bind and hydrolyze ATP to regulate channel gating (Carson *et al.,* 1995), and R-domain phosphorylation controls channel activity (Ostedgaard *et al.,* 2001, Sheppard and Welsh, 1999). Intracellular loops between TM1–12 can be involved in channel gating as well as in endocytosis from the cell surface (Silvis *et al.,* 2003).

The regulation of the CFTR chloride channel is very complex. The opening and closing of this channel is tightly controlled by the balance of kinase and phosphatase activity within the cell and by cellular ATP levels. Activation of the cAMP-dependent PKA causes the phosphorylation of multiple serine residues within the R-domain. PKA is the primary kinase that phosphorylates the R-domain (Sheppard and Welsh, 1999; Ostedgaard *et al.,* 2001). This allows binding of ATP to NBD1. When ATP is hydrolyzed by NBD1, the channel opens and anions can flow, according to the electrochemical gradient, through the pore. When the R-domain is fully phosphorylated, the NBD2 can bind ATP. This event stabilizes the open state of the chloride channel and results in longer openings. In the next step, when ATP is hydrolyzed at NBD2 and ADP and

phosphate ions are released from both NBDs, the channel will close again. For this fine-tuning of channel activity, interaction between both NBDs seems necessary (Sheppard and Welsh, 1999). As long as the R-domain is phosphorylated, the cycles of binding and hydrolysis of ATP, i.e. of the opening and closing of the channel, can continue. Once the R-domain is dephosphorylated by phosphatases, the channel will close and phosphorylation of the R-domain by PKA will be necessary in order to reactivate the channel (Gadsby and Nairn, 1994).

2.3.4. Other functions of CFTR

CFTR also acts as a regulator of other channels in the apical membrane (Figure 2). Before CFTR was identified, the defect in chloride transport found in CF epithelia was assigned to the malfunctioning of the outwardly rectifying chloride channel **(**ORCC) (Schwiebert *et al.,* 1998). Studies have shown that CFTR activates the ORCC and this process is dependent on the presence of the NBD1 and R-domain of CFTR (Gabriel *et al.,* 1993; Schwiebert *et al.,* 1998).

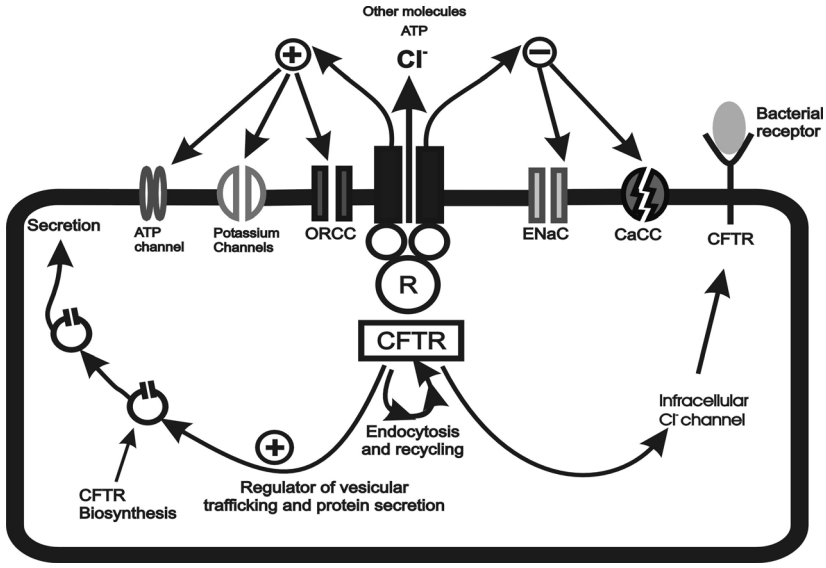

Figure 2. Possible cellular functions of CFTR. In addition to its well-known role as cAMP regulated chloride channel, CFTR may have pleiotropic roles, including: a transporter of ATP and other molecules, positive regulation of the outwardly rectifying chloride channels (ORCC), potassium channels and distinct ATP channels; negative regulation of the epithelial sodium channel (ENaC) and Ca^{2+} sensitive chloride channel (CaCC). It may also function as a regulator of vesicular traffic and as an intracellular chloride channel, and could also be a receptor for *Pseudomonas aeruginosa*. R, regulatory domain of CFTR (modified from Schwiebert *et al.,* 1998).

A Ca^{2+}-sensitve Cl^- secretory pathway is also present in the human airway, and has been found to be intact in the epithelia of patients with CF (Schwiebert *et al.,* 1998). Recent findings indicate the CFTR is able to inhibit endogenous calcium-activated chloride currents (Wei *et al.,* 1999).

In the CF lung, the Cl^- transport defects occur in association with the hyper-absorption of Na^+ ions (Welsh *et al.,* 1995). Together these two defects create an abnormally high osmotic driving force for the reabsorption of water, therefore contributing to the dehydratation of airway mucus (Schwiebert *et al.,* 1999). The Na^+ ion is absorbed via the epithelial amiloride sensitive Na^+ channel **(**ENaC**)** (Quinton*,* 1990). The mechanisms of how CFTR regulates the ENaC activity are not unequivocally understood. Based on studies using heterologous cell lines and rat ENaC, it is suggested that CFTR inhibits the rat ENaC by decreasing its open probability. This downregulation increases when CFTR is activated by PKA, and rat ENaC is in turn able to upregulate CFTR (Schwiebert *et al.,* 1998; Stutts *et al.,* 1997; Ji *et al.,* 2000). Contradictory studies of endogenous human ENaC and CFTR in the sweat ducts showed that activation of CFTR results in the stimulation of Na^+ transport through ENaC (Reddy *et al., 1999*). This might indicate that the regulation of ENaC by CFTR is tissue-specific and might require modulating cell-specific factors (Vankeerberghen *et al.,* 2002).

Recent findings indicate that CFTR also regulates an associated but distinct ATP channel (Sugita *et al.,* 1998), inhibits volume-regulated anion channels, enhances membranous potassium channels' sensitivity to sulfonylurea compounds and alters the activity of multiple ion channels endogenously expressed in *Xenopus laevis* oocytes (Schwiebert *et al.,* 1999; Vankeerberghen *et al.,* 2002).

CFTR is also present in intracellular membranes. Chloride transport through CFTR in intracellular compartments like the Golgi network, prelysosomes and endosomes is important, since it creates a driving force for the subsequent acidification of these organelles, which is important for glycosylation and ligand traffic (Vankeerberghen *et al.,* 2002). As a consequence, in CF cells an altered glycosylation pattern can be noticed in CFTR: sialylation is reduced and sulfation is increased (Barasch *et al.,* 1991). *P. aeruginosa* preferentially attaches to asialo-glycoproteins. As a consequence, the malfunctioning of CFTR could increase the colonization of CF airways by pathogens (Vankeerberghen *et al.,* 2002; Rantjen and Döring, 2003).

CFTR also regulates intracellular vesicle transport (Lukacs *et al.,* 1997; Weber *et al.,* 2001). Cell surface CFTR is constitutively internalized via clathrin-coated vesicles and recycled back into the membrane through exocytosis (Weixel and Bradbury, 2000). The stimulation of protein phosphorylation by cAMP activates CFTR channels and also increases the amount of chloride channels present in the cell membrane due to an arrest of membrane endocytosis and stimulation of exocytosis (Lukacs *et al.,* 1997).

2.4. Mutations in CFTR gene

Since the discovery of the CFTR gene in 1989, more than 1200 sequence alterations have been found in this gene (CFMDB; http://www.genet.sickkids.on.ca/cftr). Nucleotide alterations in the CFTR gene can disrupt CFTR function by different mechanisms depending on their nature and on the domain in which the alterations occur. Point mutations make up approximately 98.5% of reported mutations of the CFTR gene, and genomic rearrangements account for the remaining 1.5% of mutations. All mutation types are reported to be present in the CFTR gene. About half of the described mutations are single amino acid substitutions (missense mutations — 40%), whereas the other 3 types, nonsense (11%), frameshift (17%) and splice-site mutations (13%), are almost equally represented (CFMDB). Large deletions spanning multiple exons of the CFTR gene as well as single amino acid deletions/insertions are quite rare in the CF gene (Tsui, 1995; CFMDB http://www.genet.sickkids.on.ca/cftr). In most cases changes in nucleotide sequence are characterized as disease causing, counting the basis of the mutation and the possible theoretical structural and functional consequence. Only a small fraction of mutations have been investigated using molecular and functional studies (Vankeerberghen *et al.,* 1998).

Disease-causing sequence alterations have been reported throughout the coding region of the CFTR gene, as well as in introns, promoter and 5′– and 3′– regulatory regions. However, they are not distributed equally in the CFTR gene, and mutational hotspots can be detected in exons 4, 11 and 12 (Figure 3), suggesting that these are critical regions for normal CFTR function (Tsui, 1995; CFMDB; http://www.genet.sickkids.on.ca/cftr).

2.4.1. The most common mutation in the CFTR gene

The most common mutant allele is the F508del mutation in exon 10 (Kerem *et al.,* 1989), which is a deletion of three nucleotides resulting in the loss of a phenylalanine residue at codon 508 and subsequent defective intracellular processing of the CFTR protein. Mutant CFTR is unable to adopt a protease-resistant mature conformation (Cheng *et al.,* 1990; Zhang *et al.,* 1998), remains interacted with the chaperones calnexin and heat shock protein 70 (Pind *et al.,* 1994; Farinha *et al.,* 2002) and is prematurely degradated by the ubiquitin-proteasome pathway in a pre-Golgi compartment (Jensen *et al.,* 1995; Sato *et al.,* 1998). Reduction of temperature or the addition of chemical chaperones such as glycerol or trimethylamine-*N*-oxide (Denning *et al.,* 1992; Sato *et al.,* 1996; Roomans, 2003) can overcome impediments to the folding and allow

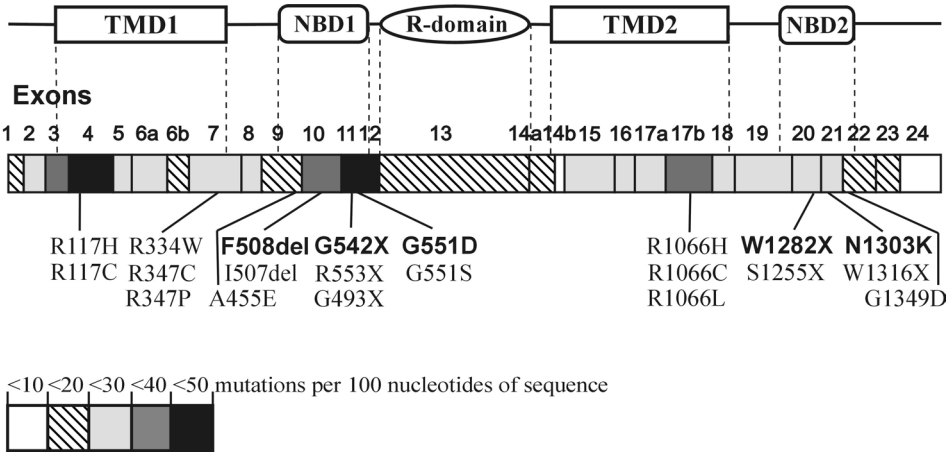

Figure 3. Density of mutations in different exons of CFTR gene. Localization of 5 more common CFTR mutations are given in bold (CFMDB, Available from http:// www.genet.sickkids.on.ca/cftr). Some important mutations linked to disease and their approximate location within the CFTR gene and protein are shown (Schwiebert et al., 1997). TMD1 and TMD2, transmembrane domain 1 and 2; NBD1 and NBD2, nucleotide binding domain 1 and 2; R–domain, regulatory domain of the CFTR protein.

proper targeting. However, at the cell surface the chloride channel formed therefrom has shown a decreased half-life and reduced open probability and sensitivity to stimulation with cAMP agonists (Vankeerberghen *et al.,* 2002).

Worldwide, the F508del mutation is responsible for approximately two-thirds (66%) of all CF chromosomes (Estivill *et al.,* 1997; Bobadilla *et al.,* 2002). The distribution of this mutation throughout Europe shows a clear north-west to south-east gradient, with a maximum in the Faroe Islands of Denmark (100% of all CF chromosomes) and a minimum of 24.5% in Turkey (European Working Group on CF Genetics, 1990; Lucotte *et al.,* 1995; Bobadilla *et al.,* 2002).

The age and place of origin of this alteration have been of interest in many investigations (Morral *et al.,* 1994; Dawson and Frossard, 2000; Mateu *et al.,* 2002). The F508del presumably has a single origin. It has been suggested that the age of F508del ranges from >50,000 years (Morral *et al.,* 1994) to 11,000–34,000 years (Wiuf *et al.,* 2001), and therefore the estimates depend greatly on genetic and demographic parameters. The current view is that F508del originated in the Middle East or the Orient, in agreement with the concept of the migration of humans across Europe (Dawson and Frossard, 2000).

The F508del mutation is accounted to be a severe mutation, as patients who are homozygous for the mutation receive a diagnosis of cystic fibrosis at an earlier age and are almost always pancreatic insufficient (Kerem *et al.,* 1990), have an increased risk for developing meconium ileus (The CF Genotype-

Phenotype Consortium, 1993) and liver disease (Colombo *et al.,* 2002), and are probably more susceptible to *P. aeruginosa* infections (Kubesh *et al.,* 1993; Parad *et al.,* 1999).

2.4.2. Other less common mutations

There is a great mutational heterogeneity in the frequency and distribution of the remaining one-third of mutant CFTR alleles, (Cystic Fibrosis Genetic Analysis Consortium, 1994; Bobadilla *et al.,* 2002; CFMDB). The vast majority are either private or limited to a small number of individuals. Only four other mutations, G542X, N1303K, G551D and W1282X, have overall allele frequencies among CF chromosomes of over 1% (Table 2), and about 17 other less common mutations exist at over 0.1% worldwide (Estivill *et al.,* 1997; Cystic

Table 2. The 20 more common CFTR mutations worldwide by the Cystic Fibrosis Genetic Analysis Consortium (1994), Estivill and co-workers (1997), Bobadilla and co-workers (2002).

Name of mutation	Frequency worldwide	Population with highest prevalence
F508del	66%	Danish, Faroe Islands
G542X	2.4%	Spanish
G551D	1.6%	English
N1303K	1.3%	Italian
W1282X	1.2%	Jewish-Ashkenazi
R553X	0.7%	German
621+1G–>T	0.7%	French-Canadian
1717–1G–>A	0.6%	Italian
R117H	0.3%	Irish
R1162X	0.3%	Italian
R347P	0.2%	Bulgarian
3849+10kbC–>T	0.2%	Jewish
I507del	0.2%	French (Britanny)
394delTT	10–30%*	Finnish, Scandinavian
G85E	0.1%	Greece (southern region)
R560T	0.1%	Irish (northern Ireland)
A455E	0.1%	The Netherlands
1078delT	0.1%	French (Brittanny)
2789+5G→A	0.1%	Greece (southern region)
3659delC	0.1%	Swedish

* detected in Scandinavian countries

Fibrosis Genetic Analysis Consortium, 1994). Some mutations can reach higher frequencies in particular populations or regions due to the founder effect (e.g. G542X, associated with the ancient Phoenicians by Loirat *et al.,* 1997, or 394delTT in Finland by Kere *et al.,* 1994) or due to genetic drift (e. g., G551D among the historic Celts by Bobadilla *et al.,* 2002). In non-Caucasian populations, a group of specific CFTR alleles are also determined as 3120+1G→A and 2307insA in African-American CF chromosomes (Macek *et al.,* 1997a).

Interestingly, the 394delTT mutation referred to as the "Nordic mutation" (Schwartz *et al.,* 1994) has been found to be present at a high frequency in countries bordering the Baltic Sea (Estivill *et al*., 1997). Many of the present day populations in this region have a common ancestry, and therefore this mutation is rarely seen outside these populations (Bobadilla *et al.,* 2002). In the 394delTT mutation, the deletion of 2 base pairs at position 394 in exon 3 leads to a chain termination in exon 4 and as a result truncated protein is synthesized.

The great mutational heterogeneity seen in different populations and geographic regions directly affects detection rates of mutated CF alleles. In principle, the central, western and northern regions of Europe have less mutational diversity than the southern parts (Bobadilla *et al.,* 2002). In these regions, an average of 10 mutations account for 78.9% of CF alleles, *versus* 25 mutations detecting 84% of all CF alleles in the Mediterranean region. Spain, Bulgaria, Greece and Turkey have some of the most diverse mutational arrays in Europe (Bobadilla *et al.,* 2002). This is most likely due to their geographic position, as they have served as historic "gateways" into Europe from the Middle East, Africa and associated waterways (Bobadilla *et al.,* 2002).

2.4.3. Classes of CFTR mutations and dysfunction of the chloride channel

Over 1200 CFTR sequence alterations have been identified to date (CFMDB; http://www.genet.sickkids.on.ca/cftr). These mutations have been classified in 6 categories (Figure 4) based on their known or predicted molecular mechanisms of dysfunction and functional consequences for the CFTR protein (Tsui, 1992; Welsh and Smith, 1993; Vankeerberghen *et al.,* 2002).

The first class of mutations includes nonsense (e.g. G542X), frameshift (e.g. 394delTT) and splice site mutations (e.g. 1717–1G→A), which produce defective protein or no protein at all. Such proteins are often unstable and are expected to be degradated relatively rapidly or to have little or no function. As a result, there is a loss of CFTR chloride channel activity in the affected epithelia and these mutations usually lead to severe disease manifestation (Welsh and Smith, 1993).

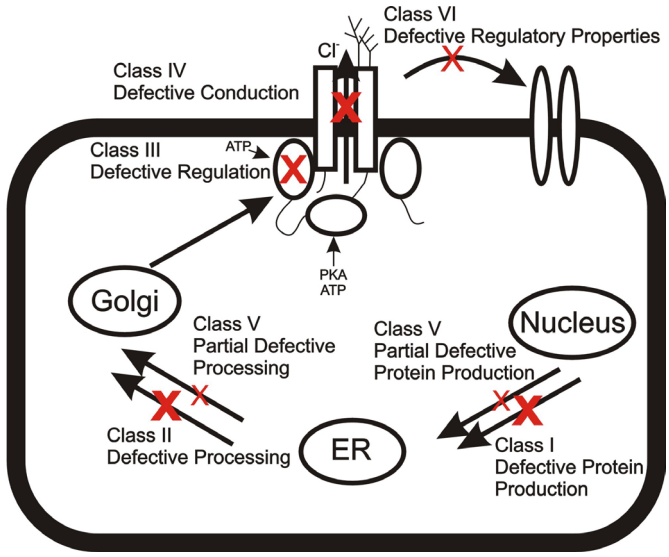

Figure 4. Schematic illustration of the different classes of CFTR mutations. ER –endoplasmatic reticulum (modified from Vankeerberghen *et al.,* 2002).

Class II mutations are associated with improper maturation of the corresponding CFTR proteins and therefore fail to reach the apical membrane under physiological conditions. As a consequence, more severe CF phenotype can be followed (Koch *et al.,* 2001). This class contains the majority of the CF mutations, including the most frequent one, F508del (Ward *et al.,* 1994). The C-terminal tail of the protein contains amino acids that influence the stability of the mature protein, and mutations in this region (e.g. 4326delTC) lead to the marked instability of an otherwise fully processed and functional variant of CFTR (Haardt *et al.,* 1999).

Class III mutant CFTR proteins include those which are inserted into the apical membrane, but fail to respond to stimulation by cAMP. Most of these mutations are found in the nucleotide binding domains (e.g. G551D) involving defective chloride channel regulation (Welsh and Smith, 1993).

Class IV mutations affect amino acids located in the pore of the channel (e.g. R334W, R347P), and a CFTR channel with defective conductive properties and channel gating will be formed. This class of mutations is in most cases associated with a milder clinical phenotype (Koch *et al.,* 2001).

Class V mutations result in reduced synthesis of normally functioning CFTR because of defective processing/maturation (e.g. A455E) or aberrant splicing at alternative sites (e.g. IVS8-5T) (Dork *et al.,* 1997; Cuppens *et al.,* 1998). As a consequence, a small amount of functional CFTR chloride channels will be generated, resulting in a mild phenotype (Koch *et al.,* 2001).

Recently, a sixth class of mutations was introduced regarding the fact that CFTR also functions as the regulator of other ion channels such as ENaC, ORCC etc. (Vankeerberghen *et al.,* 2002).

2.5. Phenotype-genotype correlations

CF is characterized by a wide variability of clinical expression: patients are diagnosed with various modes of presentation at different ages, from birth to adulthood. The large number of CFTR mutations and the variable impact of these mutations on the protein prompted the search for a correlation between the molecular defect of the CFTR gene and the clinical heterogeneity of the disease.

A CFTR genotype probably has the highest potential as a primary source of phenotypic variability (Koch *et al.,* 2001). The potential of a mutation to contribute to the severity of a CF phenotype depends on multiple factors. Firstly, clinical manifestation depends on the type of mutation. Nonsense, splice, frameshift mutations and large inframe deletions or insertions are predicted to produce grossly changed protein or prevent biosynthesis of the CFTR and therefore tend to have severe phenotypic effects (Tsui, 1995; Zielenski, 2000). The predicted or known molecular mechanisms of the alteration and its position in the gene determine the synthesis, processing, trafficking to the membrane, stability and functional consequences for the CFTR protein, especially in structurally or functionally critical regions (Zielenski, 2000).

Secondly, it depends on other intragenic factors such as the presence of other changes within the gene. For example, the complex allele R117H, associated with the IVS8-5T variant, is typically linked with the pancreatic sufficient form of CF, but only with male infertility when on the IVS8-7T background (Cuppens *et al.,* 1998, Dork *et al.,* 1997). A second mutation in the genotype also has a great influence, as one mutation is sufficient to preserve pancreatic function, irrespective of the type of the second allele (Kerem and Kerem, 1996; Zielenski, 2000)

The phenotypic impact of a mutation also depends on where the mutation is expressed and on the organ-specific pathophysiology. Overall, there is good association between specific CFTR alleles (severe or mild) and exocrine pancreatic function (pancreatic sufficiency or insufficiency) (Zielenski, 2000). For example, genotype/phenotype analysis showed a close correlation between the F508del and pancreatic insufficiency (The Cystic Fibrosis Genotype–Phenotype Consortium, 1993; Kerem *et al.,* 1990). The manifestation of other less common gastrointestinal complications such as meconium ileus, liver disease or diabetes is not so remarkably genotype-dependent, although it is observed in patients with PI and severe CFTR genotype (Koch *et al.,* 2001).

In the male reproductive tract, very mild CFTR variants can cause CBAVD (one of the forms of obstructive azoospermia). One of these, IVS8-5T, a splicing mutation in intron 8 with a variable number of thymidines (5, 7, or 9 thymidines) in the polythymidyl tract, showed a high prevalence in CBAVD patients (Cuppens *et al.,* 1998). The frequency of the IVS8-5T allele in CBAVD was found to be up to 6 times higher than in the general population, and is present in about 20–40% of these patients (Chillon *et al.,* 1995a). The penetrance of IVS8-5T is, however, incomplete, and is determined by other factors such as the polymorphic TGm locus located in front of the thymidine tract itself. The higher the number of TG repeats, the less functional CFTR will be obtained (Cuppens *et al.,* 1998), and individuals carrying a higher number of TG repeats adjacent to 5T are more likely to exhibit abnormal phenotype (Groman *et al.,* 2004). Another CFTR variant frequently found in CBAVD patients is missense mutation R117H. Analysis of T-tract variants co-segregating with this mutation revealed a tendency of the R117H allele to associate with the 5T variant in CF patients and with the 7T variant in CBAVD patients (Cuppens *et al.,* 1998; Massie *et al.,* 2001).

Pulmonary outcome is clinically the most unpredictable component of the CF phenotype. Accurate estimates of lung function decline have been difficult to define and compare because the timing of measurements and the duration of follow-up differ in various patient groups. Pancreatic sufficiency, male gender and some non-F508del mutations are associated with a slower rate of pulmonary decline (Corey *et al.,* 1997). Links between pulmonary expression and CFTR genotype are debatable. Several investigators suggest that the pulmonary phenotype is directly related to the CFTR genotype: missense mutations A455E and R117H give rise to a milder pulmonary expression (De Breakeleer *et al.,* 1997; Massie *et al*., 2001). Others suggest that F508del homozygous patients and patients' compound heterozygous for F508del and a nonsense mutation in NBD1 encoding exons are more susceptible to *P. aeruginosa* infections (Kubesh *et al.,* 1993; Parad *et al.,* 1999). Variable lung disease severity has also been reported for CF patient homozygotes for different nonsense mutations, e.g. R553X, G542X or W1282X (Castaldo *et al.,* 1997; Shoshani *et al.,* 1992).

The severity of the CF disease is also influenced by environmental factors such as social class, smoking history (Campbell, 1992), nutritional status (Koch and Hoiby, 2000), treatment regime and whether or not the patient is treated in specialized centres (Mahadeva *et al.,* 1998).

Genotype-phenotype studies have enhanced our understanding of how particular mutations could contribute to the severity of the disease and therefore may help to predict the course of the disease or offer a prognosis of the development of complications.

2.5.1. Modulator genes

Since clinical presentation varies significantly among patients with the same CFTR genotype and among various affected organs within CF patients, it is evident that factors other than CFTR genotype are involved in determining CF disease severity. There are a multitude of studies to find modifier loci in humans.

Studies of F508del homozygous twins and siblings also showed more concordant clinical findings within monozygous twins compared with dizygous pairs, implying that genes other than CFTR significantly influence the manifestation of the basic defect (Bronsveld *et al.,* 2001). A European multicentric study showed that the pulmonary expression of CF may also be influenced by a modifier gene (Mekus *et al*., 2000).

More severe lung disease was reported in CF patients bearing a polymorphic allele in the promoter region associated with enhanced production of the pro-inflammatory cytokine, tumor necrosis factor α gene (Hull and Thomson, 1998). Similarly, severe pulmonary expression was reported in CF patients bearing polymorphisms associated with lower levels of glutathione transferase M1, an enzyme involved in detoxifying hydroperoxides resulting from oxidative stress (Hull and Thomson, 1998). More severe pulmonary expression was reported in CF patients who had low levels of mannose binding protein, a lecithin involved in the opsonization and phagocytosis of micro-organisms (Gabolde *et al.,* 1999; Garred *et al.,* 1999). Arkwright and others (2000) analyzed the polymorphisms of the transforming growth factor β (TGF-β) gene in 171 CF patients bearing the same CFTR genotype, and found that a mutation in codon 10 of the TGF-β gene is a risk factor for a more severe lung phenotype in CF patients.

In some studies, mild deficiency genotypes of α-1-antitrypsin (A1AT) are associated with increased risk for *P. aeruginosa* colonisation in the airway epithelium, and therefore worse pulmonary outcome (Doring *et al.,* 1994; 1999). In contrast, other studies (Mahadeva *et al.,* 1999; Henry *et al.,* 2001) found that lung function was better in CF patients bearing the S and Z alleles of the A1AT gene. Thus the role of A1AT as a modulator of lung phenotype must be assessed in multicenter studies involving large numbers of CF patients. An increased risk of colonization with *P. aeruginosa* has been shown in CF patients with the HLA class II DR7 allele (Chauhan *et al.,* 2003) or with antineutrophil cytoplasmic antibodies directed against bactericidal/permeability-increasing protein (Sediva *et al.,* 2003; Mahadeva *et al.,* 1999).

Other studies determined the importance of the salt-sensitive innate antibacterial activity in the airway layer, which in turn depends on several antimicrobial proteins, e.g. human beta-defensins 1, 2 and 3, lactoferrin, lysozyme, histatin, and cathelicidin (Singh *et al.,* 2000; Taggart *et al.,* 2003).

In addition, mutations in the mannose binding lecithin gene, the TGF-β gene and alleles Z and S of the A1AT gene also adversely influence the

development of liver disease in CF patients (Gabolde *et al.,* 2001; Friedman *et al.,* 2001; Salvatore *et al.,* 2002).

Contradictory data are available for some locuses and polymorphisms. Some studies reported that a locus in region 19q13 probably modulates gastrointestinal expression, i.e. meconium ileus and distal intestinal obstructive syndrome (Zielenski *et al.,* 1999; Salvatore *et al.,* 2002), but others excluded the association between meconium ileus and 19q13.3 (Larriba *et al.,* 2001).

2.6. Treatment

2.6.1. Standard treatment

Therapy for CF has the following aims: preservation of lung function, optimisation of nutritional status, minimization of complications and maintenance of psychosocial well-being (Cutting, 1997). The treatment should preferably be given in special CF centres by a team of CF clinicians in collaboration with an experienced microbiologist, specially-trained nurses, physiotherapists, dieticians, social workers and psychologists (Mahadeva *et al.,* 1998; Koch and Hoiby, 2000).

Lung disease is the life-limiting factor for most CF patients, and therefore the main goal of therapy is to retard the progression of pulmonary damage through better mucus clearance and early control of infections. Special exercises should be performed by CF patients themselves every day in combination with mucolytic therapy and inhaled recombinant human DNase I (rhDNase) (Koch and Hoiby, 2000; Kearney and Wallis, 2002). Control of bacterial infection, especially *P. aeruginosa*, consists of early antibiotic therapy whenever pathogens are detected, aggressive treatment of exacerbations and continuous prophylaxis (Koch and Hoiby, 2000). Lung or other organ transplantation is the last resort in selected patients with extensive irreversible damage. This may prolong life, since the international registry reports one-year survival in 70–80% and five-year survival in 30–45% of transplantated patients (Keck *et al.,* 1999; Doull, 2001).

There is universal agreement that high-fat, high-calorie food intake is essential in patients with CF. Calorie intake of 150% of the normal recommendation or even higher may be necessary to secure normal growth and nutrition due to higher energy requirements (Koch and Hoiby, 2000; Sinaasappel *et al*., 2002). Patients with CF are at risk of multiple vitamin deficiencies and lifelong supplementation with fat-soluble vitamins A, D, E, and K is an essential part of standard care (Koch and Hoiby 2000; Sinaasappel *et al*., 2002).

Nearly all CF patients will need pancreatic enzyme supplementation with all meals, as well as fat-rich snacks. Malabsorption of fat is severe without enzyme treatment, as some 50–60% of ingested dietary fat is not absorbed without treatment (Sinaasappel *et al.,* 2002).

2.6.2. New therapies

Pharmacological treatment of ion transport defect. Advances in molecular biology, cellular biology and electrophysiology have furthered our knowledge of the consequences of mutations in different regions of the CFTR. As the physiology of the CFTR has been elucidated, different strategies are created to find a pharmacologically controlled way to correct the defect in ion transport (Table 3) (Roomans, 2003).

Table 3. Different pharmacological approach**es** to correct the ion transport defect in CF.

Strategies to repair CFTR defects	Mutations /channels involved	Drugs tested	Description of probable correcting mechanism	References
Prevention of the breakdown of mutant CFTR	F508del	4-phenyl-butyrate (4-PBA)	Breaking down the association between the F508del-CFTR and chaperones	Zeitlin *et al.,* 2002; Choo-Kang and Zeitlin, 2001
Activating the mutant CFTR with correcting folding/ trafficking	G551D F508del	Xanthines	Direct binding to the CFTR NBD1 domain	Andersson and Roomans, 2000; Powell and Zeitlin, 2002; Randak *et al.,* 1999; Andersson and Roomans, 2000
	G551D G551S F508del	Isoflavones (genistein)	Direct binding to the NBD2 domain, increasing channel´s open time	
Correction of mutated CFTR	Stop mutations	Amino-glycosides (gentamicin)	Suppressing the premature termination codons	Bedwell et al., 1997; Wil-schanski *et al.,* 2000; Zeitlin, 2003
Activate another chloride channel	Ca^{2+} acti-vated Cl^{-} channels	ATP and UTP	Stimulating chloride transport	Roomans, 2003;
Inhibit Na^{+} absorption	Epithe-lial Na^{+} channel	Amiloride, benzamil	Correcting of excessive absorption of Na^{+}, water by epithelial cells	Rodgers and Knox, 1999; Pons *et al.,* 2000

Although there is no clinically effective drug available, it is likely that a combination of these strategies will most likely be beneficial in treating CF (Powell and Zeitlin, 2002).

Gene therapy (GT). Preclinical studies showed that both viral and nonviral gene transfer agents were able to correct the chloride ion transport defect in CF transgenic mice (Hyde *et al.,* 1993; Driskell *et al.,* 2003). The best studied vectors to deliver copies of the normal CFTR gene to the airway epithelium are adenoviruses, adeno-associated viruses and cationic liposomes (Ferrari *et al*., 2002; Schwiebert, 2004). Clinically, the most relevant target for GT in CF is the lung, although many investigators started their clinical studies by investigating gene transfer efficiency in nasal mucosa. Nasal airway epithelia have a similar histology and the same CF-associated abnormalities in ion transport as pulmonary epithelia, but compared to the lung, the nasal cavity offers easier access for both gene transfer and safety measurements, and represents a reduced risk to patients in the event of side effects (Ferrari *et al.,* 2002). The first clinical trials in CF patients were carried out in 1993, and to date descriptions of 29 trial protocols have been published (Griesenbach *et al.,* 2003).

The greatest number of clinical GT studies has been performed using recombinant adenovirus as a gene transfer vector, with at least five studies involving administration of the virus to the lung (Perricone *et al.,* 2001; Ferrari *et al.,* 2002). About one third of these studies showed some changes in chloride transport in the nasal epithelia, but no functional measurements were assessed in the lung studies (Ferrari *et al.,* 2002).

Current trials of GT for CF suggest that current levels of gene transfer efficiency are probably too low to result in clinical benefit, largely as a result of various extra- and intracellular barriers faced by gene transfer vectors within airways (Griesenbach *et al.,* 2003; Ferrari *et al.,* 2002). In summary, although, the efficiency of gene transfer is generally low, steady progress has been made over the last 10 years, and the proof-of-principle for CFTR gene transfer to the lung has been established (Griesenbach *et al.,* 2003; Schwiebert, 2004).

3. AIMS OF THE STUDY

The objective of the current work was to study various aspects of cystic fibrosis in Estonia.
The specific aims were:

1. to determine the prevalence of carriers of the main mutation, F508del, in newborns in Estonia
2. to establish the incidence of CF disease in Estonia, and to compare our results with other populations in Europe
3. to identify the spectrum of CFTR mutations in Estonian CF patients
4. to analyze the demographic data of CF patients in Estonia
5. to investigate the clinical picture of CF patients in relation to their genotype
6. to introduce DNA testing for the most common CF mutations in Estonia

4. MATERIAL AND METHODS

4.1. Study subjects

4.1.1. Study population I — Newborns involved in the F508del mutation carrier screening

During the newborn screening programme, 7396 newborns born consecutively between January 1, 1993 and July 31, 1993, including 85% live births during this period, were analyzed for the presence of the F508del mutation. During the study, six blood spots on special test cards, i.e. Guthrie cards, were obtained from every newborn between 3–5 days of age. The test cards were placed separately in envelopes, registered, and stored confidentially in our database. Information pamphlets describing the screening test were sent to all of the 19 maternity hospitals in Estonia and to two pediatric intensive care units that contributed to the pilot study. Additionally, all contributing hospitals were visited at least once at the beginning of the study. Informed consent was obtained orally from mothers before the sample was taken. Information about all F508del mutation carriers identified during this study was communicated to primary care physicians and pediatricians. The study was approved by the Ethics Committee of the Medical Faculty of Tartu University.

4.1.2. Study population II — Children with CF diagnosis

In our survey we aimed to include all patients with CF born in Estonia in the period from January 1, 1974 to May 31, 2003. In a prospective study, patients with CF were identified using four different approaches. Firstly, a list of CF patients from the database of chronically ill patients registered by child pulmonologists in every county (in 1993) was available. These families were contacted and interviewed, and DNA samples were obtained. Secondly, an inquiry was set up among pediatricians, pulmonologists, child surgeons, and doctors from pediatric intensive care units working in major children's clinics and outpatient departments and in county hospitals. They were encouraged to send patients' clinical data and DNA samples to the Molecular Diagnostics Centre at the Tartu University Clinic. Thirdly, clinical information from hospital records concerning known CF patients and meconium ileus patients from the archives of Children's Clinic, Pulmonology Clinic and Surgical Clinic of Tartu University Clinics, Tallinn Children's Hospital, Nõmme Children`s Hospital, Kuressaare Hospital and Pärnu Hospital from the period of 1974–2003 were critically examined. Additionally, information was disseminated through

lectures, presentations of papers and medical seminars throughout the study period. In 1993, a special workshop devoted to the various aspects of CF was organized together with the Estonian CF Society and the Estonian Paediatric Association.

A total of 460 patients suspected of having CF were enrolled, and they all passed the preliminary screening for the two most common mutations, F508del and 394delTT. After thorough examination using diagnostic criteria and follow-up, 41 patients from 36 families were diagnosed as CF patients. All of these CF patients were included in the comprehensive analysis of CFTR gene alterations.

Retrospectively, in order to find all patients who had lived but died before DNA diagnostics was available in 1993, extensive investigation of medical records from *exitus letalis* patients and autopsy protocols from the years 1974–1993 was undertaken in the archives of the major pediatric hospitals and surgical departments in Tartu, Tallinn, Pärnu and Kuressaare. On the basis of the archival data, 35 CF patients who had died before the beginning of this study started were identified. Therefore, the final analysis for the thesis includes 76 CF patients born between 1974 and 2003. As the availability of clinical data from the latter-mentioned additional 35 CF patients was poor, and molecular analysis was not performed, they were not included in the comparison of clinical data. Therefore we only used the information of these 35 CF patients for mortality analysis and for calculating the proportion of meconium ileus newborns among CF patients and the rate of incidence of CF in Estonia.

The Ethics Committee of the Medical Faculty of Tartu University approved the study.

4.1.3. Inclusion criteria and assessment of CF phenotype

Diagnostic criteria were based on repeated positive sweat chloride tests (>60 mmol/l) and/or on typical findings of gastrointestinal and/or pulmonary disease (Table 1). Some atypical cases with borderline sweat tests, but typical clinical findings, were included. All patients were monitored at the central hospitals — the Tallinn Children's Hospital and the Children's Clinic of the Tartu University Clinics. Treatment regimes were similar in both hospitals.

The clinical phenotype was assessed on the basis of the following parameters: age at diagnosis and current age, sex, the mean value of at least 3 different sweat chloride tests, pulmonological status including data from the last spirometry and X-ray. The presence of chronic lung disease was defined as the patient having chronic bronchitis or chronic pneumonia verified by X-ray. Measures of lung volume and airflow are used to assess the degree of dysfunction in patients with CF. Patients over 6 years of age are routinely tested at least once a year for forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1). Chronic lung colonisation with bacteria was defined as at least three consecutive sputum cultures positive for *P. aeruginosa,*

S. aureus, H. influenza, K. pneumonia or others**.** Pancreatic insufficiency was determined on the basis of the fat content of stool samples and the need for pancreatic enzyme replacement therapy. Liver disease, one of the most serious complications, was assessed using functional and biochemical tests and ultrasonography. A liver biopsy was performed in the event of a suspicion of liver cirrhosis. The presence of other symptoms such as meconium ileus, anemia in early infancy (age 2–3 months), chronic sinusitis, nasal polyps, diabetes mellitus, distal intestinal obstruction syndrome, anal prolapse, gallstones and gastrooesophageal reflux were evaluated. The Shwachman–Kulczycki score, as a general score of clinical severity, consisting of general activity, physical examintion, nutrition and X-ray findings, was assessed.

Phenotype–genotype correlation. In order to compare the clinical and demographic data from patients with different genotypes, all of our patients were divided into 3 main groups. The first group harboured F508del homozygotes (n=12), and the second group (n=11) consisted of two 394delTT homozygous patients and 9 patients with genotype 394delTT/F508del. The rest of the identified mutations occurred rarely in our cohort. Therefore all other patients who were compound heterozygotes for any other mutations were classified in the third group (n=18).

4.2. Methods

4.2.1. DNA extraction

From newborns involved in the carrier screening program, six blood spots were collected on filter paper (Schleicher and Schuell filter paper No. 2992). From all CF patients, and if available also from their parents and sibs, 2–10 ml of peripheral blood was collected in tubes containing K3-EDTA or Na-citrate. Genomic DNA was extracted from peripheral blood using the standard phenol-chloroform method (Sambrook *et al.,* 1989) or a commercial DNA extraction kit (Fermentas™©, Lithuania). In the case of blood on filter paper, a polymerase chain reaction (PCR) was performed directly from the dried blood spot using the protocol described in detail in Publication I.

4.2.2. Mutational analysis

Identification of the mutations in CFTR gene was started from alterations common in European and Scandinavian countries: F508del, 394delTT, R553X, G551D, G542X, N1303K, 621+1G→A, CFTRdele2,3(21kb), etc. (Table 4). In parallel to the direct testing, scanning of all 27 exons and their flanking regions

was undertaken by single strand conformational polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) analyses. DNA samples displaying PCR fragments with altered mobility in SSCP or DGGE analyses were subsequently sequenced. A subset of patients and their families diagnosed after 1998 and where common mutations (F508del, 394delTT) were not found underwent directly the sequencing of all 27 exons and flanking regions.

Table 4. Methods used in mutational analysis of CFTR gene in Estonian CF patients.

I Mutational analysis of previously known common mutations		
Analysis method	Mutations identified	References
1. Heteroduplex analysis	F508del, 394delTT, IVS85T/7T/9T	Publication I, Chillon *et al.,* 1995a
2. Allele specific PCR	F508del, G551D, G542X, N1303K, 621+1G→A, CFTRdele2,3(21kb)	ARMS, Cellmark Diagnostics, Abingdon, UK Dork *et al.,* 2000
3. Restriction fragment length polymorphism	G551D, R553X, L206W, 1811+1.6kbA→G	Dork *et al.,* 1992; Chillon *et al.,* 1995b; Desgeorges *et al.,* 1995
II Mutational analysis of the CFTR gene using indirect methods		
Analysis method	References	
1. SSCP	Zielenski *et al.,* 1991; Shackleton *et al.,* 1994; Wall *et al.,* 1995; Cheadle *et al.,* 1993; Ravnic–Glavac *et al.,* 1994	
2. DGGE	Fanen *et al*., 1992; Macek *et al.,* 1997b; Bombieri *et al.,* 2000	
3. Direct sequencing	Zielenski *et al.,* 1991; DYEnamic™ ET terminator cycle sequencing kit, Amersham Pharmacia, UK	

SSCP, Single strand DNA conformational polymorphism analysis**;** DGGE, denaturing gradient gel electrophoresis; ARMS, amplification refractory mutation system.

4.2.3. Statistical analysis

The study's analyses were performed using the statistical package SAS Version 8.1 (SAS Institute Inc., Cary, USA). Continuous and normally distributed variables were compared with Student's t-test. A chi-square test or Fisher's exact test was used to determine if there was a relationship between different measurements. The CF patient's survival was analyzed using Kaplan-Meier estimation, and the log-rank statistic and Cox proportional hazards regression was used to assess differences between survival curves. Differences were considered statistically significant if the p-value was less than 0.05.

5. RESULTS AND DISCUSSION

5.1. Pilot screening for F508del in Estonia (Publication I)

Over a period of 7 months (from January 1, 1993 to July 31, 1993), 7396 newborns born consecutively were tested for the F508del mutation. Altogether, 85% of newborns born during the study period were covered by the monitoring (Table 5). During the screening programme 88 heterozygotes for the F508del mutation were determined. The estimated prevalence of F508del carriers for Estonia in general was 1:84 (1.19%; 95% CI 0.96-1.46%). Our results indicate a lower rate of F508del mutation carriers in Estonia than in European populations in general. In countries neighbouring Estonia, the proportion of F508del

Table 5. The data of CF newborn screening from January 1 to 31 July 1993 in 19 different regional hospitals.

Region[1]	Infants covered by test[2] (%)	Newborns screened	No. of detected F508del carriers	Prevalence of heterozygotes
Hiiumaa	97.9	95	3	1:32
Saaremaa	79.0	272	8	1:34
Lääne	94.4	206	5	1:41
Järva	65.3	183	3	1:61
Puru	100	208	3	1:68
Tallinn Pelgulinna	79.7	1029	15	1:69
Keila	96.1	212	3	1:71
Rakvere	96.6	1056	7	1:75
Kohtla-Järve	97.6	153	2	1:76
Viljandi	80.1	237	3	1:79
Põlva	93.4	257	3	1:86
Pärnu	97.8	619	7	1:88
Tallinn Keskhaigla	63.7	1073	11	1:98
Tartu	83.9	801	8	1:100
Võru	97.5	350	3	1:117
Narva	92.7	441	2	1:221
Rapla	99.3	250	1	1:250
Jõgeva	99.2	316	1	1:316
Valga	96.1	166	0	–
Total	**85.0**	**7396**	**88**	**1:84**

[1] Regions are listed in order of the prevalence of heterozygotes. [2] Percentage of tested newborns out of total number of live births in respective regional hospital during screening period.

heterozygotes is markedly different (Figure 5). The highest similarity was seen with Norway and Sweden. In Finland, which has been in long-lasting genetic isolation due to its geographic location, CF disease incidence is the lowest of all European countries, and the carrier frequency is also notably lower, i.e. 1:171 (Kere *et al.,* 1994).

No baby carrying F508del in both chromosomes was found in the study population. Applying the Hardy-Weinberg equilibrium, the incidence of F508del homozygotes was estimated to be as low as 1 in 28,246 in the Estonian population. All babies carrying the main mutation were also checked for the presence of 394delTT, the second most common mutation in Estonia, in order to find compound heterozygotes. We did not, however, find any newborns carrying the F508del/394delTT genotype. We did not later find any of these F508del heterozygous babies in our cohort of CF patients.

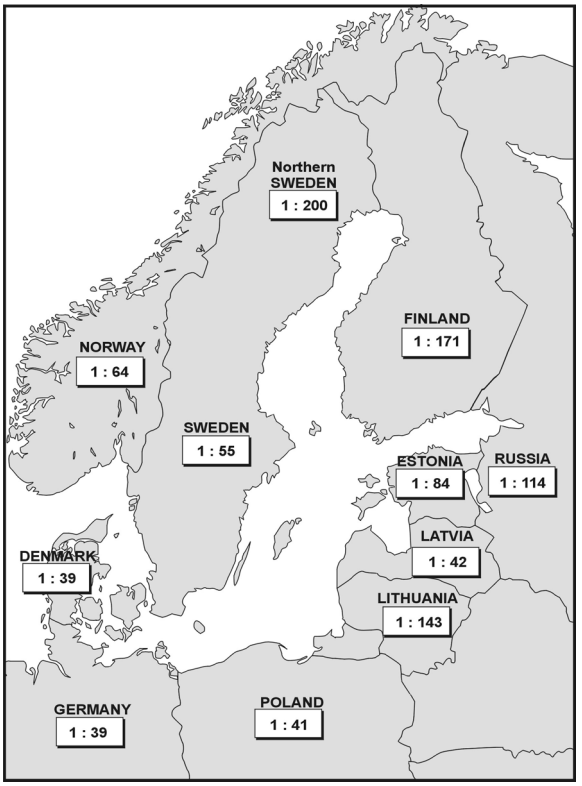

Figure 5. The incidence of F508del mutation heterozygotes in Estonia, Scandinavia and countries bordering the Baltic Sea (Kere *et al.,* 1994; Witt *et al.,* 1994; Schwartz *et al.,* 1994; Lucotte *et al.,* 1995; Krumina and Utkus, personal communication; Petrova *et al.,* 1997; Eikild *et al.,* 1993; Wennberg and Kucinskas, 1994; Lannefors and Lindgren, 2002).

5.1.1. Geographical distribution of carriers of the F508del mutation in Estonia

Significant heterogeneity was demonstrated in the proportion of F508del carriers in the 19 different regions of Estonia (Table 5). The highest proportion of F508del heterozygotes was found on the Baltic Sea islands of Hiiumaa and Saaremaa and the Western coastal part of Estonia, Lääne County, where the corresponding ratios were 1:32, 1:34 and 1:41 respectively. The lowest prevalence of the F508del alteration was detected in the eastern parts of Estonia.

Therefore, in order to increase the representativeness of the material, 8 larger regions were formed on the basis of previous somatoanthropological and population genetic studies in Estonia (Mark *et al.,* 1994) (Table 6, Figure 6). Significant differences were found in the heterozygote prevalence in these eight regions ($p=0.04$). The lowest proportion of F508del carriers in Estonia was in southeastern Estonia — 1:128, and the highest proportion was found on the Baltic Sea islands and western coastal part of Estonia, with a referring value of 1:36 (Table 6, Figure 6). When the higher prevalence in the western part of the country was compared with each of the other seven regions of Estonia, a significant difference was found with six regions ($p<0.05$); the contrast with northern Estonia (Region 6) was smaller ($p=0.06$) (Table 6). Variations in frequencies on the mainland were not statistically significant.

Table 6. Data from the newborn screening programme for CFTR mutation F508del in 8 different regions of Estonia. The homogeneity of the allele frequencies between different regions has been tested using the chi square method.

	Region	Newborns screened	No of F508del carriers	Prevalence of F508del carriers	P value[1]
1.	Western	773	16	1:36	
2.	South-western	856	10	1:86	0.024
3.	South-eastern	773	6	1:128	0.004
4.	Eastern	1117	9	1:124	0.001
5.	Central	433	4	1:108	0.036
6.	Northern	740	10	1:74	0.064
7.	Tallinn	2102	26	1:81	0.008
8.	North-eastern	802	7	1:115	0.006

[1] P value, the allele frequency in the western region was compared with allele frequencies of all other investigated regions.

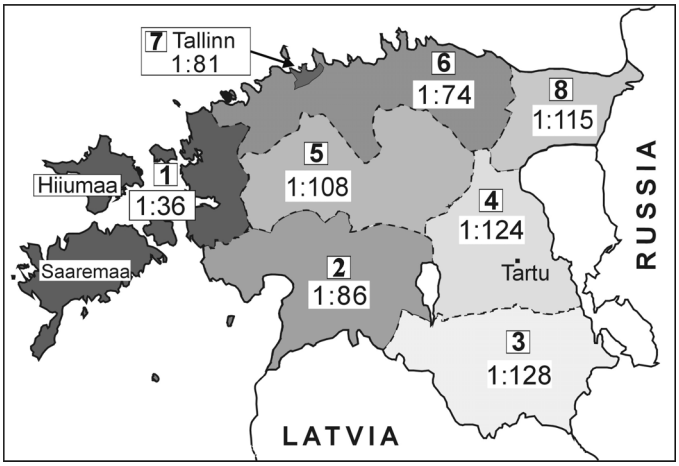

Figure 6. The carrier prevalence of the F508del mutations in different regions of Estonia. 1 Western Estonia and islands, 2 Southwest Estonia, 3 Southeast Estonia, 4 Eastern Estonia, 5 Central Estonia, 6 Northern Estonia, 7 Tallinn, 8 Northeast Estonia.

The notably increased rate of heterozygotes on the Baltic Sea islands of Saaremaa, Hiiumaa and also in the western coastal region could be explained by Swedish and Danish influences, where the carrier frequencies of the F508del mutation are higher than in Estonia, i.e. 1:55 and 1:38 respectively. This phenomenon is also supported by the islands' relative geographic isolation. It has been suggested that people from Scandinavia have settled on the islands and western coast of Estonia. There were many Swedish-speaking villages until the Second World War. The decreased frequency of heterozygotes in eastern regions could partly be explained by the vicinity of Russia and by the extensive Russian-speaking population in this region. The pilot screening of newborns in the Moscow region showed an F508del carrier frequency of 1 in 114 newborns (Petrova *et al.,* 1997).

The fact that the population of the Baltic Sea islands and western coastal region of Estonia differs considerably from the inland parts of the country, as noticed in our study, has also previously been described by anthropological and population genetic investigations (Mark *et al.,* 1994; Õunap *et al.,* 1996).

5.2. Spectrum of CFTR gene mutations in CF patients from Estonia (Publication II)

5.2.1 Detection of the most common mutations

In the present study a large-scale mutational analysis of Estonian CF patients and also patients suspected of having CF was performed. After thorough reconsideration of clinical, laboratory and molecular data**,** 41 cystic fibrosis patients from 36 families were included in the present study.

First, several more common known mutations were tested directly (Table 4). As a result of this screening, only two common mutations were revealed: F508del was found in 37 alleles (52%) and 394delTT in 11 alleles (15%) (Table 7). In agreement with the data obtained worldwide, the F508del mutation was also found to be the most common mutation detected in CF patients in Estonia. The frequency of the main mutation among other CFTR mutations in Estonia is far less than the mean value of 72.8% for the Northern and Central European countries (Estivill *et al.,* 1997) or 67% of CF chromosomes worldwide (CFGAC, 1994). Nevertheless, the frequency is relatively similar to the corresponding estimates from neighbouring countries such as Russia (54.4–56.0%) and Latvia (58.3%) (Petrova *et al.,* 1997; Bobadilla *et al.,* 2002). The proportion of F508del is also relatively low in other studied Finno-Ugric populations: 46% in Finland (Kere *et al.,* 1994) and 55% in Hungary (Bobadilla *et al.,* 2002). It has been noticed that the lower the proportion of the F508del alteration in CF chromosomes, the higher the heterogeneity in the CFTR mutation spectrum found in that region (Estivill *et al.,* 1997; Bobadilla *et al.,* 2002).

The 394delTT deletion is remarkably frequent in Estonia, accounting for 15% of all CF chromosomes and about one third (31%) of the non-F508del chromosomes. It is also the second most frequent mutation in several Scandinavian countries (Table 8), and has also been found on CF chromosomes in neighbouring countries such as Russia and Latvia (Bobadilla *et al.,* 2002).

We did not find any of the mutations that are more common in the European populations, such as G542X (relative frequency in Europe 2.6%), N1303K (1.6%), G551D (1.5%) or W1282X (1.0%) (Estivill *et al.,* 1997). This is not surprising, as the relative frequencies of these mutations are also less than 1% in the Nordic countries (Bobadilla *et al.,* 2002; Strandvik *et al.,* 2001).

In our cohort we have about 12 patients (29%) from 10 families with Slavic or mixed Estonian/Slavic backgrounds, and therefore we separately tested CF patients for whom one or two CFTR mutations remained unidentified after mutation scanning or sequencing, for a large genomic deletion, CFTRdele2,3, spanning introns 1–3 of the CFTR gene. Although this deletion is frequently observed in Central and Eastern European countries including Czech Republic

(5,5%), Russia (5.0%) and Ukraine (1.2%) (Dork *et al.,* 2000; Bobadilla *et al.,* 2002), it was not was found in our group of patients.

Table 7. Mutations identified in 36 CF families in Estonia.

Mutation	Exon	Number of chromosomes	Frequency %	Method of detection
F508del	10	37	51.4	HA
394delTT	3	11	15.2	HA
3659delC	19	2	2.8	SSCP, sequencing
1716G→A	10	2	2.8	SSCP, sequencing
359insT	3	1	1.4	SSCP, sequencing
W57R	3	1	1.4	sequencing
R117C	4	1	1.4	SSCP, sequencing
E217G	6a	1	1.4	DGGE, sequencing
S549N	11	1	1.4	sequencing
R553X	11	1	1.4	sequencing
I1005R	17a	1	1.4	SSCP, sequencing
R1066H	17b	1	1.4	DGGE, sequencing
S1196X	19	1	1.4	DGGE, sequencing
S1235R	19	1	1.4	DGGE, sequencing
Detected alleles		**62**	**86.2 %**	
Unidentified alleles		**10**	**13.8%**	
Total		**72**	**100%**	

HA-heteroduplex analysis; SSCP- single strand DNA conformational polymorphism analysis; DGGE- denaturing gradient gel electrophoresis

Table 8. Frequency of 394delTT mutations in countries neighbouring on Estonia.

Country	% of 394delTT mutation out of all CF chromosomes	References
Finland	30.0	Kere *et al.,* 1994
Estonia	**15.2**	**This study**
Sweden	7.5	Strandvik *et al.,* 2001
Norway	4.2	Estivill *et al.,* 1997
Latvia	2.8	Bobadilla *et al.,* 2002
Russia	2.1	Estivill *et al.,* 1997
Denmark	1.9	Schwartz et al., 1994
Belgium	0.5	Bobadilla et al., 2002

5.2.2 Identification of rare mutations

Subsequent to the direct testing, the scanning of all 27 exons and their flanking sequences was undertaken. All CF patients in whom one or two alleles remained unknown after direct testing then underwent mutation screening using SSCP, DGGE and/or direct sequencing of the CFTR gene. Altogether, in searching the CFTR gene exon by exon, 12 rare mutations were found: 359insT, R117C, E217G, I1005R, R1066H, S1196X, S1235R, S549N, W57R, R553X, and 3659delC, 1716G→A (Table 7). Each of these mutations was detected once, except for 3659delC and 1716G→A, which were found in two heterozygotes. All of these mutations have been described previously in other populations.

These are mainly infrequent alterations, as only three of them, R553X, S549N and 3659delC, with a relative frequency in CF chromosomes worldwide of 0.7%, 0.1% and 0.1%, respectively, belong to the list of the 24 more common mutations (CFGAC, 1994; CFMD, http://www.genet.sickkids.on.ca/cftr). Stop mutation R553X reaches relatively high frequencies in Central Europe, from 1.9% in Poland to 14.4% in Switzerland, but is atypical for Northern European countries (Estivill *et al.,* 1997; Bobadilla *et al.,* 2002). Frameshift mutation 3659delC, on the other hand, is characteristic for Scandinavia, being the third most common mutation in Sweden, with a relative frequency of 6.2% (Strandvik *et al.,* 2001).

The disease-causing changes found in the studied group were spread unevenly over 8 different exons (Figure 7). Mutation "hotspots" for Estonian patients turned out to be situated in exon 3, which is one of the exons with the highest mutation densities in the CFTR gene coding sequence, and in exon 19. Accordingly, it could be suggested that in the practical work of searching for mutations in new CF patients, the sequencing project should commence from exons 3 and 19, followed by exons 4, 10 and 11.

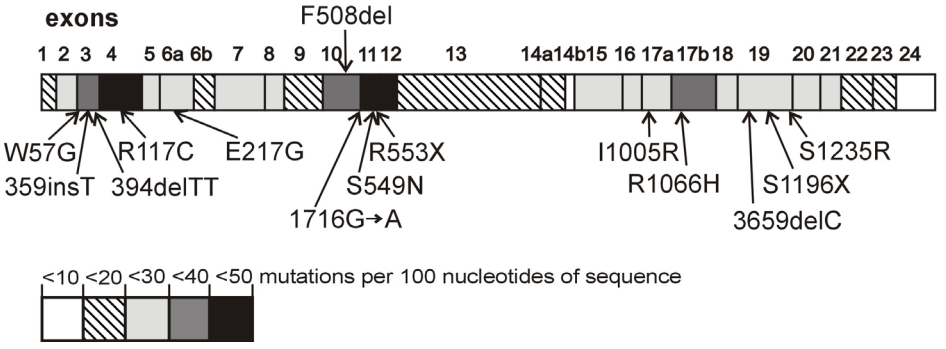

Figure 7. Schematic distribution of mutations detected in Estonian CF patients. Data about the density of the mutations in different exons of the CFTR gene originates from the Cystic Fibrosis Mutation Database (http://www.genet.sickkids.on.ca/cftr).

Altogether, 10 alleles remained unidentified in our group (Table 7). In one case (with 2 unidentified mutations) direct mutation analysis was carried out from testcard blood spots, and further analysis was not performed for technical reasons. The eight remaining uncharacterized chromosome mutations may well lie outside the investigated regions within the large introns, the positive regulatory element of the promoter, or the 3´untranslated region. Furthermore, we cannot exclude the possibility that there may be large (whole exon or greater) deletions, as these may have been missed by the sequencing approach. However, we can conclude that the vast majority of CF in Estonia is caused by molecular defects within the CFTR gene.

In conclusion, a total of 14 mutations were detected, and 86% of all mutated CF mutations were identified. As historically the population of Estonia has been influenced by several populations (Finno-Ugric, Baltic, Swedish, German and Slavic), a heterogeneous distribution of mutations could be expected. Previously, the highest allelic heterogeneity has been detected in the Mediterranean region, as reported in different countries in this region (Estivill *et al.,* 1997; Casals *et al.,* 1997; Claustres *et al.,* 2000; Bobadilla *et al.,* 2002). In these regions, an average of 25 mutations is needed to detect 84% of all CF mutations (Bobadilla *et al.,* 2002). Generally, in central and northern European countries fewer mutations (approximately 10) need to be screened in order to reach a sensitivity of 79%, but mutation distribution may still be very variable, even within the same country. For example, in Sweden the screening of the 4 most common mutations enables the detection of 81.7% of CF mutations, but accounting for all 71 mutations found in this country only raises the detection rate to 89.0%, and significant differences could be seen between different parts of Sweden (Strandvik *et al.,* 2001). In France, a total of 310 different mutations accounting for 93% of CF genes have been found, and regional variability is considerable. This spectrum of CF defects is the largest reported in one country, reflecting the ethnic heterogeneity in France (Claustres *et al.,* 2000).

From our data, it can be extrapolated that commercial kits for the routine screening of about 29 known mutations (Elucigene™ CF29 by Orchid Biosiences, or INNO-LiPA CFTR12 and CFTR17 by Innogenetics) will cover only 68.9% of mutant alleles in the CF population in Estonia, which is quite a low percentage. Thus expanding the panel of screened mutations is expected to achieve only marginal gains in sensitivity.

We can say that due to the variability of the mutation pattern in different countries/populations, it is not realistic to make one CFTR mutations testing kit suitable for worldwide or even pan-European use (for a reasonable price). Therefore, a resequencing assay should be developed for the CFTR gene in order to detect all mutations regardless of the population. The DNA microarray may be the only acceptable technique to reach a mutation detection sensitivity of close to 100%.

5.2.3. The genotypes of Estonian patients with CF

The molecular analysis of the CFTR gene has been performed in 41 CF patients, and 17 different CF genotypes have been identified (Table 9). Both mutant alleles were identified in 29 families (80%), in four families only one allele was recognized, and in three families both alleles remained unknown. Mutation F508del, the main alteration in our CF chromosomes, was found in at least one allele in 32 families (78%). Together with the second most common alteration, 394delTT, at least one common mutation could be found in 34 (85%) families. These two most common mutations are tested in all suspicious CF cases at the Molecular Diagnostics Centre of the Tartu University Clinics.

Table 9. Genotypes of 41 CF patients in Estonia. For further analysis of clinical characteristics, CF patients were divided into three groups on the basis of the genotypes identified (See 4.1.3.).

Number of patients	Genotype	Grouping
12	F508del/F508del	Group I
9	F508del/394delTT	Group II
2	394delTT/394delTT	
1	F508del/I1005R	Group III
2	F508del/359insT	
2	F508del/3659delC	
1	F508del/R117C	
1	F508del/R1066H	
1	F508del/S1196X	
1	F508del/S549N	
1	F508del/W57R	
1	F508del/1716G→A	
1	1716G→A/U	
1	S1235R/U	
1	R553X/U	
1	E217G/U	
3	U/U	

U- unidentified mutation

Out of 41 investigated patients, 12 (29%) were homozygotes and 20 (49%) were compound heterozygotes for the F508del mutation. A total of 9 patients (22%) were compound heterozygotes for other mutations. The Hardy–Weinberg equilibrium was used to determine whether the studied population was balanced. According to this equation, the number of homozygous F508del patients should be 11 (26.4%), the number of proposed heterozygotes should be

20 (50.0%) and the number of non-F508del patients should be 10 (23.6%). Thus these data correlate well with our results, offering evidence that the Estonian CF patient pool is balanced. Although our group contains 3 patients with unidentified changes in the CFTR gene, it is highly likely that the latter category is not overrepresented.

5.3. Clinical characteristics of Estonian CF patients (Publication III)

5.3.1. Analysis by gender

The clinical data of a total of 41 patients — 20 boys and 21 girls — were analyzed. In our group there were no statistically significant differences between genders as regards the segregation of CFTR mutations, mean age, mean age at diagnosis, the proportion of PI patients, results of tests of lung function, the presence of chronic lung disease, *P.aeruginosa* colonisation or liver disease. In our group we were unable to demonstrate differences between males and females in Kaplan–Meyer survival probabilities.

Reports from other countries and larger cohorts illustrate that the survival of females is generally poorer than that of males, and the male/female ratio tends to increase with increasing age (Dodge, 1997; Rosenfeld *et al.,* 1997; O'Connor *et al.,* 2002; McCormick *et al.,* 2002; Corey *et al.,* 1997). Although there was a slight overrepresentation of males in age groups above 15 years (Figure 8), the difference was statistically insignificant.

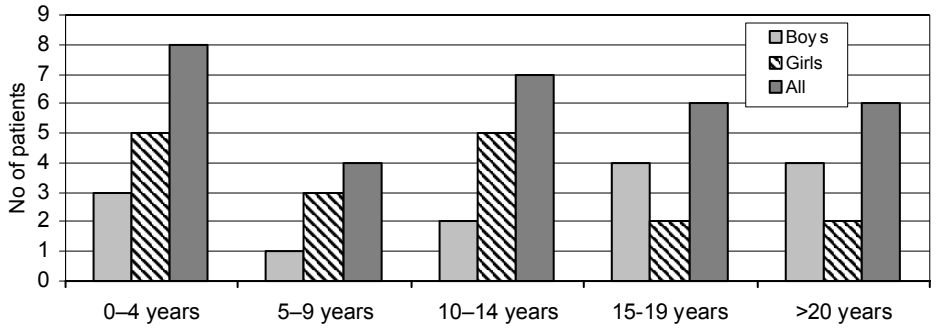

Figure 8. Age and gender profile of 31 Estonian CF patients.

The age- and sex-adjusted standard deviation score for weight was the only parameter in which we observed a statistically significant discrepancy between genders. On average the boys were significantly more underweight, with a mean standard deviation score for weight of –1.8 (±1.35SD) compared to girls' respective score of –0.96 (±1.35SD).

5.3.2. Age at diagnosis of CF

In our group of 41 patients, the mean age of establishing CF diagnosis was 2 years 1 month (95% CI 16.9 to 34.5 months), and the median age of diagnosis was 1 year 7 months (95% CI 6 to 31 months). Both figures are essentially higher than reported for other countries, i.e. mean age at diagnosis 4.6 months in Switzerland (Kraemer *et al.,* 2000), or median age at diagnosis 6 months in the USA (Cystic Fibrosis Foundation, 2002), 9 months in Sweden (Lannefors and Lindgren, 2002) and 1 year in developed European countries (European Epidemiologic Registry of CF, 2000).

Data from the CF Foundation (USA) demonstrated that 71% of CF patients were diagnosed during the first year of life (Rosenstein and Cutting, 1998), whereas in our cohort 41% of patients were only diagnosed during the first year of age. First and foremost, these patients with delayed diagnosis mostly suffer from malnutrition, and therefore also from complications of maldigestion and malabsorption (Farrell *et al.,* 2001; Koletzko and Reinhardt, 2001).

5.3.3. Analysis of survival and mortality

Of 41 patients, 31 were alive on the census date, May 31, 2003. The mean age of known living CF patients in Estonia on the census date was 12 years and 3 months (ranged 1 month to 24.5 years; 95% CI 9.4–15.1 years), which is about 1,5 times lower than the mean age in Sweden (18 years) or other European countries (16 years) (Lannefors and Lindgren, 2002; European Epidemiologic Registry of CF, 2000). About 39% of patients (12/31) are over 15 years of age (Figure 8), which is lower than similar data from European countries, 45%, respectively (European Epidemiologic Registry of CF, 2000). In the UK 51% of CF patients are over 15 years of age (McCormick *et al.,* 2002).

Only 9 of 31 patients (29%) in our CF population are over 18 years old. In Sweden, 45% of the CF population was ≥18 years old (Lannefors and Lindgren, 2002). According to the recent Cystic Fibrosis Foundation Patient Registry (USA) report, there is a significant increase in the percentage of CF patients classified as adults (≥18 years), with the total percentage rising from 29.5% in 1988 to 40% in 2002, and the continuous increase of adult patients numbers was predicted (Cystic Fibrosis Foundation, 2003). Unfortunately, we have no living patients alive aged over 25 years, whereas in 1997 11.3% of patients in the UK

were over 25 years of age (Dodge, 1997), and in 2001 11% of CF patients in the UK were over 30 years old (McCormick *et al.,* 2002), thereby illustrating the worldwide tendency of improved survival of CF patients.

In Estonia a steady improvement in the survival of patients with CF can also be observed. In 1993, when the study began, the mean age of living patients was 8 years 2 months (95% CI 6 years 1 month to 10 years 1 month), but ten year later, in 2003, the mean age of our patients had increased to 12 years 3 months. In 1993 there was no adult patient in Estonia, but in 2003 one third of our patients (9/31), had reached the age of 18 years. However, this raises the question of the urgent need for adult CF centres, as today all follow-up and treatment in Estonia takes place at the Children's Hospitals in Tartu and Tallinn. In the United States and in developed European countries the median survival age has reached 31.6 and 32 years, respectively (Cystic Fibrosis Foundation, 2003; European Epidemiologic Registry of CF, 2000). The factors responsible for improved longevity are not entirely clear, but exocrine pancreatic sufficiency, male sex, white race, higher social classes, absence of colonisation with mucoid *P. aeruginosa*, presentation with predominantly gastrointestinal symptoms, balanced family functioning and coping, and compliance with treatment regimens correlate with a more favourable outcome (Rosenstein and Zeitlin*,* 1998; Hamosh *et al.,* 1998; Doull, 2001; O'Connor *et al.,* 2002).

The lower mean age of our patients may also be influenced by the fact that some adult CF patients with mild disease manifestation are probably not recognized in Estonia, and therefore are not included in this study. In Sweden about 6% and in European countries about 3% of the entire CF-population were diagnosed after the age of 18 (Lannefors and Lindgren, 2002; European Epidemiologic Registry of CF, 2000). Therefore, continuous information and reminders about CF within various areas of adult health care remains important.

Mortality analysis. Of the 41 CF patients clinically described in this study, 10 have died. Four of these patients were F508del homozygotes; 3 were compound heterozygotes for mutations 394delTT/F508del; 1 was a 394delTT homozygote; two were compound heterozygotes for F508del/359insT. Therefore all patients carried severe CFTR mutations characterized at the molecular level as class I or class II alterations. On the basis of other studies, it has been confirmed that homozygosity for the F508del (class II) mutation is associated with a higher risk of death and a lower mean age (Koch *et al.,* 2001; O'Connor *et al.,* 2002).

The median age at death in our cohort (n=10) was 6 years (range 1 month to 20 years), which is considerably lower than the median age at death in Sweden (26 years) or in European countries (24 years) (Lannefors and Lindgren, 2002; European Epidemiologic Registry of CF, 2000). Nevertheless, the situation in Estonia has improved in recent years. During the last 5 years, only one patient in Estonia has died, at the age of 20 years.

For an analysis of the **mortality rates (MR)** of our patients during the last 20 years, in addition to the group of 41 CF patients clinically described in this

study, the data of 35 dead CF patients were included after investigation in the medical archives (See 4.1.2. Study population II). Comparing mortality rates during the last 20 years, a significant decrease can be seen (Figure 9). During the period from 1983 to 1987, the mortality rate was calculated to be as high as 12.2% (95% CI 6.9–21.5) per 100 person-years. Throughout the following periods, a significant decline in MR has been detected. For the period 1998–2002, MR was as low as 0.7% (95% CI 0.1–5.1) per 100 person-years. In other countries a decrease in MR has also been shown. In Sweden the mean MR was 2.0% during the period 1981–1990 and decreased to 0.9% throughout 1991–1998, (Lannefors and Lindgren, 2002).

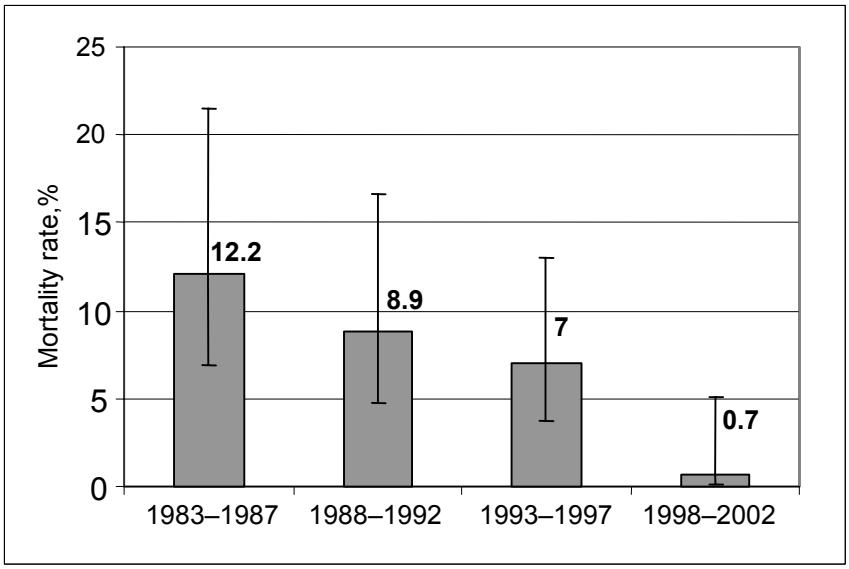

Figure 9. Mortality rates of CF patients in Estonia during the period 1983–2002 (% with 95% CI per 100 person-years).

Several hypotheses about the reasons for lower life expectancy and higher mortality in Estonia may be advanced, starting with a lack of specialized care centres for CF until 1994–1995 and therefore the follow-up, prophylaxis and treatment schedules in different hospitals were variable. In addition to this, there were periods of poor availability of pancrease enzyme supplement formulas, lasting until the beginning of the nineties. A new generation of pancreatic enzyme preparations (e.g. Creon®), mucolytic therapy (inhaled rhDNase), more aggressive antibacterial treatment and new devices for mucus clearance from airways (e.g. Flutter™) became available from 1994–1995. In 1993 the Estonian CF Society was established to support CF patients and their families. One of the major tasks of the Society was the spreading of knowledge about the importance of everyday care such as physiotherapy, exercises, nutrition and treatment with

preparations. All of these principles are known today as part of the standard care of CF patients, and were gradually implemented in Estonia during the last 10 years.

5.3.4. Pancreatic insufficiency

Thirty three out of 41 patients (80%) had pancreatic insufficiency. Worldwide, an average of about 10–15% of CF patients have some extant pancreatic enzyme activity. The higher proportion of pancrease-sufficient patients in the studied group can be explained by the early death of almost all meconium ileus patients, in whome pancreatic insufficiency is observed almost exclusively (Kerem *et al.,* 1990; Zielenski, 2000). It is also likely that a subset of patients with the most severe disease manifestation, born in an earlier period, died prior to the commencement of this study in 1993.

Nutrition plays an essential role in the management of cystic fibrosis. We observed that the age- and sex-adjusted standard deviation (SD) scores for weight and height were consistently lower in our group than in the Estonian reference population (Grünberg *et al.,* 1998). The mean age- and sex-adjusted SD score for weight was –1.4 (±1.4SD), the mean SD score for height was slightly better: –0.8 (±1.5SD). Most CF infants are already malnourished at the time of diagnosis due to energy imbalance with decreased intake and increased losses and needs, which is difficult to compensate even with increased energy intake for long periods afterwards. According to the guidelines consensus report of European nutritional experts, the weight and height of CF patients should remain above 90%, and ideally should be over 95% of the normal values for their age (Sinaasappel *et al.,* 2002). Better nutritional status is associated with better pulmonary outcome and can influence long-term survival (Kraemer *et al.,* 2000; Koletzko and Reinhardt, 2001).

5.3.5. Pulmonary manifestation

Chronic lung disease has developed in 30 out of 41 patients (73.2%; 95% CI, 57.1–85.8%). In 25 patients, chronic disease was detected even before 10 years of age. Most patients with chronic lung disease (22/30) also had persistent bacterial colonisation with microbes typical for CF patients, such as *S. aureus, H. influenza, P. aeruginosa* and others. *P. aeruginosa* was found at least once in sputum cultures in 20 patients, but in 7 patients *P. aeruginosa* was detected consistently. The onset of chronic *P. aeruginosa* infection is a turning point for the individual patient, indicating that the present treatment is ineffective in eliminating the organism from the lower respiratory tract (Koch and Hoiby, 2000). Only one patient has been infected with *Burkholderia cepacia,* and another patient with *Aspergillus fumigatus*, both strongly suggesting poorer

prognosis and shorter survival, as it is extremely difficult to eradicate them from the lungs. Extra measures (isolation) must be followed if patients colonized with the latter organisms visit an outpatient department or are hospitalized in CF clinics.

The decline of pulmonary function with increasing age is universal in CF. FEV_1 is the variable that best reflects the status of lung function throughout the course of CF lung disease and is the best clinical predictor of death (Corey *et al.,* 1997). FVC reflects the reduction in functional lung volume as obstructive lung disease progresses. For patients aged over six years (n=22), the mean scores of lung function tests corrected for height were lower than normal, for FVC 80.3% (95% CI 63.7–96.9) and for FEV_1 82.0% (95% CI 65.8–98.3). Differences in terms of FEV_1 and FVC were not observed between males and females or different genotype groups, although in other studies diversities between the latter-mentioned cohorts were observed (Corey *et al.,* 1997; Loubieres *et al.,* 2002).

5.3.6. Meconium ileus

Meconium ileus is the earliest most serious complication of CF, occurring in about 10–20% of CF patients (Welsh *et al.,* 1995). Initially, of 41 patients investigated, only 2 (4.7%) MI patients were found. Thus it was suspected that the majority of MI patients died in early infancy. By medical records from *exitus letalis* patients and death certificates from the years 1974–1993, an additional 12 CF patients with *meconium ileus* were identified. Overall, 76 patients with CF had been diagnosed during the period of the study, and a total of 14 (18.4%; 95% CI 10.5–29.0%) were diagnosed at birth as having MI, which is quite similar to reports from other studies, i.e. 22.8% in northern Italy (Mastella *et al.,* 2001) or 22% in a Wisconsin study (Farrell *et al.,* 2001).

All but one MI patient in Estonia died during the neonatal period. The only living MI patient, born in April 2003, was treated surgically on the second day of life. The 394delTT mutation was detected in both alleles in this patient. At age 1.5 months she was underweight (–1SD), but her age and sex–adjusted height was normal. She needed a pancreatic enzyme supplement, but at the age of six months, she has no pulmonary symptoms.

5.3.7. Early anaemia in infancy

Clinically significant anaemia, as one of the most prominent initial symptoms of CF in early infancy, was found relatively frequently in our group, i.e. in 5 patients (12%; 95% CI, 4.1–26.2%). In the investigated group, clinically significant anaemia at the age of 2 to 4 months was not connected to infections or prematurity. In all cases anaemia and failure to thrive were the reasons for

hospitalization at this age, and the CF diagnosis was established after differential diagnostics. Unfortunately, in one case, although an infant was hospitalized due to severe anaemia at the age of 3 months, the diagnosis of CF was only established after 17 months, when severe lung involvement and liver enlargement were already present. His weight score was more than –4.0SD lower than predicted for his sex and age. This case clearly indicates that CF must always be considered in the differential diagnosis of anaemic babies. Four out of five patients with early anaemia died at an early age. Data from our group demonstrates that severe anaemia in early infancy in CF patients may be a sign of poor prognosis, but more clinical evidence from CF patients is needed to determine the possible specificity of this sign.

The cause of early anaemia remains unclear, but most previous studies describe anaemia as haemolytic, implicating the malabsorption of fat-soluble vitamin E (Wilfond *et al.,* 1994; Sasavan *et al.,* 1997; Farrell *et al.,* 1977; Swann and Kendra, 1998). It is suggested that vitamin E acts as a protective antioxidant toward peroxidation of membranous polyunsaturated fatty acids. Among infants newly diagnosed with CF, the frequency of vitamin E deficiency has been found to be very high, ranging from 38% to 59% (Sokol *et al.,* 1989; Marcus *et al.,* 1991), but at the same time the anaemia associated with failure to thrive, hypoabuminuria and hypoproteinaemia is a relatively rare finding, detected in about 4% of CF patients (Wilfond *et al.,* 1994; Sinaasappel *et al.,* 2002), suggesting that additional factors most likely contribute to the manifestation of severe anaemia in CF patients.

5.3.8. Other complications

Complications observed in our patients were similar to those described elsewhere. The most serious complication, liver disease, was revealed in 16 patients, and of them, liver cirrhosis was detected in 11 patients (26%), confirmed by ultasonography and/or liver biopsy. Five of these patients died aged 5, 6, 16, 17 and 20 years. Higher risk for developing severe liver disease was revealed for patients of male sex and for those with a history of meconium ileus or severe mutations (Colombo *et al.,* 2002). All but one of our cirrhotic patients carried severe mutations (class I or II) in both alleles.

Diabetes and acute pancreatitis becoming prevalent with increasing age (Koch and Hoiby, 2000) were both noticed only once in our patients. The low incidence of diabetes and acute pancreatitis is probably due to the lower mean age of the patients in our group.

Overall, chronic sinusitis was one of the most frequent complications, and was found in 20 patients (47.6%), which is consistent with other studies (Hadfield *et al.,* 2000). The prevalence of nasal polyposis has been estimated to be very variable, ranging from 7–56% (Hadfield *et al.,* 2000; Henriksson *et al.,* 2002), with a higher prevalence when patients are studied by endoscopic

procedure. In our group the endoscopic technique was not practiced in all patients, and nasal polyposis was diagnosed in only 3 patients, all of whom underwent surgical treatment.

5.4. Genotype/phenotype correlation of patients

The clinical characteristics of patients with different genotypes were compared. Group 1 (n=12) consisted of only F508del homozygous patients, Group 2 (n=11) included patients with 394delTT mutations in one or two alleles, and Group 3 (n=18) comprises all other patients with different genotypes (Table 9, 10).

The F508del homozygous patients (Group 1) were on average diagnosed earlier, which probably offers evidence of their more severe disease manifestation. It is generally accepted that F508del is a "severe" mutation associated with pancreatic insufficiency, and homozygous patients have an increased risk of developing meconium ileus, tend to receive the diagnosis of CF at an earlier age and are probably more susceptible to *P. aeruginosa* infections (Kerem *et al.,* 1990; CF Genetic Analysis Consortium, 1994; O'Connor *et al.,* 2002).

The patients in Group 2 had a higher mean level of sweat chlorides, and more patients were diagnosed as having clinically significant anemia at an early age. The clinical data concerning patients carrying the 394delTT alteration has only been published once in the literature, and our results agreed with the previous description (Strandvik *et al.,* 2001). This is in correlation with the prediction made on the basis of the character of the mutation. 394delTT is defined as a class I mutation, in which the non-functional protein, truncated from the first transmembrane helix, is synthesized (Andersson *et al.,* 2002).

Overall, the severity of disease manifestation in 394delTT patients (Group 2) was similar to that in homozygotes for F508del (Group 1). Patients from both groups showed severe disease manifestation with pancreatic insufficiency, highly elevated sweat chlorides, prevailing pulmonary symptoms and a high proportion of *P. aeruginosa* colonisation and liver cirrhosis.

Altogether, Group 3 (n=18), including patients with other different genotypes, tended to have a milder course of the CF disease, but in particular cases the severity of the disease depended upon the individual alteration (Table 11). There were significantly more patients with pancreatic sufficiency, and the mean age at diagnosis was notably higher than in other groups. The higher mean age of establishing CF diagnosis is most likely associated with the greater number of pancreas-sufficient patients. This group tended to have a higher mean age, lower level of sweat chlorides, decreased rate of liver disease and chronic sinusitis, but the differences were not statistically significant.

Table 10. Comparison of the clinical data of CF patients with different genotypes.

Grouping	Group 1 n=12	Group 2 n=11	Group 3 n=18
Distribution by mutations	F508del	394delTT	Other
Male/Female	5/7	6/5	10/8
Number of patients who died	4	4	2
Mean age (years, SD)	11.0(±6.9)	11.5 (±9.3)	13.3 (±7.9)
Mean age at diagnosis (years, SD)	1.0 (±1.0)	1.6 (±2.4)	3.2 (±2.45)*
Sweat chloride mmol/l (mean, SD)	95.2 (±28.3)	113.6 (±17.9)**	80.0 (±24.8)
Pancreatic insufficiency	12	11	11***
Chronic pulmonary disease	9	8	14
PA colonisation at least once in sputum	7	6	7
Liver disease with cirrhosis	4	3	4
Chronic sinusitis	7	8	5
Moderate or severe anemia in early childhood before diagnosis	1	4****	0
Meconium ileus	1	1	0
Shwachman–Kulczycki score (mean, SD)	74.0 (±13.0)	74.1 (±17.19)	81.5(±15.9)

PA —*Pseudomonas aeruginosa* colonisation. *Mean age at diagnosis is associated with genotype. Mean age at diagnosis is significantly higher in Group 3 than in Group 1 (Tukey test, $p<0.03$); **Mean value of sweat chlorides is significantly higher in patients carrying the 394delTT mutation than in patients homozygous for F508del (Tukey test; $p<0.01$); *** A significantly lower number of patients with PI was observed in Group 3 than in other mutation groups (Fisher's exact test $p<0.01$); **** A statistically significant difference in the presence of anaemia in early childhood was observed between Group 1 and Group 2 (Fisher's exact test $p<0.01$).

Patients' survival was also analyzed in distinct genotype groups and found to be differ significantly (Figure 10). Survival probabilities differed significantly in Groups 1 and 3 (log-rank p= 0.03). A statistical comparison of curves was performed using Cox proportional hazard regression analysis, which gave a 2.4-fold higher relative risk of death (RR=2.4; 95% CI 1.0 to 5.7) in Group 1. Group 2 (patients with the deletion F508del) and Group 3 (patients with different genotypes) were also found to be statistically divergent (log-rank p=0.03), with a 5.5-fold higher relative risk of death in patients carrying 394delTT (RR=5.5; 95% CI 1.0 to 30.9). Survival probabilities in Groups 1 and 2 were similar. This shows that the alterations F508del and 394delTT both are severe mutations and patients homozygous for these mutations have a higher relative risk of death.

Table 11. Clinical profiles of the patients with rare genotypes (Group 3).

Genotypes	**Patients' clinical data**					
	M/F	Age, years	PS/PI	Lung involvement	Sweat Cl^- mmol/l	Other symptoms
3659delC/F508del	M	17	PI	moderate	91	Liver cirrhosis at age 9
3659delC/F508del	F	4.5	PI	severe	57	Chronic PA colonization at age 3
359insT/F508del	F	Died, 17y	PI	severe	112	Liver cirrhosis
359insT/F508del	M	Died 22y	PI	severe	115	Chronic PA colonization, liver cirrhosis, nasal polyps
S1196X/F508del	M	22.5	PI	moderate	80	Diabetes mellitus
I1005R/F508del	M	17.5	PI	severe, *Burkholderia cepacia*	105	Acute pancreatitis, GOR, live cirrhosis, chronic PA, asthma bronchiale
R1066H/F508del	M	11	PS	severe	62	Chronic PA colonization, nasal polyps
R553X/ U	F	3	PI	mild	95	Liver enlargement +2.25SD
S549N/F508del	M	0.75	PI	moderate	91	Malabsorption
W57R/F508del	F	5.5	PI	severe	NA	Digital clubbing
R117C/F508del	F	24.5	PS	mild	130	Recurrent pneumonias and abdominal pain in childhood
1716G→A/ F508del	M	24	PS	mild	62	Recurrent pneumonias
1716G→A/ U	F	4.5	PI	mild	42	Rectal prolaps, GOR
S1235R/ U	F	12.5	PS	moderate	62	
E217G/U	M	22.5	PS	mild	60	Chronic sinusitis
U/U	F	11.5	PI	moderate	70	DIOS, abdominal pain
U/U	F	18.5	PS	mild	63	
U/U	M	14	PI	moderate	62	DIOS

[1]Chronic PA — chronic *Pseudomonas aeruginosa* colonization; [2]GOR-gastrooesophageal reflux; U-unidentified mutation

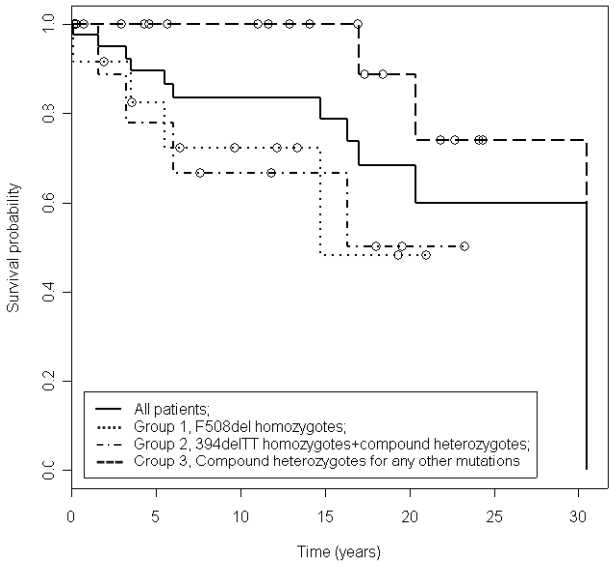

Figure 10. Kaplan-Meier survival estimates for CF patients in whome molecular analysis has been performed (n=41). Group 1 (n=12), Group 2 (n=11) and Group 3 (n= 18) were compared. The Kaplan-Meier curves of patients with different mutations are significantly divergent: Group 1 *versus* Group 3 (log-rank p = 0.03) and Group 2 *versus* Group 3 (log-rank p=0.03). ○ living index cases.

5.5. Determination of incidence of CF disease

Taking into account the F508del carrier frequency in the whole population (1:84) and the relative frequency of the F508del mutation among all CF chromosomes (51.4%), the frequency of CF in Estonia can be calculated. According to the Hardy–Weinberg equilibrium the incidence of CF disease is about 1:7457. With current birth rate (13 000–14 000 live births per year) we might have about 2 new CF patients per year.

After careful investigation of medical and autopsy records (see 4.1.2.), a total of 76 patients with CF disease were identified during the period from January 1974 to May 2003. Thereby, the incidence of CF is estimated at 1 in 7743 live births (Table 12).

CF incidence rates were calculated for different time periods based on patients' birthdates. Data about live births were obtained from the authorized statistics of the Estonian Statistical Office (www.stat.ee). It is possible that we missed some patients born in the period 1974–1979, as Estonian law specifies that medical records should be preserved for 25 years, and we had difficulty acquiring all of the medical records needed. Therefore the incidence rate,

Table 12. CF cases and incidence in Estonia from January 1, 1974 to May 31, 2003.

Years	No. of CF cases	No of live births	Incidence of CF	95% CI
1974–1979	16	152,524	1:9532	1:6396–18,691
1980–1989	34	238,858	1:7025	1:5257–10,582
1990–1999	21	153,104	1:7290	1:5106–12,738
2000–2003[a]	5	44,016	1:8803[b]	1:4691–71,275
Total	**76**	**588,502**	**1:7743**	**1:6322–9989**
CF incidence by Hardy-Weinberg calculation			**1:7457**	

[a] For the year 2003, only live births from January to May were included. [b]Ascertainment is inevitably incomplete for patients born in recent years, because they have not yet been diagnosed.

1:9532, for this period is probably underestimated. The incidences for the periods 1980–1989 and 1990–1999 are very similar, 1:7025 and 1:7290 respectively. The CF incidence of the last period (2000–2003), 1:8803, is probably also misjudged, as the mean age of establishing CF diagnosis in our group is 2 years 1 month, and thus some patients have not yet been diagnosed. As DNA diagnostics of CF patients was available from 1993, there were 6 cases of prenatal diagnostics in CF families during the period 1998–2001, and three pregnancies were aborted. This fact could also influence the incidence rates of CF in the most recent periods. The total incidence rate of CF disease for the whole period 1974–2003 (1:7743) is comparable to the incidence calculated using the Hardy-Weinberg equation — 1:7457.

An analysis of the data for Estonia indicates a lower incidence of CF than in many European populations (Figure 11). The closest similarity was seen with Scandinavian countries, excluding Finland where the prevalence of CF differs more than threefold. As Finland has long been genetically isolated owing to its geographical location, the frequency of CF is exceptionally low there, i.e. 1:25,000. Similar discrepancies have also been seen for other diseases (e.g phenylketonuria). A decreased frequency of CF has also been detected in northwestern and central regions of Russia (1:12,300) (Petrova *et al.,* 1997). Interestingly, the incidence reported for Latvia is relatively high, 1:3300, but this can be explained by the fact that only ethnically (three generation) Latvians were investigated in the pilot screening (Krumina, personal communication). In this manner they sought to eliminate the influence of the Slavic population.

In conclusion, the results of our work indicate that both CF prevalence and the proportion of F508del carriers in Estonia are lower than is generally the case in Caucasians (Figure 11). The relative incidence of CF and in particular the low proportion of the F508del mutation in CF chromosomes means that medical screening using DNA diagnostic tests cannot be suggested in our population.

The age of CF diagnosis must, however, be lowered significantly based on clinical symptoms by increasing knowledge of CF among general practitioners and paediatricians.

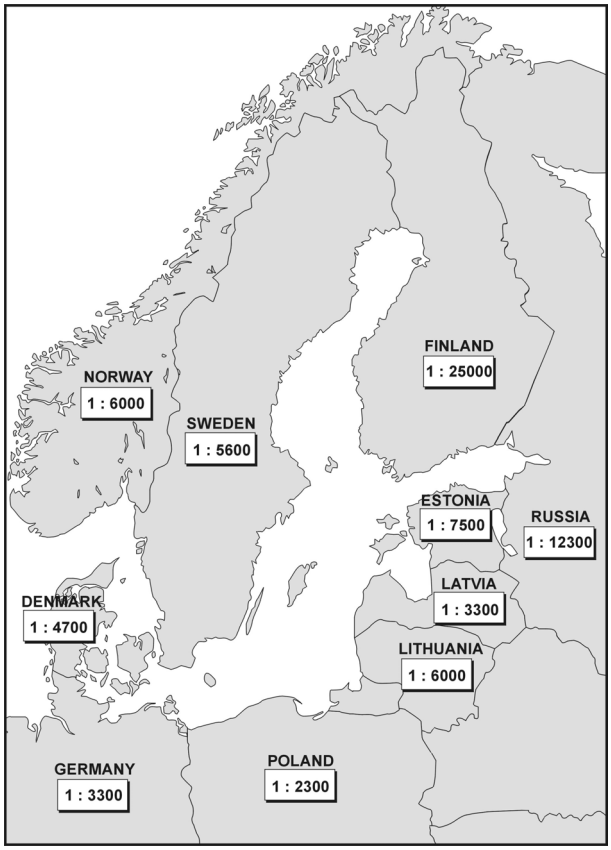

Figure 11. The incidence of CF disease in Estonia and in countries bordering the Baltic Sea (Kere *et al.,* 1994; Petrova *et al.,* 1997; Lannefors and Lindgren, 2002; Eikild *et al.,* 1993; Krumina, personal communication; Schwartz *et al.,* 1994; Witt *et al.,* 1994; Lucotte *et al.,* 1995; Utkus, personal communication).

6. CONCLUSIONS

1. The prevalence of F508del mutation carriers in Estonia is 1:84, but demonstrates statistically significant heterogeneity within Estonia. The highest proportion of F508del heterozygotes was identified in the western region (1 in 36 newborns), and the lowest incidence was detected in the southeastern region (1 in 128 newborns).

2. According to the Hardy–Weinberg calculations, the incidence of CF in Estonia is 1 in 7457 live births. In conformity with retrospective data from all known CF patients born from January 1974 to May 2003, the incidence rate is 1 CF patient in 7743 live births.

3. The two most common CFTR gene alterations in Estonia are F508del and 394delTT, accounting for 52% and 15% of all CF chromosomes respectively. Twelve more CFTR mutations (359insT, R117C, E217G, I1005R, R1066H, S1196X, S1235R, S549N, W57R, R553X, 3659delC, 1716G→A) were found in Estonian CF patients.

4. The median age at diagnosis of CF in Estonian patients (1 year 7 months) is considerably higher and the mean age of our patients (12 years and 3 months) is lower than in other European countries. However, the survival rate of our CF patients is increasing. In 1993 there were no adult patients in Estonia, but in 2003 one third of our patients had reached the age of 18 years. This therefore indicates the urgent need to reorganize the provision of specialized care for adult CF patients.

5. The mortality rate of CF patients in Estonia is decreasing, being 12.2% during the period 1983–1987 and dropping to 0.7% in the period 1997–2002. There is considerable diversity in survival probabilities between groups of different genotypes analyzed using Kaplan-Meier curves. Patients homozygous for F508del and with 394delTT/394delTT or 394delTT/F508del genotypes showed a significantly higher relative risk of mortality, 2.4 and 5.5 times respectively, than patients with other mutations.

6. The phenotype-genotype correlation showed severe disease manifestation of patients harbouring 394delTT/394delTT and 394delTT/F508del genotypes. Their clinical characteristics were similar to the group of patients with the F508del/F508del genotype, which was previously known to result in severe disease manifestation.

7. In our cohort, there were 33 patients (80%) with pancreatic insufficiency. Chronic lung disease developed in 30 out of 41 patients (73%). In 18% of patients, meconium ileus was diagnosed at birth. Clinically significant anaemia in early infancy was found relatively frequently (12%) in our group, and should always be considered in the differential diagnosis of anaemia.

8. DNA testing for common CFTR mutations has been introduced in the practical work of the Molecular Diagnostics Centre of United Laboratories, Tartu University Clinics.

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SUMMARY IN ESTONIAN

Tsüstiline fibroos Eestis

Tsüstiline fibroos (TF) on sagedasemaid pärilik letaalne autosoomretsessiivne haigus. Valge rassi hulgas on TF esinemissageduseks ligikaudu 1:2500, kuid isegi Euroopas on antud haiguse sagedused väga erinevad kõikudes 1:1700 Põhja-Iirimaal kuni 1:25000 Soomes. TF iseloomustatakse klassikalise sümptomite triaadiga: krooniline obstruktiivne kopsuhaigus, pankrease eksokriinne puudulikkus ning klooriioonide kontsentratsiooni tõus higis. Haigus on põhjustatud muutustest geenis, mida nimetatakse tsüstilise fibroosi transmembraanse juhtivuse regulaatoriks (TFTR). TFTR geen asub regioonis 7q31.2 ning kodeerib 1494 aminohappe pikkust transmembraanset valku, mis funktsioneerib peamiselt klooriioone transportiva kanalina. On näidatud ka, et TFTR valk osaleb teiste rakumembraani kanalite töö reguleerimises (näit. epiteliaalne naatriumiooni kanal, kaltsiumiooni tundlik klooriiooni kanal). TFTR valk ekspresseerub paljude organite epiteelkudedes, näiteks kopsudes, sooles, kõhunäärmes, maksas, sapipõies, süljenäärmetes, higinäärmetes, testistes ja emakas ning kõigi nende organite töö häirumist on kirjeldatud ka TF patsientidel.

TFTR geen on suhteliselt suur hõlmates 27 eksonit. Praeguseks on geenis kirjeldatud üle 1200 muutuse. Enamik neist on väga harvad, ülemaailmselt ulatub vaid 5 mutatsiooni osakaal kõigist TFTR geeni muutustest üle 1%. Ülemaailmselt on TFTR geeni põhimutatsiooniks F508del, mida on leitud ligikaudu 67% TF haigete kromosoomidest. Erinevates populatsioonides või geograafilistest piirkondades võib leida ka regioonispetsiifilisi mutatsioone nagu 394delTT Skandinaaviamaades või CFTRdele2,3 slaavlastel. Erinevates maades võib haigetel identifitseeritud mutatsioonide muster olla väga erinev.

Kuigi TF loetakse klassikaliseks monogeenseks haiguseks, võib TF olla väga varieeruva kliinilise pildiga. Kõige suuremat rolli arvatakse selles olevat patsiendi genotüübil. Konkreetsete mutatsioonide mõju sõltub paljudest faktoritest: muutuse tüübist (näit. asendus- või stoppmutatsioon), molekulaarsest mehhanismist ja lokalisatsioonist geenis. Mõjutavad ka intrageensed faktorid, näiteks polümorfismid, mis asuvad samas alleelis tuvastatud muutusega. Olulised on ka keskkonna faktorid nagu patsiendi toitumus, ravirežiim (sh. spetsiaalse TF keskuse olemasolu), haige sotsiaalne klass ning suitsetamine. Perekonna ja kaksikute uuringutest on selgunud, et sama genotüübiga ning sarnastes keskkonna oludes kasvanud patsientidel esineb siiski olulisi erinevusi haiguse raskuses ja kulus. Seetõttu on viimastel aastatel järjest suuremat tähelepanu pööratud võimalike sekundaarsete, haigust moduleerivate geneetiliste fakorite leidmiseks.

Töö eesmärgid:

1) teha kindlaks TFTR geeni põhimutatsiooni F508del kandjate sagedus Eestis
2) määrata kindlaks TF haiguse sagedus Eestis

3) identifitseerida Eesti TF patsientidel TFTR geenis esinevad muutused
4) analüüsida Eesti patsientide demograafilisi näitajaid
5) hinnata TF patsientide kliinilist pilti ning luua seoseid genotüübiga
6) juurutada sagedasemate TF mutatsioonide DNA põhine analüüs.

Materjal ja metoodika

Mutatsiooni F508del kandjate sageduse kindlakstegemisel uuriti 01.01.1993–31.07.1993 järjestikuliselt sündinud 7396 last. TF haigete uurimisgrupi moodustasid kõik Eestis teadaolevad TF haiged (n=76), kes olid sündinud ajavahemikus 01.01.1974–31.05.2003. Kliiniliste andmete ja TFTR geeni molekulaarne analüüs osutus võimalikuks 41 patsiendil. Ülejäänud 35 haige (kes olid surnud enne uuringu algust) andmeid kasutati ainult haiguse sageduse, mekooniumiileuse osakaalu arvutamisel ning suremuse analüüsil. Patsientide andmeid koguti Tallinna Kesklinna, Lasnamäe, Kopli, Nõmme Pärnu ja Tartu lastepolikliinikutest, TÜ Kliinikumi Lastekliiniku, Kirurgiakliiniku, Kopsukliiniku, Tallinna Lastehaigla, Nõmme Lastehaigla, Viljandi, Põlva, Pärnu ja Kuressaare haiglate arhiividest.

TFTR geeni molekulaarne analüüs viidi läbi TÜMRI Biotehnoloogia õppetoolis, Eesti Biokeskuses ja TÜ Kliinikumi Ühendlabori Molekulaardiagnostika Keskuses. Haigete DNA analüüsimisel rakendati: 1) enam levinud mutatsioonide otsest detekteerimist, 2) kaudseid meetodeid nagu ühe-ahelalise DNA konformatsiooni polümorfismide analüüs; denatureeriva gradiendiga geelelektroforees; 3) otsest DNA järjestuse sekveneerimist.

Tulemused ja arutelu

1. Eestis on TFTR geeni põhimutatsiooni F508del kandjate sagedus 1:84, mis on madalam kui keskmiselt Lääne-Euroopa maades (näit. Saksamaal 1:35), kuid kõrgem kui meie naabermaades Soomes (1:171) või Venemaal (1:114). Uurimisel leiti, et F508del kandjate levimus Eesti erinevates piirkondades on statistiliselt oluliselt erinev. Kõrgeim on mutatsioonikandjate osakaal Lääne-Eestis ja saartel — 1:36 ning madalaim Kagu-Eestis vastavalt 1:128 vastsündinu kohta.
2. Antud uurimistöö tulemusena saadi TF sageduseks Eestis 1:7743 (95% CI 1:6322–1:9989) ning Hardy-Weinbergi reegli alusel arvutati sageduseks 1: 7457. Seega on haiguse sagedus Eestis ligikaudu 3 korda madalam kui Kesk-Euroopas (Poolas 1:2300, Saksamaal 1:3300), kuid tunduvalt kõrgem kui näiteks Soomes (1:25 000).
3. Eestis on TF patsientide hulgas kõige sagedamini haigust põhjustavaks muutuseks F508del esinedes 52%-l TF haigete kromosoomides. Selle mutatsiooni suhteline sagedus meie grupis on tunduvalt madalam kui Skandinaavias (Rootsis 67%) või Kesk-Euroopas (Saksamaal 72%), kuid on võrreldav sama näitajaga Soomes (46%). Sageduselt teine mutatsioon, 394delTT, hõlmab ligi-

kaudu 15% haiguslikest alleelidest Eestis. Antud muutus on iseloomulik kõigile Skandinaaviamaadele. DNA testid nende muutuste määramiseks on juurutatud ka TÜ Kliinikumi Ühendlabori Molekulaardiagnostika osakonnas.

4. Eesti TF patsientidel leiti veel 12 erinevat TFTR geeni muutust: 359insT, R117C, E217G, I1005R, R1066H, S1196X, S1235R, S549N, W57R, R553X, 3659delC, 1716G→A.

5. Meie haigete TF diagnoosi püstitamise vanuse mediaan, 1 aasta 7 kuud, on tunduvalt kõrgem kui näiteks Rootsis (9 kuud) või USA-s (6 kuud). Meie patsientide grupi keskmiseks vanuseks on 12 aastat ja 3 kuud, mis on ligikaudu 1,5 korda väiksem kui teistes Euroopa maades (Rootsis 18 aastat). Viimase 10 aasta jooksul on meie patsientide elulemus siiski paranenud. Kui 1993. a. polnud teada ühtki üle 18-aastast patsienti, siis mais 2003 oli neid 9, seega üks kolmandik patsientidest. Seetõttu on tekkinud vajadus spetsiaalse täiskasvanud TF patsientidele loodud ambulatoorse ja statsionaarse abi keskuse järele. Hetkel toimub kõigi TF haigete ravi ja jälgimine lastehaiglate juures.

6. Suremuse määr Eesti TF patsientide hulgas on langustendentsiga, olles aastatel 1983–1987 keskmiselt 12,2% ning aastatel 1998–2002 0,7% inimaasta kohta. Analüüsides patsientide elulemust erineva genotüübiga haigete gruppides võis märgata olulisisi erinevusi. Mutatsiooniga F508delTT patsientide grupis oli suremise risk 2,4 korda ja mutatsiooniga 394delTT patsientide grupis 5,5 korda suurem kui grupis, kuhu olid koondatud erinevate harvemate genotüüpidega patsiendid.

7. Mutatsiooniga 394delTT patsientide (n=11) haiguse kulg oli raske ning võrreldav F508del homosügootidest (n=12) patsientidega. Eelnevates uurimustes on F508del tunnistatud nn. "raskeks" mutatsiooniks nii geenimuutuse iseloomu tõttu kui ka arvukates genotüüp-fenotüübi võrdlusuuringutes.

8. Meie haigete hulgas oli 80% (33/41) lastest pankrease puudulikkusega. Kroonilist kopsukahjustust diagnoositi 30 lapsel 41-st, seejuures 25 haigel olid kroonilised muutused kopsudes välja kujunenud juba enne 10. eluaastat. Enamikul kroonilise kopsukahjustusega lastest (22/30) oli püsiv bakteriaalne kolonisatsioon (sh. *P. aeruginosa* 7 haigel). Maksatsirroosi esines uuritutest 11 patsiendil. Suhteliselt sagedasti esines grupis mõõdukat kuni rasket varast imikuea aneemiat (5/41) ning seetõttu peaks imikuea aneemiate põhjuste selgitamisel alati kaaluma TF diagnoosi.

Lõpetuseks võib öelda, et antud uurimistöö selgitas välja TF haiguse sageduse ja selle põhimutatsiooni F508del kandjate sageduse Eestis. Saadud informatsioon Eesti patsientidel esinevatest mutatsioonidest on väga oluline igapäevases TF haigete DNA diagnostikas sh. sünnieelses diagnostikas ning samuti perekondade nõustamises ja haiguse kulu prognoosimisel. Käesoleva töö raames on koostatud üle-eestiline TF patsientide register ja juurutatud DNA diagnostika. Haiguse kulu, tüsistuste tekke ning patsientide demograafiliste näitajate detailne analüüs loob eeldused parema meditsiiniabi korraldamiseks TF patsientidele.

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Alates 1992. aastast olen tegelenud Prof. Andres Metspalu laboris geneetiliste haiguste molekulaarse diagnostikaga ning sünnieelse diagnostikaga seotud uute mitteinvasiivsete meetodite väljatöötamisega. Peamiseks uurimisvaldkonnaks on olnud tsüstiline fibroos.

Aastatel 2002 kuni 2004 olin Euroopa Ühenduse 5. Raamprojekti granti No QLRT-2001-02876 “Quality of life and management of living recources; Thematic Network around Cystic Fibrosis and related Diseases (CF Network)” grantihoidja Eestis.

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